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Rozprawa doktorska

Rola metalotionein owsa zwyczajnego (*Avena sativa* L.) w odpowiedzi na czynniki środowiskowe

Praca wykonana w Katedrze Genetyki
pod kierunkiem
prof. dr hab. Grażyny Dąbrowskiej
i dr Agnieszki Mierek-Adamskiej.

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Paniom prof. dr hab. Grażynie Dąbrowskiej i dr Agnieszce Mierek-Adamskiej

za wsparcie merytoryczne, cenne wskazówki oraz całą pomoc udzieloną na każdym etapie przygotowywania niniejszej pracy doktorskiej,

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Moim Rodzicom i Mężowi

za cierpliwość i wiarę we mnie,

serdecznie dziękuję.

Spis treści

Wykaz skrótów

ABA - kwas abscysynowy, *ang. abscisic acid*

ABTS - 2,2'-azobis(3-etylobenzotiazolino-6-sulfonianu)

AC - zdolność antyoksydacyjna, *ang. antioxidant capacity*

CAT - katalaza, *ang. catalase*

FRAP - zdolność redukowana jonów żelaza, *ang. ferric reducing antioxidant power*

HM - metale ciężkie, *ang. heavy metals*

HSAB - teoria twardych i miękkich kwasów i zasad, *ang. hard and soft acids and bases theory*

IPTG - izopropylo β-d-1-tiogalaktopiranozyd

MT - metalotioneina, *ang. metallothionein*

MT1 - metalotioneina typu 1, *ang. MT type 1*

MT2 - metalotioneina typu 2, *ang. MT type 2*

MT3 - metalotioneina typu 3, *ang. MT type 3*

MT4 - metalotioneina typu 4, *ang. MT type 4*

PEG - poliglikol etylenowy

PX - peroksydaza, *ang. peroxidase*

RFT - reaktywne formy tlenu, *ang. reactive oxygen species*

RWC - względna zawartość wody, *ang. relative water content*

SOD - dysmutaza ponadtlenkowa, *ang. superoxide dismutase*

TPC - całkowita zawartość fenoli, *ang. total phenolic content*

I. Streszczenia

1. Streszczenie w języku polskim

Rośliny są narażone na ciągły stres, który może być wywołany czynnikami abiotycznymi, np. niedoborem lub nadmiarem wody, obecnością zanieczyszczeń oraz obecnością mikroorganizmów lub insektów. W odpowiedzi na niekorzystne czynniki środowiskowe w komórkach roślinnych wytwarzane są między innymi reaktywne formy tlenu, które w nadmiarze prowadzą do uszkodzeń białek, lipidów i DNA, co może prowadzić do śmierci komórki. Rośliny wytworzyły szereg mechanizmów, które minimalizują negatywne efekty zmieniającego się środowiska. Metalotioneiny (MT) to białka o niskiej masie cząsteczkowej bogate w reszty cysteinowe (Cys). MT zostały wykryte u bakterii, ssaków i roślin. Ze względu na swoje zdolności do wiązania jonów metali, białka te biorą udział w utrzymaniu homeostazy metali, ale także w procesach ich detoksyfikacji. U roślin MT dzieli się na cztery typy, w zależności od liczby i ułożenia Cys. Grupa tiolowa -SH Cys może reagować z reaktywnymi formami tlenu, chroniąc komórki przed stresem oksydacyjnym.

Celem niniejszych badań było zidentyfikowanie genów *MT* owsa *Avena sativa* L. (*AsMT*) i określenie roli kodowanych przez nie białek w reakcji owsa na czynniki abiotyczne (stres suszy, stres osmotyczny, metale ciężkie) oraz biotyczne (obecność grzybów *Trichoderma viride*).

W genomie *A. sativa* L. zidentyfikowano 21 genów *MT*, należących do czterech typów (*AsMT1-4*). W sekwencjach promotorowych tych genów stwierdzono obecność *cis*-elementów odpowiedzialnych za reakcję rośliny na: metale ciężkie, fitohormony, światło, niedobór wody i czynniki biotyczne oraz elementów regulatorowych związanych z rozwojem roślin. Założono, że MT pełnią szczególną rolę i są niezbędne do prawidłowego wzrostu i rozwoju roślin. Wykazano zmiany ekspresji *AsMT1-4* w pierwszych godzinach kiełkowania nasion owsa, przy czym całkowita ilość transkryptów *AsMT* pozostawała taka sama. Analiza funkcjonalna, wykazała, że bakterie niosące geny *AsMT1-4* charakteryzowały się większą tolerancją na stres osmotyczny i stres wywołany obecnością jonów Zn i Cd. Obecność jonów metali (Zn, Cd oraz mieszaniny Zn i Cd) powodował w różnym stopniu zmiany ilości transkryptów *AsMT1-4* w korzeniach i pędach siewek owsa. W warunkach stresu wywołanego metalami

ciężkimi w 21-dniowych roślinach owsa obserwowano wzrost zawartości fenoli oraz antyoksydantów hydrofilowych i lipofilowych, a zmiany te korelowały z ekspresją *AsMT*. W eksperymencie donicowym wykazano, że wzrost zawartości Cd w glebie powoduje zmniejszenie biomasy owsa i liczby wytworzonych nasion oraz wzrost zawartości Cd w części nadziemnej roślin, nie stwierdzono istotnych statystycznie zmian ekspresji *AsMT*. Natomiast stwierdzono zmiany ekspresji *AsMT1-3* w obecności *T. viride*, co wskazuje na udział AsMT w interakcji tego gatunku z grzybem saprofitycznym. Stresy osmotyczny i suszy powodowały zmiany ekspresji genów *AsMT* w pędach i korzeniach oraz wzrost aktywności enzymów antyoksydacyjnych, zawartości kwasu abscysynowego, związków fenolowych i cukrów.

Przeprowadzone badania wskazały, że MT owsa biorą udział nie tylko w odpowiedzi na obecność jonów metali w środowisku, ale są nieodłącznym elementem odpowiedzi na stresy osmotyczny i suszy oraz biorą udział w interakcji roślina-grzyb. U owsa zwyczajnego poszczególne typy *AsMT* pełnią zróżnicowane funkcje, a ich ekspresja koreluje ze zmianami biochemicznymi zachodzącymi w komórkach roślinnych pod wpływem stresów. Metalotioneiny AsMT2 i AsMT3 mogą stanowić odpowiednio markery molekularne suszy i stresu osmotycznego. Natomiast *AsMT1*, którego ekspresja jako pierwsza zmienia się w stresie wywołanym obecnością Cd, może być wskaźnikiem stresu powodowanego metalami ciężkimi. W przyszłości uzyskane wyniki mogą stanowić podstawę do wytworzenia transgenicznego owsa zwyczajnego o podwyższonej tolerancji na stresy zwłaszcza spowodowanego odwodnieniem i zanieczyszczeniem środowiska metalami ciężkimi, co ma istotne znaczenie w dobie zmian klimatycznych.

2. Streszczenie w języku angielskim (abstract)

Plants are exposed to constant stress caused by abiotic factors, such as water deficiency or excess, pollutants and biotic factors. In response to unfavourable environmental factors, plant cells produce, among others, reactive oxygen species, which in excess lead to damage to proteins, lipids and DNA, which can lead to cell death. Plants have developed several mechanisms that minimise the adverse effects of a changing environment. Metallothioneins (MTs) are low molecular weight proteins rich in cysteine residues (Cys). MTs have been detected in bacteria, mammals and plants. Due to their ability to bind metal ions in cells, these proteins participate in maintaining metal homeostasis and in detoxification processes. In plants, MTs are divided into four types, depending on the number and arrangement of Cys. The thiol group -SH of Cys can react with reactive oxygen species, protecting cells from oxidative stress.

This study aimed to identify the *MT* genes of *Avena sativa* L. (*AsMT*) and determine the role of the proteins they encode in the response of oat to abiotic (drought stress, osmotic stress, heavy metals) and biotic (presence of *Trichoderma viride* fungi) factors.

In *A. sativa* L. genome, 21 MT genes belonging to four types (*AsMT1-4*) were identified. The promoter sequences of these genes contained *cis*-elements responsible for the plant response to heavy metals, phytohormones, light, water deficiency and biotic factors, and those related to plant development. MTs are necessary for the proper plant growth and development. In the first hours of oat seed germination, changes in the expression of *AsMT1-4* were observed, while total number of *AsMT* transcripts remained the same. An analysis carried out to understand the function of AsMT, showed that bacteria carrying *AsMT1-4* were characterised by greater tolerance to osmotic stress and stress induced by the presence of Zn and Cd. The presence of metal ions (Zn, Cd and mixture of Zn and Cd) caused variations in the level of *AsMT1-4* transcripts in the roots and shoots of oat seedlings. Under stress conditions induced by heavy metals, an increase in the content of phenols and hydrophilic and lipophilic antioxidants was observed in 21-day-old oat plants, and these changes correlated with the expression of *AsMT*. Oat plants growing with *T. viride* and without the fungus were subjected to long-term stress induced by Cd ions in the soil. It was shown that with the increase in Cd in the soil, the biomass of plants and the number of seeds produced decreased, while the concentration of Cd in

the above-ground part of the plants increased. Changes in the expression of *AsMT1-3* were observed in the presence of *T. viride*. In contrast, the presence of Cd did not cause statistically significant changes in the content of transcripts of these genes, which indicates the participation of AsMT in the interaction of plants with microorganisms. Osmotic and drought stresses caused changes in the expression of *AsMT* genes in shoots and roots. Moreover, those stresses increased the activity of antioxidant enzymes, the content of abscisic acid, phenolic compounds and sugars.

The studies indicate that oat MTs participate not only in response to the presence of metal ions in the environment but are also an integral element of the response to osmotic and drought stresses and presence of microorganisms. In common oats, individual types of AsMT perform diverse functions, and their expression correlates with biochemical changes occurring in plant cells under stress. AsMT2 may be a molecular marker of drought, and AsMT3 may be a marker of osmotic stress. In response to Cd the expression of *AsMT1* changed first. Thus, this gene may indicate stress induced by heavy metals. The results may be used for producing transgenic varieties of oat with increased tolerance to stresses, especially those caused by dehydration and environmental pollution, which is important in the era of climate change.

II. Wykaz publikacji będących podstawą rozprawy doktorskiej

Praca doktorska obejmuje wyniki badań zawartych w czterech pracach eksperymentalnych, opublikowanych w recenzowanych, międzynarodowych czasopismach naukowych, których wykaz zawarto poniżej.

1	Konieczna W., Mierek-Adamska A., Warchoł M., Skrzypek E., i Dąbrowska G. B. The involvement of metallothioneins and stress markers in response to osmotic stress in *Avena sativa* L. *Journal of Agronomy and Crop Science*, 2023, 209(3), 371-389. doi.org/10.1111/jac.12633	IF: 3,7 $IF_{5\text{-year}}$: 4,0 MNiSW: 140
2	Konieczna W., Warchoł M., Mierek-Adamska A., Skrzypek E., Waligórski P., Piernik A., i Dąbrowska G. B. Changes in physio-biochemical parameters and expression of metallothioneins in *Avena sativa* L. in response to drought. *Scientific Reports*, 2023, 13(1), 2486. doi.org/10.1038/s41598-023-29394-2	IF: 3,8 $IF_{5\text{-year}}$: 4,3 MNiSW: 140
3	Konieczna W., Mierek-Adamska A., Chojnacka N., Antoszewski M., Szydłowska-Czerniak A. i Dąbrowska G. B. Characterization of the metallothionein gene family in *Avena sativa* L. and the gene expression during seed germination and heavy metal stress. *Antioxidants*, 2023, 12(10), 1865. doi.org/10.3390/antiox12101865	IF: 6,0 $IF_{5\text{-year}}$: 6,7 MNiSW: 100
4	Konieczna W. Turkan S. Warchoł M. Skrzypek E. Dąbrowska G. B., i Mierek-Adamska A. The contribution of *Trichoderma viride* and metallothioneins in enhancing the seed quality of *Avena sativa* L. in Cd-contaminated soil. *Foods*, 2024, 13(15), 2469. doi.org/10.3390/foods13152469	IF: 4,7 $IF_{5\text{-year}}$: 5,1 MNiSW: 100
	Suma IF Suma $IF_{5\text{-year}}$ Suma MNiSW	18,2 20,1 480

III. Wstęp

Ziemia jest planetą roślin - to one (oraz inne organizmy przeprowadzające fotosyntezę) ukształtowały atmosferę Ziemi, pozwalając na rozwój życia. Pierwsza roślina lądowa pojawiła się około 500 milionów lat temu w okresie kambru (Morris i in., 2018). Od tego czasu rośliny cały czas ewoluują, by dostosować się do warunków w jakich przyszło im żyć. W przeciwieństwie do zwierząt, rośliny nie są w stanie przenieść się w inne miejsce, gdzie panują korzystniejsze warunki. Rośliny są wrażliwe na zmiany środowiska, a każde warunki, które nie są optymalne dla ich wzrostu, są definiowane jako warunki stresowe (Zhang i in., 2020). Mimo wielu lat badań nie są w pełni poznane mechanizmy stojące za odpowiedzią roślin na czynniki powodujące stres. Ich zrozumienie jest kluczowe nie tylko dla dalszego rozwoju nauki, ale również ma znaczenie dla rolnictwa oraz zapewnienia bezpieczeństwa żywnościowego świata (Zhang i in., 2020). Poznanie kluczowych procesów niezbędnych dla przetrwania roślin w trudnych warunkach umożliwi lepsze zrozumienie funkcjonowania roślin. Wiedza ta w przyszłości może zostać wykorzystana do rozwiązania problemów związanych ze stabilnością produkcji roślinnej.

Jednym z takich problemów jest niedostateczna produkcja żywności. Kwestia ta dotyka głównie krajów z dużą populacją i/lub trudnymi warunkami środowiskowymi. Innym problemem, z którym przyszło się nam zmierzyć jest tak zwany głód utajony. Jest to sytuacja, w której, mimo dostarczania organizmowi odpowiedniej (lub zbliżonej do odpowiedniej) liczby kalorii, ilości składników odżywczych takich jak białko, tłuszcze, witaminy czy mikroelementy są niedostateczne. Szacuje się, że ukryty głód dotyczy ponad dwóch miliardów ludzi na całym świecie (Lowe, 2021; Weffort i Lamounier, 2023).

1. Stres u roślin

Stres roślin odnosi się do czynników zewnętrznych, w których efekcie dochodzi do nagłych zmian wpływających na wzrost, kwitnienie, rozwój i kiełkowanie nasion, fotosyntezę, starzenie się oraz metabolizm komórkowy. W konsekwencji dochodzi do obniżenia plonowania i produktywności upraw (Fernandes i Ghag, 2022). Stres u roślin dzieli się na ten wywołany czynnikami abiotycznymi (wysoka lub niska temperatura, światło, zmiany w ilości dostępnej wody, obecność zanieczyszczeń

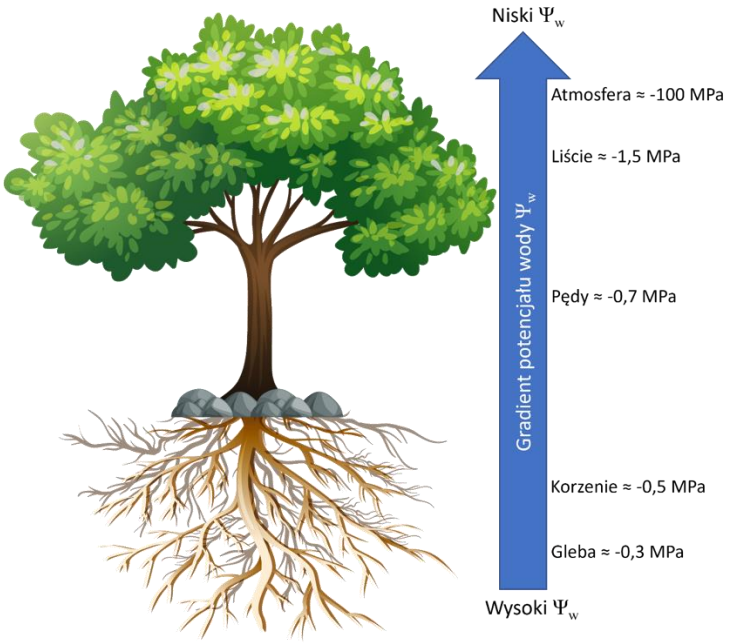
na przykład metali ciężkich) oraz biotycznymi (obecność insektów czy mikroorganizmów) (Fernandes i Ghag, 2022; Szmidt-Jaworska i Kopcewicz, 2024).

Rośliny wytworzyły skomplikowane mechanizmy, mające na celu zwiększenie tolerancji na czynniki zewnętrzne, aby przetrwać w ciągle zmieniającym się środowisku mimo osiadłego trybu życia oraz braku układu odpornościowego jaki występuje u zwierząt. Podstawą odpowiedzi na środowisko jest odbieranie informacji. Do kluczowych cząsteczek sygnałowych zaangażowanych w odpowiedź roślin na stres zalicza się jony Ca^{2+}, tlenek azotu (NO) i reaktywne formy tlenu (RFT, *ang. reactive oxygen species*). Podstawowe bodźce, np. światło, mogą powodować depolaryzację błon komórkowych roślin w ciągu kilku sekund, co jest powiązane z napływem Ca^{2+} i wypływem anionów przez kanały jonowe. Wapń jest podstawową cząsteczką sygnałową, a wzrost jego wewnątrzkomórkowego stężenia jest jedną z najwcześniejszych reakcji na bodźce egzogenne. Sygnalizacja Ca^{2+} reguluje wiele procesów w roślinach, w tym regulację transkrypcyjną i późniejsze reakcje fizjologiczne oraz rozwojowe (Xu i in., 2022a). Tlenek azotu jest powszechną cząsteczką sygnałową, która działa poprzez modulację stresu oksydacyjnego, transportu metali a także aktywności niektórych czynników transkrypcyjnych (Kumar i Ohri, 2023). Również RFT działają jako cząsteczki sygnałowe - kontrolują i regulują procesy biologiczne takie jak wzrost, cykl komórkowy, programowana śmierć komórki, sygnalizacja hormonalna, reakcje na stres biotyczny i abiotyczny (Mansoor i in., 2022). W większych ilościach RFT powodują uszkodzenia DNA czy peroksydację lipidów, co w efekcie może prowadzić do śmierci komórki (Storey i Storey, 2000; Yoshida i in., 2014).

Zmiany środowiska indukują różne odpowiedzi komórek roślinnych. Na przykład wysokie zasolenie czy stres suszy powodują zmiany w osmolarności komórek roślinnych poprzez zwiększenie stężenia substancji rozpuszczonych, czego efektem jest zmniejszenie potencjału osmotycznego i zwiększenie zdolności rośliny do zatrzymywania wody (Tuteja i Sopory, 2008; Zhang i in., 2023). W odpowiedzi na czynniki zewnętrzne syntetyzowane są w komórkach roślinnych hormony roślinne takie jak kwas jasmonowy, etylen, kwas salicylowy czy kwas abscysynowy (ABA). Zmieniające się poziomy tych cząsteczek wywołują różne reakcje komórek roślinnych – na przykład wzrost stężenia ABA powoduje zamykanie aparatów szparkowych (Zia i in., 2021).

1.1. Stres suszy

Zmiany klimatyczne towarzyszą roślinom od samego początku ich istnienia, jednak nigdy wcześniej nie zachodziły one w tak szybkim tempie jak teraz. Zmiany temperatur, dystrybucji opadów i składu atmosfery negatywnie wpływają na wzrost oraz plonowanie roślin (Raza i in., 2019; Chaudhry i Sidhu, 2022). Na skutek globalnego ocieplenia coraz częściej dochodzi do suszy. W roku 2022 średnia obszarowa temperatura powietrza wynosiła w Polsce 9,8 °C i była wyższa o 0,8 °C od średniej rocznej wieloletniej (Miętus, 2023). Dodatkowo, w kluczowych dla uprawy rolnej miesiącach (od marca do września) opady były niższe, przyczyniając się do zmniejszenia zapasów wody w glebie (Główny Urząd Statystyczny, Departament Rolnictwa i Środowiska, 2023). Susza jest problemem dotykającym wszystkich rolników w Polsce, a straty w plonach z powodu deficytu wody mogą wynieść w bieżącym roku nawet 20% (Bernat, 2024). Woda jest niezbędna do funkcjonowania wszelkich istot żywych. Już Tales z Miletu prowadził rozważania na temat istotności wody na świecie i określał ją jako *arche*, czyli praprzyczynę wszystkich bytów (Legutko, 2017). To, jak ważna jest woda wspaniale podsumowuje cytat z powieści Antoine de Saint-Exupér'ego „Ziemia, planeta ludzi" - „*Wodo, […] Nie jesteś niezbędna do życia: jesteś samym życiem*". Na każdym etapie rozwoju rośliny jest ona niezbędnym substratem. To od dostępu do wody zależy czy nasiono wykiełkuje. Na wczesnym etapie wzrostu i rozwoju rośliny zapotrzebowanie na wodę nie maleje, gdyż to waśnie jej dostępność umożliwia szybki wzrost elongacyjny komórek (Szmidt-Jaworska i Kopcewicz, 2024). Potencjał wody (Ψ_w) w glebie musi być wyższy niż w korzeniu wówczas mogą one pobierać wodę, która przemieszcza się w kierunku malejącego potencjału wody (Ryc. 1). Im gleba jest bardziej sucha, tym ma niższy potencjał wody (Van Loon, 2015; Szmidt-Jaworska i Kopcewicz, 2024).



Ryc. 1. Schematyczne przedstawienie gradientu potencjału wody (Ψ_w) między glebą, rośliną i atmosferą. Rycina zaprojektowana z wykorzystaniem programu freepik.com.

W warunkach naturalnych rośliny doświadczają okresów, gdy woda jest niedostępna. Susza atmosferyczna występuje, jeśli w danym analizowanym okresie suma opadów atmosferycznych jest niższa niż wieloletnia średnia, lub gdy opad wcale nie występuje. W jej efekcie może dojść do suszy glebowej i hydrologicznej. Ta pierwsza ma miejsce wtedy, gdy wilgotność gleby jest zbyt niska by zaspokoić potrzeby roślin. Stwierdza się ją dopiero po zaobserwowaniu reakcji roślin w postaci spadku biomasy czy ograniczeniu plonowania. Susza hydrologiczna wynika z obniżenia zawartości wody powierzchniowej, przez co dochodzi do zanikania źródeł i cieków wodnych. Innym typem suszy jest susza fizjologiczna, która wynika z niemożności pobrania przez roślinę wody obecnej w podłożu (Van Loon, 2015; Szmidt-Jaworska i Kopcewicz, 2024). Brak dostępu do wody powoduje, że rośliny mają krótsze pędy i korzenie oraz niższą biomasę. Dodatkowo susza prowadzi do ograniczenia aktywności fotosyntetycznej roślin sterowanej mechanizmem roślinnej odpowiedzi ścisłej (Zia i in., 2021). W celu ochrony przed negatywnymi skutkami niedoboru wody rośliny wytworzyły szereg mechanizmów obronnych, z których trzy główne strategie opierają się na:

1) produkcji i akumulacji osmoprotektanów, takich jak prolina, cukry rozpuszczalne czy betaina, w celu utrzymania turgoru komórek (Hanson i in., 1994; Hoekstra

i in., 2001; Mohanty i in., 2002; Ashraf i Foolad, 2007; Gong i in., 2010; Fang i Xiong, 2015);

2) syntezie kwasu abscysynowego (ABA), czyli hormonu odpowiedzialnego za zamykanie aparatów szparkowych i hamowanie wzrostu roślin, a dodatkowo hormon ten reguluje ekspresję wielu genów na przykład akwaporyn (Dąbrowska i Głowacka, 2004; Mordaka i Dąbrowska, 2007), metalotionein (Koszucka i Dąbrowska, 2006), u rzodkiewnika (*Arabidopsis thaliana* (L.) Heynh.) genu *soc1* związanego z kwitnieniem, genów *abi2* i *abi4/5* związanych z kiełkowaniem nasion, czy genu *rd29B* związanego z odpowiedzią na suszę (Uno i in., 2000; Yamaguchi-Shinozaki i Shinozaki, 2005; Bhargava i Sawant, 2013; Liu i in., 2018; Mahmood i in., 2019; Yao i in., 2021);

3) ograniczeniu negatywnego wpływu reaktywnych from tlenu powstających w odpowiedzi na stres, włączając stres suszy. W celu ochrony przed RFT, rośliny w drodze ewolucji wytworzyły enzymatyczne i nieenzymatyczne systemy antyoksydacyjne. Najważniejsze enzymy antyoksydacyjne to katalazy (CAT, ang. *catalase*), peroksydazy (PX, ang. *peroxidase*) i dysmutazy ponadtlenkowe (SOD, ang. *superoxide dismutase*), zaś fenole, witamina C, glutation, tokoferole to najważniejsze antyoksydanty nieenzymatyczne (Ashraf, 2009).

1.2. Wpływ zanieczyszczenia środowiska na wzrost i rozwój roślin

Działalność człowieka prowadzi do nieustannych zmian środowiska. Mimo rosnącej świadomości ekologicznej ludzi, duża część działań człowieka powoduje wzrost zanieczyszczenia środowiska; począwszy od zanieczyszczenia świetlnego, przez zanieczyszczenia tworzywami sztucznymi do zanieczyszczeń różnego rodzaju pierwiastkami (Briffa i in., 2020). Przemysł metalurgiczny, górniczy, petrochemiczny, transport oraz stosowanie niektórych nawozów mineralnych powoduje, że do środowiska przedostają się szkodliwe metale ciężkie (HM, *ang. heavy metals*) takie jak ołów (Pb), kadm (Cd), cynk (Zn), miedź (Cu) i inne (Mirsal, 2004; Möller i in., 2005; Stefanowicz i in., 2020). Metale ciężkie utrzymują się w środowisku przez stulecia, a nawet tysiąclecia, a dodatkowo mogą rozprzestrzeniać się na odległe obszary. Stanowi to potencjalne zagrożenie dla zdrowia ludzkiego, ponieważ metale te przedostają się do łańcucha pokarmowego ludzi poprzez bioakumulację w tkankach roślin i zwierząt.

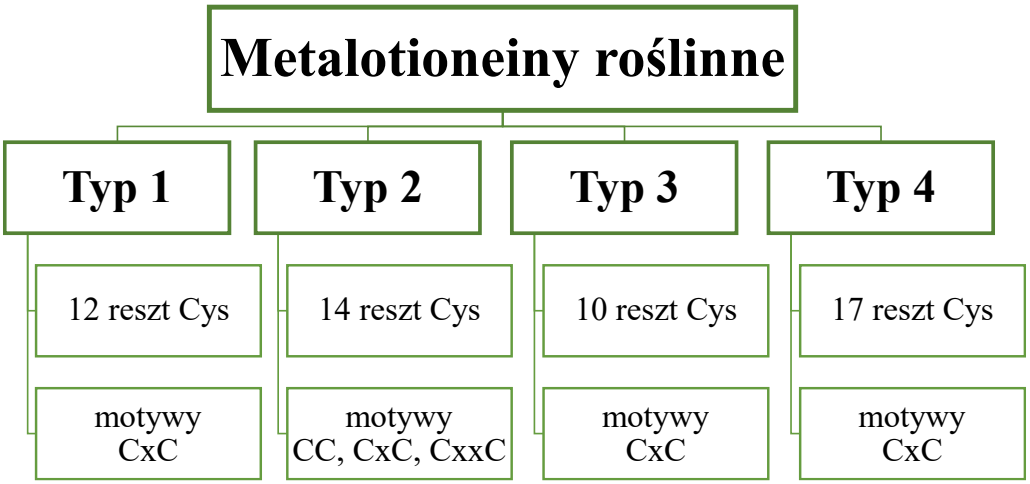
Metale ciężkie mogą powodować wady wrodzone, problemy z przewodem pokarmowym, wątrobą, nerkami i centralnym układem nerwowym (Zhang i in., 2020; Stefanowicz i in., 2020). Wiele z nich ma toksyczny wpływ na organizmy żywe już w niewielkich ilościach, jak na przykład kadm (dla człowieka ważącego 70 kg, najniższa śmiertelna dawka Cd wynosi 5 g). Cynk jest mikroelementem niezbędnym do poprawnego funkcjonowania wszystkich organizmów, ale w nadmiarze staje się szkodliwy (powyżej 200 mg na dzień) (Ngole i Ekosse, 2012; Rafati Rahimzadeh i in., 2017; Ryu i Aydemir, 2020; Charkiewicz i in., 2023). U roślin stres wywołany metalami ciężkimi przejawia się zahamowaniem fotosyntezy, ograniczeniem wzrostu i może wpływać na asymilację składników odżywczych oraz przyspieszać starzenie się roślin. W skrajnych przypadkach może dojść nawet do śmierci rośliny (Rabêlo i Borgo, 2016; Singh i in., 2016). Rolę w ochronie komórek roślinnych przed tym stresem odgrywają między innymi białka metalotioneiny, które wiążą jony metali i pomagają utrzymać ich homeostazę w komórkach (Emamverdian i in., 2015). Podobnie jak w przypadku stresu suszy, w obecności toksycznych metali ciężkich dochodzi do produkcji i akumulacji RFT. Rośliny, które tolerują obecność metali ciężkich w swoim środowisku mają dobrze funkcjonujące systemy antyoksydacyjne, które chronią komórki przed negatywnym działaniem RFT (Gill i Tuteja, 2010).

2. Metalotioneiny

Metalotioneiny (MT) występują u roślin, zwierząt i bakterii (Koszucka i Dąbrowska, 2006; Blindauer, 2011; Capdevila i Atrian, 2011; Mierek-Adamska i in., 2017, Mierek-Adamska i in., 2018). MT są białkami o niskiej masie cząsteczkowej (<10 kDa) i wysokiej zawartości reszt cysteinowych (nawet do 30% w MT2A człowieka) (Isani i Carpenè, 2014). To właśnie cysteiny, ułożone w charakterystyczne i konserwowane ewolucyjnie motywy, odpowiadają za wiązanie jonów metali, a w szczególności jonów Cu(I), Zn(II) i Cd(II) (Leszczyszyn i in., 2013).

Zidentyfikowano wiele MT roślinnych, które na podstawie liczby i ułożenia reszt Cys podzielono na cztery typy (MT1-4) (Ryc. 2). Typ pierwszy (MT1) stanowią białka, które w swojej sekwencji zawierają 12 reszt Cys. W typie drugim (MT2) występuje 14 Cys, a w typie trzecim (MT3) jest ich 10. Z kolei metalotioneiny typu 4 mają w sekwencji aminokwasowej aż 17 Cys. Do MT4 należy białko E_c (*ang. early cysteine-labeled*) - pierwsza odkryta metalotioneina roślinna wyizolowana z zarodków pszenicy

(*Triticum aestivum* L.). W przypadku pierwszych trzech typów MT, Cys są zgrupowane w dwóch regionach na N- i C-końcu białka połączonych fragmentem niezawierającym reszt Cys. W typie czwartym MT cysteiny są zlokalizowane w trzech regionach (Lane i in., 1987; Koszucka i Dąbrowska, 2006; Freisinger, 2008; Capdevila i in., 2012; Leszczyszyn i in., 2013).



Ryc. 2. Podział metalotionein roślinnych na podstawie Cobbett i Goldsbrough, 2002. C - cysteina, x – dowolny inny niż cysteina aminokwas.

Cysteina stanowi wyjątkowy aminokwas, gdyż wraz z glicyną, proliną i tryptofanem jest jednym z najczęściej konserwowanych aminokwasów w białkach, a jednocześnie jest najrzadziej występującym aminokwasem (Marino i Gladyshev, 2010; Krick i in., 2014). Co ciekawe, badania wskazują, że jeśli cysteiny są konserwowane, to stopień ich konserwacji wynosi 90% lub więcej, co podkreśla jak ważne są dla funkcjonalności białek (Marino i Gladyshev, 2010). Dzięki obecności grupy tiolowej (-SH) w swojej strukturze, Cys mogą ulegać różnym reakcjom nukleofilowym. W związku z tym Cys jest popularnym aminokwasem występującym w miejscach aktywnych enzymów (Ulrich i Jakob, 2019). Dodatkowo Cys wiąże metale takie jak Zn, Fe, Cu czy Cd z wysokim powinowactwem (Poole, 2015). Ponadto ważną cechą Cys jest jej zdolność do przechodzenia odwracalnej i nieodwracalnej oksydacji. Dzięki temu białka posiadające ten aminokwas w swojej strukturze mogą reagować z RFT chroniąc komórki przed stresem oksydacyjnym (Ulrich i Jakob, 2019). Inną ciekawą obserwacją z ewolucyjnego punktu widzenia jest częstość występowania Cys w białkach organizmów eukariotycznych. Jako aminokwas kodowany przez dwa kodony, to jej częstość występowania jest znacząco niższa niż teoretycznie wyliczona wartość, która

równa jest 3,3%. Ponadto częstość występowania Cys wzrasta wraz ze stopniem skomplikowania organizmu, i tak u *Archea* wartość ta jest równa 0,4%, natomiast w ssaczym proteomie jest równa 2,3% (Miseta i Csutora, 2000). Ulrich i in. (2019) sugerują, że fenomen ten może być związany z koniecznością posttranslacyjnej regulacji aktywności białek u wyższych organizmów.

2.1. Funkcje metalotionein w komórkach roślinnych

Główną funkcją MT jest utrzymanie homeostazy mikroelementów, zwłaszcza cynku, ale także detoksyfikacja szkodliwych jonów metali takich jak kadm (Blindauer i Leszczyszyn, 2010). Metalotioneiny wiążą metale poprzez grupy tiolowe Cys (Freisinger, 2008; Hassinen i in., 2011; Joshi i in., 2016). Dodatkowo w przypadku MT typu 4 potwierdzono udział reszt histydynowych w wiązaniu jonów metali (Blindauer i in., 2007; Blindauer, 2008; Leszczyszyn i in., 2013). Liczba jonów metali wiązanych przez MT zależy od liczby Cys. Na przykład MT3 z najmniejszą liczbą Cys są w stanie związać maksymalnie cztery jony Zn (Leszczyszyn i in., 2013). W warunkach *in vitro,* inkubacja MT w wysokich stężeniach jonów metali powoduje powstanie form w pełni wysyconych, czyli związanych z maksymalną możliwą liczbą jonów metali. W komórkach zwierzęcych udowodniono istnienie MT, które miały związane mniej niż maksymalną liczbę jonów metali – były w formie niewysyconej metalami (Petering i in., 2006; Krezel i Maret, 2008). Przypuszcza się, że również w komórkach roślinnych występują MT, które związały mniej niż maksymalną możliwą liczbę jonów metali. Wydaje się to być szczególnie istotne w przypadku MT biorących udział w odpowiedzi na stres oksydacyjny. W sytuacji, gdy nie wszystkie reszty tiolowe są zaangażowane w wiązanie jonów, wolne grupy -SH mogą brać udział w innych procesach, na przykład w neutralizowaniu RFT (Leszczyszyn i in., 2013).

W przeszłości wielokrotnie wykazano, że ekspresja *MT* wzrasta w wyniku traktowania roślin jonami metali. Na przykład u rzepaku (*Brassica napus* L.) ekspresja *BnMT1-4* wzrastała pod wpływem arsenu (Pan i in., 2018), a u *Brassica rapa* ekspresja *BrMT1-3* była indukowana przez traktowanie Fe, Cu, Zn i Mn (Ahn i in., 2012). U topoli (*Populus alba* L.) ekspresja *PaMT1* i *PaMT3* w liściach wzrosła w odpowiedzi na traktowanie Zn, zaś ekspresja *PaMT2* pozostała bez zmian (Castiglione i in., 2007). U wierzby wiciowej (*Salix viminalis* L.) obserwowano zmiany ekspresji *SvMT1* w obecności mikroorganizmów (Hrynkiewicz i in., 2012). W wyniku traktowania roślin

Salicornia brachiata L. jonami Zn i Cu wzrosła ekspresja *SbMT2*. Nadekspresja *SbMT2* w bakteriach *Escherichia coli* spowodowała, że bakterie te miały zwiększoną tolerancję wobec Zn, Cu i Cd (Chaturvedi i in., 2012). Ekspresja *MT4* z ogórka (*Cucumis sativus* L.) indukowana była obecnością zarówno Zn jak i Cd, a bakterie *E. coli* eksprymujące ten gen charakteryzowały się nie tylko zwiększoną tolerancją na te pierwiastki, ale także były w stanie akumulować więcej Cd (Duan i in., 2019). Rośliny transgeniczne nadeskprymujące *MT* wykazują większą tolerancję na kadm (Gu i in., 2014; Zhi i in., 2020),

Oprócz utrzymania homeostazy mikroelementów i detoksykacji toksycznych jonów metali ciężkich wykazano też inne funkcje MT. Przeprowadzono wiele badań, w których wykazano zmiany ekspresji *MT* pod wpływem różnych czynników środowiskowych takich jak niska temperatura (Singh i in., 2011), susza (Ozturk i in., 2002; Akashi i in., 2004), zasolenie (Kim i Kang, 2018), obecność metali ciężkich (Kim i in., 2014; Kim i Kang, 2018), a także obecność mikroorganizmów (Dauch i Jabaji-Hare, 2006; Miles i in., 2011; Dąbrowska i in., 2012a; Hrynkiewicz i in., 2012; Dąbrowska i in., 2014; Dąbrowska i in., 2021a). Ponadto rośliny transgeniczne nadeskprymujące *MT* charakteryzowały się większą tolerancją na stresy środowiskowe np. na suszę (Kumar i in., 2022) i stres osmotyczny (Feng i in., 2022). Co ciekawe ekspresja *MT* zmienia się również w trakcie wzrostu i rozwoju roślin (Moyle i in., 2005; Yuan i in., 2008), w tym podczas kiełkowania nasion (Yuan i in., 2008; Zhou i in., 2012; Dąbrowska i in., 2013). Ta uniwersalność działania MT w reakcji roślin na stresy może być związana z właściwościami antyoksydacyjnymi grup tiolowych cystein. Wcześniejsze badania przeprowadzone w Katedrze Genetyki wykazały, że nadekspresja *MT* rzepaku w komórkach *E. coli* ograniczała negatywny wpływ RFT na podziały komórkowe bakterii (Mierek-Adamska i in., 2019). Inni badacze wykazali, że transgeniczne rośliny *A. thaliana*, które nadeksprymowały *MT2* daktylowca (*Phoenix dactylifera* L.) wykazywały zwiększoną zdolność neutralizowania negatywnych skutków działania RFT (Patankar i in., 2019).

3. Owies zwyczajny

Owies zwyczajny (*Avena sativa* L.) jest rośliną o dużym znaczeniu gospodarczym. W 2022 roku w Europie zbiory owsa wyniosły 7,5 milionów ton (Eurostat, 2023). Popularność tego zboża wynika między innymi z prozdrowotnych właściwości owsa. Roślina ta była od dawna stosowana w medycynie ludowej do leczenia nerwicy i bezsenności. Owies stosowany był także na skórę, w celu jej nawilżenia oraz złagodzenia zaczerwień i podrażnień (Singh i in., 2013).

W Polsce uprawa owsa rozpoczęła się w VII-IX wieku, zaś hodowlę odmian rozpoczęto w końcówce XIX wieku (Spiss, 2003; Zarzecka i in., 2018). Wówczas owies stanowił podstawę w żywieniu koni, ale już wtedy zaczęto zwracać uwagę na potencjał nasion owsa w żywieniu ludzi (Spiss, 2003). Zboże to było szczególnie popularne w rejonach górzystych i o trudnym klimacie, bowiem roślina ta dobrze przystosowuje się do trudnych warunków środowiska (Zarzecka i in., 2018). Występuje zarówno w formie jarej jak i ozimej, w Polsce uprawia się głównie tę pierwszą. Wiązkowy system korzeniowy owsa jest w stanie wydajnie pobierać składniki odżywcze z gleby; pojedyncze korzenie mogą sięgać nawet do dwóch metrów w głąb gleby. Dzięki temu owies dobrze plonuje także na słabszych glebach (Zarzecka i in., 2018). Jest to roślina o niewielkich wymaganiach cieplnych, ale wysokich potrzebach wodnych, szczególnie w okresie wyrzucania wiech i kwitnienia, który to w Polsce przypada na maj/czerwiec (Michalski i in., 1999). Owies jest również znany jako roślina fitosanitarna. Uważany jest za dobry przedplon dla innych roślin zbożowych, gdyż charakteryzuje się on dużą odpornością na choroby grzybowe, a także dużą naturalną konkurencyjnością w stosunku do chwastów (Pawłowski i Deryło, 1988).

Do rodzaju *Avena* należą gatunki roślin diploidalnych, tetraploidalnych i heksaploidalnych. Gatunki diploidalne mają genomy AA lub CC, tetraploidalne AABB, AACC, CCCC lub CCDD, a heksaploidalne – AACCDD. Owies zwyczajny jest gatunkiem heksaploidalnym (2n = 6x = 42), który powstał najprawdopodobniej poprzez hybrydyzację tetraploidu (CCDD) i diploidu (AA) (Yan i in., 2016a; Yan i in., 2016b; Jiang i in., 2021). Poziom skomplikowania genomu owsa, podnosi fakt, że chromosomy tej rośliny uległy wielu rearanżacjom. Co więcej, proces ten wciąż trwa a chromosomy współczesnych odmian owsa mogą być różnie zorganizowane (Tinker i in., 2022). W 21 chromosomach owsa zwyczajnego zlokalizowanych jest ponad

80 tysięcy genów. Z tego powodu prace nad zsekwencjonowaniem genomu owsa prowadzone były przez długi czas, a pełna sekwencja genomu została opublikowana dopiero w 2021 roku (PepsiCo, 2021; Kamal i in., 2022).

W ostatnich latach wzrasta zainteresowanie żywnością ekologiczną i zrównoważonym odżywaniem się. Nasiona owsa i produkty pochodzenia owsianego idealnie wpisują się w ten trend, bowiem są one bardzo dobrym źródłem wapnia, tłuszczy, białek, witamin (A, B i E) oraz błonnika (Butt i in., 2008). W porównaniu do ziaren pszenicy, ziarna owsa zawierają więcej aminokwasów egzogennych, takich jak treonina, metionina, lizyna, fenyloalanina, tyrozyna, walina i leucyna (Zarzecka i in., 2018). Nasiona owsa zawierają też więcej błonnika niż nasiona pszenicy, ryżu (*Oryza sativa* L.) czy kukurydzy (*Zea mays* L.) (Singh i in., 2013). Wykazano, że błonnik pochodzenia owsianego efektywnie obniża poziom cholesterolu oraz normuje poziom cukru we krwi (Wood i in., 1990; Wood, 1991; Kahlon i Chow, 1997). Ważnym składnikiem błonnika jest β-glukan, czyli polisacharyd o wyjątkowych właściwościach. Już w stężeniu 1% roztwory β-glukanu wykazują wysoką lepkość, która jest stała w szerokim zakresie pH (2-10), ale zmienia się wraz ze wzrostem temperatury (Butt i in., 2008). Dzięki temu, w przewodzie pokarmowym tworzy lepkie żele, które zaspokajają uczucie łaknienia i spowalniają pasaż treści pokarmowej. Dodatkowo zwiększona lepkość ogranicza wchłanianie cukrów, tłuszczu i cholesterolu oraz zwiększa wydalanie kwasów żółciowych z organizmu, obniża stężenie glukozy we krwi po posiłku (Wood i in., 1994; Zarzecka i in., 2018). Dotychczasowe badania oparte o analizy genomu owsa pozwoliły wykazać, że produkty spożywcze uzyskiwane z owsa są bezpiecznym pokarmem dla osób chorych na celiakię. Wynika to z faktu, że w porównaniu do innych zbóż jak pszenica, czy jęczmień (*Hordeum vulgare* L.), w genomie owsa jest niewielka liczba kopii genów kodujących epitopy glutenu oraz innych wysoce immunogennych białek (Kamal i in., 2022; Leišová-Svobodová i in., 2022).

Poznanie genomu rośliny umożliwia dogłębne zrozumienie mechanizmów leżących u podstaw wszystkich procesów zachodzących w roślinach, w tym wzrostu i rozwoju oraz odpowiedzi na stresy środowiskowe. Pełne pojęcie procesów zachodzących w roślinach w wyniku stresu, na przykład suszy, jest kluczowe dla dalszego rozwoju nowoczesnego rolnictwa. W obliczu zmian klimatycznych oraz rosnącej światowej populacji niezwykle ważne jest scharakteryzowanie szlaków biorących udział

w odpowiedzi na stresy środowiskowe, które w największym stopniu zagrażają roślinom i zmniejszają plony.

IV. Cel pracy

Celem pracy było zidentyfikowanie genów metalotionein owsa (*AsMT1-4*) i określenie roli kodowanych przez nie białek w odpowiedzi na stresy abiotyczne (stres osmotyczny, suszy i metali ciężkich) oraz biotyczne (obecność mikroorganizmów).

Szczegółowe cele polegały na:

1) Poznaniu i scharakteryzowaniu sekwencji nukleotydowych i aminokwasowych MT owsa zwyczajnego;

2) Sprawdzeniu udziału AsMT we wzroście i rozwoju roślin owsa zwyczajnego;

3) Ustaleniu roli AsMT w odpowiedzi roślin owsa zwyczajnego na obecność jonów metali ciężkich (Cd i Zn) i grzybów z rodzaju *Trichoderma*;

4) Ocenie wpływu stresów suszy i osmotycznego na ekspresję *AsMT* i parametry biochemiczne roślin owsa zwyczajnego.

V. Wyniki i dyskusja

1. Identyfikacja i charakterystyka rodziny genów *AsMT*

Od momentu opublikowania sekwencji genomu *A. sativa* liczba publikacji dotyczących analizy genów owsa rośnie. Dostępność sekwencji genomu pozwala na lepsze zrozumienie molekularnych podstaw mechanizmów regulacji wzrostu i rozwoju czy odpowiedzi na stres u owsa (PepsiCo, 2021; Kamal i in., 2022; Liu i in., 2022; Ghorbel i in., 2023; Ling i in., 2023; Pan i in., 2023; Chen i in., 2024; Pan i in., 2024; Sandro i in., 2024; Zhang i in., 2024; Zhou i in., 2024). Znajomość molekularnych mechanizmów stojących za podstawowymi procesami zachodzącymi w roślinach jest kluczowa dla dalszego rozwoju rolnictwa. Wiedza ta umożliwi skrócenie czasu niezbędnego do otrzymania nowych odmian. Dodatkowo, poznanie mechanizmów stojących za odpowiedzią na stresy środowiskowe umożliwi szybsze i lepsze dopasowanie działań rolników, co w konsekwencji może ograniczyć nadmierne zużycie środków ochrony roślin i nawozów, ale także przyczynić się do ograniczenia strat w uprawach do minimum. W niniejszej pracy doktorskiej postawiono hipotezę badawczą, która zakłada, że MT to białka konserwowane ewolucyjnie, o potencjalnej roli w wielu procesach zachodzących w roślinach w tym we wzroście i rozwoju oraz odpowiedzi na stres.

Przeprowadzone analizy genomu owsa *in silico* wykazały obecność 21 genów kodujących MT (*AsMT*). W zidentyfikowanych sekwencjach odnalazłam charakterystyczne dla MT motywy bogate w reszty cysteinowe. Na ich podstawie sekwencje zaklasyfikowałam do czterech typów roślinnych MT – pięć z nich koduje MT1, dziewięć MT2, trzy MT3 oraz cztery MT4. Metalotioneiny *A. sativa* rozmieszczone są na 12 z 21 chromosomów owsa (publikacja 3). Ta mnogość genów MT u owsa związana jest z jego heksaploidalnością. Każdy z trzech subgenomów owsa (subgenom A, C i D) zawiera przynajmniej po jednym genie kodującym każdy z czterech typów MT. Poszczególne subgenomy *A. sativa* musiały zawierać wszystkie geny niezbędne do przetrwania gatunku (publikacja 3). To pokazuje jak ważną rolę w roślinach pełnią MT. Ponadto wcześniejsze badania wskazują, że liczba genów *MT* koreluje z ploidalnością i nie zależy od wielkości genomu (Yu i in., 2020). Przykładem są rośliny diploidalne: *A. thaliana* o genomie 135 Mb posiada siedem genów *MT* (Guo i in., 2003), ryż o genomie 420 Mb posiada 11 genów *MT* (Cheng i in., 2021), a kukurydza o genomie

2500 Mb posiada tylko 9 genów *MT*. U tetraploidalnych *B. napus* (975 Mb) i *B. juncea* L. (920 Mb) obecnych jest odpowiednio 16 i 12 genów *MT* (Yu i in., 2020).

Dzięki dostępności pełnego genomu owsa możliwe było wykonanie analiz *in silico* sekwencji promotorowych genów *AsMT* pod kątem obecności potencjalnych *cis*-elementów (publikacja 3). Analiza ta pozwoliła na określenie prawdopodobnych funkcji białek kodowych przez poszczególne geny *AsMT*. Na przykład wśród *cis*-elementów obecnych w sekwencjach promotorowych *MT* owsa obecne były elementy odpowiedzi na fitohormony np. na kwas abscysynowy (element ABRE). Najwięcej elementów związanych z odpowiedzią na fitohormony posiadały promotory genów *AsMT2* i *AsMT4*. Kolejną liczną grupą *cis*-elementów obecnych w promotorach *AsMT1-4* były *cis*-elementy zaangażowane w odpowiedź na stres suszy (elementy DRE, MBS, MYB), stres wywołany obecnością metali ciężkich (MRE, CuRE), czy stresy biotyczne wywołane obecnością mikroorganizmów (W-box, AT-rich sequences, TC-rich repeats). Co ciekawe, suma *cis*-elementów związana z odpowiedzią na stresy abiotyczne była podobna wśród wszystkich genów *AsMT* i średnio wynosiła 11. Jednak w przypadku elementów związanych z odpowiedzią na stres suszy najwięcej elementów obecnych było w promotorach genów *AsMT4*. W promotorach *AsMT1-4* obecne były też elementy związane z rozwojem rośliny, na przykład związane z rozwojem merystemów (CAT-box, NON-box) czy powstawaniem nasion (RY-element) (publikacja 3). Przeprowadzone analizy z dużym prawdopodobieństwem wskazują, że AsMT są zaangażowane we wzrost i rozwój roślin, a także w odpowiedź rośliny na stresy środowiskowe. Podobne obserwacje przedstawiono odnośnie MT u innych roślin: kukurydzy, ryżu i rzodkiewnika (Zhou i in., 2006; Dąbrowska i in., 2012b; Yu i in., 2020). Udowodniono, że promotor MT typu 1 ryżu może być zaindukowany przez zranienie oraz traktowanie miedzią i PEG (poliglikolem etylenowym) (Lü i in., 2007). Ponadto promotor MT typu 1 ryżu gwarantuje specyficzność tkankową oraz jest indukowany przez wiele czynników, w tym kwas abscysynowy (ABA), ciemność, suszę i jony metali (Cu, Zn, Pb, Al, Cd i Co) (Dong i in., 2010). Niewiele jest jednak doniesień potwierdzających funkcjonalność przewidzianych *cis*-elementów w MT roślinnych, dlatego uzyskane wyniki stanowią ważną część charakterystyki genów *AsMT* i są istotnym wyznacznikiem dalszego kierunku badań.

2. Udział AsMT w kiełkowaniu owsa zwyczajnego

Wyniki analizy *in silico* wskazujące, że w promotorach genów *AsMT* typu 1 i 3 obecne są *cis*-elementy RY-element i GCN4_motif, biorące udział w rozwoju nasion ukierunkowały moje badania, w których sprawdziłam ekspresję czterech genów *AsMT1-4* owsa w kiełkujących nasionach (publikacja 3). Kiełkowanie nasion jest jednym z najważniejszych etapów w cyklu życia rośliny, gdyż od tego etapu uzależniona jest także wielkość plonu. Założyłam, że ekspresja *AsMT* będzie zmieniać się trakcie kiełkowania nasion, a poziom ekspresji będzie różny w zależności od analizowanego typu MT oraz czasu kiełkowania. Geny te były zróżnicowanie eksprymowane. W drugiej dobie kiełkowania ekspresja *AsMT1* była najwyższa. W przypadku *AsMT2* najwięcej transkryptów było w 6. i 12. Godzinie kiełkowania. Ilość transkryptów *AsMT3* była najwyższa w suchych nasionach i w 48. godzinie kiełkowania, a genu *AsMT4* w 3., 9. i 12. godzinie kiełkowania. Po 24. i 48. godzinach od rozpoczęciu kiełkowania ekspresja *AsMT4* obniżyła się ponad 20-krotnie. W pierwszych 12. godzinach kiełkowania w nasionach obecnych było najwięcej mRNA genu *AsMT4*. W kolejnych godzinach ilość transkryptów tego genu gwałtownie zmalała, a na wysokim poziomie utrzymywało się mRNA *AsMT1-3* (publikacja 3). Wysoka ekspresja genów *AsMT* w czasie kiełkowania sugeruje ich ważną rolę w tym procesie. Podobne wyniki uzyskano dla rzepaku, gdzie w kiełkujących nasionach stwierdzono ekspresję genów należących do czterech typów MT (Dąbrowska i in., 2013). Z kolei Zhou i in. (2012) wykazali, że nasiona transgenicznych *A. thaliana* niosący geny *MT* typu 2 lub 3 lotosu zwyczajnego (*Nelumbo nucifera* Gaertn.) kiełkowały szybciej w porównaniu z roślinami typu dzikiego. W przypadku badań prowadzonych na ryżu zaobserwowano, że OsMT2b pełni kluczową rolę w kiełkowaniu nasion poprzez regulowanie poziomu cytokinin (Yuan i in., 2008). W dojrzewających nasionach gryki zwyczajnej (*Fagopyrum esculentum* L.) zaobserwowano ekspresję *MT3*, a także wzrost ekspresji tego genu po indukcji jonami Zn (Brkljačić i in., 2004).

W kiełkowaniu nasion najlepiej zbadana jest rola MT4. Zespół Kawashima i in. (1992) pracujący na pszenicy dostrzegł, że geny E_c są eksprymowane w trakcie embriogenezy, a ekspresja zanika krótko po rozpoczęciu kiełkowania. Transkrypty *MT4* są akumulowane w zarodkach czasie dojrzewania nasion kukurydzy, a ich poziom wzrasta po traktowaniu ABA i po indukcji stresu osmotycznego (White i Rivin, 1995).

W dojrzewających nasionach jęczmienia występowanie zarówno transkryptów jak i białek MT4 było ograniczone do warstwy zarodkowej i aleuronowej nasiona. Dodatkowo wykazano, że MT4 wiąże preferencyjnie jony Zn. Sugeruje to rolę MT4 jako magazynu Zn w dojrzewających i dojrzałych nasionach (Hegelund i in., 2012). Zespół Schiller i in. (2014) wykazał, że u jęczmienia ekspresja *MT4* była najwyższa 28 dni po zapyleniu. Co ciekawe, zaobserwowali oni też zmiany w ekspresji genów kodujących MT pozostałych typów, sugerując rolę wszystkich typów MT w powstawaniu nasion. U rzodkiewnika wykazano, że geny *AtMT4a* i *AtMT4b* są eksprymowane w dojrzewających zarodkach. Ich wyciszenie z użyciem RNAi zmniejszyło masę nasion i spowodowało wolniejszy wzrost siewek, zaś nadekspresja tych genów wywołała odwrotny efekt. Dodatkowo wykazano, że ekspresja *AtMT4a* i *AtMT4b* koreluje pozytywnie z zawartością jonów Zn w nasionach oraz tkankach wegetatywnych. Wyniki wskazują na kluczową rolę MT4 jako magazynu jonów Zn, które są wykorzystywane we wczesnych etapach wzrostu siewek po kiełkowaniu nasion (Ren i in., 2012).

Oczywistą rolą MT w procesie dojrzewania i kiełkowania nasion jest regulowanie i utrzymanie odpowiedniej ilości mikroelementów (Cobbett i Goldsbrough, 2002; Kranner i Colville, 2011), jednakże ta funkcja została wykazana jedynie dla MT4 i jonów Zn. W dojrzewających nasionach jęczmienia zaobserwowano, że transkrypty *MT3* utrzymywały się na stałym poziomie podczas rozwoju nasion i były obecne we wszystkich tkankach nasion. Sugeruje to rolę MT3 w utrzymaniu homeostazy metali, a nie jako białka zapasowe (Hegelund i in., 2012). Wskazuje się też na korelacje między kiełkowaniem nasion, poziomem MT a poziom RFT. Reaktywne formy tlenu odgrywają kluczową rolę w regulacji procesu kiełkowania (Bailly, 2019). W nasionach pomidora traktowanych zmiennym polem magnetycznym zauważono wzrost poziomu nadtlenku wodoru oraz ekspresji MT typu 1 i 4 (Anand i in., 2019). Nasiona rzodkiewnika, u których gen *AtMT2a* został wyciszony, były bardziej wrażliwe na chłód podczas kiełkowania i miały podwyższony poziom H_2O_2. Co ciekawe, u roślin rzodkiewnika z wyciszonym genem kodującym katalazę zaobserwowano wzrost ekspresji *AtMT2a* pod wpływem działania niskiej temperatury. Sugeruje to wzajemnie uzupełniające się działanie AtMT2a i katalazy w regulacji poziomu RFT (Zhu i in., 2009).

3. Zaangażowanie AsMT w reakcję siewek owsa zwyczajnego na obecność metali ciężkich

Obecność w promotorach genów *AsMT cis*-elementów związanych z reakcją na działanie metali ciężkich wskazuje na udział tych genów w odpowiedź rośliny na działanie HM (publikacja 3). W związku z tym postawiłam hipotezę zakładającą, że metalotioneiny owsa są zaangażowane w odpowiedź na Zn i Cd, jednakże rola poszczególnych typów MT może być odmienna. W celu sprawdzenia zdolności AsMT do ochrony komórek przed stresem wywołany obecnością metali ciężkich w wektor ekspresyjny wklonowałam sekwencje kodujące AsMT1-4. Bakterie *E. coli* niosące geny *AsMT3* i *AsMT4* lepiej tolerowały obecność jonów Zn i Cd w środowisku, w porównaniu do bakterii niosących pusty wektor ekspresyjny. W przypadku bakterii transformowanych wektorem ekspresyjnym niosącym geny *AsMT1* i *AsMT2* tempo wzrostu było na takim samym poziomie jak u bakterii niosących wektor ekspresyjny nie zawierający wstawki (publikacja 4). Podobnie, transgeniczne drożdże niosące geny *B. rapa BrMT1-3* lepiej tolerowały obecność Cd i Zn w porównaniu do drożdży niosących pusty wektor, a największą tolerancją na oba pierwiastki charakteryzowały się bakterie eksprymujące *BrMT3* (Liu i in. 2021). Z kolei *E. coli* nadeksprymujące MT jęczmienia *HvMT2b2* i *HvMT4* akumulowały więcej Zn i Cd. Wyższe tempo wzrostu transformowanych bakterii obserwowano jedynie w obecności Zn. W przypadku Cd tempo wzrostu było niższe niż dla bakterii kontrolnych (Pourjalali i in., 2022). Podobne obserwacje opisano dla bakterii transformowanych wektorem niosącym sekwencję kodującą *MT* typu 4 z rzepaku, gdzie zaobserwowano szybsze tempo wzrostu bakterii transformowanych niż kontrolnych w obecności Zn, zaś w obecności Cd nie obserwowano zmian (Mierek-Adamska i in., 2018). Obserwowane różnice tj. lepszy wzrost bakterii eksprymujących *AsMT3* i *AsMT4* w porównaniu do bakterii kontrolnych i brak takich różnic w przypadku *AsMT1* i *AsMT2,* wskazują na ochronę komórek bakterii przed szkodliwym działaniem Zn i Cd przez AsMT3 i AsMT4, czego nie potwierdzono dla AsMT1 i AsMT2 (publikacja 4). Pośrednio może to sugerować zaangażowanie AsMT3 i AsMT4 w ochronę komórek owsa zwyczajnego przed stresem spowodowanym obecnością Zn i Cd. Co istotne, w przypadku *B. rapa* i *H. vulgare* MT1 i MT2 również pełnią funkcje ochronne (Liu i in., 2021; Pourjalali i in., 2022).

W kolejnych eksperymentach, mających na celu ustalenie roli MT podczas stresu powodowanego obecnością HM sprawdziłam czy ekspresja *MT* owsa będzie zmieniać się w odpowiedzi na traktowanie jonami Zn i Cd, oraz czy będzie ona różna w zależności od czasu trwania stresu, typu MT, części rośliny i zastosowanego metalu. Siewki owsa w uprawie hydroponicznej traktowałam 100 μM $CdSO_4$ i 200 μM $ZnSO_4$ i mieszaninie obu pierwiastków (publikacja 3). Pod względem fizykochemicznym Zn i Cd są do siebie podobne i w środowisku często występują razem. Ich fizjologiczne role i oddziaływanie na organizmy żywe jest natomiast zupełnie odmienne, dlatego też ważne jest badanie wpływu obu tych pierwiastków na organizmy żywe (Almeida i in., 2018). Co ciekawe, wykazano zarówno antagonistyczne jak i synergistyczne interakcje pomiędzy tymi pierwiastkami (Moustakas i in., 2011; Puga i in., 2015). Ilość transkryptów *AsMT* była różna w pędach i korzeniach roślin rosnących w warunkach kontrolnych (bez obecności HM). Ekspresja *AsMT* zmieniała się w czasie oraz na skutek działania HM. Pod wpływem działania Cd i mieszaniny Zn i Cd wzrastała ekspresja *AsMT1* i *AsMT2* w pędach. W korzeniach zaś w wyniku działania tych czynników ekspresja *AsMT1* malała. Obecność samego Zn stymulowała ekspresję *AsMT2* i *AsMT3* w korzeniach, ale nie pędach roślin. W oparciu o przedstawione wyniki zakładamy, że AsMT1 pełni rolę w detoksykacji Cd, a AsMT2 i AsMT3 są zaangażowane w utrzymanie homeostazy Zn. Jednakże role te są prawdopodobnie organospecyficzne oraz zależą od etapu rozwoju i/lub czasu trwania stresu (publikacja 3). Największe fluktuacje w ekspresji odnotowano dla *AsMT4*. Ekspresja tej *AsMT* w pędach najwyższa była trzeciego dnia traktowania roślin mieszaniną Zn i Cd, w korzeniach zaś najwyższą ekspresję odnotowano po czternastu dniach traktowania mieszaniną jonów (publikacja 3). Wzrost ekspresji *AsMT4* tylko w odpowiedzi na jednoczesne działanie Cd i Zn, a nie samego Zn lub Cd sugeruje rolę MT typu 4 jako filtrów, które dzięki obecności reszt histydyny w swojej strukturze, są w stanie rozróżnić niezbędny do życia Zn od toksycznego Cd (Leszczyszyn i in., 2007; Mierek-Adamska i in., 2018).

Wielokrotnie wykazano, że na ekspresję *MT* wypływa obecność HM w środowisku jednakże odpowiedź zależy od rodzaju metalu, typu MT, gatunku rośliny, organu czy etapu rozwoju (Hsieh i in., 1995; Zhou i Goldsbrough, 1995; Cobbett i Goldsbrough, 2002). Na przykład u kawy (*Coffea arabica* L.) zauważono, że ekspresja *MT2* wzrastała wraz ze wzrostem stężenia Zn (Barbosa i in., 2017). Gao i in. (2022) wykazali zróżnicowaną ekspresję dziewięciu *MT* kukurydzy zależną od HM

(Cu, Cd i Pb). Inne przykłady zmian ekspresji *MT* pod wpływem HM można znaleźć u *A. thaliana* (Guo i in., 2003), rzepaku (Ahn i in., 2012), jadłoszynu baziowatego (*Prosopis juliflora* L.) (Usha i in., 2009), czy wilca ziemniaczanego (*Ipomea batatas* L.) (Kim i in., 2014). W dwóch pracach, gdzie zbadano ekspresję czterech typów MT w odpowiedzi na stres wywołany HM, podkreślone zostały różnice pomiędzy poszczególnymi typami MT. Każdy z analizowanych genów w inny sposób reagował na dany pierwiastek, a ekspresja była różna w różnych tkankach roślinnych (Pan i in., 2018; Gao i in., 2022). Wszystkie te analizy wskazują na to, że u owsa MT rożnych typów odpowiadają za utrzymanie homeostazy mikroelementów i detoksykację toksycznych jonów, a dodatkowo wykazują też swego rodzaju organospecyficzność.

3.1. Ocena zawartości Zn i Cd w *A. sativa* rosnącym w obecności HM i *Trichoderma viride*

Niektóre rośliny mają wyższą niż inne tolerancję na obecność HM w glebie, ale istnieją różne strategie na ograniczanie toksyczności HM. Po pierwsze rośliny ograniczają pobieranie HM poprzez immobilizację HM z udziałem mikroorganizmów mikoryzowych oraz sekwestrację i kompleksowanie metali przez związki obecne w eksudacie korzeniowym. Kolejno, jeśli HM dostaną się do wnętrza rośliny, dochodzi do sekwestracji i kompartmentacji HM w komórkach korzenia w przedziałach komórkowych np. wakuolach, dzięki czemu ograniczone jest ich toksyczne działanie. Część roślin transportuje HM do nadziemnych części roślin gdzie są następnie magazynowane (Emamverdian i in., 2015; Ghori i in., 2019). Poprzednio wykazano, że rośliny owsa mogą nie tylko wzrastać w obecności jonów Cd, ale także mogą go akumulować (Tanhuanpää i in., 2007; Tuma i in., 2014; Rolka, 2015). Założyłam, że wraz ze wzrostem stężenia Cd w glebie, stężenie Cd w roślinach owsa będzie wzrastało. W celu oceny tolerancji oraz stopnia akumulacji Cd przez rośliny owsa wykonałam doświadczenie donicowe, w którym rośliny owsa były hodowane w glebie zawierającej od 1 mg do 20 mg Cd na kg gleby. Dodatkowo zamierzałam zweryfikować hipotezę zakładającą, że obecność grzyba *Trichoderma viride* ogranicza akumulację Cd w tkankach roślinnych (publikacja 4). Po 6 miesiącach hodowli nie zaobserwowano różnic w długościach pędu i korzenia między roślinami rosnącymi w różnych stężeniach Cd. Różnice zaobserwowano jedynie w biomasie pędów i korzeni – wraz ze wzrostem stężenia Cd w glebie, biomasa roślin malała. Ponadto, wraz ze wzrostem stężenia

Cd w glebie malała liczba wytworzonych wiech, a co za tym idzie, liczba powstałych nasion (publikacja 4).

Rośliny owsa są w stanie wydajnie transportować jony Cd z podłoża do części nadziemnej rośliny (Ebbs i Kochian, 1998; Gutiérrez-Ginés i in., 2010; Tuma i in., 2014; Marchel i in., 2018). Analiza zawartości pierwiastków w części nadziemnej owsa potwierdziła, że wraz ze wzrastającym stężeniem Cd w glebie, wzrasta stężenie Cd w roślinie. Zaobserwowałam też pozytywną korelację między stężeniem Zn w roślinie, a stężeniem Cd w glebie (publikacja 4). Jest to o tyle ciekawe, że Zn i Cd są podobne pod względem fizykochemicznym i zwykle im wyższe stężenie Cd w glebie, tym mniejsze jest pobieranie Zn przez rośliny (Vasiliadou i Dordas, 2009; Murtaza i in., 2017). Zawartość Cu pozostawała na takim samym poziomie i korelowała negatywnie z poziomem Cd w glebie (publikacja 4). Obserwowana przeze mnie zawartość pierwiastków może być związana z tym, że rośliny lepiej akumulują Zn i Cd niż Cu (Pusz i in., 2021). Ponadto, na biodostępność metali w glebie ma wpływ wiele czynników takich jak na przykład: pH gleby, przewodność elektryczna, zdolność wymiany kationów i zawartość materii organicznej (Bayouli i in., 2020; Yang i in., 2021). Obecność grzybów *T. viride* wpłynęła pozytywnie na biomasę pędów i korzeni oraz liczbę wyprodukowanych nasion w obecności Cd, ale nie wpłynęła na zawartość Zn, Cd i Cu w części nadziemnej roślin. Mikroorganizmy te są znane ze swoich właściwości promujących wzrost roślin (Znajewska i in., 2018; Turkan i in., 2023). Choć tu nie zaobserwowaliśmy wpływu *T. viride* na ilość zakumulowanego Cd w części nadziemnej owsa, to dostępne są raporty, w których obecność grzybów z tego rodzaju spowodowała wzrost akumulacji Cd w roślinach takich jak *B. juncea* (Cao i in., 2008) czy *H. vulgare* (Taghavi Ghasemkheili i in., 2022). Dodatkowo, w obecności *T. viride* plon owsa był wyższy, co sugeruje, że obecność tych grzybów ograniczała negatywny wpływ Cd na rośliny. Jest to istotne nie tylko w sytuacji, gdy poszukuje się rozwiązań związanych z fitoekstrakcją metali z gleby, czyli usuwaniem zanieczyszczeń z gleby lub wody z użyciem roślin. Ma to również znaczenie ze względu na zdrowie ludzi, bowiem jeśli Cd akumuluje się roślinach wykorzystywanych do żywienia ludzi i zwierząt, to może dochodzić do bioakumulacji Cd w łańcuchu pokarmowym. Nasze wyniki pokazują, że *T. viride* zwiększa wytwarzanie nasion przez rośliny owsa, a jednocześnie nie zwiększa akumulacji Cd w częściach nadziemnych roślin. Jeśli chodzi o akumulację HM w nasionach roślin uprawnych rosnących w glebach skażonych

metalami ciężkimi, to wyniki nie są jednoznaczne. Dla soi rosnącej w glebie skażonej Zn i Pb nie zaobserwowano wzrostu tych HM w nasionach (Salazar i in., 2012). Podobnie dla rzepaku rosnącego w glebie skażonej Cu, Zn, Pb, Cd i Mn nie zaobserwowano akumulacji HM w nasionach, która stanowiłaby zagrożenie dla ludzi. Jednak dla nasion ryżu i pszenicy rosnących w tej samej glebie przekroczone zostały maksymalne stężenia Pb i Cd (Xu i in., 2022b). Dlatego też by ograniczyć konsumpcję HM przez ludzi wprowadzono limity zawartości HM w glebach uprawnych (Edelstein i Ben-Hur, 2018).

W celu określenia roli AsMT1-4 w interakcji roślin z *T. viride* sprawdziłam ekspresję genów *MT* u roślin owsa zainokulowanych lub niezainokulowanych sporami *T. viride*, rosnących w glebie zawierającej od 1 mg do 20 mg Cd na kg gleby (publikacja 4). W roślinach zainokulowanych sporami grzybów ekspresja *AsMT1-3* w obecności 1 mg/kg Cd była dwukrotnie większa w porównaniu z próbkami nieinokulowanymi. Natomiast w wyższych stężeniach Cd (5, 10 i 20 mg/kg) ekspresja w obu wariantach była na podobnym poziomie. Obserwowano pozytywne korelacje pomiędzy inokulacją *T. viride* a ekspresją *AsMT1-3*, natomiast negatywne korelacje zaobserwowałam pomiędzy poziomem Cd w glebie a ekspresją *AsMT1-3*. W przypadku *AsMT4* ekspresja wzrastała po inokulacji sporami grzyba w roślinach rosnących w glebie zawierającej 10 mg Cd na kg gleby. Ekspresja tego genu negatywnie korelowała zarówno z inokulacją *T. viride*, jaki i zawartością Cd w glebie i ekspresją pozostałych *AsMT* (publikacja 4). Istnieją dowody na to, że MT biorą udział w interakcjach między roślinami a mikroorganizmami – w przeszłości zaobserwowano, że po inokulacji roślin rzepaku grzybami arbuskularnymi wzrosła ekspresja *MT* typu 2 w liściach tych roślin w porównaniu do roślin nieinokulowanych (Dąbrowska i in., 2014). Cicatelli i in. (2010) wykazali, że inokulacja topoli białej grzybami arbuskularnymi nie tylko powodowała zwiększenie biomasy rośliny w glebie zanieczyszczonej Cu i Zn, ale także zaindukowana została ekspresja *MT* typu 1, 2 i 3. Co ciekawe, badania Garsteckiej i in. (2023) wskazują, że różne szczepy *T. viride* w odmienny sposób wpływają na ekspresję *MT* rzepaku. W przypadku MT owsa wykazałam w promotorach tych genów obecność elementów związanych z odpowiedzią na stresy biotyczne. Wzrost ekspresji *AsMT1-3* odnotowałam tylko dla roślin inokulowanych rosnących w obecności 1 mg Cd natomiast wzrost ekspresji *AsMT4* tylko dla roślin inokulowanych rosnących w obecności 10 mg Cd. Wskazuje to na odmienne role AsMT1-3 i AsMT4 w interakcji roślina - mikroorganizm w glebie skażonej Cd. Ponadto, prawdopodobnie w warunkach

intensywniejszego stresu (wyższych stężeń Cd w glebie) grzyby *T. viride* nie wchodzą w interakcje z owsem w takim samym stopniu jak w warunkach bardziej korzystnych, gdyż ich metabolizm może być wówczas skierowany na ochronę własnych komórek przed negatywnym działaniem HM. Hipoteza ta jednak wymaga dalszej weryfikacji.

4. Zaangażowanie AsMT w reakcję siewek owsa zwyczajnego na stres osmotyczny i suszy

Analizy *in silico* wykazały w promotorach *AsMT* obecność wielu elementów związanych z odpowiedzią na stres suszy oraz odpowiedzią na kwas abscysynowy. Fitohormon ten jest ściśle związany ze stresem suszy u roślin i odpowiada m. in. za zamykanie aparatów szparkowych (Szmidt-Jaworska i Kopcewicz, 2024). W związku z tym zweryfikowałam hipotezę zakładającą udział MT owsa w reakcji na działanie stresów osmotycznego i suszy. Zmiany w wartości potencjału wody w środowisku wynikające czy to z suszy atmosferycznej, czy też z suszy fizjologicznej wywołują u roślin stres osmotyczny. Wymusza to na roślinach zmiany potencjału osmotycznego ich komórek, by móc pobierać wodę z otoczenia. Niedobór wody u roślin powoduje zmiany m. in. w morfologii, wymianie gazowej i zawartości chlorofilu (Marcińska i in., 2013). W warunkach naturalnych to właśnie susza i nadmierne zasolenie gleby są najczęstszymi przyczynami stresu osmotycznego (Xiong i Zhu, 2002). Jednak w pracach eksperymentalnych stosuje się inne metody indukcji stresu osmotycznego u roślin np. związki chemiczne obniżające potencjał wody takie jak poliglikol etylenowy (PEG) i D-mannitol (Udawat i in., 2014; Yadav i in., 2014; Ghosh i in., 2019;). PEG i D-mannitol działają poprzez zmniejszenie dostępności wody, naśladując wpływ suszy na rośliny. Stosowanie PEG i D-mannitolu w hodowli hydroponicznej jest prostsze niż prowadzenie doświadczeń donicowych, jednak wymaga pewnym kompromisów. Nie są to warunki identyczne z naturalnymi – brak wpływu rodzaju i heterogeniczności gleby, intensywności światła, dostępności składników odżywczych i innych. Jest to uproszczona wersja stresu suszy, która skupia się jedynie na deficycie wody (Reyes i in., 2023).

W pierwszym etapie wykonałam analizę funkcjonalną AsMT eksprymowanych w bakteriach *E. coli*, rosnących w pożywce zawierającej PEG lub D-mannitol. W warunkach stresu wywołanego przez D-mannitol nie zaobserwowano różnic istotnych statystycznie między bakteriami niosącymi geny *AsMT1-3* a bakteriami niosącymi pusty

wektor. W przypadku PEG, bakterie niosące gen *AsMT2* charakteryzowały się szybszym tempem wzrostu w porównaniu do pozostałych bakterii (publikacja 1). W literaturze niewiele jest doniesień o wpływie nadekspresji roślinnych MT w bakteriach na tolerancję stresu osmotycznego. Jednym z dostępnych jest badanie, w którym *E. coli* eksprymujące MT typu 4 z ogórka (*Cucumis sativus* L.) charakteryzowały się wyższym współczynnikiem przeżycia w obecności sorbitolu i chlorku sodu niż bakterie kontrolne (Zhou i in., 2019).

Kolejnym krokiem było sprawdzenie jak stres osmotyczny wpłynie na ekspresję *AsMT1-3*. Siewki owsa hodowałam w hodowli hydroponicznej z dodatkiem PEG i D-mannitolu przez 4 dni, a następnie sprawdziłam ekspresję genów *AsMT1-3*. W warunkach stresu wywołanego PEG odnotowałam 1,5-krotny wzrost ekspresji *AsMT2* w pędach. W przypadku roślin traktowanych D-mannitolem – w pędach stwierdziłam wzrost ekspresji genów *AsMT1-3*, a w korzeniach *AsMT2* i *AsMT3*. Najwyższy, bo prawie 9-krotny, wzrost ekspresji *AsMT2* zaobserwowałam w korzeniach roślin traktowanych D-mannitolem (publikacja 1). Następnie, w eksperymencie donicowym, rośliny owsa poddałam stresowi suszy przez 14 dni. Nie odnotowałam zmian w ekspresji *AsMT1* w pędach, a w korzeniach ekspresja tego genu był o połowę niższa niż w roślinach kontrolnych. Ekspresja *AsMT2* była wyższa 12-krotnie w pędach i 27-krotnie w korzeniach roślin poddanych stresowi suszy w porównaniu do próby kontrolnej. Ekspresja *AsMT3* była 2,6-krotnie wyższa w korzeniach i 2,6-krotnie niższa w pędach roślin poddanych stresowi suszy w porównaniu do roślin kontrolnych (publikacja 2).

W literaturze znajdują się doniesienia wykazujące, że w odpowiedzi na stres osmotyczny dochodzi do podwyższenia ekspresji *MT*. Na przykład u bawełny kosmatej (*Gossypium hirsutum* L.) zaobserwowano wzrost ekspresji *MT* typu 3 w obecności NaCl i PEG (Xue i in., 2009). Pod wpływem działania PEG odnotowano też wzrost ekspresji *MT1* u ryżu (Yang i in., 2009) i głożyny pospolitej (*Ziziphus jujuba* Mill.) (Yang i in., 2015). Ponadto, zwiększoną tolerancję na stres osmotyczny zaobserwowano u transgenicznego tytoniu (*Nicotiana tabacum* L.) nadeksprymującego *MT3* z bawełny kosmatej (Xue i in., 2009) czy *MT1* z ryżu (Kumar i in., 2012). Podobnie stres suszy powoduje wzrost ekspresji *MT* na przykład u ciecierzycy poddanej stresowi suszy przez 7 dni zaobserwowano ośmiokrotny wzrost ekspresji *MT1* (Kumar i in., 2022), a u gryki zwyczajnej pod wpływem 15-dniowego stresu suszy ekspresja *MT3* wzrosła ponad

trzykrotnie w dojrzałych liściach (Samardžić i in., 2010). Wzrost ekspresji *MT1* w wyniku suszy zaobserwowano również u *Cyamopsis tetragonoloba* L. Taub. (Jaiswal i in., 2018) i *MT2* u arbuza (*Citrullus lanatus* (Thunb.) Mansf) (Akashi i in., 2004). Transgeniczny rzodkiewnik nadeksprymujacy *MT2* z ciecierzycy (*Cicer arietinum* L.) w warunkach suszy charakteryzował się lepszym wzrostem i zwiększoną zawartością antyoksydantów w porównaniu do rośliny dzikiej (Dubey i in., 2019). Podobne wyniki uzyskano dla *A. thaliana* nadeksprymujacego *MT2A* daktylowca (*Phoenix dactylifera* L.) (Patankar i in., 2019) i *MT3* ryżu (Mekawy i in., 2020). Stres osmotyczny indukowany z wykorzystaniem PEG i D-mannitolu jest uproszczoną wersją stresu suszy, dlatego mechanizmy zachodzące w roślinach w wyniku działania tych dwóch stresów mogą się różnić. Nasze badania to potwierdzają, bo widoczne są różnice w ekspresji genów *AsMT*. Odmienna ekspresja *AsMT1-3* w odpowiedzi na stresy podkreśla ich różne role w roślinach.

5. Zmiany parametrów biometrycznych owsa zwyczajnego w reakcji na czynniki abiotyczne

Działanie czynników stresowych powoduje w roślinach zmiany wielu procesów metabolicznych. Zbadanie zmian zawartości barwników fotosyntetycznych, względnej zawartości wody (RWC, *ang. relative water content*), aktywności enzymów antyoksydacyjnych oraz zawartości cukrów, fenoli i antyoksydantów pozwoliło na określenie, które szlaki są uruchamiane w komórkach owsa w odpowiedzi na metale ciężkie, stres suszy i stres osmotyczny. Ponadto, w celu określenia udział AsMT1-4 w szlakach metabolicznych, wykonałam analizy korelacji tych parametrów z ekspresją badanych MT. Założyłam, że ekspresja *AsMT1-4* będzie pozytywnie korelować z parametrami związanymi ściśle z odpowiedzią na stres antyoksydacyjny.

5.1. Metale ciężkie

Różnice w parametrach biometrycznych siewek owsa traktowanych Zn i Cd w stosunku do roślin kontrolnych pojawiły się już w 7. dni traktowania (publikacja 3). Obecność Zn w podłożu wpłynęła pozytywnie na produkcję świeżej i suchej biomasy pędu i korzenia, w 14. dniu stresu masa była 1,3 razy wyższa niż u roślin kontrolnych. Rośliny traktowane Cd lub mieszaniną Zn i Cd miały niższe tempo wzrostu i były znacząco mniejsze od roślin kontrolnych – po 14 dniach hodowli pędy tych roślin były ponad dwukrotnie krótsze w porównaniu do roślin kontrolnych (publikacja 3).

Podobny efekt zaobserwowano dla innych gatunków roślin – m. in. rzepaku, słonecznika (*Helianthus annuus* L.), soi (*Glycine max* L. Merr.), orzecha ziemnego (*Arachis hypogaea* L.) czy kukurydzy (Shi i Cai, 2010; Krishna i in., 2023). Choć w przeszłości wykazano, że suplementacja Zn może obniżać negatywny wpływ działania kadmu (McKenna i in., 1993; Cherif i in., 2011), to w tym doświadczeniu nie zaobserwowaliśmy znoszenia negatywnego działania Cd kiedy rośliny traktowano mieszaniną Zn+Cd. Takie obserwacje uzyskano na przykład dla pszenicy, gdzie traktowanie roślin opryskiem Zn obniżyło zawartość Cd w części nadziemnej roślin, w tym w nasionach, zwiększając jednocześnie zawartość Zn (Qian i in., 2023). W przypadku badań na owsie brak takiego ochronnego działania Zn może wynikać z zastosowania zbyt wysokiego stężenia Cd lub sposobu aplikacji Zn.

Traktowanie roślin jonami Zn w eksperymencie prowadzonym w hodowli hydroponicznej nie wywołało istotnych zmian zawartości związków fenolowych w siewkach owsa (publikacja 3). Fenole akumulują się w komórkach roślinnych w odpowiedzi na różne stresy, w tym stres suszy, co zaobserwowano między innymi dla pszenicy (Hura i in., 2016), grochu (*Pisum sativum* L.) (Juzoń i in., 2013), kukurydzy (Hura i in., 2008; Warzecha i in., 2023) czy łubinu żółtego (*Lupinus luteus* L.) (Juzoń i in., 2013). Mogą one chronić komórki przed RFT, a ich wydajność antyoksydacyjna może być nawet wyższa niż ta tokoferolu czy witaminy C (Weidner i in., 2011). Można dlatego przypuszczać, że w roślinach owsa traktowanych jonami cynku poziom stresu nie był na tyle wysoki by doszło do akumulacji tych związków. W przypadku roślin owsa traktowanych mieszaniną Zn i Cd stwierdziłam wzrost zawartości związków fenolowych już po trzech dniach stresu, zaś w przypadku traktowania samym Cd dopiero po siedmiu dniach traktowania (publikacja 3). Wzrost zawartości związków fenolowych pod wpływem działania Cd zaobserwowano już wcześniej na przykład u pszenicy (Kobyletska i in., 2022) i u bazylii (*Ocimum basilicum* L), gdzie zaobserwowano, że zawartość związków fenolowych rosła wraz ze wzrostem stężenia Cd w środowisku (Korkmaz i in., 2018). Dla bazylii traktowanej Zn zaobserwowano, że wraz ze wzrastającym stężeniem tego pierwiastka, zawartość fenoli malała (Mahmoudi i in., 2021). Odwrotnych obserwacji dokonano dla czarnuszki siewnej (*Nigella sativa* L.), dla której wraz ze wzrostem stężenia Zn rosła zawartość fenoli (Marichali i in., 2016). Fernàndez-Martínez z zespołem (2014) zbadali odpowiedź dwóch odmian topoli na traktowanie Zn w dwóch różnych stężeniach. Zauważyli oni, że u odmiany Eridano

doszło do wzrostu zawartości fenoli, zaś u bardziej wrażliwej odmiany I-214 zawartość fenoli spadła. Wyniki te wskazują, że rośliny bardziej tolerancyjne na Zn zwiększają produkcję fenoli w momencie kiedy dawka Zn zaczyna być dla nich toksyczna. U roślin bardziej wrażliwych na Zn mechanizm ten nie działa tak sprawnie.

W wielu roślinach, na przykład u pomidora (*Lycopersicum esculentum* Mill.) i grochu, obserwowano zmiany w zawartości chlorofilu i karotenoidów pod wpływem działania Zn (Yadav i in., 2014; Manzoor i in., 2022). Jednak zmiana ta jest zależna od stężenia tego pierwiastka – niższe stężenia Zn powodują wzrost zawartości barwników, zaś wyższe – ich spadek (Jain i in., 2010; Fatima i in., 2011). W przypadku owsa traktowanego Zn nie zaobserwowałam zmian w ilości barwników fotosyntetycznych, ale u roślin traktowanych mieszaniną Zn i Cd ich poziom wzrósł, a w szczególności zawartość chlorofilu a. Dla roślin traktowanych wyłącznie Cd poziom barwników utrzymywał się na podobnym poziomie, jedynie w 14 dniu traktowania zaobserwowano wzrost zawartości chlorofilu a (publikacja 3). Odmiennych obserwacji dokonano między innymi dla truskawki (*Fragaria* x *ananassa* Duch), gdzie wraz ze wzrastającym stężeniem Cd, zawartość chlorofilu a i b spadała. Spadek stężenia barwników fotosyntetycznych ma prawdopodobnie związek z toksycznym wpływem Cd na enzymy biorące udział w syntezie barwników (Muradoglu i in., 2015). Jednak w przypadku kukurydzy zaobserwowano, że w wyniku traktowania roślin jonami Cd wzrosła zawartość chlorofilu a w młodych liściach, zaś w liściach dojrzałych i starych zawartość chlorofilu a utrzymywała się na tym samym poziomie (Drążkiewicz i Baszyński, 2005).

W siewkach owsa traktowanych jonami cynku przez 3 dni zaobserwowałam wzrost zawartości antyoksydantów zmierzony metodą FRAP, jednak w kolejnych dniach stresu różnica ta zanikła. Zmiany tej nie zaobserwowałam wcale w przypadku analizy wykonanej metodą ABTS przez cały czas trwania eksperymentu (publikacja 3). Wzrost zawartości antyoksydantów zmierzony metodą FRAP i ABTS zaobserwowałam dla siewek traktowanych Cd lub mieszaniną Zn i Cd. Interpretując wyniki należy pamiętać, że obie metody bazują na różnych mechanizmach i uzyskiwane wyniki mogą się od siebie różnić. Test ABTS jest bardziej specyficzny dla tak zwanych przeciwutleniaczy pierwotnych czyli donorów wodoru lub elektronów. W metodzie FRAP na wartość pomiaru wpływają też przeciwutleniacze, które działają pośrednio

poprzez wychwytywanie tlenu i chelatowanie jonów metali przejściowych. Stąd też wynika różnica w pomiarach między tymi dwoma metodami – wartości uzyskiwane metodą ABTS są o dwa rzędy wielkości większe w porównaniu do metody FRAP. W przeciwieństwie do metody ABTS, metoda FRAP uniemożliwia zmierzenie antyoksydantów tiolowych, np. glutationu czy metalotionein. Ponadto ekstrakty roślinne, dla których wartość ABTS jest wysoka najprawdopodobniej zawierają więcej antyoksydantów pierwotnych (Szydłowska-Czerniak i Łaszewska, 2015; Munteanu i Apetrei, 2021). Wyższe wartości FRAP niż ABTS mogą być związane z faktem, że metoda ta wyklucza substancje o dużej masie molekularnej, przez co jony żelaza mogą reagować z innymi antyoksydantami (Cecchini i Fazio, 2020). .

W celu zrozumienia potencjalnego udziału MT w szlakach biochemicznych wykonałam analizę korelacji Pearsona. Stwierdziłam wysoką pozytywną korelację między ekspresją *AsMT1* i *AsMT2* a zawartością związków fenolowych (TPC) i pojemnością antyoksydacyjną (AC), a także między *AsMT3* a AC w pędach owsa. Natomiast w korzeniach, stwierdziłam ujemne korelacje ekspresji *AsMT1-3* z TPC i AC. W pędach ekspresja *AsMT4* korelowała pozytywnie z TPC i negatywnie z AC, zaś w korzeniach korelował dodatnio z AC. Można przypuszczać zatem, że *AsMT1* i *AsMT2* pełnią rolę w odpowiedzi na stres oksydacyjny w pędach, ale nie w korzeniach roślin, zaś w przypadku *AsMT4* sytuacja jest odwrotna (publikacja 3). Sugeruje się,że MT mogą działać jako pierwotne przeciwutleniacze, ponieważ wykazano bezpośrednią reakcję MT z RFT, kiedy nie wszystkie grupy tiolowe są połączone z metalami. Z drugiej strony, MT wiążą się z jonami metali przejściowych, w tym miedzi, ograniczając w ten sposób reakcje Fentona i Habera-Weissa (Xue i in., 2009; Vašák i Meloni, 2011).

5.2. Stres osmotyczny i suszy

Pod wpływem stresu osmotycznego w siedmiodniowych roślinach owsa z hodowli hydroponicznej z dodatkiem PEG zaobserwowałam 1,8-krotny wzrost zawartości związków fenolowych w korzeniach roślin traktowanych w porównaniu do roślin kontrolnych (publikacja 1). Jednak w warunkach stresu suszy nie obserwowałam różnic (publikacja 2). W literaturze są dostępne dane, w których pokazano, że pod wpływem stresu suszy w roślinach może dojść do zmniejszenia zawartości związków fenolowych na przykład u *Rehmannia glutinosa* (Chung i in., 2006) i winorośli (*Vitis vinifera* L.) (Król i in., 2014) lub do ich wzrostu na przykład u soi

(Swigonska i in., 2014) i kukurydzy (Latif i in., 2016). Obserwowane różnice między danymi literaturowymi a danymi uzyskanymi przeze mnie wynikać mogą z faktu, że do analiz wykorzystano inne części roślin lub materiał roślinny pochodzący z innej fazy rozwojowej czy poddany innym czynnikom indukującym stres wodny.

W przeprowadzonych badaniach w siewkach owsa rosnących w warunkach stresu osmotycznego wywołanego działaniem PEG, D-mannitolu oraz w stresie suszy, stwierdziłam wzrost zawartości cukrów rozpuszczalnych (publikacje 1 i 2). Akumulacja cukrów, które działają jak osmoprotektanty, przez rośliny zwiększa zdolność komórek do zatrzymywania wody, co może zmniejszać stres związany z odwodnieniem (Hoekstra i in., 2001, Arabzadeh, 2012). Dodatkowo cukry rozpuszczalne pełnią rolę w utrzymaniu równowagi redoks w roślinach, przyczyniając się do eliminacji nadtlenku wodoru i wraz ze związkami fenolowymi mogą tworzyć zintegrowany system usuwania RFT (Bolouri-Moghaddam i in., 2010). Cukry mogą również działać jako cząsteczki sygnałowe i regulować ekspresję genów. U rzodkiewnika udowodniono, że cukry wpływają na ekspresję między innymi genów zaangażowanych w metabolizm węgla (Lloyd i Zakhleniuk, 2004). Co ciekawe egzogenna aplikacja cukrów zwiększyła tolerancję rzepaku, kukurydzy, ryżu i rzodkiewnika na stres suszy (Kaur i in., 2021). Ponadto u soi poddanej stresowi suszy zaobserwowano wzrost ekspresji genów związanych z metabolizmem i transportem cukrów (Du i in., 2020). Jako, że cukry rozpuszczalne pełnią tak ważną rolę w regulacji mechanizmów związanych ze stresem suszy, zasugerowano, że można użyć ich jako marker do selekcji odmian pszenicy i lucerny siewnej (*Medicago sativa* L.) odpornych na suszę (Hakimi i in., 1995; Maghsoodi i Razmjoo, 2015).

Rośliny owsa poddane stresowi suszy charakteryzowały obniżone zawartości chlorofilu a i karotenoidów (publikacja 2). Podobne obserwacje odnotowano dla 13 odmian pszenicy twardej (*Triticum durum* L.) poddanych stresowi suszy, gdzie odmiany bardziej wrażliwe na suszę miały obniżoną zawartość chlorofilu (Zaefyzadeh i in., 2009). Postuluje się, że obniżona fluorescencja chlorofilu może być narzędziem do oceny poziomu stresu suszy u truskawek (Razavi i in., 2008). W warunkach stresu osmotycznego wywołanego PEG nie zaobserwowałam zmian w ilości barwników fotosyntetycznych, zaś w obecności D-mannitolu poziom chlorofilu a wzrósł (publikacja 1). W innych badaniach zaobserwowano, że pod wpływem działania PEG zmniejsza się zawartość chlorofilu w ryżu (Hsu i Kao, 2003) i orzechu ziemnym (Meher

i in., 2018). Podobnie pod wpływem działania D-mannitolu obserwuje się spadek zawartości barwników fotosyntetycznych u eukaliptusa (*Eucalyptus camaldulensis* Dehnh.) (Cha-Um i Kirdmanee, 2010), ryżu (Cha-Um i in., 2010) i kukurydzy (Możdżeń i in., 2015). Wyniki te podkreślają, że choć we wszystkich przypadkach rośliny są wystawione na działanie deficytu wody, to występują znaczące różnice w niektórych parametrach, wskazując na różne mechanizmy odpowiedzi na stres suszy i stres osmotyczny wywołany działaniem PEG i D-mannitolu. W warunkach suszy obserwuje się spadek parametrów związanych z fotosyntezą, gdyż rośliny chcąc ochronić chloroplasty przed uszkodzeniem obniżają tempo fotosyntezy, co jak pokazują najnowsze dane sterowane jest wzrostem zawartości sygnałowych alarmonów, nietypowych ufosforylowanych nukleotydów (Lichtenthaler, 1987; Boniecka i in., 2017; Dąbrowska i in., 2021b).

Zarówno w warunkach stresu suszy jak i w warunkach stresu osmotycznego wywołanego działaniem PEG lub D-mannitolu zaobserwowałam wzrost poziomu enzymów antyoksydacyjnych (publikacje 1 i 2). W odpowiedzi na różne stresy obserwuje się wzrost zawartości RFT, a w konsekwencji też enzymów antyoksydacyjnych. Jednak, jak pokazują badania, w zależności od rośliny oraz czasu trwania i rodzajów bodźców wywołujących stres, rośliny wykorzystują enzymatyczne i nieenzymatyczne systemy antyoksydacyjne. Działanie tych cząsteczek wzajemnie się uzupełnia i zapewnia roślinom ochronę przed RFT (You i Chan, 2015; Nadarajah, 2020). W warunkach stresu deficytu wody (wywołanego suszą albo stresem osmotycznym) zaobserwowano wzrost aktywności enzymów antyoksydacyjnych między innymi u lucerny (*Medicago sativa* L.) (Zhang i in., 2019), jabłoni (*Malus domestica* Borkn. cv. Red Fuji) (Wang i in., 2018), ryżu (Liu i in., 2019), czy gerbery (*Gerbera jamesonii* Bolus) (Lai i in., 2007).

Charakterystyczną zmianą zachodzącą w komórkach roślinnych w odpowiedzi na odwodnienie jest wzrost stężenia ABA. Hormon ten odpowiada między innymi za zamykanie aparatów szparkowych, ograniczając transpirację. Dodatkowo ABA indukuje ekspresję wielu genów, w tym metalotionein (Zhou i in., 2005; Dong i in., 2010; Ahn i in., 2012; Szmidt-Jaworska i Kopcewicz, 2024). W pędach siewek owsa traktowanych zarówno D-mannitolem jak i PEG zaobserwowałam gwałtowny wzrost zawartości ABA, przy czym najwyższe stężenie tego fitohormonu obserwowano w roślinach traktowanych D-mannitolem (publikacja 2). W korzeniach

stężenie ABA było na porównywalnym poziomie zarówno w kontroli jak i u roślin traktowanych PEG i D-mannitolem. Podobnie dla roślin owsa z eksperymentu donicowego poddanych stresowi suszy przez 14 dni w pędach zaobserwowałam znaczący wzrost stężenia ABA, z tą różnicą, że w tym przypadku wzrost stężenia tego fitohormonu miał też miejsce w korzeniach roślin (publikacja 1). Analizy biochemiczne roślin poddanych stresowi osmotycznemu i stresowi suszy potwierdzają, że rośliny te znalazły się w warunkach stresowych. Szczególną uwagę należy zwrócić na zawartość kwasu abscysynowego, hormonu kluczowego w odpowiedzi roślin na stresy abiotyczne, w tym stres suszy (Vishwakarma i in., 2017). Zaobserwowałam pozytywne korelacje między ilością ABA i ekspresją *AsMT2* i *AsMT3*, a negatywne z ekspresją *AsMT1*, co wskazuje pośrednio na to, że ABA reguluje ekspresję tych genów. Dla wielu innych roślin odnotowano wzrost ekspresji *MT* po traktowaniu ABA czego przykładem są badania przeprowadzone na *P. juliflora* (Usha i in., 2009), *G. hirsutum* (Xue i in., 2009) i *O. sativa* (Zhou i in., 2005). Sugeruje to, że ABA swoją kluczową rolę w odpowiedzi na stres osmotyczny odgrywa nie tylko regulując transpirację, ale także poprzez regulację ekspresji *MT*. Ponadto ekspresja *AsMT* korelowała pozytywnie z zawartością fenoli i aktywnością enzymów antyoksydacyjnych. Uzyskane wyniki są zgodne z obserwacjami zespołu Li i in. (2016), którzy wykazali, że nadekspresja genów *MT* może znacząco poprawić tolerancję roślin na suszę i towarzyszy jej podwyższona aktywność enzymów przeciwutleniających. Powyższe dane potwierdzają, że MT owsa, podobnie jak i innych roślin biorą udział w regulacji zawartości RFT w komórkach.

VI. Podsumowanie i wnioski

- Genom heksaploidalnego owsa zwyczajnego (*Avena sativa* L.) zawiera 21 genów metalotionein, należące do czterech typów (AsMT1-4) (publikacja 3).
- Promotory genów *AsMT1-4* zawierają sekwencji regulatorowe odpowiedzialne za reakcję owsa m.in. na: fitohormony, światło, suszę i stresy wywołane metalami ciężkimi. Analizy sekwencji promotorowych wskazały na potencjalną rolę MT w adaptacji owsa do warunków stresowych, i ich rolę w regulacji rozwoju i wzrostu rośliny (publikacja 3).
- Ekspresja *AsMT* owsa wszystkich czterech typów ulega zmianom podczas kiełkowania nasion. Całkowita zawartość transkryptów *AsMT1-4* utrzymuje się na podobnym poziomie podczas 48 godzin kiełkowania, natomiast zmieniały się ilości transkryptów poszczególnych typów *AsMT*. Wskazuje to, że metalotioneiny owsa odgrywają zróżnicowane i uzupełniające się role w czasie kiełkowania nasion (publikacja 3).
- Wykazałam zmiany ekspresji *AsMT1-4* w siewkach owsa rosnącego w obecności Zn, Cd i mieszaniny Zn i Cd po 3., 7. i 14. dniach od indukcji stresu. Ekspresja *AsMT1-4* była różna dla każdego typu MT w pędach i korzeniach roślin. Zaobserwowałam pozytywne jak i negatywne korelacje między ekspresją *AsMT1-4* a parametrami pokazującymi potencjał antyoksydacyjny roślin, a rodzaj korelacji zależał od części rośliny i porównywanego parametru (publikacja 3).
- Długotrwały stres wywołany obecnością Cd w glebie nie wpływa negatywnie na wzrost siewek owsa, ale powoduje spadek liczby nasion. Wraz ze wzrostem ilości kadmu w glebie wzrasta jego zawartość w części nadziemnej owsa. Aplikacja spor grzybów *T. viride* oddziałuje pozytywnie na wzrost plonowania *A. sativa* i co ważne nie powoduje wzrostu zawartości Cd w roślinie oraz wpływa na zmiany ekspresji *AsMT1-4* (publikacja 4).
- Analiza funkcjonalna wykazała, że *E. coli* eksprymujące *AsMT1-4* owsa w warunkach stresu wywołanego obecnością Zn i Cd oraz PEG i D-mannitolu wykazują zwiększone tempo wzrostu w porównaniu do bakterii transformowanych pustym wektorem ekspresyjnym. Wskazuje to na udział AsMT2 w ochronie komórek bakteryjnych przed działaniem stresu osmotycznego oraz AsMT3 i AsMT4 w ochronie przed stresem wywołanym Zn i Cd (publikacje 1 i 4).

- W warunkach stresu osmotycznego spowodowanego działaniem PEG i D-mannitolu w siewkach owsa wzrasta zawartość ABA, fenoli, cukrów rozpuszczalnych i enzymów antyoksydacyjnych oraz zmienia się ekspresja *AsMT*. Obecność D-mannitolu powodował wzrost ekspresji *AsMT1-3* w pędach i *AsMT2-3* w korzeniach owsa zwyczajnego. Aplikacja PEG wpływała na wzrost ekspresji *AsMT2* w korzeniach. Zmiany ekspresji *AsMT1-3* korelowały ze zmianami parametrów biochemicznych, co wskazuje na zaangażowanie AsMT1-3 w odpowiedź owsa na stres osmotyczny. Ekspresja poszczególnych typów MT była odmienna, co sugeruje, że pełnią one różne i uzupełniające się role w komórkach owsa (publikacja 1).
- W warunkach stresu suszy u owsa wzrastają zawartości kwasu abscysynowego, cukrów i enzymów antyoksydacyjnych oraz zmian ulega ekspresja genów *AsMT1-3*, w szczególności *AsMT2,* co wskazuje na rolę tych genów w odpowiedzi *A. sativa* na suszę (publikacja 2).
- Zmiany ekspresji genów *AsMT1-3* korelują ze zmianami poziomu ABA i aktywności enzymów antyoksydacyjnych. Sugeruje to, że ekspresja *AsMT* jest regulowana przez kwas abscysynowy a metalotioneiny stanowią element systemu antyoksydacyjnego (publikacja 2).

VII. Literatura

Ahn YO, Kim SH, Lee J, Kim H, Lee H-SS, Kwak S-S. **2012**. Three *Brassica rapa* metallothionein genes are differentially regulated under various stress conditions. *Molecular Biology Reports* 39:2059–2067. DOI: 10.1007/s11033-011-0953-5.

Akashi K, Nishimura N, Ishida Y, Yokota A. **2004**. Potent hydroxyl radical-scavenging activity of drought-induced type-2 metallothionein in wild watermelon. *Biochemical and Biophysical Research Communications* 323:72–78. DOI: 10.1016/j.bbrc.2004.08.056.

Almeida LG, Silva DMD, Jorge ADP, Souza KRDD, Guilherme LRG, Alves JD. **2018**. Synergy between cadmium and zinc in bean plants cultivated in multi contaminated soils. *Acta Scientiarum. Agronomy* 41:35829. DOI: 10.4025/actasciagron.v41i1.35829.

Anand A, Kumari A, Thakur M, Koul A. **2019**. Hydrogen peroxide signaling integrates with phytohormones during the germination of magnetoprimed tomato seeds. *Scientific Reports* 9:1–11. DOI: 10.1038/s41598-019-45102-5.

Arabzadeh N. **2012**. The effect of drought stress on soluble carbohydrates (sugars) in two species of *Haloxylon persicum* and *Haloxylon aphyllum*. *Asian Journal of Plant Sciences* 11:44–51. DOI: 10.3923/AJPS.2012.28.35.

Ashraf M. **2009**. Biotechnological approach of improving plant salt tolerance using antioxidants as markers. *Biotechnology Advances* 27:84–93. DOI: 10.1016/j.biotechadv.2008.09.003.

Ashraf M, Foolad MR. **2007**. Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany* 59:206–216. DOI: 10.1016/J.ENVEXPBOT.2005.12.006.

Bailly C. **2019**. The signalling role of ROS in the regulation of seed germination and dormancy. *Biochemical Journal* 476:3019–3032. DOI: 10.1042/BCJ20190159.

Barbosa BCF, Silva SC, de Oliveira RR, Chalfun A. **2017**. Zinc supply impacts on the relative expression of a metallothionein-like gene in *Coffea arabica* plants. *Plant and Soil* 411:179–191. DOI: 10.1007/s11104-016-2983-1.

Bayouli IT, Gómez-Gómez B, Bayouli HT, Pérez-Corona T, Meers E, Ammar E, Ferchichi A, Albarrán YM. **2020**. Heavy metal transport and fate in soil-plant system: study case of industrial cement vicinity, Tunisia. *Arabian Journal of Geosciences* 13:75. DOI: 10.1007/s12517-019-4898-7.

Bernat P. **2024**. Raport IUNG-PIB – susza rolnicza i 20% strat w plonach. *Dostępne na: https://www.topagrar.pl/articles/pogoda/raport-iung-pib-susza-rolnicza-i-20-strat-w-plonach-2511736* (Dostęp: 26 wrzesień 2024).

Bhargava S, Sawant K. **2013**. Drought stress adaptation: metabolic adjustment and regulation of gene expression. *Plant Breeding* 132:21–32. DOI: 10.1111/pbr.12004.

Blindauer CA. **2008**. Metallothioneins with unusual residues: histidines as modulators of zinc affinity and reactivity. *Journal of Inorganic Biochemistry* 102:507–521. DOI: 10.1016/j.jinorgbio.2007.10.032.

Blindauer CA. **2011**. Bacterial metallothioneins: past, present, and questions for the future. *Journal of Biological Inorganic Chemistry* 16:1011–1024. DOI: 10.1007/s00775-011-0790-y.

Blindauer CA, Leszczyszyn OI. **2010**. Metallothioneins: unparalleled diversity in structures and functions for metal ion homeostasis and more. *Natural Product Reports* 27:720–741. DOI: 10.1039/B906685N.

Blindauer CA, Razi MT, Campopiano DJ, Sadler PJ. **2007**. Histidine ligands in bacterial metallothionein enhance cluster stability. *Journal of Biological Inorganic Chemistry* 12:393–405. DOI: DOI 10.1007/s00775-006-0196-4.

Bolouri-Moghaddam MR, Le Roy K, Xiang L, Rolland F, Van Den Ende W. **2010**. Sugar signalling and antioxidant network connections in plant cells. *FEBS Journal* 277:2022–2037. DOI: 10.1111/j.1742-4658.2010.07633.x.

Boniecka J, Prusińska J, Dąbrowska GB, Goc A. **2017**. Within and beyond the stringent response-RSH and (p)ppGpp in plants. *Planta* 246:817–842. DOI: 10.1007/s00425-017-2780-y.

Briffa J, Sinagra E, Blundell R. **2020**. Heavy metal pollution in the environment and their toxicological effects on humans. *Heliyon* 6:e04691. DOI: 10.1016/j.heliyon.2020.e04691.

Brkljačić JM, Samardžić JT, Timotijević GS, Maksimović VR. **2004**. Expression analysis of buckwheat (*Fagopyrum esculentum* Moench) metallothionein-like gene (*MT3*) under different stress and physiological conditions. *Journal of Plant Physiology* 161:741–746. DOI: 10.1078/0176-1617-01211.

Butt MS, Tahir-Nadeem M, Khan MKI, Shabir R, Butt MS. **2008**. Oat: Unique among the cereals. *European Journal of Nutrition* 47:68–79. DOI: 10.1007/s00394-008-0698-7.

Cao L, Jiang M, Zeng Z, Du A, Tan H, Liu Y. **2008**. *Trichoderma atroviride* F6 improves phytoextraction efficiency of mustard (*Brassica juncea* (L.) Coss. var. foliosa Bailey) in Cd, Ni contaminated soils. *Chemosphere* 71:1769–1773. DOI: 10.1016/j.chemosphere.2008.01.066.

Capdevila M, Atrian S. **2011**. Metallothionein protein evolution: a miniassay. *JBIC Journal of Biological Inorganic Chemistry* 16:977–989. DOI: 10.1007/s00775-011-0798-3.

Capdevila M, Bofill R, Palacios Ò, Atrian S. **2012**. State-of-the-art of metallothioneins at the beginning of the 21st century. *Coordination Chemistry Reviews* 256:46–62. DOI: 10.1016/j.ccr.2011.07.006.

Castiglione S, Franchin C, Fossati T, Lingua G, Torrigiani P, Biondi S. **2007**. High zinc concentrations reduce rooting capacity and alter metallothionein gene expression in white

poplar (*Populus alba* L. cv. Villafranca). *Chemosphere* 67:1117–1126. DOI: 10.1016/j.chemosphere.2006.11.039.

Cecchini S, Fazio F. **2020**. Assessment of total antioxidant capacity in serum of heathy and stressed hens. *Animals* 10:2019. DOI: 10.3390/ani10112019.

Charkiewicz AE, Omeljaniuk WJ, Nowak K, Garley M, Nikliński J. **2023**. Cadmium toxicity and health effects—a brief summary. *Molecules* 28:6620. DOI: 10.3390/molecules28186620.

Chaturvedi AK, Mishra A, Tiwari V, Jha B. **2012**. Cloning and transcript analysis of type 2 metallothionein gene (*SbMT-2*) from extreme halophyte *Salicornia brachiata* and its heterologous expression in *E. coli*. *Gene* 499:280–287. DOI: 10.1016/j.gene.2012.03.001.

Chaudhry S, Sidhu GPS. **2022**. Climate change regulated abiotic stress mechanisms in plants: a comprehensive review. *Plant Cell Reports* 41:1–31. DOI: 10.1007/s00299-021-02759-5.

Cha-Um S, Kirdmanee C. **2010**. Effects of water stress induced by sodium chloride and mannitol on proline accumulation, photosynthetic abilities and growth characters of eucalyptus (*Eucalyptus camaldulensis* Dehnh.). *New Forests* 40:349–360. DOI: 10.1007/s11056-010-9204-1.

Cha-Um S, Nhung NTH, Kirdmanee C. **2010**. Effect of mannitol- and salt-induced iso-osmotic stress on proline accumulation, photosynthetic abilities and growth characters of rice cultivars (*Oryza sativa* L. spp. indica). *Pakistan Journal of Botany* 42:927–941.

Chen Y, Li A, Yun P, Chen Q, Pan D, Guo R, Zhang H, Ahmed HAI, Hu H, Peng Y, Wang C, Dong H, Qiu C, Shabala L, Shabala S, Luo B, Hou P. **2024**. Genome-wide analysis of *MYB* transcription factor family and *AsMYB1R* subfamily contribution to ROS homeostasis regulation in *Avena sativa* under PEG-induced drought stress. *BMC Plant Biology* 24:632. DOI: 10.1186/s12870-024-05251-w.

Cheng M, Yuan H, Wang R, Zou J, Liang T, Yang F, Li S. **2021**. Genome-wide identification and analysis of the metallothionein genes in *Oryza* genus. *International Journal of Molecular Sciences* 22. DOI: 10.3390/ijms22179651.

Cherif J, Mediouni C, Ammar WB, Jemal F. **2011**. Interactions of zinc and cadmium toxicity in their effects on growth and in antioxidative systems in tomato plants (*Solarium lycopersicum*). *Journal of Environmental Sciences* 23:837–844. DOI: 10.1016/S1001-0742(10)60415-9.

Chung IM, Kim JJ, Lim JD, Yu CY, Kim SH, Hahn SJ. **2006**. Comparison of resveratrol, SOD activity, phenolic compounds and free amino acids in *Rehmannia glutinosa* under temperature and water stress. *Environmental and Experimental Botany* 56:44–53. DOI: 10.1016/J.ENVEXPBOT.2005.01.001.

Cicatelli A, Lingua G, Todeschini V, Biondi S, Torrigiani P, Castiglione S. **2010**. Arbuscular mycorrhizal fungi restore normal growth in a white poplar clone grown on heavy metal-contaminated soil, and this is associated with upregulation of foliar

metallothionein and polyamine biosynthetic gene expression. *Annals of Botany* 106:791–802. DOI: 10.1093/aob/mcq170.

Cobbett C, Goldsbrough P. **2002**. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology* 53:159–182. DOI: 10.1146/annurev.arplant.53.100301.135154.

Dąbrowska G, Baum C, Trejgell A, Hrynkiewicz K. **2014**. Impact of arbuscular mycorrhizal fungi on the growth and expression of gene encoding stress protein - metallothionein *BnMT2* in the non-host crop *Brassica napus* L. *Journal of Plant Nutrition and Soil Science* 177:459–467. DOI: 10.1002/jpln.201300115.

Dąbrowska GB, Garstecka Z, Trejgell A, Dąbrowski HP, Konieczna W, Szyp-Borowska I. **2021a**. The Impact of Forest Fungi on Promoting Growth and Development of *Brassica napus* L. *Agronomy* 11:1–15. DOI: https://doi.org/10.3390/agronomy11122475.

Dąbrowska G, Głowacka B. **2004**. Akwaporyny - nowe spojrzenie na transport wody w roślinach. *Postępy Biochemii* 50:383–7.

Dąbrowska G, Hrynkiewicz K, Trejgell A. **2012a**. Do arbuscular mycorrhizal fungi affect metallothionein *MT2* expression in *Brassica napus* L. roots? *Acta Biologica Cracoviensia Series Botanica* 54:34–39. DOI: 10.2478/v10182-012-0003-1.

Dąbrowska G, Mierek-Adamska A, Goc A. **2012b**. Plant metallothioneins: Putative functions identified by promoter analysis *in silico*. *Acta Biologica Cracoviensia Series Botanica* 54:109–120. DOI: 10.2478/v10182-012-0030-y.

Dąbrowska G, Mierek-Adamska A, Goc A. **2013**. Characterisation of *Brassica napus* L. metallothionein genes (BnMTs) expression in organs and during seed germination. *Australian Journal of Crop Science* 7:1324–1332.

Dąbrowska GB, Turkan S, Tylman-Mojżeszek W, Mierek-Adamska A. **2021b**. *In silico* study of the *RSH* (RelA/SpoT homologs) gene family and expression analysis in response to PGPR bacteria and salinity in *Brassica napus*. *International Journal of Molecular Sciences* 22. DOI: 10.3390/ijms221910666.

Dauch AL, Jabaji-Hare SH. **2006**. *Metallothionein* and *bZIP* transcription factor genes from velvetleaf and their differential expression following *Colletotrichum coccodes* infection. *Phytopathology* 96:1116–1123. DOI: 10.1094/PHYTO-96-1116.

Dong C-JJ, Wang Y, Yu S-SS, Liu J-YY. **2010**. Characterization of a novel rice metallothionein gene promoter: its tissue specificity and heavy metal responsiveness. *Journal of Integrative Plant Biology* 52:914–924. DOI: 10.1111/j.1744-7909.2010.00966.x.

Drążkiewicz M, Baszyński T. **2005**. Growth parameters and photosynthetic pigments in leaf segments of *Zea mays* exposed to cadmium, as related to protection mechanisms. *Journal of Plant Physiology* 162:1013–1021. DOI: 10.1016/j.jplph.2004.10.010.

Du Y, Zhao Q, Chen L, Yao X, Zhang W, Zhang B, Xie F. **2020**. Effect of drought stress on sugar metabolism in leaves and roots of soybean seedlings. *Plant Physiology and Biochemistry* 146:1–12. DOI: 10.1016/j.plaphy.2019.11.003.

Duan L, Yu J, Xu L, Tian P, Hu X, Song X, Pan Y. 2019**.** Functional characterization of a type 4 metallothionein gene (*CsMT4*) in cucumber. *Horticultural Plant Journal* 5:120–128. DOI: 10.1016/j.hpj.2019.04.002.

Dubey AK, Kumar N, Kumar A, Ansari MA, Ranjan R, Gautam A, Meenakshi, Sahu N, Pandey V, Behera SK, Mallick S, Pande V, Sanyal I. **2019**. Over-expression of *CarMT* gene modulates the physiological performance and antioxidant defense system to provide tolerance against drought stress in *Arabidopsis thaliana* L. *Ecotoxicology and Environmental Safety* 171:54–65. DOI: 10.1016/j.ecoenv.2018.12.050.

Ebbs SD, Kochian LV. **1998**. Phytoextraction of zinc by oat *Avena sativa*, barley *Hordeum vulgare*, and indian mustard *Brassica juncea*. *Environmental Science & Technology* 32:802–806. DOI: 10.1021/es970698p.

Edelstein M, Ben-Hur M. **2018**. Heavy metals and metalloids: Sources, risks and strategies to reduce their accumulation in horticultural crops. *Scientia Horticulturae* 234:431–444. DOI: 10.1016/j.scienta.2017.12.039.

Emamverdian A, Ding Y, Mokhberdoran F, Xie Y. **2015**. Heavy metal stress and some mechanisms of plant defense response. *Scientific World Journal* 2015. DOI: 10.1155/2015/756120.

Eurostat. **2023**. Agricultural production - crops. *Dostępne na: https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Agricultural_production_-_crops* (Dostęp 13 styczeń 2024).

Fang Y, Xiong L. **2015**. General mechanisms of drought response and their application in drought resistance improvement in plants. *Cellular and Molecular Life Sciences* 72:673–689. DOI: 10.1007/s00018-014-1767-0.

Fatima N, Ahmad N, Anis M. **2011**. Enhanced *in vitro* regeneration and change in photosynthetic pigments, biomass and proline content in *Withania somnifera* L. (Dunal) induced by copper and zinc ions. *Plant Physiology and Biochemistry* 49:1465–1471. DOI: 10.1016/j.plaphy.2011.08.011.

Feng M, Yu Q, Chen Y, Fu Z, Xu L, Guo J. **2022**. *ScMT10*, a metallothionein-like gene from sugarcane, enhances freezing tolerance in *Nicotiana tabacum* transgenic plants. *Environmental and Experimental Botany* 194:104750. DOI: 10.1016/J.ENVEXPBOT.2021.104750.

Fernandes LB, Ghag SB. **2022**. Molecular insights into the jasmonate signaling and associated defense responses against wilt caused by *Fusarium oxysporum*. *Plant Physiology and Biochemistry* 174:22–34. DOI: 10.1016/j.plaphy.2022.01.032.

Fernàndez-Martínez J, Zacchini M, Fernández-Marín B, García-Plazaola JI, Fleck I. **2014**. Gas-exchange, photo- and antioxidant protection, and metal accumulation in I-214

and Eridano *Populus* sp. clones subjected to elevated zinc concentrations. *Environmental and Experimental Botany* 107:144–153. DOI: 10.1016/j.envexpbot.2014.06.004.

Freisinger E. **2008**. Plant MTs - Long neglected members of the metallothionein superfamily. *Dalton Transactions*: 6663–6675. DOI: 10.1039/b809789e.

Gao C, Gao K, Yang H, Ju T, Zhu J, Tang Z, Zhao L, Chen Q. **2022**. Genome-wide analysis of metallothionein gene family in maize to reveal its role in development and stress resistance to heavy metal. *Biological Research* 55:1–13. DOI: 10.1186/s40659-021-00368-w.

Garstecka Z, Antoszewski M, Mierek-Adamska A, Krauklis D, Niedojadło K, Kaliska B, Hrynkiewicz K, Dąbrowska GB. **2023**. *Trichoderma viride* colonizes the roots of *Brassica napus* L., alters the expression of stress-responsive genes, and increases the yield of canola under field conditions during drought. *International Journal of Molecular Sciences* 24:15349. DOI: 10.3390/ijms242015349.

Ghorbel M, Zribi I, Chihaoui M, Alghamidi A, Mseddi K, Brini F. **2023**. Genome-wide investigation and expression analysis of the catalase gene family in oat plants (*Avena sativa* L.). *Plants* 12:3694. DOI: 10.3390/plants12213694.

Ghori N-H, Ghori T, Hayat MQ, Imadi SR, Gul A, Altay V, Ozturk M. **2019**. Heavy metal stress and responses in plants. *International Journal of Environmental Science and Technology* 16:1807–1828. DOI: 10.1007/s13762-019-02215-8.

Ghosh D, Gupta A, Mohapatra S. **2019**. A comparative analysis of exopolysaccharide and phytohormone secretions by four drought-tolerant rhizobacterial strains and their impact on osmotic-stress mitigation in *Arabidopsis thaliana*. *World Journal of Microbiology and Biotechnology* 35. DOI: 10.1007/s11274-019-2659-0.

Gill SS, Tuteja N. **2010**. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry: PPB* 48:909–930. DOI: 10.1016/j.plaphy.2010.08.016.

Główny Urząd Statystyczny, Departament Rolnictwa i Środowiska. **2023**. Rolnictwo w 2022 r.

Gong DS, Xiong YC, Ma BL, Wang TM, Ge JP, Qin XL, Li PF, Kong HY, Li ZZ, Li FM. **2010**. Early activation of plasma membrane H+-ATPase and its relation to drought adaptation in two contrasting oat (*Avena sativa* L.) genotypes. *Environmental and Experimental Botany* 69:1–8. DOI: 10.1016/j.envexpbot.2010.02.011.

Gu CS, Liu LQ, Zhao YH, Deng YM, Zhu XD, Huang SZ. **2014**. Overexpression of *Iris. lactea* var. chinensis metallothionein *llMT2a* enhances cadmium tolerance in *Arabidopsis thaliana*. *Ecotoxicology and Environmental Safety* 105:22–28. DOI: 10.1016/j.ecoenv.2014.04.002.

Guo WW-JJWJ, Bundithya W, Goldsbrough PB. **2003**. Characterization of the *Arabidopsis* metallothionein gene family: Tissue-specific expression and induction during senescence and in response to copper. *New Phytologist* 159:369–381. DOI: 10.1046/j.1469-8137.2003.00813.x.

Gutiérrez-Ginés MJ, Pastor J, Hernández AJ. **2010**. Effect of heavy metals from mine soils on *Avena sativa* L. and education strategies. *Fresenius Environmental Bulletin* 19:2083–2086.

Hakimi AA, Monneveux P, Galiba G. **1995**. Soluble sugars, proline, and relative water content (RCW) as traits for improving drought tolerance and divergent selection for RCW from *T. polonicum*. *Journal of Genetics and Breeding* 49:237–244.

Hanson AD, Rathinasabapathi B, Rivoal J, Burnet M, Dillon M 0 O 0, Gage DA. **1994**. Osmoprotective compounds in the *Plumbaginaceae*: a natural experiment in metabolic engineering of stress tolerance. *Proceedings of the National Academy of Sciences of the United States of America* 91:306–310. DOI: 10.1073/pnas.91.1.306.

Hassinen VH, Tervahauta AI, Schat H, Kärenlampi SO. **2011**. Plant metallothioneins - metal chelators with ROS scavenging activity? *Plant Biology* 13:225–232. DOI: 10.1111/j.1438-8677.2010.00398.x.

Hegelund JN, Schiller M, Kichey T, Hansen TH, Pedas P, Husted S, Schjoerring JK. **2012**. Barley metallothioneins: MT3 and MT4 are localized in the grain aleurone layer and show differential zinc binding. *Plant Physiology* 159:1125–1137. DOI: 10.1104/pp.112.197798.

Hoekstra FA, Golovina EA, Buitink J. **2001**. Mechanisms of plant desiccation tolerance. *Trends in Plant Science* 6:431–438. DOI: 10.1016/S1360-1385(01)02052-0.

Hrynkiewicz K, Dąbrowska G, Baum C, Niedojadlo K, Leinweber P. **2012**. Interactive and single effects of ectomycorrhiza formation and *Bacillus cereus* on metallothionein *MT1* expression and phytoextraction of Cd and Zn by willows. *Water, Air, and Soil Pollution* 223:957–968. DOI: 10.1007/s11270-011-0915-5.

Hsieh H-M, Liu W-K, Huang PC. **1995**. A novel stress-inducible metallothionein-like gene from rice. *Plant Molecular Biology* 28:381–389. DOI: 10.1007/BF00020388.

Hsu S-Y, Kao CH. **2003**. Differential effect of sorbitol and polyethylene glycol on antioxidant enzymes in rice leaves. *Plant Growth Regulation* 39:83–90. DOI: 10.1023/A:1021830926902.

Hura T, Dziurka M, Hura K, Ostrowska A, Dziurka K. **2016**. Different allocation of carbohydrates and phenolics in dehydrated leaves of *triticale*. *Journal of Plant Physiology* 202:1–9. DOI: 10.1016/J.JPLPH.2016.06.018.

Hura T, Hura K, Grzesiak S. **2008**. Contents of total phenolics and ferulic acid, and pal activity during water potential changes in leaves of maize single-cross hybrids of different drought tolerance. *Journal of Agronomy and Crop Science* 194:104–112. DOI: 10.1111/J.1439-037X.2008.00297.X.

Isani G, Carpenè E. **2014**. Metallothioneins, unconventional proteins from unconventional animals: a long journey from nematodes to mammals. *Biomolecules* 4:435–457. DOI: 10.3390/biom4020435.

Jain R, Srivastava S, Solomon S, Shrivastava AK, Chandra A. **2010**. Impact of excess zinc on growth parameters, cell division, nutrient accumulation, photosynthetic pigments and oxidative stress of sugarcane (*Saccharum* spp.). *Acta Physiologiae Plantarum* 32:979–986. DOI: 10.1007/s11738-010-0487-9.

Jaiswal PS, Mittal N, Randhawa GS. **2018**. *Cyamopsis tetragonoloba* type 1 metallothionein (*CtMT1*) gene is upregulated under drought stress and its protein product has an additional C-X-C motif and unique metal binding pattern. *International Journal of Biological Macromolecules* 119:1324–1334. DOI: 10.1016/j.ijbiomac.2018.08.027.

Jiang W, Jiang C, Yuan W, Zhang M, Fang Z, Li Y, Li G, Jia J, Yang Z. **2021**. „A universal karyotypic system for hexaploid and diploid *Avena* species brings oat cytogenetics into the genomics era. *BMC Plant Biology* 21:1–15. DOI: 10.1186/s12870-021-02999-3.

Joshi R, Pareek A, Singla-Pareek SL. **2016**. Chapter 9 - Plant metallothioneins: classification, distribution, function, and regulation. W: Ahmad P ed. *Plant Metal Interaction*. Elsevier, 239–261. DOI: 10.1016/B978-0-12-803158-2.00009-6.

Juzoń K, Skrzypek E, Czyczło-Mysza I, Marcińska I. **2013**. Effect of soil drought on the yield structure, protein and phenolics content in *Pisum sativum* and *Lupinus luteus*. *Acta Agronomica Hungarica* 61, 4:267-278. DOI: 10.1556/AAgr.61.2013.4.3.

Kahlon T, Chow FI. **1997**. Hypocholesterolemic effects of oat, rice, and barley dietary fibers and fractions. *Cereal Foods World*.

Kamal N, Tsardakas Renhuldt N, Bentzer J, Gundlach H, Haberer G, Juhász A, Lux T, Bose U, Tye-Din JA, Lang D, van Gessel N, Reski R, Fu Y-B, Spégel P, Ceplitis A, Himmelbach A, Waters AJ, Bekele WA, Colgrave ML, Hansson M, Stein N, Mayer KFX, Jellen EN, Maughan PJ, Tinker NA, Mascher M, Olsson O, Spannagl M, Sirijovski N. **2022**. The mosaic oat genome gives insights into a uniquely healthy cereal crop. *Nature* 606:113–119. DOI: 10.1038/s41586-022-04732-y.

Kaur H, Manna M, Thakur T, Gautam V, Salvi P. **2021**. Imperative role of sugar signaling and transport during drought stress responses in plants. *Physiologia Plantarum* 171:833–848. DOI: 10.1111/ppl.13364.

Kawashima I, Kennedy TD, Chino M, Lane BG. **1992**. Wheat *Ec* metallothionein genes: like mammalian Zn^{2+} metallothionein genes, wheat Zn^{2+} metallothionein genes are conspicuously expressed during embryogenesis. *European Journal of Biochemistry* 209:971–976. DOI: 10.1111/j.1432-1033.1992.tb17370.x.

Kim SH, Jeong JC, Ahn YO, Lee H-S, Kwak S-S. **2014**. Differential responses of three sweet potato metallothionein genes to abiotic stress and heavy metals. *Molecular Biology Reports* 41:6957–6966. DOI: 10.1007/s11033-014-3582-y.

Kim Y-O, Kang H. **2018**. Comparative expression analysis of genes encoding metallothioneins in response to heavy metals and abiotic stresses in rice (*Oryza sativa*) and *Arabidopsis thaliana*. *Bioscience, Biotechnology, and Biochemistry* 82:1656–1665. DOI: 10.1080/09168451.2018.1486177.

Kobyletska M, Kavulych Y, Romanyuk N, Korchynska O, Terek O. **2022**. Exogenous salicylic acid modifies cell wall lignification, total phenolic content, pal-activity in wheat (*Triticum aestivum* L.) and buckwheat (*Fagopyrum esculentum* Moench) plants under cadmium chloride impact. *Biointerface Research in Applied Chemistry* 13:117. DOI: 10.33263/BRIAC132.117.

Korkmaz K, Erturk O, Ayvaz MC, Ozcan MM, Akgun M, Kirli A, Odabas Alver D. **2018**. Effect of cadmium application on antimicrobial, antioxidant and total phenolic content of basil genotypes. *Indian Journal of Pharmaceutical Education and Research* 52:s108–s114. DOI: 10.5530/ijper.52.4s.84.

Koszucka AM, Dąbrowska G. **2006**. Plant metallothioneins. *Postępy Biologii Komórki* 33:285–302.

Kranner I, Colville L. **2011**. Metals and seeds: Biochemical and molecular implications and their significance for seed germination. *Environmental and Experimental Botany* 72:93–105. DOI: 10.1016/j.envexpbot.2010.05.005.

Krezel A, Maret W. **2008**. Thionein/metallothionein control Zn(II) availability and the activity of enzymes. *Journal of Biological Inorganic Chemistry* 13:401–409. DOI: 10.1007/s00775-007-0330-y.

Krick T, Verstraete N, Alonso LG, Shub DA, Ferreiro DU, Shub M, Sánchez IE. **2014**. Amino acid metabolism conflicts with protein diversity. *Molecular Biology and Evolution* 31:2905–2912. DOI: 10.1093/molbev/msu228.

Krishna AV, Mehera B, Kumar P. **2023**. Effect of plant growth regulators and zinc on growth and yield of baby corn (*Zea mays* L.). *International Journal of Plant & Soil Science* 35:78–83. DOI: 10.9734/ijpss/2023/v35i72865.

Król A, Amarowicz R, Weidner S. **2014**. Changes in the composition of phenolic compounds and antioxidant properties of grapevine roots and leaves (*Vitis vinifera* L.) under continuous of long-term drought stress. *Acta Physiologiae Plantarum* 36:1491–1499. DOI: 10.1007/s11738-014-1526-8.

Kumar G, Kushwaha HR, Panjabi-Sabharwal V, Kumari S, Joshi R, Karan R, Mittal S, Pareek SLS, Pareek A. **2012**. Clustered metallothionein genes are co-regulated in rice and ectopic expression of *OsMT1e-P* confers multiple abiotic stress tolerance in tobacco via ROS scavenging. *BMC Plant Biology* 12:1. DOI: 10.1186/1471-2229-12-107.

Kumar D, Ohri P. **2023**. Say NO to plant stresses: Unravelling the role of nitric oxide under abiotic and biotic stress. *Nitric Oxide* 130:36–57. DOI: 10.1016/j.niox.2022.11.004.

Kumar S, Yadav A, Verma R, Dubey AK, Narayan S, Pandey A, Sahu A, Srivastava S, Sanyal I. **2022**. Metallothionein (MT1): A molecular stress marker in chickpea enhances drought and heavy metal stress adaptive efficacy in transgenic plants. *Environmental and Experimental Botany* 199. DOI: 10.1016/j.envexpbot.2022.104871.

Lai Q, Bao Z, Zhu Z, Qian Q, Mao B. **2007**. Effects of osmotic stress on antioxidant enzymes activities in leaf discs of *PSAG12-IPT* modified gerbera. *Journal of Zhejiang University. Science. B* 8:458–464. DOI: 10.1631/jzus.2007.B0458.

Lane B, Kajioka R, Kennedy T. **1987**. The wheat-germ Ec protein is a zinc-containing metallothionein. *Biochemistry and Cell Biology. 65:1001-1005.*

Latif F, Ullah F, Mehmood S, Khattak A, Khan AU, Khan S, Husain I. **2016**. Effects of salicylic acid on growth and accumulation of phenolics in *Zea mays* L. under drought stress. *Acta Agriculturae Scandinavica, Section B — Soil & Plant Science* 66:325–332. DOI: 10.1080/09064710.2015.1117133.

Legutko R. **2017**. Tales z Miletu o wodzie. *Peitho / Examina Antiqua 1* 8.

Leišová-Svobodová L, Sovová T, Dvořáček V. 2022. Analysis of oat seed transcriptome with regards to proteins involved in celiac disease. *Scientific Reports* 12:8660. DOI: 10.1038/s41598-022-12711-6.

Leszczyszyn OI, Imam HT, Blindauer CA. **2013**. Diversity and distribution of plant metallothioneins: A review of structure, properties and functions. *Metallomics* 5:1146–1169. DOI: 10.1039/c3mt00072a.

Leszczyszyn OI, Schmid R, Blindauer CA. **2007**. Toward a property/function relationship for metallothioneins: Histidine coordination and unusual cluster composition in a zinc-metallothionein from plants. *Proteins* 68:922–935. DOI: 10.1002/prot.21463.

Li Z, Zhang Y, Zhang X, Peng Y, Merewitz E, Ma X, Huang L, Yan Y. **2016**. The alterations of endogenous polyamines and phytohormones induced by exogenous application of spermidine regulate antioxidant metabolism, metallothionein and relevant genes conferring drought tolerance in white clover. *Environmental and Experimental Botany* 124:22–38. DOI: 10.1016/j.envexpbot.2015.12.004.

Lichtenthaler HK. **1987**. Chlorophylls and Carotenoids: Pigments of Photosynthetic Biomembranes. *Methods in Enzymology* 148:350–382.

Ling L, Li M, Chen N, Xie X, Han Z, Ren G, Yin Y, Jiang H. **2023**. Genome-wide identification of *NAC* gene family and expression analysis under abiotic stresses in *Avena sativa*. *Genes* 14:1186. DOI: 10.3390/GENES14061186.

Liu J, Hasanuzzaman M, Wen H, Zhang J, Peng T, Sun H, Zhao Q. **2019**. High temperature and drought stress cause abscisic acid and reactive oxygen species accumulation and suppress seed germination growth in rice. *Protoplasma* 256:1217–1227. DOI: 10.1007/s00709-019-01354-6.

Liu K, Ju Z, Jia Z, Liang G, Ma X, Liu W. **2022**. Genome-wide identification and characterization of the oat (*Avena sativa* L.) *WRKY* transcription factor family. *Genes* 13:1918. DOI: 10.3390/genes13101918.

Liu S, Lv Z, Liu Y, Li L, Zhang L. **2018**. Network analysis of ABA-dependent and ABA-independent drought responsive genes in *Arabidopsis thaliana*. *Genetics and Molecular Biology* 41:624–637. DOI: 10.1590/1678-4685-gmb-2017-0229.

Liu J, Zhang J, Kim SH, Lee HS, Marinoia E, Song WY. **2021**. Characterization of *Brassica rapa* metallothionein and phytochelatin synthase genes potentially involved in heavy metal detoxification. *PLoS ONE* 16:e0252899. DOI: 10.1371/JOURNAL.PONE.0252899.

Lloyd JC, Zakhleniuk OV. **2004**. Responses of primary and secondary metabolism to sugar accumulation revealed by microarray expression analysis of the *Arabidopsis* mutant, *pho3*. *Journal of Experimental Botany* 55:1221–1230. DOI: 10.1093/jxb/erh143.

Lowe NM. **2021**. The global challenge of hidden hunger: perspectives from the field. *Proceedings of the Nutrition Society* 80:283–289. DOI: 10.1017/S0029665121000902.

Lü S, Gu H, Yuan X, Wang X, Wu AM, Qu L, Liu JY. **2007**. The GUS reporter-aided analysis of the promoter activities of a rice metallothionein gene reveals different regulatory regions responsible for tissue-specific and inducible expression in transgenic *Arabidopsis*. *Transgenic Research* 16:177–191. DOI: 10.1007/S11248-006-9035-1/FIGURES/4.

Maghsoodi M, Razmjoo J. **2015**. Identify physiological markers for drought tolerance in alfalfa. *Agronomy Journal* 107:149–157. DOI: 10.2134/AGRONJ14.0255.

Mahmood T, Khalid S, Abdullah M, Ahmed Z, Shah MKN, Ghafoor A, Du X. **2019**. Insights into drought stress signaling in plants and the molecular genetic basis of cotton drought tolerance. *Cells* 9. DOI: 10.3390/cells9010105.

Mahmoudi H, Salah IB, Zaouali W, Zorrig W, Smaoui A, Ali T, Gruber M, Ouerghi Z, Hosni K. **2021**. Impact of zinc excess on germination, growth parameters and oxidative stress of sweet basil (*Ocimum basilicum* L.). *Bulletin of Environmental Contamination and Toxicology* 106:899–907. DOI: 10.1007/s00128-021-03188-6.

Mansoor S, Ali Wani O, Lone JK, Manhas S, Kour N, Alam P, Ahmad A, Ahmad P. **2022**. Reactive oxygen species in plants: from source to sink. *Antioxidants* 11:225. DOI: 10.3390/antiox11020225.

Manzoor H, Mehwish, Bukhat S, Rasul S, Rehmani MIA, Noreen S, Athar H-R, Zafar ZU, Skalicky M, Soufan W, Brestic M, Habib-ur-Rahman M, Ogbaga CC, EL Sabagh A. **2022**. Methyl jasmonate alleviated the adverse effects of cadmium stress in pea (*Pisum sativum* L.): A nexus of photosystem II activity and dynamics of redox balance. *Frontiers in Plant Science* 13. DOI: 10.3389/fpls.2022.860664.

Marchel M, Kaniuczak J, Hajduk E, Właśniewski S. **2018.** Response of oat (*Avena sativa*) to the addition cadmium to soil inoculation with the genus *Trichoderma* fungi. *Journal of Elementology* 2. DOI: 10.5601/jelem.2017.22.1.1391.

Marcińska I, Czyczyło-Mysza I, Skrzypek E, Filek M, Grzesiak S, Grzesiak MT, Janowiak F, Hura T, Dziurka M, Dziurka K, Nowakowska A, Quarrie SA. **2013**. Impact of osmotic stress on physiological and biochemical characteristics in drought-susceptible and drought-resistant wheat genotypes. *Acta Physiologiae Plantarum* 35:451–461. DOI: 10.1007/s11738-012-1088-6.

Marichali A, Dallali S, Ouerghemmi S, Sebei H, Casabianca H, Hosni K. **2016**. Responses of *Nigella sativa* L. to zinc excess: focus on germination, growth, yield and yield components, lipid and terpene metabolism, and total phenolics and antioxidant activities. *Journal of Agricultural and Food Chemistry* 64:1664–1675. DOI: 10.1021/acs.jafc.6b00274.

Marino SM, Gladyshev VN. **2010**. Cysteine function governs its conservation and degeneration and restricts its utilization on protein surfaces. *Journal of Molecular Biology* 404:902–916. DOI: 10.1016/j.jmb.2010.09.027.

McKenna IM, Chaney RL, Williams FM. **1993**. The effects of cadmium and zinc interactions on the accumulation and tissue distribution of zinc and cadmium in lettuce and spinach. *Environmental Pollution* 79:113–120. DOI: 10.1016/0269-7491(93)90060-2.

Meher, Shivakrishna P, Ashok Reddy K, Manohar Rao D. **2018**. Effect of PEG-6000 imposed drought stress on RNA content, relative water content (RWC), and chlorophyll content in peanut leaves and roots. *Saudi Journal of Biological Sciences* 25:285–289. DOI: 10.1016/j.sjbs.2017.04.008.

Mekawy AMM, Assaha DVM, Ueda A. **2020**. Constitutive overexpression of rice metallothionein-like gene *OsMT-3a* enhances growth and tolerance of *Arabidopsis* plants to a combination of various abiotic stresses. *Journal of Plant Research* 133:429–440. DOI: 10.1007/s10265-020-01187-y.

Michalski T, Idziak R, Menzel L. **1999**. Wpływ warunków pogodowych na plonowanie owsa. *Żywność* 1:46–52.

Mierek-Adamska A, Dąbrowska GB, Blindauer CA. **2018**. The type 4 metallothionein from *Brassica napus* seeds folds in a metal-dependent fashion and favours zinc over other metals. *Metallomics* 10:1430–1443. DOI: 10.1039/c8mt00161h.

Mierek-Adamska A, Kotowicz K, Goc A, Boniecka J, Berdychowska J, Dąbrowska GB. **2019**. Potential involvement of rapeseed (*Brassica napus* L.) metallothioneins in the hydrogen peroxide-induced regulation of seed vigour. *Journal of Agronomy and Crop Science* 205:598–607. DOI: 10.1111/jac.12361.

Mierek-Adamska A, Tylman-Mojzeszek W, Znajewska Z, Dąbrowska GB. **2017**. Metalotioneiny bakteryjne. *Postępy Mikrobiologii* 56:171–179.

Miętus M. **2023**. Charakterystyka wybranych elementów klimatu w Polsce w 2022 roku – podsumowanie. *Dostępne na: https://imgw.pl/wydarzenia/charakterystyka-wybranych-elementow-klimatu-w-polsce-w-2022-roku-podsumowanie*

Miles TD, Day B, Schilder AC. **2011**. Identification of differentially expressed genes in a resistant versus a susceptible blueberry cultivar after infection by *Colletotrichum acutatum*. *Molecular Plant Pathology* 12:463–477. DOI: 10.1111/j.1364-3703.2010.00687.x.

Mirsal IA. **2004**. *Soil Pollution Origin, Monitoring & Remediation*. Berlin: Springer Berlin.

Miseta A, Csutora P. **2000**. Relationship between the occurrence of cysteine in proteins and the complexity of organisms. *Molecular Biology and Evolution* 17:1232–1239. DOI: 10.1093/oxfordjournals.molbev.a026406.

Mohanty AK, Misra M, Drzal LT. **2002**. Sustainable bio-composites from renewable resources: opportunities and challenges in the green materials world. *Journal of Polymers and the Environment* 10:19–26. DOI: 10.1023/A:1021013921916.

Möller A, Müller HW, Abdullah A, Abdelgawad G, Utermann J. **2005**. Urban soil pollution in Damascus, Syria: Concentrations and patterns of heavy metals in the soils of the Damascus Ghouta. *Geoderma* 124:63–71. DOI: 10.1016/j.geoderma.2004.04.003.

Mordaka P, Dąbrowska G. **2007**. Różnorodność i regulacja kanałów wodnych w świecie roślin. *Postępy Biochemii* 53:84–90.

Morris JL, Puttick MN, Clark JW, Edwards D, Kenrick P, Pressel S, Wellman CH, Yang Z, Schneider H, Donoghue PCJ. **2018**. The timescale of early land plant evolution. *Proceedings of the National Academy of Sciences* 115:E2274–E2283. DOI: 10.1073/pnas.1719588115.

Moustakas NK, Akoumianaki -Ioannidou A., Barouchas PE. **2011**. The effects of cadmium and zinc interactions on the concentration of cadmium and zinc in pot marigold (*Calendula officinalis* L.). *Australian Journal of Crop Science* 5:277–282. DOI: 10.3316/informit.279657561038210.

Moyle R, Fairbairn DJ, Ripi J, Crowe M, Botella JR. **2005**. Developing pineapple fruit has a small transcriptome dominated by metallothionein. *Journal of Experimental Botany* 56:101–112. DOI: 10.1093/jxb/eri015.

Możdżeń K, Bojarski B, Rut G, Migdałek G, Repka P, Rzepka A. **2015**. Effect of drought stress induced by mannitol on physiological parameters of maize (*Zea mays* L.) seedlings and plants. *Journal of Microbiology, Biotechnology and Food Sciences* 04:86–91. DOI: 10.15414/jmbfs.2015.4.special2.86-91.

Munteanu IG, Apetrei C. **2021**. Analytical methods used in determining antioxidant activity: a review. *International Journal of Molecular Sciences* 22:3380. DOI: 10.3390/ijms22073380.

Muradoglu F, Gundogdu M, Ercisli S, Encu T, Balta F, Jaafar HZ, Zia-Ul-Haq M. **2015**. Cadmium toxicity affects chlorophyll a and b content, antioxidant enzyme activities and mineral nutrient accumulation in strawberry. *Biological Research* 48:11. DOI: 10.1186/s40659-015-0001-3.

Murtaza G, Javed W, Hussain A, Qadir M, Aslam M. **2017**. Soil-applied zinc and copper suppress cadmium uptake and improve the performance of cereals and legumes. *International Journal of Phytoremediation* 19:199–206. DOI: 10.1080/15226514.2016.1207605.

Nadarajah KK. **2020**. ROS homeostasis in abiotic stress tolerance in plants. *International Journal of Molecular Sciences* 21:1–29. DOI: 10.3390/IJMS21155208.

Ngole VM, Ekosse GIE. **2012**. Copper, nickel and zinc contamination in soils within the precincts of mining and landfilling environments. *International Journal of Environmental Science and Technology* 9:485–494. DOI: 10.1007/s13762-012-0055-5.

Ozturk ZN, Talamé V, Deyholos M, Michalowski CB, Galbraith DW, Gozukirmizi N, Tuberosa R, Bohnert HJ. **2002**. Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. *Plant Molecular Biology* 48:551–573. DOI: 10.1023/A:1014875215580.

Pan J, Ju Z, Ma X, Duan L, Jia Z. **2024**. Genome-wide characterization of *TCP* family and their potential roles in abiotic stress resistance of oat (*Avena sativa* L.). *Frontiers in Plant Science* 15. DOI: 10.3389/fpls.2024.1382790.

Pan J, Zhou Q, Wang H, Chen Y, Wang Z, Zhang J. **2023**. Genome-wide identification and characterization of abiotic stress responsive *GRAS* family genes in oat (*Avena sativa*). *PeerJ* 11:e15370. DOI: 10.7717/PEERJ.15370.

Pan Y, Zhu M, Wang S, Ma G, Huang X, Qiao C, Wang R, Xu X, Liang Y, Lu K, Li J, Qu C. **2018**. Genome-wide characterization and analysis of metallothionein family genes that function in metal stress tolerance in *Brassica napus* L. *International Journal of Molecular Sciences* 19:1–18. DOI: 10.3390/ijms19082181.

Patankar HV, Al-Harrasi I, Al Kharusi L, Jana GA, Al-Yahyai R, Sunkar R, Yaish MW. **2019**. Overexpression of a metallothionein 2A gene from date palm confers abiotic stress tolerance to yeast and *Arabidopsis thaliana*. *International Journal of Molecular Sciences* 20. DOI: 10.3390/ijms20122871.

Pawłowski F, Deryło S. **1988**. Plonowanie i wartość przedplonowa owsa w zmianowaniach o zróżnicowanej koncentracji zbóż. *Zeszyty Problemowe Postępów Nauk Rolniczych* 331:101–109.

PepsiCo. **2021**. *Avena sativa* - OT3098 v2, PepsiCo, https://wheat.pw.usda.gov/jb?data=/ggds/oat-ot3098v2-pepsico. *Dostępne na: https://wheat.pw.usda.gov/jb/?data=%2Fggds%2Foat-ot3098v2-pepsico&loc=chr7A%3A5130802..5132383&tracks=genes&highlight=* (Dostęp 1 Maj 2022).

Petering DH, Zhu J, Krezoski S, Meeusen J, Kiekenbush C, Krull S, Specher T, Dughish M. **2006**. Apo-metallothionein emerging as a major player in the cellular activities of metallothionein. *Experimental Biology and Medicine (Maywood, N.J.)* 231:1528–1534. DOI: 10.1177/153537020623100912.

Poole LB. **2015**. The basics of thiols and cysteines in redox biology and chemistry. *Free Radical Biology and Medicine* 80:148–157. DOI: 10.1016/j.freeradbiomed.2014.11.013.

Pourjalali Z, Shahpiri A, Golkar P. **2022**. Barley metallothionein isoforms, MT2b2 and MT4, differentially respond to phytohormones in barley aleurone layer and their recombinant forms show different affinity for binding to zinc and cadmium. *BioMetals* 36:3–18. DOI: 10.1007/S10534-022-00452-Y/FIGURES/7.

Puga AP, Abreu CA, Melo LCA, Beesley L. **2015**. Biochar application to a contaminated soil reduces the availability and plant uptake of zinc, lead and cadmium. *Journal of Environmental Management* 159:86–93. DOI: 10.1016/j.jenvman.2015.05.036.

Pusz A, Wiśniewska M, Rogalski D. **2021**. Assessment of the accumulation ability of *Festuca rubra* L. and *Alyssum saxatile* L. tested on soils contaminated with Zn, Cd, Ni, Pb, Cr, and Cu. *Resources* 10:46. DOI: 10.3390/resources10050046.

Qian L, Dawar K, Ullah I, Irfan M, Zhang Z, Mian IA, Khan B, Gul N, Fahad S, Jalal A, Danish S, Iqbal RK, Alarfaj AA. **2023**. Zinc foliar application mitigates cadmium-induced growth inhibition and enhances wheat growth, chlorophyll contents, and yield. *ACS Omega* 8:32372–32381. DOI: 10.1021/acsomega.3c01511.

Rabêlo FHS, Borgo L. **2016**. Changes caused by heavy metals in micronutrient content and antioxidant system of forage grasses used for phytoremediation: an overview. *Ciência Rural* 46:1368–1375. DOI: 10.1590/0103-8478cr20151291.

Rafati Rahimzadeh M, Rafati Rahimzadeh M, Kazemi S, Moghadamnia A. **2017**. Cadmium toxicity and treatment: An update. *Caspian Journal of Internal Medicine* 8:135–145. DOI: 10.22088/cjim.8.3.135.

Raza A, Razzaq A, Mehmood SS, Zou X, Zhang X, Lv Y, Xu J. **2019**. Impact of climate change on crops adaptation and strategies to tackle its outcome: a review. *Plants* 8:34. DOI: 10.3390/plants8020034.

Razavi F, Pollet B, Steppe K, van Labeke MC. **2008**. Chlorophyll fluorescence as a tool for evaluation of drought stress in strawberry. *Photosynthetica* 46:631–633. DOI: 10.1007/s11099-008-0108-7.

Ren Y, Liu Y, Chen H, Li G, Zhang X, Zhao J. **2012**. Type 4 *metallothionein* genes are involved in regulating Zn ion accumulation in late embryo and in controlling early seedling growth in *Arabidopsis*. *Plant, Cell & Environment* 35:770–789. DOI: 10.1111/j.1365-3040.2011.02450.x.

Reyes JAO, Casas DE, Gandia JL, Parducho MJL, Renovalles EM, Quilloy EP, Delfin EF. **2023**. Polyethylene glycol-induced drought stress screening of selected Philippine high-yielding sugarcane varieties. *Journal of Agriculture and Food Research* 14:100676. DOI: 10.1016/j.jafr.2023.100676.

Rolka E. **2015**. Effect of soil contamination with cadmium and application of neutralizing substances on the yield of oat (*Avena sativa* L.) and the uptake of cadmium by this crop. *Journal of Elementology* 20:975–986. DOI: 10.5601/jelem.2014.19.4.810.

Ryu M-S, Aydemir TB. **2020**. Chapter 23 - Zinc. W: Marriott BP, Birt DF, Stallings VA, Yates AA eds. *Present Knowledge in Nutrition (Eleventh Edition)*. Academic Press, 393–408. DOI: 10.1016/B978-0-323-66162-1.00023-8.

Salazar MJ, Rodriguez JH, Nieto GL, Pignata ML. **2012**. Effects of heavy metal concentrations (Cd, Zn and Pb) in agricultural soils near different emission sources on quality, accumulation and food safety in soybean [*Glycine max* (L.) Merrill]. *Journal of Hazardous Materials* 233–234:244–253. DOI: 10.1016/j.jhazmat.2012.07.026.

Samardžić JT, Nikolić DB, Timotijević GS, Jovanović ŽS, Milisavljević ME, Maksimović VR. **2010**. Tissue expression analysis of *FeMT3*, a drought and oxidative stress related metallothionein gene from buckwheat (*Fagopyrum esculentum*). *Journal of Plant Physiology* 167:1407–1411. DOI: 10.1016/j.jplph.2010.05.016.

Sandro P, Bhatta M, Bower A, Carlson S, Jannink J-L, Waring DJ, Birkett C, Smith K, Wiersma J, Caffe M, Kleinjan J, McMullen MS, English L, Gutierrez L. **2024**. Genomic prediction for targeted populations of environments in oat (*Avena sativa*). *Crop and Pasture Science* 75. DOI: 10.1071/CP23126.

Schiller M, Hegelund JN, Pedas P, Kichey T, Laursen KH, Husted S, Schjoerring JK. **2014**. Barley metallothioneins differ in ontogenetic pattern and response to metals. *Plant, Cell & Environment* 37:353–367. DOI: 10.1111/pce.12158.

Shi G, Cai Q. **2010**. Zinc Tolerance and accumulation in eight oil crops. *Journal of Plant Nutrition* 33:982–997. DOI: 10.1080/01904161003728669.

Singh RK, Anandhan S, Singh S, Patade VY, Ahmed Z, Pande V. **2011**. Metallothionein-like gene from *Cicer microphyllum* is regulated by multiple abiotic stresses. *Protoplasma* 248:839–847. DOI: 10.1007/s00709-010-0249-y.

Singh R, De S, Belkheir A. **2013**. *Avena sativa* (Oat), A potential neutraceutical and therapeutic agent: an overview. *Critical Reviews in Food Science and Nutrition* 53:126–144. DOI: 10.1080/10408398.2010.526725.

Singh S, Parihar P, Singh R, Singh VP, Prasad SM. **2016**. Heavy metal tolerance in plants: role of transcriptomics, proteomics, metabolomics, and ionomics. *Frontiers in Plant Science* 6:1–36. DOI: 10.3389/fpls.2015.01143.

Spiss L. **2003**. Historia hodowli owsa w Polsce. *Biuletyn Instytutu Hodowli i Aklimatyzacji Roślin* 229:7–11.

Stefanowicz AM, Kapusta P, Zubek S, Stanek M, Woch MW. **2020**. Soil organic matter prevails over heavy metal pollution and vegetation as a factor shaping soil microbial communities at historical Zn–Pb mining sites. *Chemosphere* 240. DOI: 10.1016/j.chemosphere.2019.124922.

Storey KB, Storey JM. **2000**. *Environmental stressors and gene responses*. Elsevier.

Swigonska S, Amarowicz R, Król A, Mostek A, Badowiec A, Weidner S. **2014**. Influence of abiotic stress during soybean germination followed by recovery on the phenolic compounds of radicles and their antioxidant capacity. *Acta Societatis Botanicorum Poloniae* 83:209–218. DOI: 10.5586/asbp.2014.026.

Szmidt-Jaworska A, Kopcewicz J. **2024**. *Fizjologia roślin*. Warszawa: Wydawnictwo Naukowe PWN.

Szydłowska-Czerniak A, Łaszewska A. **2015**. Effect of refining process on antioxidant capacity, total phenolics and prooxidants contents in rapeseed oils. *LWT Lebensmittel-Wissenschaft & Technologie* 64:853–859. DOI: 10.1016/j.lwt.2015.06.069.

Taghavi Ghasemkheili F, Ekelund F, Johansen JL, Pirdashti H, Ghadirnezhad Shiade SR, Fathi A, Kjøller R. **2022**. Ameliorative effects of *Trichoderma harzianum* and rhizosphere soil microbes on cadmium biosorption of barley (*Hordeum vulgare* L.) in Cd-polluted soil. *Journal of Soil Science and Plant Nutrition* 22:527–539. DOI: 10.1007/s42729-021-00666-y.

Tanhuanpää P, Kalendar R, Schulman AH, Kiviharju E. **2007**. A major gene for grain cadmium accumulation in oat (*Avena sativa* L.). *Genome* 50:588–594. DOI: 10.1139/G07-036.

Tinker NA, Wight CP, Bekele WA, Yan W, Jellen EN, Renhuldt NT, Sirijovski N, Lux T, Spannagl M, Mascher M. **2022**. Genome analysis in *Avena sativa* reveals hidden breeding barriers and opportunities for oat improvement. *Communications Biology* 5. DOI: 10.1038/s42003-022-03256-5.

Tuma J, Skalicky M, Tumova L, Flidr J. **2014**. Influence of cadmium dose and form on the yield of oat (*Avena sativa* L.) and the metal distribution in the plant. *Journal of Elementology* 19. DOI: 10.5601/jelem.2014.19.3.448.

Turkan S, Mierek-Adamska A, Kulasek M, Konieczna WB, Dąbrowska GB. **2023**. New seed coating containing *Trichoderma viride* with anti-pathogenic properties. *PeerJ* 11:e15392. DOI: 10.7717/PEERJ.15392.

Tuteja N, Sopory SK. **2008**. Plant signaling in stress. *Plant Signaling & Behavior* 3:79–86.

Udawat P, Mishra A, Jha B. **2014**. Heterologous expression of an uncharacterized universal stress protein gene (*SbUSP*) from the extreme halophyte, *Salicornia brachiata*, which confers salt and osmotic tolerance to *E. coli*. *Gene* 536:163–170. DOI: 10.1016/j.gene.2013.11.020.

Ulrich K, Jakob U. **2019**. The role of thiols in antioxidant systems. *Free Radical Biology & medicine* 140:14. DOI: 10.1016/J.FREERADBIOMED.2019.05.035.

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K. **2000**. *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proceedings of the National Academy of Sciences of the United States of America* 97:11632–11637. DOI: 10.1073/pnas.190309197.

Usha B, Venkataraman G, Parida A. **2009**. Heavy metal and abiotic stress inducible metallothionein isoforms from *Prosopis juliflora* (SW) D.C. show differences in binding to heavy metals *in vitro*. *Molecular and Genetics Genomics* 281:99–108. DOI: 10.1007/s00438-008-0398-2.

Van Loon AF. **2015**. Hydrological drought explained. *WIREs Water* 2:359–392. DOI: 10.1002/wat2.1085.

Vašák M, Meloni G. **2011**. Chemistry and biology of mammalian metallothioneins. *Journal of Biological Inorganic Chemistry* 16:1067–1078. DOI: 10.1007/S00775-011-0799-2/FIGURES/4.

Vasiliadou S, Dordas C. **2009**. Increased concentration of soil cadmium affects on plant growth, dry matter accumulation, Cd, and Zn uptake of different tobacco cultivars (*Nicotiana tabacum* L.). *International Journal of Phytoremediation* 11:115–130. DOI: 10.1080/15226510802378400.

Vishwakarma K, Upadhyay N, Kumar N, Yadav G, Singh J, Mishra RK, Kumar V, Verma R, Upadhyay RG, Pandey M, Sharma S. **2017**. Abscisic acid signaling and abiotic stress tolerance in plants: A review on current knowledge and future prospects. *Frontiers in Plant Science* 8:161. DOI: 10.3389/FPLS.2017.00161/BIBTEX.

Wang Z, Li G, Sun H, Ma L, Guo Y, Zhao Z, Gao H, Mei L. **2018**. Effects of drought stress on photosynthesis and photosynthetic electron transport chain in young apple tree leaves. *Biology Open* 7. DOI: 10.1242/BIO.035279.

Warzecha T, Bocianowski J, Warchoł M, Bathelt R, Sutkowska A, Skrzypek E. **2023**. Effect of soil drought stress on selected biochemical parameters and yield of oat × maize addition (OMA) lines. *International Journal of Molecular Sciences* 24:13905. DOI: 10.3390/ijms241813905.

Weffort VRS, Lamounier JA. **2023**. Hidden hunger – a narrative review. *Jornal de Pediatria*. DOI: 10.1016/j.jped.2023.08.009.

Weidner S, Brosowska-Arendt W, Szczechura W, Karamać M, Kosińska A, Amarowicz R. **2011**. Effect of osmotic stress and post-stress recovery on the content of phenolics and properties of antioxidants in germinating seeds of grapevine *Vitis californica*. *Acta Societatis Botanicorum Poloniae* 80:11–19.

White CN, Rivin CJ. **1995**. Characterization and expression of a cDNA encoding a seed-specific metallothionein in maize. *Plant Physiology* 108:831–832.

Wood PJ. **1991**. Oat β-glucan-physicochemical properties and physiological effects. *Trends in Food Science & Technology* 2:311–314. DOI: 10.1016/0924-2244(91)90733-Y.

Wood PJ, Braaten J, Scott F, Riedel D, Poste L. **1990**. Comparisons of viscous properties of oat and guar gum and the effects of these and oat bran on glycemic index. *Journal of Agricultural and Food Chemistry* 38:753–757.

Wood PJ, Braaten JT, Scott FW, Riedel KD, Wolynetz MS, Collins MW. **1994**. Effect of dose and modification of viscous properties of oat gum on plasma glucose and insulin following an oral glucose load. *The British Journal of Nutrition* 72:731–743. DOI: 10.1079/bjn19940075.

Xiong L, Zhu J-K. **2002**. Molecular and genetic aspects of plant responses to osmotic stress. *Plant, Cell & Environment* 25:131–139. DOI: 10.1046/j.1365-3040.2002.00782.x.

Xu T, Niu J, Jiang Z. **2022a**. Sensing mechanisms: calcium signaling mediated abiotic stress in plants. *Frontiers in Plant Science* 13. DOI: 10.3389/fpls.2022.925863.

Xu D, Shen Z, Dou C, Dou Z, Li Y, Gao Y, Sun Q. **2022b**. Effects of soil properties on heavy metal bioavailability and accumulation in crop grains under different farmland use patterns. *Scientific Reports* 12:9211. DOI: 10.1038/s41598-022-13140-1.

Xue T, Li X, Zhu W, Wu C, Yang G, Zheng C. **2009**. Cotton metallothionein *GhMT3a*, a reactive oxygen species scavenger, increased tolerance against abiotic stress in transgenic tobacco and yeast. *Journal of Experimental Botany* 60:339–349. DOI: 10.1093/jxb/ern291.

Yadav NS, Singh VK, Singh D, Jha B. **2014**. A novel gene *SbSI-2* encoding nuclear protein from a halophyte confers abiotic stress tolerance in *E. coli* and tobacco. *PLoS ONE* 9. DOI: 10.1371/journal.pone.0101926.

Yamaguchi-Shinozaki K, Shinozaki K. **2005**. Organization of *cis*-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends in Plant Science* 10:88–94. DOI: 10.1016/j.tplants.2004.12.012.

Yan H, Bekele WA, Wight CP, Peng Y, Langdon T, Latta RG, Fu YB, Diederichsen A, Howarth CJ, Jellen EN, Boyle B, Wei Y, Tinker NA. **2016a**. High-density marker profiling confirms ancestral genomes of *Avena* species and identifies D-genome chromosomes of hexaploid oat. *Theoretical Applied Genetics* 129:2133–2149. DOI: 10.1007/s00122-016-2762-7.

Yan H, Martin SL, Bekele WA, Latta RG, Diederichsen A, Peng Y, Tinker NA. **2016b**. Genome size variation in the genus *Avena*. *Genome* 59:209–220. DOI: 10.1139/gen-2015-0132.

Yang Y, Li Y, Wang M, Chen W, Dai Y. **2021**. Limestone dosage response of cadmium phytoavailability minimization in rice: A trade-off relationship between soil pH and amorphous manganese content. *Journal of Hazardous Materials* 403:123664. DOI: 10.1016/j.jhazmat.2020.123664.

Yang Z, Wu Y, Li Y, Ling HQ, Chu C. **2009**. OsMT1a, a type 1 metallothionein, plays the pivotal role in zinc homeostasis and drought tolerance in rice. *Plant Molecular Biology* 70:219–229. DOI: 10.1007/s11103-009-9466-1.

Yang M, Zhang F, Wang F, Dong Z, Cao Q, Chen M. **2015**. Characterization of a type 1 *metallothionein* gene from the stresses-tolerant plant *Ziziphus jujuba*. *International Journal of Molecular Sciences* 16:16750–16762. DOI: 10.3390/ijms160816750.

Yao T, Zhang J, Xie M, Yuan G, Tschaplinski TJ, Muchero W, Chen J-G. **2021**. Transcriptional regulation of drought response in *Arabidopsis* and woody plants. *Frontiers in Plant Science* 11. DOI: 10.3389/fpls.2020.572137.

Yoshida T, Mogami J, Yamaguchi-Shinozaki K. **2014**. ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. *Current Opinion in Plant Biology* 21:133–139. DOI: 10.1016/j.pbi.2014.07.009.

You J, Chan Z. **2015**. ROS regulation during abiotic stress responses in crop plants. *Frontiers in Plant Science* 6:1092. DOI: https://doi.org/10.3389/fpls.2015.01092.

Yu Q, He L, Huo C, Jiang X, Chen H, Wang R, Tang M, Dong L, Chen J, Li Y, Zhu S, Liu W. **2020**. Genome-wide identification and expression analysis of heavy metal stress–responsive metallothionein family genes in *Nicotiana tabacum*. *Plant Molecular Biology Reporter* 39:443–454. DOI: 10.1007/s11105-020-01262-7.

Yuan J, Chen D, Ren Y, Zhang X, Zhao J. **2008**. Characteristic and expression analysis of a metallothionein gene, *OsMT2b*, down-regulated by cytokinin suggests functions in root development and seed embryo germination of rice. *Plant Physiology* 146:1637–1650. DOI: 10.1104/pp.107.110304.

Zaefyzadeh M, Quliyev R, Babayeva S, Abbasov M. **2009**. The effect of the interaction between genotypes and drought stress on the superoxide dismutase and chlorophyll content in durum wheat landraces. *Turkish Journal of Biology* 33:1–7. DOI: 10.3906/biy-0801-12.

Zarzecka K, Gugała M, Mystkowska I, Baranowska A, Sikorska A, Zarzecka M. **2018**. Odżywcze i prozdrowotne właściwości ziarna owsa i przetworów owsianych. *Kosmos* 67:409–414. DOI: 10.36921/kos.2018_2399.

Zhang S, Hu H, Cui S, Yan L, Wu B, Wei S. **2024**. Genome-wide identification and functional analysis of the cellulose synthase-like gene superfamily in common oat (*Avena sativa* L.). *Phytochemistry* 218:113940. DOI: 10.1016/j.phytochem.2023.113940.

Zhang C, Shi S, Liu Z, Yang F, Yin G. **2019**. Drought tolerance in alfalfa (*Medicago sativa* L.) varieties is associated with enhanced antioxidative protection and declined lipid peroxidation. *Journal of Plant Physiology* 232:226–240. DOI: 10.1016/j.jplph.2018.10.023.

Zhang Y, Xu J, Li R, Ge Y, Li Y, Li R. **2023**. Plants' Response to Abiotic Stress: Mechanisms and Strategies. *International Journal of Molecular Sciences* 24:10915. DOI: 10.3390/ijms241310915.

Zhang H, Zhao Y, Zhu J-K. **2020**. Thriving under Stress: how plants balance growth and the stress response. *Developmental Cell* 55:529–543. DOI: 10.1016/j.devcel.2020.10.012.

Zhi J, Liu X, Yin P, Yang R, Liu J, Xu J. **2020**. Overexpression of the metallothionein gene *PaMT3-1* from *Phytolacca americana* enhances plant tolerance to cadmium. *Plant Cell, Tissue and Organ Culture* 143:211–218. DOI: 10.1007/s11240-020-01914-2.

Zhou Y, Chu P, Chen H, Li Y, Liu J, Ding Y, Tsang EWT, Jiang L, Wu K, Huang S. **2012**. Overexpression of *Nelumbo nucifera* metallothioneins 2a and 3 enhances seed germination vigour in *Arabidopsis*. *Planta* 235:523–537. DOI: 10.1007/s00425-011-1527-4.

Zhou J, Goldsbrough PB. **1995**. Structure, organization, and expression of the metallothionein gene family in *Arabidopsis*. *Molecular and General Genetics* 248:318–328. DOI: https://doi.org/10.1007/BF02191599.

Zhou Y, Liu J, Liu S, Jiang L, Hu L. **2019**. Identification of the metallothionein gene family from cucumber and functional characterization of *CsMT4* in *Escherichia coli* under salinity and osmotic stress. *3 Biotech* 9:1–11. DOI: 10.1007/s13205-019-1929-8.

Zhou G, Xu Y, Li J, Yang L, Liu JY. **2006**. Molecular analyses of the metallothionein gene family in rice (*Oryza sativa* L.). *Journal of Biochemistry and Molecular Biology* 39:595–606. DOI: 10.5483/bmbrep.2006.39.5.595.

Zhou GK, Xu YF, Liu JY. **2005**. Characterization of a rice class II metallothionein gene: tissue expression patterns and induction in response to abiotic factors. *Journal of Plant Physiology* 162:686–696. DOI: 10.1016/J.JPLPH.2004.11.006.

Zhou X, Yi D, Ma L, Wang X. **2024**. Genome-wide analysis and expression of the aquaporin gene family in *Avena sativa* L. *Frontiers in Plant Science* 14. DOI: 10.3389/fpls.2023.1305299.

Zhu W, Zhao DX, Miao Q, Xue TT, Li XZ, Zheng CC. **2009**. *Arabidopsis thaliana* metallothionein, *AtMT2a*, mediates ROS balance during oxidative stress. *Journal of Plant Biology* 52:585–592. DOI: 10.1007/S12374-009-9076-0/FIGURES/4.

Zia R, Nawaz MS, Siddique MJ, Hakim S, Imran A. **2021**. Plant survival under drought stress: Implications, adaptive responses, and integrated rhizosphere management strategy for stress mitigation. *Microbiological Research* 242:126626. DOI: 10.1016/j.micres.2020.126626.

Znajewska Z, Narbutt O, Dąbrowska GB, Narbutt O. **2018**. *Trichoderma viride* strains stimulating the growth and development of winter rapeseed (*Brassica napus* L.). *Progress in Plant Protection* 58:264–269. DOI: 10.14199/ppp-2018-036.

VIII. Załączniki

Załącznik 1. - Publikacja I

Załącznik 2. - Publikacja II

Załącznik 3. - Publikacja III

Załącznik 4. - Publikacja IV

Załącznik 5. - Dorobek naukowy

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DROUGHT STRESS

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The involvement of metallothioneins and stress markers in response to osmotic stress in *Avena sativa* L.

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Abstract

Osmotic stress frequently caused by drought is the most threatening environmental stress that remarkably reduces crop yield. Oat (*Avena sativa* L.) is sensitive to water deficiency during growth. Plant metallothioneins (pMTs) show tremendous promise in enhancing general stress tolerance in plants. This study aimed to verify whether pMTs and elements of the antioxidant defence system protect oat against osmotic stress. Coding and genomic regions of *A. sativa* L. *MT*s belonging to three different types were cloned. To evaluate the role of MTs in osmotic stress, the expression of genes encoding AsMT1-3 was checked by qRT-PCR in the roots and shoots of oat plants growing in a hydroponic culture in the presence of polyethylene glycol (PEG 6000) and mannitol. The expression of *AsMT1-3* changed in response to osmotic stress; however, the changes depended on the type of *MT* and the treatment. The amount of *AsMT3* transcript was about five-fold and nine-fold higher in shoots and roots, respectively, in the presence of mannitol. To further analyse the response of oat to osmotic stress, the level of phenolic compounds, soluble sugars, abscisic acid, and the activity of antioxidant enzymes were tested using spectrophotometric and chromatographic methods. During osmotic stress, the content of phenols, soluble sugars, abscisic acid, and the activity of catalase and peroxidase in shoots increased. In roots treated with PEG 6000, the amount of phenolic compounds was higher than that in roots treated with mannitol. The activity of superoxide dismutase was about 5-fold higher in roots than in shoots. MTs are involved in plant response to osmotic stress. In the future, insight provided in this study will lead to application in agriculture either by using MTs as molecular markers for stress-resistant crop cultivars or by a generation of genetically modified crops overexpressing *MT*s.

KEYWORDS
abscisic acid, antioxidant system capacity, metallothioneins, osmoprotectants, osmotic stress, photosynthetic pigments

Key points

- Three types of *Avena sativa* metallothioneins were cloned.

Wiktoria Konieczna and Agnieszka Mierek-Adamska contributed equally to this work.

- Osmotic stress increased the content of phenols, abscisic acid, sugars, and the activity of antioxidant enzymes.
- Expression of oat *MT*s correlates with the antioxidant enzymes activity and abscisic acid content.
- Oat metallothioneins can serve as markers of the occurrence of osmotic stress.

1 | INTRODUCTION

Oat (*Avena sativa* L.) is an important grain and forage crop in many parts of the world, grown on about 13.2 million hectares. It is used in the food, biomedical and cosmetics industries and as a biofuel (Barcchiya et al., 2017; Rines et al., 2006). Oat grains have the highest protein level among cereals ranging from 9% to 20% in the whole grain and high soluble fibre content (Peterson, 2015). This oat fibre can attenuate blood postprandial glycaemic and insulinemic responses, lower total blood cholesterol and help to maintain a healthy body weight (Daou & Zhang, 2012). A very important advantage of oat is the fact that this species requires less intensive fertilization and less chemical protection in comparison to the other cereals. *A. sativa* L. is also characterized by a high forecrop value, constituting a positive "anti-fatigue" element in the crop rotation. However, compared to other cereal crops, oat has higher water requirements and is more susceptible to drought (Pisulewska et al., 2011). Osmotic stress occurs due to the lowering of soil water potential, which reduced the uptake of water. It is usually caused by drought, salinity and cold. Osmotic stress leads to DNA damage, membrane distortion and protein aggregation. It affects cell division and may lead to cell apoptosis (Storey & Storey, 2000; Yoshida et al., 2014).

Osmotic stress that causes water deficiency is one of the most significant stressors for crops, such as oat (Llanes et al., 2016; Shao et al., 2009), limiting plant development from seeds to mature plants (Islam et al., 2011). Osmotic stress causes the increased production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide ion ($O_2^{\cdot-}$) and hydroxyl radical ($HO^{\cdot}$) (Szechyńska-Hebda et al., 2012). At low concentrations, ROS are essential components in cell signalling pathways, but the excessive generation of ROS results in toxicity, can damage macromolecules and ultimately might lead to cell death (Gechev et al., 2006). To minimize the deleterious effect of oxidative stress, plants have evolved a complex antioxidant system, which includes antioxidant enzymes and non-enzymatic antioxidants, for example, glutathione, ascorbate, osmoprotectants, sugars, phenolic compounds and small proteins metallothioneins (MTs; Mierek-Adamska et al., 2019; Skrzypek et al., 2008; Yang et al., 2009). Antioxidant enzymes, such as catalase (CAT), peroxidases (PXs) and superoxide dismutase (SOD), form the first line of defence against ROS, and thus, their level in plant tissues can be used as an indicator of oxidative stress. SOD catalyses the dismutation of the superoxide anion radical to oxygen (O_2) or hydrogen peroxide (Sikora & Świeca, 2018). PXs catalyse the decomposition of H_2O_2 to H_2O with a reducing agent (e.g. ascorbic acid for ascorbate peroxidase; Dąbrowska et al., 2007). CAT does not require a reducing agent, and it catalyses the dismutation of H_2O_2 to H_2O and O_2 (Alscher et al., 2002; Das & Roychoudhury, 2014). The level of oxidative stress can be measured indirectly by the assessment of the level of oxidative by-products of lipids and proteins and by the analysis of the content of non-enzymatic antioxidants such as phenolic compounds. Moreover, the accumulation of low-molecular-weight, like carbohydrates, might serve as the determinant of oxidative stress level (Chiappero et al., 2021; Mohammadkhani & Heidari, 2008; Skrzypek et al., 2008; Szymańska et al., 2019).

Environmental stresses cause changes in the level of phenolic compounds, which are considered one of the stress indicators in plants (Hura et al., 2016). Phenolic compounds, which are metabolites derived from the phenylpropanoid pathway, are the most common secondary metabolites in plants (Vogt, 2010). Plant phenolic content changes in response to water deficit; however, the nature of the response depends on species, plant organs and the drought tolerance of particular genotypes (Aninbon et al., 2016). In oat, an increase in phenolic compound content was observed under cold stress (Goyal & Kaur, 2018) and drought stress (Perveen et al., 2019).

Soluble sugars can act as osmolytes by maintaining the leaf cell turgor, protecting the integrity of membranes and preventing the denaturation of proteins (Keunen et al., 2013). It has been shown that the accumulation of sugars in plant organs occurs in response to a variety of environmental stresses. Moreover, in the selection of drought-tolerant wheat cultivar, the content of soluble sugars was proved to be a better marker than the content of proline (Nayer & Reza, 2008). Sugars such as sucrose, glucose, fructose and trehalose-6-phosphate can act as signalling molecules that can modulate plant development and stress response. They can interact with other signalling pathways or act directly (Ruan, 2014).

Sucrose plays an important role in plant metabolism at both cellular and whole-organism level (Pluskota et al., 2015). It not only participates in plant response to abiotic stresses but also serves as a nutrient and signalling molecule that modulates the expression of a wide range of genes (Gibson, 2005). Sucrose is produced primarily in the cytosol of mature leaves, but it can be resynthesized in sink tissues. Sucrose can be degraded into glucose and fructose or into UDP glucose and fructose (Ruan, 2014).

Plant metallothioneins (pMTs) are a family of low-molecular-weight, cysteine-rich proteins. Based on the content and the arrangement of cysteine (Cys) motifs (CC, CXC, CXXC), plant MTs are divided into four types (Koszucka & Dąbrowska, 2006; Mierek-Adamska et al., 2018). Type 1 MTs contain 12 Cys residues, type 2 MTs contain 14 Cys residues, type 3 MTs contain 10 Cys residues, and type 4 MTs possess 17 Cys residues. Thiolate groups of cysteine

residues can bind a variety of metal ions, in particular, copper, zinc and cadmium, allowing MTs to maintain the homeostasis of metals (Cobbett, 2000; Evans et al., 1992; Freisinger, 2011; Leszczyszyn et al., 2013). Some plant MTs have one or more histidine residues, which also participate in metal binding (Leszczyszyn et al., 2013; Tomas et al., 2014). MTs can protect plants from oxidative stress since thiol groups of cysteine residues directly react with different ROS (Mierek-Adamska et al., 2019). Moreover, MTs can reduce the amount of produced ROS by binding copper and therefore decreasing the level of the Fenton reaction (Dąbrowska et al., 2013; Hrynkiewicz et al., 2012; Lee et al., 2004; Leszczyszyn et al., 2013; Miller et al., 2010; Mir et al., 2004). There are many reports about the response of plant MTs to various factors. It has been shown that *MT* transcripts were upregulated in plants under heavy metals (Dąbrowska, 2014; Guo et al., 2003; Murphy & Taiz, 1995; Turchi et al., 2012; Zhou & Goldsbrough, 1994), salt (Kawasaki et al., 2001; Ozturk et al., 2002; Rabbani et al., 2003; Soda et al., 2016) and drought/osmotic (Bae et al., 2010; Ozturk et al., 2002; Rabbani et al., 2003; Samardžić et al., 2010) stresses. Moreover, the expression of *MTs* is increased by abscisic acid (ABA), which is a plant stress hormone (Singh et al., 2011; Xue et al., 2009). In addition, in the promoters of *MT* genes, multiple regulatory elements of response to various abiotic and biotic factors are present (Dąbrowska, Hrynkiewicz, & Trejgell, 2012; Dąbrowska, Mierek-Adamska, & Goc, 2012).

Inspired by the postulated role of pMTs in stress response, our objectives were to clone the coding and genomic sequences of *A. sativa* L. metallothioneins (*AsMTs*), analyse the function of AsMT1-3 in a prokaryotic system and study the expression of *AsMT1-3* in oat seedlings in response to osmotic stress. Additionally, the activity of antioxidant enzymes (SOD, CAT and PX), the content of low-molecular-weight osmoprotectants (soluble sugars and phenolic compounds) and ABA were determined. We hypothesized that MTs are involved in the response of oat to osmotic stress and they contribute to the protection of plants against dehydration. Moreover, it is highly plausible that oat MTs are the part of oxidative stress–response system, and their expression will correlate with other elements of the antioxidant system. This work presents novel information about the oat MT genes family since the oat's genome has been recently sequenced but has not yet been fully annotated and gives insight into the reaction of oat to osmotic stress. Our results reported here will provide a framework for further studies to gain a deeper understanding of the relationship between pMTs and antioxidant systems.

2 | MATERIALS AND METHODS

2.1 | Hydroponic cultures of oat

A. sativa L. seeds *cv.* Bingo were disinfected in 70% ethanol (3 min) and then washed three times in sterile distilled water. Then, seeds were transferred to 90 mm Petri dishes containing filter paper soaked in sterile distilled water (2 mL per dish). For germination, the seeds were incubated in the darkness at 21 ± 2°C for 48 hours and then under the light intensity of 100 μmol m^{-2} s^{-1} (16/8 h light/dark). The 5-day-old seedlings were placed in 500 mL plastic vessels containing Hoagland liquid medium (Hoagland & Arnon, 1938) and maintained in hydroponic culture in a growth chamber at 21 ± 2°C under the light intensity of 100 μmol m^{-2} s^{-1} (16/8 h light/dark). For stress induction, polyethylene glycol (PEG 6000) at a concentration of 180 g L^{-1} and mannitol at a concentration of 69 g L^{-1} of Hoagland liquid mediums were used. To avoid hypoxia occurrence, the medium was aerated consistently by an air pump (Hailea ACO-2201, Happet, Poland). Seedlings growing in Hoagland liquid medium without mannitol or PEG 6000 were used as a control. Six seedlings were cultivated in five replications for each treatment (control, PEG 6000 and mannitol). After 4 days of stress treatment, oat shoots and roots were washed in sterile distilled water and collected separately. Samples were frozen in liquid nitrogen and kept at −80°C for further analyses.

2.2 | Isolation of nucleic acid and reverse transcription reaction

Genomic DNA was isolated from 1-week-old oat seedlings using Gene MATRIX Plant and Fungi DNA Purification Kit (EURx) according to the manufacturer's protocol. Total RNA was isolated separately from roots and shoots of 9-day-old oat seedlings using RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's protocol. The quality and quantity of the extracted RNA and DNA were checked by agarose gel electrophoresis and by spectrophotometric measurement using NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific).

To remove any DNA contamination from RNA samples, 1.5 μg of total RNA was treated with 1 U of DNase I (Thermo Fisher Scientific) and incubated at 37°C for 30 min. The enzyme was inactivated by the addition of 1 μL 50 mM EDTA and incubation at 65°C for 10 min. Next, reverse transcription reaction using RevertAid™ Reverse Transcriptase (Thermo Fisher Scientific) was performed according to the manufacturer's protocol.

2.3 | Cloning and sequencing oat *MTs*

Coding sequences of *MTs* from *Triticum aestivum* L. (*MT1* – AY688468.1, *MT2* – AF470355.1) and *Hordeum vulgare* L. (*MT3* – AJ555613.1) deposited in NCBI (National Center for Biotechnology Information) GenBank were used for searching *A. sativa* L. EST (expressed sequence tags) database. From numerous EST sequences found, the following were used to design gene-specific primers (Table 1): CN818146.1 (*MT1*), GO588763.1 (*MT2*) and CN818499.1 (*MT3*). PCR mixture contained cDNA or gDNA as a template, 0.3 μL of 10 μM primers, 0.4 μL of 10 mM dNTPs and 1 U of Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) in a total volume of 20 μL. The thermal cycling conditions were as follows: 30 s of

TABLE 1 Primers used for cloning of *AsMTs*.

Primer name	Sequence 5′→3′	Amplicon name	Amplicon size [bp]
AsMT1_f	TGTGCTCATCATCTTCTCCGA	cAsMT1	404[a]
AsMT1_r	GCGAGCAAGTAAACCACACA	gAsMT1	667[b]
AsMT2_f	AGAAAGCAGATCGAGGTGGT	cAsMT2	423[a]
AsMT2_r	GCACACAGTAAGTACAGGCG	gAsMT2	712[b]
AsMT3_f	GCTCTCTCTCTCCGTTCACA	cAsMT3	453[a]
AsMT3_r	ACTCACATACGATCCGAAGCT	gAsMT3	788[b]

[a]cDNA as PCR template.
[b]Genomic DNA as PCR template.

TABLE 2 Primers for amplification of open reading frames of *AsMT1-3*.

Primer name	Sequence 5′→3′
eAsMT1_f_Nde	AAACATATGTCTTGCAGCTG TGGACT
eAsMT1_r_Xho	AAACTCGAGTTAACAGT TGCAGGGGTTGC
eAsMT2_f_Nde	AAACATATGTCGTGCTG CGGAGGCAAC
eAsMT2_r_Xho	AAACTCGAGTTACTTGC AAGTGCACGGG
eAsMT3_f_Nde	AAACATATGTCGAACACCTG CGGCAAC
eAsMT3_r_Xho	AAACTCGAGTCAGTGGC CGCAGGTGCAG

denaturation at 98°C, followed by 30 cycles of amplification (98°C for 5 s, 63°C for 10 s and 72°C for 20 s). The amplified PCR products were separated in 1.5% agarose gel stained with EtBr in TAE Buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA). PCR products were purified from the gel using Gene MATRIX Agarose-Out DNA Purification Kit (EURx) according to the manufacturer's protocol and ligated into the pJET1.2 vector (Thermo Fisher Scientific) according to the manual. The ligation mixture was used to transform *Escherichia coli* DH5α bacteria using the heat shock method (Sambrook & Russell, 2001). Plasmid DNA was isolated using the Gene MATRIX Plasmid Miniprep DNA Purification Kit (EURx) according to the manufacturer's protocol. The DNA sequencing was performed in Genomed, Poland.

2.4 | In silico analysis of oat *MT* genes

The similarity of oat *MT* nucleotide sequences to other plant MTs was verified using NCBI's nBLAST software (Altschul et al., 1990). ExPASy Translate (Gasteiger et al., 2003) was used to obtain putative amino acid sequences of AsMT1-3. Predicted amino acid sequences were further analysed using NCBI's pBLAST to find their homologs. Putative amino acid sequences of AsMT1-3 and their homologous sequences were used in phylogenetic analysis via the MEGA-X Neighbour-Joining algorithm (Kumar et al., 2018).

2.5 | Preparation of expression constructs

Coding regions of *AsMT1-3* were amplified with sequence-specific primers containing *Nde*I (forward primer) and *Xho*I (reverse primer) restriction sites (Table 2). PCR products were digested with *Nde*I and *Xho*I and ligated with pET21a(+) (Novagen) linearized with *Nde*I and *Xho*I. The ligation mixture was used to transform *E. coli* DH5α bacteria. Plasmid DNA was isolated (Gene MATRIX Plasmid Miniprep DNA Purification Kit; EURx) and sequenced to confirm the presence of a correct open reading frame without any point and indel mutations (Genomed). The obtained constructs were named pET-*AsMT1-3*.

2.6 | Functional analysis of *AsMT1-AsMT3* in *Escherichia coli*

The *E. coli* Rosetta(DE3) cells (Novagen) were transformed using the heat shock method (Sambrook & Russell, 2001) with an empty pET21a vector (control) or pET-*AsMT1-3* constructs. Overnight cultures of transformed cells were diluted (1:100, v/v) in LB medium with antibiotics (50 μg mL^{-1} ampicillin and 34 μg mL^{-1} chloramphenicol) and 100 g L^{-1} PEG-6000 or 109 g L^{-1} mannitol to $OD_{600} \approx 0.2$. To induce the expression of *AsMT1-3*, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM (to avoid high overexpression of transgene). Cultures without PEG 6000 or mannitol and with IPTG served as controls. Bacteria cells were collected every hour for 7 h, and OD_{600} was measured. The analysis was performed in three biological replicates, and for each biological replicate, three technical replicates were done. The growth rate of bacteria cultures was expressed as the slope of the linear proportion of the growth curve and was calculated using Microsoft Excel.

2.7 | Analysis of *AsMT1-3* expression in response to mannitol and PEG 6000

For analysis of changes in expression during osmotic stress, RNA isolated from roots and shoots of oat treated with PEG 6000, mannitol and control was used. Quantitative PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). The reaction contained 5 μL of Master Mix, forward

and reverse primer at final concertation of 0.3 μM each, and 4 μL of 10x diluted cDNA in a total volume of 10 μL. Three replicates were performed for each reaction. The qPCR reaction was conducted in LightCycler® 480 Instrument (Roche) according to the following protocol: 10 min denaturation at 95°C, followed by 40 cycles of amplification (95°C for 15 s, 60°C for 30 s and 72°C for 30 s). The SYBR Green I fluorescence signal was recorded at the end of each extension step. The specificity of each reaction was verified by performing a melting curve analysis using the following thermal cycling profile: 95°C for 60 s, 55°C for 30 s and ramping to 95°C with stepwise signal acquisition. Differences in the target genes expression were evaluated by a relative quantification method normalizing the data to the reference genes encoding eukaryotic initiation factor 4A-3 (*EIF4A*) and heterogeneous nuclear ribonucleoprotein 27C (*HNR*; Yang et al., 2020). The fold-change in gene expression was calculated using LightCycler 480 Software (ver. 1.5.1.62). Primers used for qPCR reactions are shown in Table 3.

2.8 | Biochemical analysis

For determination of the level of photosynthetic pigments, phenolic compounds, soluble sugars and abscisic acid, plant material was freeze-dried, and samples were ground in a ball mill (MM400; Retsch) in 2 mL Eppendorf vials. Antioxidant enzymes activities were determined in fresh plant material.

2.8.1 | Determination of photosynthetic pigments (chlorophyll *a* and *b* and carotenoids)

The content of photosynthetic pigments in shoots was determined via spectrophotometric measurement using a microplate reader (Synergy II; BioTek). Five milligram of plant material was used for extraction in 80% ethanol. The samples were shaken for 15 min (30 Hz) and then centrifuged (20,000 ×g). The spectrophotometric measurement was carried out at the wavelengths of 470, 648 and 664 nm. The content of chlorophyll *a* and *b* and the total amount of carotenoids were calculated according to Lichtenthaler and Wellburn (1983):

$$\text{Chl. } a = 13.36A_{664} - 5.19\,A_{648}$$

$$\text{Chl. } b = 27.43A_{648} - 8.12A_{664}$$

$$\text{Car} = \left(1000A_{470} - 2.13\,\text{Chl. } a - 97.64\,\text{Chl. } b\right) / 209$$

where $A\lambda$ is the absorbance value for the wavelength [λ], Chl. *a* is the concentration of chlorophyll *a*, Chl. *b* is the concentration of chlorophyll *b*, and Car is the concentration of total carotenoid.

2.8.2 | Soluble sugar content

The content of soluble sugars was determined spectrophotometrically according to Dubois et al. (1956) using the phenol-sulphuric acid method. The extraction was carried out from 5 mg of plant material in 80% ethanol. The reaction mixture consisted of 150 μL of distilled water, 50 μL of the ethanolic plant extract, 200 μL of 5% phenol solution and 1 mL of concentrated H_2SO_4. The absorbance at 490 nm was measured using a microplate reader (Synergy II; Bio-Tek).

2.8.3 | Phenolic compound content

The total content of phenolic compounds was determined spectrophotometrically by the method of Singleton and Rossi (1965). Phenolic compounds were extracted from 5 mg of plant material in 1 mL of 80% ethanol. The reaction mixture consisted of 150 μL of distilled water, 20 μL of the ethanolic plant extract, 250 μL of 25% Na_2CO_3, and 125 μL of Folin–Ciocalteau reagent (diluted with distilled water 1:1 before use). Absorbance measurements were performed at a wavelength of 760 nm (Synergy II; Bio-Tek). Phenolic content was determined in milligram of chlorogenic acid per 1 g of the dry weight of plant tissue.

2.9 | Abscisic acid content

Extraction and quantification of abscisic acid (ABA) were performed according to Dziurka et al. (2019). One hundred milligram of plant

TABLE 3 Primers used for qPCR reactions.

Primer name	Sequence 5′→3′	Amplicon size [bp]
AsMT1_qPCR_f	CAAACTGCAAGTGCGGGAAG	103
AsMT1_qPCR_r	TTGTTCTCATGAGCCACGCC	
AsMT2_qPCR_f	CTGCGGAGGGTGCAAGATG	96
AsMT2_qPCR_r	AACGATGGCTTGGAAGAGGG	
AsMT3_qPCR_f	TCCACCATGTCGAACACCTG	107
AsMT3_qPCR_r	TGGCTCTTCTCGGTGTCAAC	
EIF4A_f	TCTCGCAGGATACGGATGTCG	88
EIF4A_r	TCCATCGCATTGGTCGCTCT	
HNR_f	ATTGGGTTTGTCACTTTCCGTAG	134
HNR_r	CTTGGAGGGTGTCTCGCATCT	

material spiked with stable isotope-labelled internal standards was extracted using a methanol/water/formic acid mixture (MeOH/H_2O/HCOOH 15:4:1, v/v/v) (Dobrev & Kamínek, 2002). The extraction was repeated twice, and combined extracts were evaporated under N_2, then resuspended in 5% MeOH in 1 M HCOOH. The fraction containing ABA was evaporated under N_2, reconstituted in 70 µL of ACN, filtered (0.22 µm nylon membrane), and used for UHPLC analyses. The system consisted of UHPLC (Agilent Infinity 1260; Agilent) and a triple quadrupole mass spectrometer (Agilent 6410; Agilent) with electrospray ionization (ESI). Separation was performed on Ascentis Expres RP-Amide analytical column (2.7 µm, 2.1 mm × 150 mm; Supelco) at the linear gradient of water vs. ACN both with 0.01% HCOOH. ABA standard was from OlChemim (Olomunc) at the highest available purity, whereas solvents were of HPLC grade from Sigma-Aldrich.

2.10 | Determination of antioxidant enzyme activity

One hundred milligrams of plant material was homogenized at 4°C with 0.05 M phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The homogenate was centrifuged at 10,000 × g for 15 min. The activity of antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT) and peroxidase (PX) were measured spectrophotometrically (Synergy II; Bio-Tek). SOD activity was measured by the cytochrome method (McCord & Fiodovich, 1969) at $\lambda = 550$ nm. CAT activity was assessed by measuring the rate of H_2O_2 decomposition using the method of Aebi (1984) at $\lambda = 240$ nm. PX activity was determined by measuring the amount of oxidation products of 1% p-phenylenediamine in the presence of H_2O_2 at $\lambda = 485$ nm (Lűck, 1962). The enzymatic activity was expressed relatively to the total amount of proteins present in shoots and roots. The total amount of proteins was determined according to the method of Bradford (1976).

2.11 | Statistical analysis

Data analysis was performed using two-way ANOVA and post hoc Duncan test in the statistical package STATISTICA 13.0 (Stat-Soft, Inc.) or RStudio using Kruskal–Wallis and Mann–Whitney tests (RStudio Team, 2020). Significant differences between treatments were marked at $p \leq .01$ or $p \leq .05$. Pearson's simple correlation coefficients were estimated between values to determine the relative share of each trait in the multivariate variation of the treatment. Principal component analysis (PCA) was used to assess the relationships between the expression level of *AsMT1-3*, the content of soluble sugars, phenolic compounds, abscisic acid and the activity of SOD, CAT and PX in shoots and roots considering treatments. An acute angle on the biplot between the measured parameters means that a positive correlation was observed, an obtuse angle – a negative correlation, and a right angle – no correlation. This orthogonal transformation is defined in such a way that the first principal component has the largest possible variance.

3 | RESULTS

3.1 | Analysis of nucleotide and amino acid sequences of oat MT

The genomic and coding sequences of *AsMT* types 1–3 were cloned and sequenced. The comparison of coding and genomic sequences revealed that in *AsMT1* and *AsMT2*, there were single introns 267 and 289 bp long, respectively. The *AsMT3* has two introns of 171 and 164 bp in length (Table 4). The predicted amino acid sequences of AsMT1-3 differed in length, with AsMT2 being the longest (79 aa) and AsMT3 being the shortest (64 aa). BLASTP analysis of amino acid sequences showed that AsMT1 shows the highest similarity to sequences of MT1 from *Festuca rubra* L., and *H. vulgare* L. AsMT2 was most similar to MT2 from *Poa secunda* J.Presl and *Phalaenopsis equestris* (Schauer) Rchb.f. AsMT3 shares the highest number of identical amino acids with eudicot *Aquilegia coerulea* E. James and monocot *Oryza coarctata* Roxb. (*Porteresia coarctata* (Roxb.) Tateoka) (Table 4). Putative amino acid sequences of AsMT1-3 are rich in Cys residues arranged in two domains characteristic for each type of plant MTs. AsMT1 contains 12 Cys arranged in two domains of six cysteine residues. AsMT2 contains 14 cysteines arranged in two domains of eight and six Cys. AsMT3 has 10 Cys in two clusters of four and six cysteines (Table 4, Figure 2). Moreover, histidine residues were present in AsMT1 and AsMT3 (Figure 2). Phylogenetic analysis further confirmed that proteins coded by *AsMT1-3* genes belong to specific types of plant MTs (Figure 1). The number and position of Cys residues are the most conserved in MTs belonging to type 2 (Figure 2). Type 1 MTs from dicots Brassicaceae family, *Brassica napus* L. and *Arabidopsis thaliana* (L.) Heynh., have a shorter stretch between Cys-rich domains and have 13 cysteine residues. Type 1 MTs from monocots *A. sativa* L., *H. vulgare* L. and *Oryza sativa* L. have 12 Cys residues and a long spacer between cysteine domains. Type 3 MTs from some dicots belonging to the Brassicaceae family have 12 Cys residues. Monocotyledonous type 3 MTs have only 10 Cys residues, and they also contain His residues (Figure 2).

3.2 | Functional analysis of *AsMT1-3* genes under osmotic stress

To evaluate whether oat MTs limit the negative impact of osmotic stress on *E. coli* bacteria, the impact of heterologous overexpression of *AsMT1-3* on the growth of *E. coli* was investigated (Figures 3 and 4). To obtain negative water potential, LB medium was supplemented with mannitol (109 g L^{-1}) or PEG 6000 (100 g L^{-1}). Figure 3 shows the growth of transformed bacteria without IPTG, that is, the expression of the transgene was not induced. In the control environment (LB medium) bacteria transformed with pET_*AsMT3* had a lower growth rate than bacteria transformed with pET_empty. In PEG 6000, no differences between bacteria transformed with an empty vector and bacteria transformed with a vector containing *AsMT1-3* were observed. In presence of mannitol, bacteria transformed with pET_*AsMT2* and pET_*AsMT3* had a lower growth rate compared to

TABLE 4 The characteristics of cloned *AsMT* genes and their homology to other plant MT.

Gene name and NCBI accession number	Intron/s length [bp]	Amino acid length [aa]	Cys residue number	Cys sequence pattern	Length of spacer between Cys domains [aa]	Protein with highest homology and % of identical aa
AsMT1 LC741351.1	267	72	12 (6 + 6)	C-X-C	41	*F. rubra* – 87.93% (O24528.1) *H. vulgare* –78.67% (CAD54078.1)
AsMT2 LC741352.1	289	79	14 (8 + 6)	CC, C-X-C, C-X-X-C	41	*P. secunda* – 84.72% (AAK38824.1) *P. equestris* – 69.14% (XP_020580705.1)
AsMT3 LC741353.1	171, 164	64	10 (4 + 6)	C, C-X-C	32	*A. coerulea* – 75.00% (PIA55396.1) *O. coarctata* – 73.44% (AAF68995.1)

pET_empty. After the addition of IPTG to the cell cultures, a decrease in the growth rate of all tested cells was observed, which is related to the toxic effect of IPTG (Figure 4). Bacteria cells expressing *AsMT1-3* in the control environment grew better than cells transformed with empty pET. In the presence of PEG 6000 and IPTG, bacteria transformed with pET_*AsMT2* had a higher growth rate compared to the control. No significant differences were observed for the LB medium supplemented with mannitol (Figure 4).

3.3 | Analysis of *AsMT* expression level in oat under osmotic stress

The exposure of oat seedlings to mannitol increased the expression of all tested *MT*s (Figure 5). The exception was the expression of *AsMT1* in the roots, that is, it remains at the same level as in control regardless of the treatment used. In shoots treated with mannitol, the level of *AsMT1* increased 2.1 times compared to the control. The greatest increase in expression was observed for *AsMT2*, 4.7 times higher in shoot and 8.9 times higher in root in comparison to the control. In response to mannitol, *AsMT3* expression increased two-fold in shoots (Figure 5a) and 4.4-fold in roots (Figure 5b). PEG 6000 treatment did not cause changes in *AsMT1-3* expression in oat roots (Figure 5b). In shoots after PEG 6000 treatment, the level of *AsMT2* transcripts increased 1.5 times, when compared to control (Figure 5a).

3.4 | Chlorophyll *a*, chlorophyll *b*, and carotenoid content

Oat treatment with PEG 6000 did not affect the content of chlorophyll *a*, chlorophyll *b* and carotenoids in oat seedlings (Figure 6). Treatment with mannitol caused a significant increase in the content of chlorophyll *a* (26.5 mg g^{-1} DW), compared to the control (14.1 mg g^{-1} DW).

3.5 | Phenolic compounds, soluble sugars, and ABA content

Levels of phenolic compounds in shoots were unaffected by the osmotic stress, whereas in roots, PEG 6000 treatment increased the content of these compounds to the level of 3.4 mg g^{-1} DW, which is 1.8 times higher than that in the control (Figure 7a).

As a result of osmotic stress, the content of soluble sugars (Figure 7b) increased in shoots of seedlings exposed to PEG 6000 and mannitol and decreased in roots. Generally, the highest content of soluble sugars was observed in shoots treated with PEG 6000. In contrast, in roots, a decrease in sugar content was observed in the presence of PEG 6000 and mannitol compared to the control.

The treatment of oat seedlings with PEG 6000 and mannitol caused no difference in the content of ABA in roots (Figure 7c). In contrast, osmotic stress increased the content of ABA in shoots. As a result of PEG 6000 and mannitol treatments, in shoots ABA accumulation was more than five-fold and nine-fold higher, respectively, than in the control.

3.6 | Antioxidant enzyme activity

Catalase activity increased significantly in response to PEG 6000 and mannitol treatments in shoots and was higher than in the control seedlings by 1.3 times and 1.5 times, respectively (Figure 8a). In roots, CAT activity was around 3.5-fold lower than in shoots. CAT activity was not affected by osmotic stress in oat roots.

The greatest increase in PX activity was observed in shoots treated with PEG 6000, 2.5-fold higher than in control shoots and shoots of seedlings treated with mannitol (Figure 8b). In roots, the osmotic stress did not affect the level of PX activity.

SOD activity was significantly higher in roots compared to shoots for all treatments (Figure 8c). In shoots, osmotic stress caused by PEG 6000 or mannitol did not change SOD activity. In roots treated with mannitol, SOD activity increased 1.3-fold compared to the control.

3.7 | Correlation analysis

Correlations coefficients between studied parameters under two stress treatments are presented in Table 5. The statistically significant correlations in seedlings between the relative expression level of *AsMT1-3*, the content of soluble sugars (SUG), the content of phenolic compounds (PHE), the content of ABA and the activity of SOD,

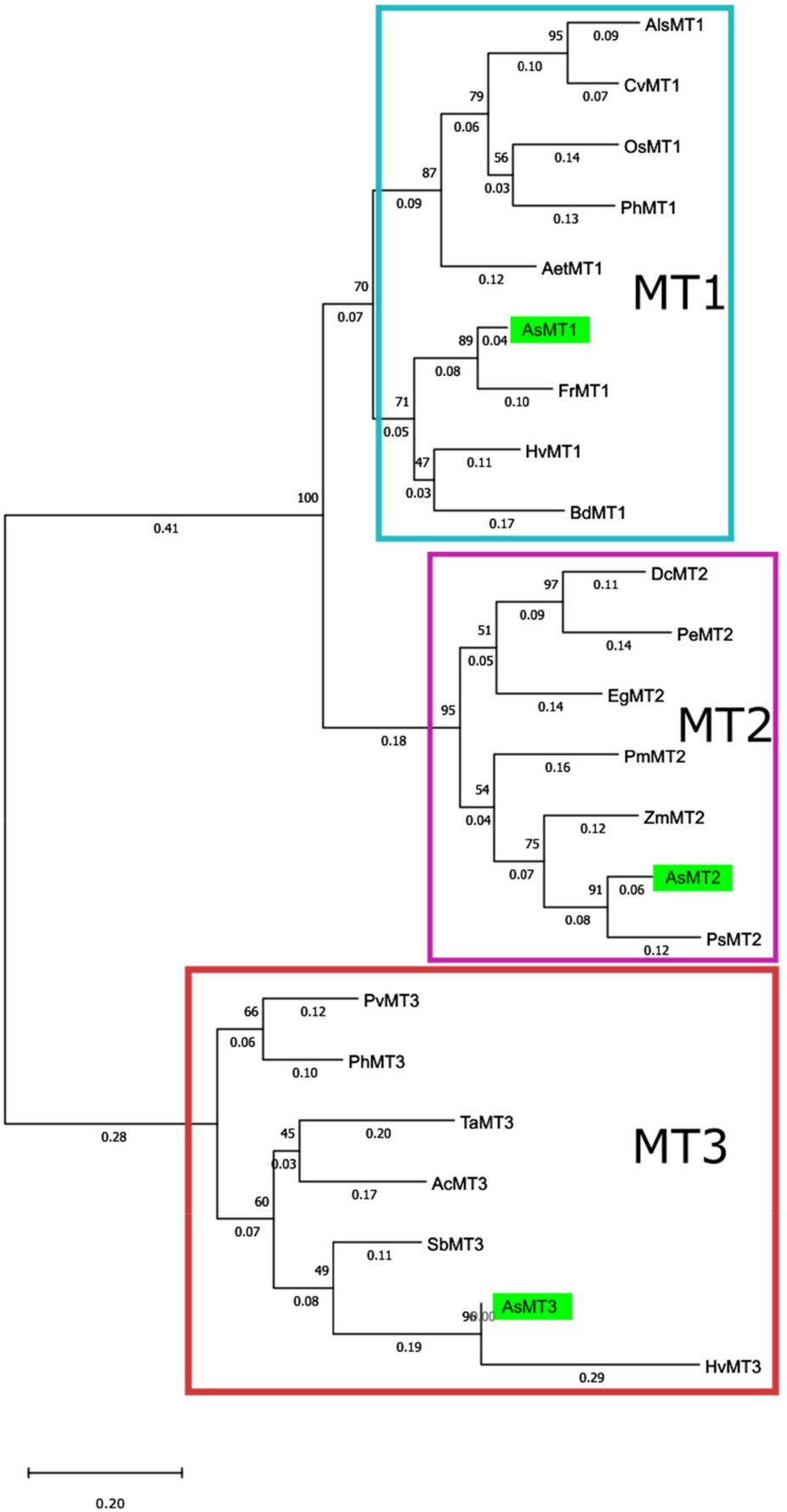
AlsMT1
CvMT1
OsMT1
PhMT1
AetMT1
MT1
AsMT1
FrMT1
HvMT1
BdMT1
DcMT2
PeMT2
EgMT2
MT2
PmMT2
ZmMT2
AsMT2
PsMT2
PvMT3
PhMT3
TaMT3
AcMT3
MT3
SbMT3
AsMT3
HvMT3
0.20

FIGURE 1 The phylogenetic analysis of plant MT proteins based on predicted amino acid sequences of MTs from monocots. The evolutionary history was inferred using MEGA-X software. Evolutionary distances were computed using the Poisson correction method, presented using the units of the number of amino acid substitutions per site. A bootstrap test with 1000 replicates was applied to assess the reliability of the phylogenetic tree. The sequences of AsMT1-3 are highlighted. The following amino acid sequences deposited in NCBI GenBank were used: AcMT3 – *Ananas comosus* (L.) Merr. (OAY84410.1), AetMT1 – *Aegilops tauschii* Coss. (XP 020173251.1), AlsMT1 – *Allium sativum* L. (AAL13057.1), BdMT1 – *Brachypodium distachyon* (L.) P.Beauv. (XP 014757938.1), CvMT1 – *Chloris virgata* Sw. (BAF73618.1), DcMT2 – *Dendrobium catenatum* Lindl. (XP 020694706.1), EgMT2 – *Elaeis guineensis* Jacq. (XP 010937631.1), FrMT1 – *Festuca rubra* L. (O24528.1), HvMT1 – *Hordeum vulgare* L. (CAD54078.1), HvMT3 – *H. vulgare* (CAD88266.1), OsMT1 – *Oryza sativa* L. (XP 015617237.1), PeMT2 – *Phalaenopsis equestris* (Schauer) Rchb.f (XP 020571140.1), PhMT1 – *Panicum hallii* Vasey (XP 025826729.1), PhMT3 – *P. hallii* (XP 025815067.1), PmMT2 – *Panicum miliaceum* L. (RLM91775.1), PsMT2 – *Poa secunda* J.Presl (AAK38824.1), PvMT3 – *Panicum virgatum* L. (XP 039846084.1), SbMT3 – *Sorghum bicolor* (L.) Moench (XP 021313180.1), TaMT3 – *Typha angustifolia* L. (ACV51811.1) and ZmMT2 – *Zea mays* L. (ONM30027.1).

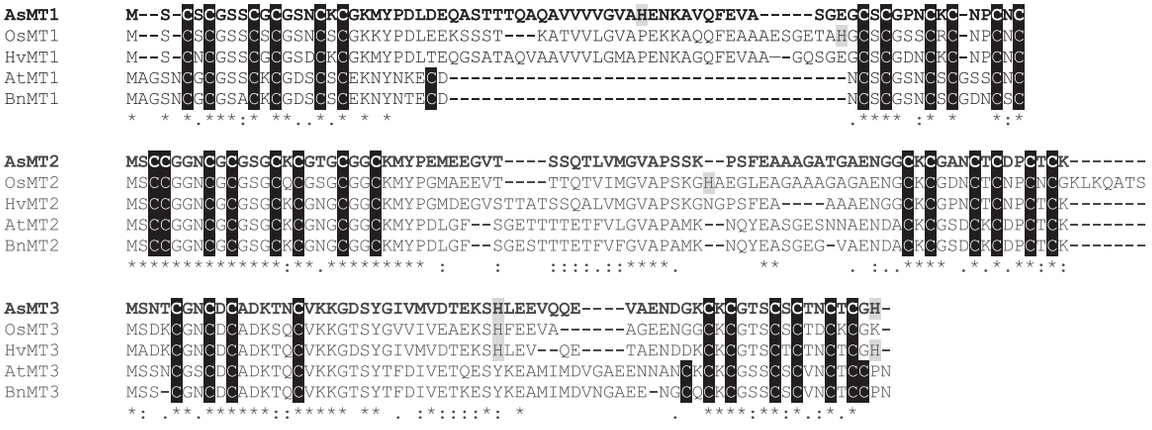

FIGURE 2 Alignment of amino acid sequences of AsMT1-3 and representative members of types 1–3 plant MTs. Conserved Cys residues are highlighted in black, and His residues are highlighted in grey. The following amino acid sequences deposited in NCBI GenBank were used: *Arabidopsis thaliana* (L.) Heynh: AtMT1 Q38804.1, AtMT2 OAP04976.1, AtMT3 O22433.1; *Brassica napus* L.: BnMT1 AFO70132.1, BnMT2 AFP57434.1, BnMT3 AFP57435.1; *Hordeum vulgare* L.: HvMT1 CAD54078.1, HvMT2 XP_044974743.1, HvMT3 CAD88266.1; *Oryza sativa* L.: OsMT1 XP_015617237.1, OsMT2 XP_015645105.1, OsMT3 A1YTM8.1.

CAT and PX were found. The expression level of *AsMT1* showed strong significant correlations with the expression level of *AsMT2*, positive in shoots (1.000) and negative (−1.000) in roots. Moreover, strong positive correlations in shoots and negative in roots were observed between the expression levels of *AsMT1* and *AsMT3*. The expression of *AsMT2* and *AsMT3* was strongly positively correlated both in roots and shoots.

A significant negative correlation was observed between the expression level of *AsMT1-2* in shoots and PX activity in roots ($r = -.999$ and $r = -.998$, respectively). In addition, very high (≤0.9) correlations between transcript levels of *AsMT1* and *AsMT2* in roots and PX activity in roots ($r = .993$ for AsMT1 and $r = -.992$ for AsMT2) were observed. Transcript levels of *AsMT1* and *AsMT2* in shoots were positively correlated with SOD in roots ($r = .914$ and $r = .926$, respectively). Moreover, high and very high positive correlations between the expression level of *AsMT1-2* and ABA concentration in shoots were observed ($r = .898$ and $r = .910$, respectively).

The expression level of *AsMT3* in roots showed a strong significant positive correlation with SOD activity in roots ($r = .999$) and with phenolic compound level, CAT activity and ABA content in shoots ($r = .989$, $r = .975$ and $r = .996$, respectively). Additionally, very high negative correlations between *AsMT3* in roots, SUG in roots and PHE in roots ($r = -.903$ and $r = -.966$, respectively) and *AsMT3* in shoots and PX activity in roots ($r = -.920$) were found.

In oat shoots, a strong positive correlation between PHE content, CAT activity and ABA concentration ($r = .997$ and $r = .998$, respectively) was also recorded. Additionally, PHE in shoots was negatively correlated with SUG in roots ($r = -.957$). The high negative correlations between soluble sugar concentration in roots and SOD activity in roots ($r = -.924$) and ABA concentration in roots ($r = -.957$) were also observed. In shoots, a negative correlation between SUG and SOD activity ($r = -.948$) was found. A significant positive correlation was shown between SOD activity in roots and ABA concentration in shoots ($r = .999$), while the activity of SOD in shoots was negatively correlated with the activity of CAT in shoots ($r = -.914$).

Principal component analysis and biplot analysis were used to visualize correlations among the analysed in this study variables (Figure 9a). Positive correlations among the expression of *AsMTs* are visible beside the expression of *AsMT1* in roots. The mRNA level of *AsMT1* in roots is strongly positively correlated with the activity of PX in roots. The expression PCA indicated that the expression level of *AsMT1-3*, the activity of SOD, CAT and PX, and the content of SUG, PHE and ABA in oat

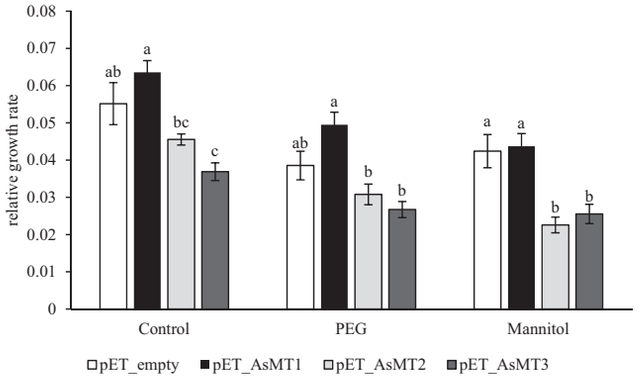

FIGURE 3 Comparison of the growth of *Escherichia coli* transformed with empty pET vector (white bars) and pET vectors bearing coding regions of *AsMT1-3* (black, grey and dark grey bars, respectively) in the presence of 100 g L^{-1} PEG or 109 g L^{-1} mannitol without IPTG. Control was LB medium. Bars show the slope of bacterial growth curves obtained from plotting optical density against time. Bars represent mean values ± SE of three independent replicates. The results obtained for a given condition (i.e. control, PEG and mannitol) were compared, and different letters indicate significant differences between *E. coli* carrying different plasmids (Kruskal–Wallis, Mann–Whitney; $p \leq .05$).

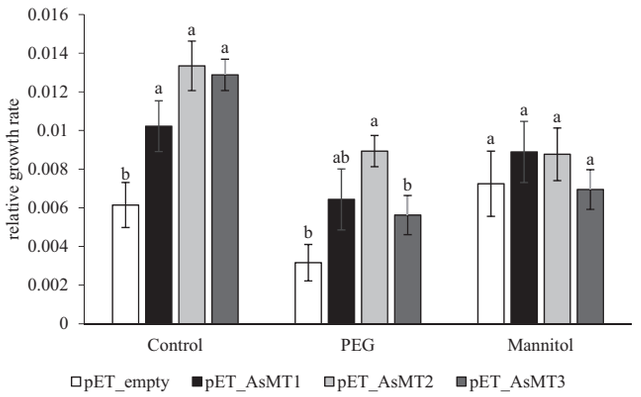

FIGURE 4 Comparison of the growth of *E. coli* transformed with empty pET vector (white bars) and pET vectors bearing coding regions of *AsMT1-3* (black, grey and dark grey bars, respectively) in the presence of 100 g L^{-1} PEG or 109 g L^{-1} mannitol and 0.1 mM IPTG. Control was LB medium. Bars show the slope of bacterial growth curves obtained from plotting optical density against time. Bars represent mean values ± SE of three independent replicates. The results obtained for a given condition (i.e. control, PEG and mannitol) were compared, and different letters indicate significant differences between *E. coli* carrying different plasmids (Kruskal–Wallis, Mann–Whitney; $p \leq .05$).

seedlings were differentiated by treatments (control, mannitol and PEG 6000; Figure 9b).

4 | DISCUSSION

Environmental water deficit caused by drought, cold and salinity leads to osmotic stress in plants. The consequence of osmotic stress has reduced plant growth and significant loss of yield. Plants possess mechanisms to sense osmotic stress and to trigger the signal transduction pathways that activate the physiological, morphological and

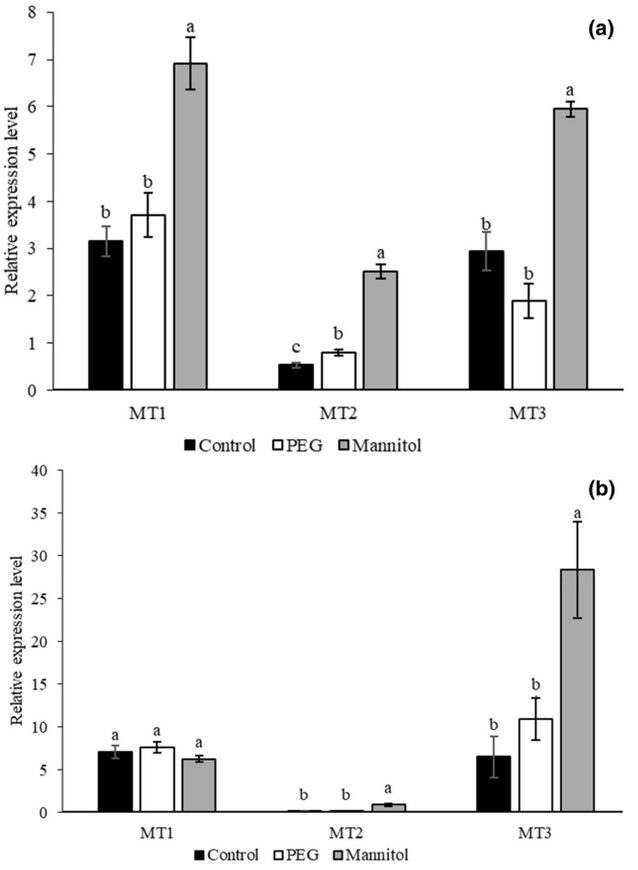

FIGURE 5 Relative gene expression of *AsMT1-3* (a) in shoots and (b) in roots of oat seedlings in control and osmotic stress conditions induced by PEG (180 g L^{-1}) and mannitol (69 g L^{-1}). The *AsMT1-3* genes were quantified with RT-qPCR and normalized using the housekeeping gene *EIF4A*. Bars represent mean values ($n = 3$) ± SE. The results obtained for each gene were compared separately for shoots and roots, and different letters indicate significant differences (Kruskal–Wallis, Mann–Whitney; $p \leq .01$).

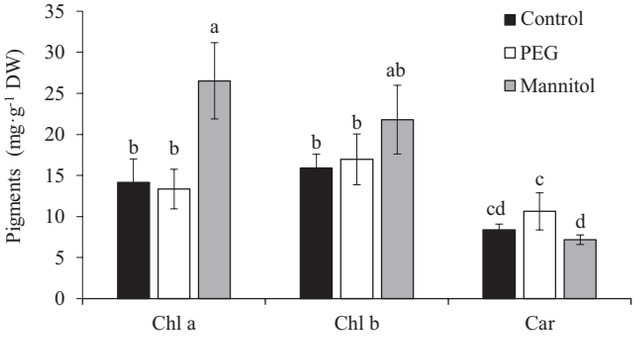

FIGURE 6 Effect of osmotic stress on the content of chlorophyll *a*, *b* (Chl *a*, *b*) and carotenoids (Car) in the leaves of oat. Bars represent mean values ($n = 5$) ± SE. Different letters indicate significant differences between means (Duncan's multiple range test; $p \leq .05$).

biochemical mechanisms allowing adaptation to water-deficit stress (Fàbregas et al., 2020). The effectiveness of these mechanisms under drought stress depends on the plant species and plant cultivar, as well as on the duration and the intensity of stress (Reddy et al., 2004). Consequently, the potency of these mechanisms should be determined not only for each species but also for genotypes within species. These analyses are a potent tool for obtaining a set of molecular markers of drought-tolerant species/cultivars useful in breeding programmes.

Phenolic compounds can accumulate in plant cells as a result of stress. They can protect the cells from ROS, and their antioxidant efficiency tends to be higher than that of tocopherol or ascorbate (Weidner et al., 2011). In leaves of crops such as rice and maize, the levels of phenolic compounds increased under drought stress conditions (Ayaz & Bertoft, 2001; Hura et al., 2008). In our study, the osmotic stress did not change the content of phenolic compounds in oat leaves. However, the Król et al. (2014) study showed that the leaves of *Vitis vinifera* L. had lower content of phenolic compounds than the roots. Moreover, the content of total phenolic compounds in *V. vinifera* L. plants subjected to drought stress was lower than that in the control plants. Drought stress reduced total phenolic content also in roots of *Rehmannia glutinosa* (Gaertn.) Steud. (Chung et al., 2006), and that result corresponds with our study, where the lowest concentration of phenolic compounds was noted in oat roots treated with mannitol. However, in soybean roots subjected to osmotic stress, the level of phenolic compounds increased (Swigonska et al., 2014). The inconsistent results concerning the impact of osmotic stress on the content of phenolic compounds might depend on plant parts used for analyses, on the stage of plant development and on conditions during the stress period.

The plant tolerance mechanism in water-deficit conditions is associated with the accumulation of osmoprotectants such as soluble sugars (Hoekstra et al., 2001). Soluble sugars serve as regulators of gene expression and as signal molecules (Rosa et al., 2009; Tarkowski & Van den Ende, 2015). In the Wilmer et al. (2022) study, plants treated with PEG-induced osmotic stress produced more sugars compared to plants without PEG. Moreover, compared to the roots, more sugars were accumulated in the shoots, which could indicate an inhibited sugar transportation. In our study, we observed that osmotic stress increased the content of soluble sugars in oat shoots and decreased in oat roots. Similarly, the content of these osmoprotectants in the two oat cultivars, drought-resistant DA92-2F6 and drought-susceptible Longyan, slightly increased during the first 7 days of PEG treatment. Moreover, the soluble sugar content in DA92-2F6 leaves was significantly higher than that in the leaves of Longyan (Gong et al., 2022). In *Zea mays* L., the concentration of soluble sugars increased in roots and shoots treated with PEG (Nayer & Reza, 2008). As soluble sugars play an important role in the mechanisms of stress tolerance in the plants through osmotic adjustment (Mostajeran & Rahimi-Eichi, 2009), it was suggested that soluble sugars might be used as a marker for the selection of the drought-tolerant cultivars of *Triticum durum* Desf. (Hakimi et al., 1995) and *Medicago sativa* L. (Maghsoodi & Razmjoo, 2015). Moreover, soluble sugars act as key players in the maintenance of redox balance in plants, they contribute to H_2O_2 elimination, and together with phenolic compounds can make an integrated redox system for quenching reactive oxygen species (Bolouri-Moghaddam et al., 2010).

Antioxidant enzymes, such as CAT, PX and SOD, form the first line of defence against ROS under stress conditions (Chiappero et al., 2021). SOD is responsible for the dismutation of the superoxide radicals to molecular oxygen and hydrogen peroxide, while CAT and PX decompose H_2O_2. We observed the highest activity of CAT and PX in the seedling shoots, whereas SOD activity was significantly higher in roots. A similar observation was reported by Pastuszak et al. (2021) in the wheat seedlings, where greater SOD activity was detected in the roots than in the leaves. Gong et al. (2022) reported different reactions of antioxidant enzymes in oat cultivars under PEG-induced drought stress. In leaves of drought-resistant cultivar DA92-2F6, first the activity of CAT, SOD and PX increased but then it decreased. In contrast, in the drought-susceptible cultivar Longyan, the activity of those three antioxidant enzymes continuously increased. In our experiment, in the shoots, the activity of CAT was increased by both PEG 6000 and mannitol, whereas the activity of PX increased only in seedlings treated with PEG 6000. Interestingly, in the roots, only the activity of SOD increased under mannitol treatment. In contrast to our study, Zhou et al. (2022) observed in *Glycine max* (L.) Merr. roots treated with PEG the increasing activities of CAT and PX. Although an increase in ROS content and as a consequence an increase in the activity of antioxidant enzymes are widely observed for various stress treatments (reviewed in Nadarajah, 2020; You & Chan, 2015), there are differences in the action of antioxidant mechanisms depending on plant species, plant organs, and type and duration of stress stimuli.

MTs, small cysteine-rich proteins, are known for their role in maintaining micronutrient (mostly zinc and copper) homeostasis in cells. In silico analyses of plant MT genes showed that their promoter regions have multiple sites for binding various transcription factors that are known to be involved in response to abiotic stress in plants (Dąbrowska, Hrynkiewicz, & Trejgell, 2012; Dąbrowska, Mierek-Adamska, & Goc, 2012; Patankar et al., 2019). Several studies indicate that pMTs are also involved in ROS scavenging via cysteine residues (Gu et al., 2014; Guo et al., 2008; Liu et al., 2015; Mekawy et al., 2020; Woo & Lazo, 1997; Xia et al., 2012; Xue et al., 2009). In this work, we characterized three novel MT genes from *A. sativa* L. – *AsMT1*, *AsMT2* and *AsMT3*. Their predicted amino acid sequences are rich in cysteine residues which are organized in Cys-rich motifs typical for pMTs. Moreover, His residues, which are shown to also take a part in binding metal ions (Leszczyszyn et al., 2013; Tomas et al., 2014), are present in amino acid sequences of AsMT1 and AsMT3.

Polyethylene glycol and mannitol decrease the availability of water molecules, inducing osmotic stress in bacteria (Ghosh et al., 2019; Udawat et al., 2014; Yadav et al., 2014). Our analyses showed that *E. coli* bacteria were more sensitive to osmotic stress induced by PEG 6000 than by mannitol. Functional analysis of *AsMT1-3* in *E. coli* Rosetta cells showed that overexpression of the *AsMT2* gene

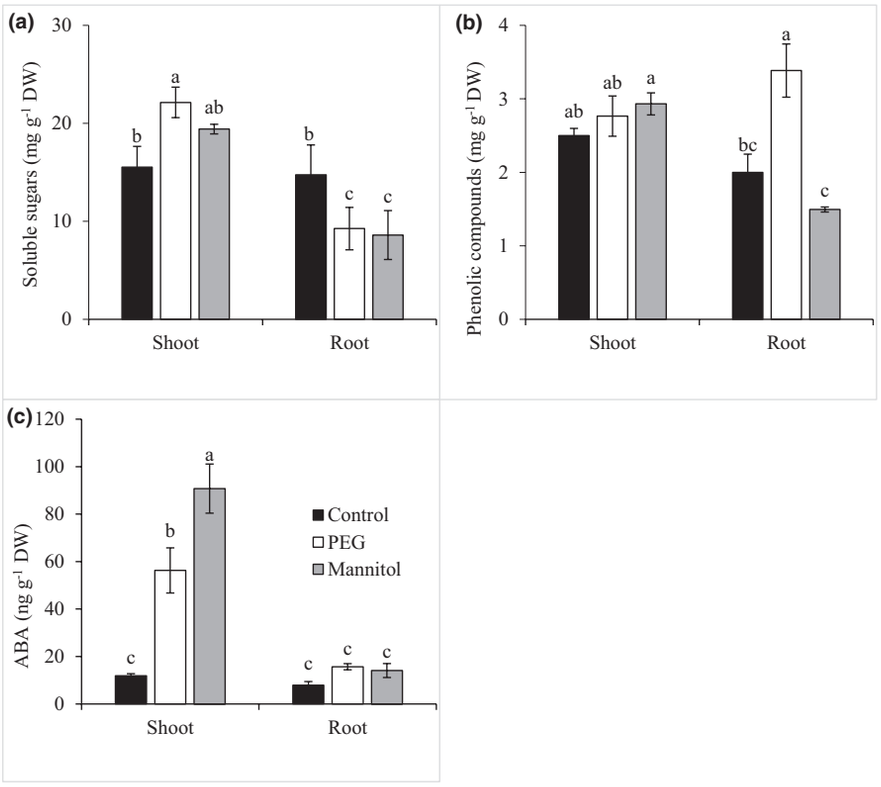

FIGURE 7 Effect of osmotic stress on the content of (a) soluble sugars, (b) phenolic compounds and (c) ABA in the shoots and roots of oat. Bars represent mean values ($n = 5$) ± SE. Different letters for each organ indicate significant differences between means (Duncan's multiple range test; $p \leq .05$).

increased bacteria tolerance to osmotic stress caused by PEG 6000. In the literature, only a few reports are showing the effect of the heterologous expression of pMTs on the tolerance of microorganisms to osmotic stress. *E. coli* bacteria expressing *MT* type 4 from *Cucumis sativus* L. had a higher survival rate than control *E. coli* in the presence of NaCl and sorbitol (Zhou et al., 2019). Similarly, yeast expressing the *Phoenix dactylifera* L. *MT2* gene grew better under osmotic stress (Patankar et al., 2019). Increased tolerance to osmotic stress was also shown for transgenic plants overexpressing pMTs. *Nicotiana tabacum* L. overexpressing *MT1* from *Oryza sativa* L. had a higher tolerance for PEG-induced stress (Kumar et al., 2012). Transgenic *N. tabacum* L. overexpressing *MT3* from *Gossypium hirsutum* L. had a higher tolerance towards 25% PEG and 300 mM NaCl (Xue et al., 2009). Similarly, transgenic *A. thaliana* (L.) Heynh. overexpressing *MT3a* from *O. sativa* L. had increased tolerance to NaCl, PEG and $CdCl_2$ individually and as combined stresses (Mekawy et al., 2020). *A. thaliana* (L.) Heynh. overexpressing *MT* type 2 from *P. dactylifera* L. had a better tolerance towards drought and oxidative stresses (Patankar et al., 2019). Transgenic *N. tabacum* L. overexpressing *OsMT1e-P* from *O. sativa* L. had a better tolerance towards multiple abiotic stresses, including drought stress (Kumar et al., 2012).

The expression of MTs is often shown to increase under various abiotic stresses, including drought stress. For example, a three-fold increase in the expression of *MT* type 3 from *Fagopyrum esculentum* Moench was observed during drought stress (Samardžić et al., 2010). During drought stress, an increase in the expression of type 2 *MT* from *Citrullus lanatus* (Thunb.) Matsum. & Nakai was observed (Akashi et al., 2004). Similarly, the expression of *MT3* from *G. hirsutum* L. increased after the application of NaCl and PEG (Xue et al., 2009). An increase in the expression of *OsMT1* was observed under PEG stress (Yang et al., 2009). The expression of *ZjMT1* from stress-tolerant *Ziziphus jujuba* Mill. increases under PEG, NaCl and $CdCl_2$ stress (Yang et al., 2015). An increase in the expression of type 1 *MT* under PEG-induced drought stress has been observed for *Trifolium repens* L. (Li et al., 2016). We have shown that the expression of *AsMT1-3* genes increases under osmotic stress. The expression of oat *MT* genes is more responsive towards osmotic stress induced by mannitol than by PEG 6000. Only the expression of *AsMT1* in the root did not change after the application of mannitol, but in shoots, the expression of this gene increased 2.1 times. Of all three tested oat *MTs*, the highest increase in the expression was observed for *AsMT2* for both shoots and roots. Osmotic stress induced by PEG 6000 resulted in an increase in *AsMT2* expression in the shoots. Similarly, an increase of *MT* type 2 under osmotic stress was observed for *MT* from *C. sativus* L. (*CsMT2*) (Zhou et al., 2019), *Cicer arietinum* L. (*CmMet-2*) (Singh et al., 2011) and *Populus* sp. (Bae et al., 2010). It is generally accepted that plant MTs belonging to different types can fulfil different physiological roles (Leszczyszyn et al., 2013). We observed in our study that different *AsMT1-3* expression responses to osmotic stress and the different impact of the heterologous expression of *AsMT1-3* on bacterial tolerance to osmotic stress support the concept that oat MT1-3 plays varied possibly complementary roles in plant stress response.

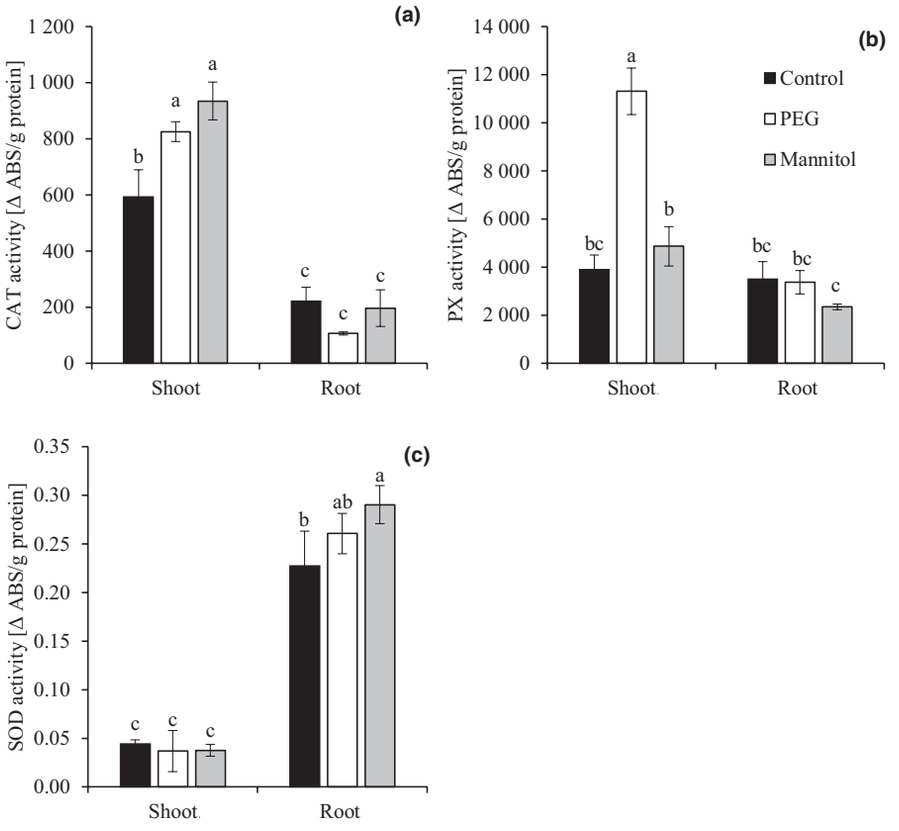

FIGURE 8 Effects of osmotic stress on (a) catalase, (b) peroxidases and (c) superoxide dismutase activity in the leaves and roots of oat. Bars represent mean values ($n = 5$) ± SE. Different letters for each organ indicate significant differences between means (Duncan's multiple range test; $p \leq .05$).

TABLE 5 Pearson coefficients of linear correlation ($p \leq .05$) between relative expression level of metallothioneins (*MT1-3*), soluble sugars (SUG), phenolic compounds (PHE), the activity of superoxide dismutase (SOD), catalase (CAT), peroxidases (PX) and abscisic acid (ABA) content in shoots (S) and roots (R) of oat seedlings.

Variable	MT1 S	MT1 R	MT2 S	MT2 R	MT3 S	MT3 R	SUG S	SUG R	PHE S	PHE R	SOD S	SOD R	CAT S	CAT R	PX S	PX R	ABA S	ABA R
MT1 S	1.000																	
MT1 R	−.988	1.000																
MT2 S	1.000	−.983	1.000															
MT2 R	.986	−1.000	.981	1.000														
MT3 S	.786	−.872	.768	.878	1.000													
MT3 R	.934	−.868	.944	.862	.514	1.000												
SUG S	.238	−.087	.267	.074	−.412	.570	1.000											
SUG R	−.690	.571	−.711	−.560	−.095	−.903	−.868	1.000										
PHE S	.871	−.785	.885	.777	.381	.989	.685	−.957	1.000									
PHE R	−.601	.716	−.577	−.725	−.966	−.275	.633	−.165	−.130	1.000								
SOD S	−.534	.399	−.559	−.387	.102	−.801	−.948	.981	−.881	−.355	1.000							
SOD R	.914	−.842	.926	.835	.469	.999	.611	−.924	.995	−.225	−.831	1.000						
CAT S	.831	−.736	.847	.728	.310	.975	.738	−.976	.997	−.055	−.914	.985	1.000					
CAT R	.183	−.331	.154	.344	.751	−.180	−.911	.586	−.324	−.896	.733	−.231	−.394	1.000				
PX S	−.261	.405	−.233	−.417	−.802	.101	.875	−.519	.248	.929	−.677	.152	.320	−.997	1.000			
PX R	−.999	.993	−.998	−.992	−.808	−.920	−.203	.663	−.852	.629	.503	−.899	−.810	−.219	.296	1.000		
ABA S	.898	−.820	.910	.812	.434	.996	.642	−.938	.998	−.187	−.852	.999	.991	−.269	.191	−.881	1.000	
ABA R	.449	−.307	.475	.295	−.199	.739	.975	−.957	.831	.444	−.995	.773	.870	−.796	.745	−.417	.797	1.000

0≤|r|≤.2	.2<|r|≤.4	.4<|r|≤.7	.7<|r|≤.9	.9<|r|≤1.0	−.9<|r|≤−1.0	−.7<|r|≤−.9	−.4<|r|≤−.7	−.2<|r|≤−.4	0≤|r|≤−.2

Correlation analysis has shown very strong positive correlations between the activities of analysed antioxidant enzymes (CAT, PX and SOD) and the expression level of *MTs*; however, those correlations were dependent on the type of *AsMT* and the type of antioxidant enzyme. Kumar et al. (2012) showed that MT1 protects against oxidative stress primarily through efficient scavenging of ROS in rice.

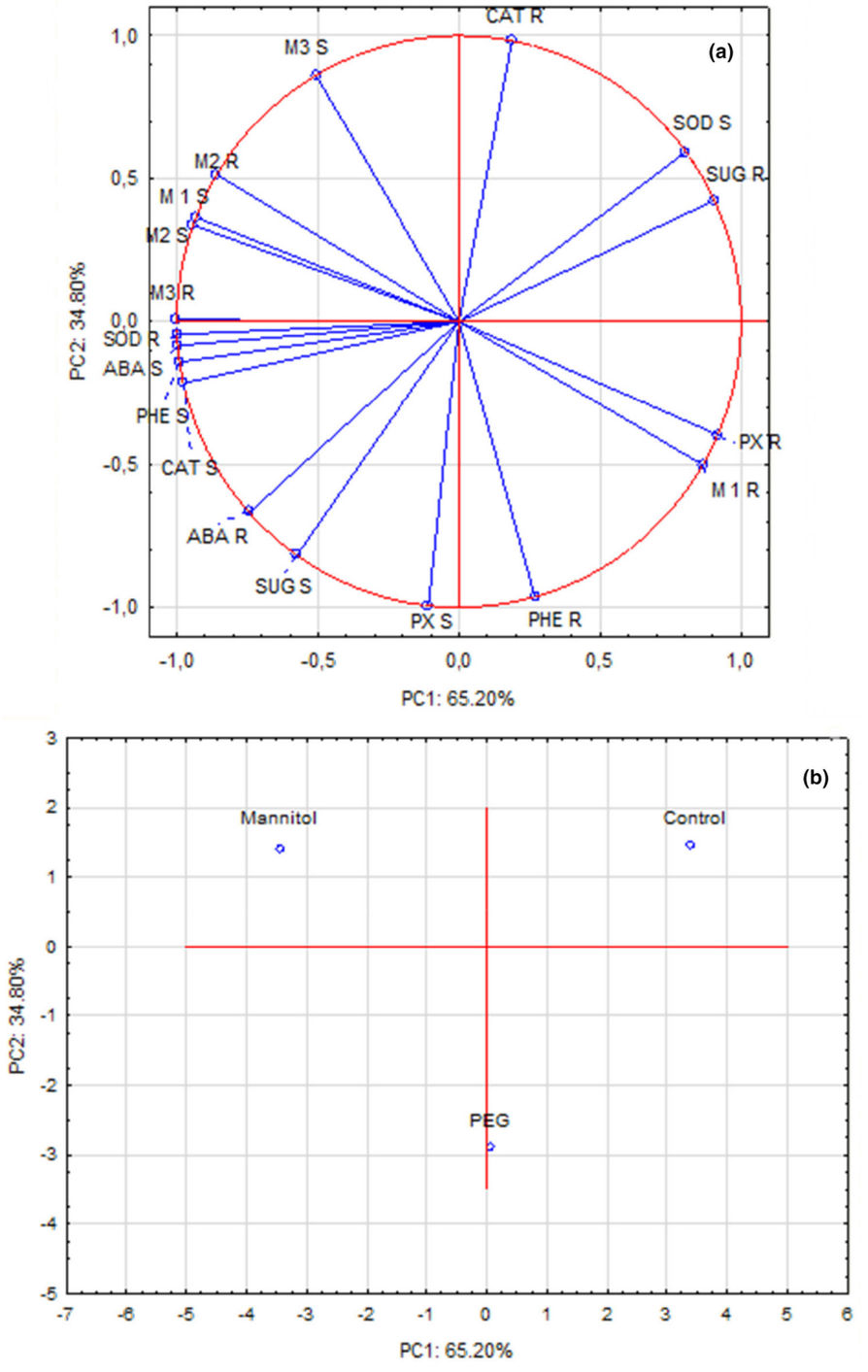

FIGURE 9 Biplot of the first two principal components (PC1 and PC2) for (a) the expression level of *AsMT1-3*; the content of SUG, PHE and ABA; and the activity of SOD, CAT and PX in shoots (S) and roots (R) of oat seedlings and (b) the distribution of treatments (control, mannitol and PEG) based on PC1 and PC2 obtained from the principal component analysis.

Also, Usha et al. (2009) reported the involvement of plant MT3 in maintaining the redox balance in wild rice exposed to heavy metals. MT type 2 from watermelon was shown to efficiently detoxify hydroxyl radicals (Akashi et al., 2004). The number of research studies indicating the antioxidant properties of plant MTs is constantly increasing, but the link between metals and redox metabolism in the plant cell remains elusive. Both CAT and SOD need metal ions for their function. Interestingly, it has been shown that MTs can transfer metal ions that can be used by SOD and CAT (Deljoona & Shahpiri, 2018). Experiments on transgenic *A. thaliana* (L.) Heynh. showed that MTs and CAT complement each other in the process of ROS scavenging. Overexpression of *AtMT2a* in *Arabidopsis cat2* mutant increased the CAT activity under cold stress (Zhu et al., 2009). SOD activity was elevated in plants overexpressed *Tamarix androssowii* Litv. *MT3* gene

in response to Cd^{2+} stress (Zhou et al., 2014). Several other studies showed that the plant antioxidant system is more efficient in plants overexpressing *MTs* (Rahman et al., 2020; Yang et al., 2009). In vitro studies on animal MTs showed that they can effectively scavenge free radicals reacting with ROS even faster than typical antioxidant enzymes (Kumari et al., 1998; Pan & Zhang, 2006; Ruttkay-Nedecky et al., 2013; Zeitoun-Ghandour et al., 2011). The release of Zn^{2+} bound to MTs via oxidation may have also a significant impact on general cell metabolism. In animals, it is now widely accepted that Zn^{2+} serves as a signalling molecule (Maret, 2017) and a possibly similar mechanism exists also in plants. Therefore, it might be hypothesized that zinc ions are the indirect link between MTs and antioxidant enzymes.

The accumulation of ABA under unfavourable environmental conditions such as drought, heat stress, salinity and cold is well documented in the literature (Vishwakarma et al., 2017). ABA protects plants under abiotic stress, in particular through induction of the production of osmoprotective proteins and metabolites, and the regulation of stomatal conductance (Zhu et al., 2010). Gong et al. (2022) showed that in oat drought-tolerant cultivar DA92-2F6, ABA signalling pathways were activated, but in drought-sensitive Longyan, they were suppressed. In our study, the highest concentration of ABA was observed in oat shoots where stress was induced by mannitol. Moreover, there was a very strong positive correlation between the ABA and the soluble sugar concentration in shoots. The exact mechanism of interaction between sugars and ABA remains to be elucidated since they form a complex cascade regulating thousands of genes involved in photosynthesis and metabolism (Chandrasekaran et al., 2020). Furthermore, we recorded a strong positive correlation between the expression level of *AsMT1* and *AsMT2* and the content of ABA. The upregulation of *MT* expression after the application of ABA was observed for *Prosopis juliflora* (Sw.) DC. *MT1-3* (Usha et al., 2009), *G. hirsutum MT3a* (Xue et al., 2009), *Hevea brasiliensis* Muli. Arg. *MT2a* (Li et al., 2015), and *O. sativa* L. *MT1a* and *MT2b* (Yang et al., 2009; Yuan et al., 2008). Moreover, ABA is a strong inductor of the expression of seed-specific type 4 MT (Kawashima et al., 1992; Ren et al., 2012). These results may suggest that ABA plays a crucial role not only in regulating the state of hydration in plants through the stomatal opening but also through the induction of the expression of *MT* genes (Li et al., 2016).

5 | CONCLUSIONS

Three new *MT* genes from *Avena sativa* L. were cloned, and their response to osmotic stress using qPCR and heterologous expression in a prokaryotic system was assessed. The observed upregulation of the expression of oat *MTs* in response to osmotic stress caused by the presence of mannitol indicates that these MTs are involved in the protection of the oat against osmotic stress. We found significant positive correlations between the expression of oat *MTs* with osmoprotectants and antioxidant enzymes. For the first time, we showed that pMTs are implicated in the network for plant osmotic stress response. Further work is needed to elucidate whether oat MTs are involved in ABA-dependent or ABA-independent signalling pathways in water-deficit stress response.

AUTHOR CONTRIBUTIONS

Wiktoria Konieczna: Data curation; formal analysis; investigation; visualization; writing – original draft; writing – review and editing. **Agnieszka Mierek-Adamska:** Data curation; formal analysis; investigation; methodology; visualization; writing – original draft; writing – review and editing. **Marzena Warchoł:** Data curation; formal analysis; investigation; methodology; validation; visualization; writing – original draft; writing – review and editing. **Edyta Skrzypek:** Conceptualization; data curation; formal analysis; funding acquisition; methodology; resources; supervision; writing – original draft; writing – review and editing. **Grażyna B. Dąbrowska:** Conceptualization; data curation; funding acquisition; methodology; project administration; resources; supervision; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are available from the corresponding author on request.

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REFERENCES

Aebi, H. (1984). Catalase in vitro. *Methods in Enzymology*, *105*, 121–125. https://doi.org/10.1016/S0076-6879(84)05016-3

Akashi, K., Nishimura, N., Ishida, Y., & Yokota, A. (2004). Potent hydroxyl radical-scavenging activity of drought-induced type-2 metallothionein in wild watermelon. *Biochemical and Biophysical Research Communications*, *323*(1), 72–78. https://doi.org/10.1016/j.bbrc.2004.08.056

Alscher, R. G., Erturk, N., & Heath, L. (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *Journal of Experimental Botany*, *53*(372), 1331–1341. https://doi.org/10.1093/jexbot/53.372.1331

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Aninbon, C., Jogloy, S., Vorasoot, N., Patanothai, A., Nuchadomrong, S., & Senawong, T. (2016). Effect of end of season water deficit on phenolic compounds in peanut genotypes with different levels of

resistance to drought. *Food Chemistry*, *196*, 123–129. https://doi.org/10.1016/j.foodchem.2015.09.022

Ayaz, F. A., & Bertoft, E. (2001). Sugar and phenolic acid composition of stored commercial oleaster fruits. *Journal of Food Composition and Analysis*, *14*(5), 505–511. https://doi.org/10.1006/JFCA.2001.1004

Bae, E. K., Lee, H., Lee, J. S., & Noh, E. W. (2010). Isolation and characterization of osmotic stress-induced genes in poplar cells by suppression subtractive hybridization and cDNA microarray analysis. *Plant Physiology and Biochemistry*, *48*(2–3), 136–141. https://doi.org/10.1016/j.plaphy.2009.11.002

Barcchiya, J., Meena, R. K., & Lal, N. (2017). Oat is a multifunctional cereal crop. *Innovative Farming*, *2*(2), 114–116.

Bolouri-Moghaddam, M. R., Le Roy, K., Xiang, L., Rolland, F., & Van Den Ende, W. (2010). Sugar signalling and antioxidant network connections in plant cells. *FEBS Journal*, *277*(9), 2022–2037. https://doi.org/10.1111/j.1742-4658.2010.07633.x

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254. https://doi.org/10.1006/abio.1976.9999

Chandrasekaran, U., Luo, X., Zhou, W., & Shu, K. (2020). Multifaceted signaling networks mediated by abscisic acid insensitive 4. *Plant Communications*, *1*(3), 100040. https://doi.org/10.1016/j.xplc.2020.100040

Chiappero, J., Cappellari, L. R., Palermo, T. B., Giordano, W., Khan, N., & Banchio, E. (2021). Antioxidant status of medicinal and aromatic plants under the influence of growth-promoting rhizobacteria and osmotic stress. *Industrial Crops and Products*, *167*, 113541. https://doi.org/10.1016/J.INDCROP.2021.113541

Chung, I. M., Kim, J. J., Lim, J. D., Yu, C. Y., Kim, S. H., & Hahn, S. J. (2006). Comparison of resveratrol, SOD activity, phenolic compounds and free amino acids in *Rehmannia glutinosa* under temperature and water stress. *Environmental and Experimental Botany*, *56*(1), 44–53. https://doi.org/10.1016/J.ENVEXPBOT.2005.01.001

Cobbett, C. S. (2000). Phytochelatins and their roles in heavy metal detoxification. *Plant Physiology*, *123*(3), 825–832. https://doi.org/10.1104/pp.123.3.825

Dąbrowska, G., Hrynkiewicz, K., & Trejgell, A. (2012). Do arbuscular mycorrhizal fungi affect metallothionein *MT2* expression in *Brassica napus* L. roots? *Acta Biologica Cracoviensia Series Botanica*, *54*(1), 34–39. https://doi.org/10.2478/v10182-012-0003-1

Dąbrowska, G., Kata, A., Goc, A., Szechyńska-Hebda, M., & Skrzypek, E. (2007). Characteristics of the plant ascorbate peroxidase family. *Acta Biologica Cracoviensia Series Botanica*, *49*(1), 7–17.

Dąbrowska, G., Mierek-Adamska, A., & Goc, A. (2012). Plant metallothioneins: Putative functions identified by promoter analysis in silico. *Acta Biologica Cracoviensia Series Botanica*, *54*(2), 109–120. https://doi.org/10.2478/v10182-012-0030-y

Dąbrowska, G., Mierek-Adamska, A., & Goc, A. (2013). Characterisation of *Brassica napus* L. metallothionein genes (*BnMTs*) expression in organs and during seed germination. *Australian Journal of Crop Science*, *7*(9), 1324–1332.

Dąbrowska, G. B. (2014). The role of metallothionein genes and microorganisms in the reaction of rape to stress factors. *Oilseed Crops*, *35*, 49–58. https://doi.org/10.5604/12338273.1137526

Daou, C., & Zhang, H. (2012). Oat beta-glucan: Its role in health promotion and prevention of diseases. *Comprehensive Reviews in Food Science and Food Safety*, *11*(4), 355–365. https://doi.org/10.1111/J.1541-4337.2012.00189.X

Das, K., & Roychoudhury, A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science*, *2*, 1–13. https://doi.org/10.3389/fenvs.2014.00053

Deljoona, R., & Shahpiri, A. (2018). Enhancement of catalase and superoxide dismutase activities in transgenic *Escherichia coli* expressing rice metallothionein isoforms. *Journal of BioScience and Biotechnology*, *7*(1), 5–10.

Dobrev, P., & Kamínek, M. (2002). Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. *Journal of Chromatography A*, *950*(1–2), 21–29. https://doi.org/10.1016/S0021-9673(02)00024-9

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350–356. https://doi.org/10.1021/ac60111a017

Dziurka, K., Dziurka, M., Warchoł, M., Czyczyło-Mysza, I., Marcińska, I., Noga, A., Kapłoniak, K., & Skrzypek, E. (2019). Endogenous phytohormone profile during oat (*Avena sativa* L.) haploid embryo development. *In Vitro Cellular and Developmental Biology - Plant*, *55*(2), 221–229. https://doi.org/10.1007/s11627-019-09967-5

Evans, K. M., Gatehouse, J. A., Lindsay, W. P., Shi, J., Tommey, A. M., & Robinson, N. J. (1992). Expression of the pea metallothionein-like gene *PsMTA* in *Escherichia coli* and *Arabidopsis thaliana* and analysis of trace metal ion accumulation: Implications for PsMTA function. *Plant Molecular Biology*, *20*(6), 1019–1028. https://doi.org/10.1007/BF00028889

Fàbregas, N., Yoshida, T., & Fernie, A. R. (2020). Role of Raf-like kinases in SnRK2 activation and osmotic stress response in plants. *Nature Communications*, *11*(1), 6184. https://doi.org/10.1038/s41467-020-19977-2

Freisinger, E. (2011). Structural features specific to plant metallothioneins. *Journal of Biological Inorganic Chemistry*, *16*(7), 1035–1045. https://doi.org/10.1007/s00775-011-0801-z

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R., & Bairoch, A. (2003). ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Research*, *31*(13), 3784–3788. https://doi.org/10.1093/NAR/GKG563

Gechev, T. S., Van Breusegem, F., Stone, J. M., Denev, I., & Laloi, C. (2006). Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *BioEssays*, *28*(11), 1091–1101. https://doi.org/10.1002/bies.20493

Ghosh, D., Gupta, A., & Mohapatra, S. (2019). A comparative analysis of exopolysaccharide and phytohormone secretions by four drought-tolerant rhizobacterial strains and their impact on osmotic-stress mitigation in *Arabidopsis thaliana*. *World Journal of Microbiology and Biotechnology*, *35*(6), 90. https://doi.org/10.1007/s11274-019-2659-0

Gibson, S. I. (2005). Control of plant development and gene expression by sugar signaling. *Current Opinion in Plant Biology*, *8*(1), 93–102. https://doi.org/10.1016/J.PBI.2004.11.003

Gong, W., Ju, Z., Chai, J., Zhou, X., Lin, D., Su, W., & Zhao, G. (2022). Physiological and transcription analyses reveal the regulatory mechanism in oat (*Avena sativa*) seedlings with different drought resistance under PEG-induced drought stress. *Agronomy*, *12*(5), 1005. https://doi.org/10.3390/AGRONOMY12051005

Goyal, M., & Kaur, N. (2018). Low temperature induced oxidative stress tolerance in oats (*Avena sativa* L.) genotypes. *Indian Journal of Plant Physiology*, *23*(2), 316–324. https://doi.org/10.1007/s40502-018-0371-y

Gu, C.-S., Liu, L.-Q., Zhao, Y.-H., Deng, Y.-M., Zhu, X.-D., & Huang, S.-Z. (2014). Overexpression of *Iris lactea* var. *chinensis* metallothionein *IlMT2a* enhances cadmium tolerance in *Arabidopsis thaliana*. *Ecotoxicology and Environmental Safety*, *105*(1), 22–28. https://doi.org/10.1016/j.ecoenv.2014.04.002

Guo, W.-J., Bundithya, W., & Goldsbrough, P. B. (2003). Characterization of the *Arabidopsis* metallothionein gene family: Tissue-specific expression and induction during senescence and in response to copper. *New Phytologist*, *159*(2), 369–381. https://doi.org/10.1046/j.1469-8137.2003.00813.x

Guo, W. J., Meetam, M., & Goldsbrough, P. B. (2008). Examining the specific contributions of individual Arabidopsis metallothioneins to copper distribution and metal tolerance. *Plant Physiology*, *146*(4), 1697–1706. https://doi.org/10.1104/pp.108.115782

Hakimi, A., Monneveux, P., & Breeding, G. G. (1995). Soluble sugars, proline, and relative water content (RCW) as traits for improving

drought tolerance and divergent selection for RCW from *T. polonicum*. *Journal of Genetics and Breeding*, *49*, 237–244.

Hoagland, D., & Arnon, D. (1938). The water-culture method for growing plants without soil. *California Agricultural Experiment Station*, *347*, 1–39.

Hoekstra, F. A., Golovina, E. A., & Buitink, J. (2001). Mechanisms of plant desiccation tolerance. *Trends in Plant Science*, *6*(9), 431–438. https://doi.org/10.1016/S1360-1385(01)02052-0

Hrynkiewicz, K., Dąbrowska, G., Baum, C., Niedojadlo, K., & Leinweber, P. (2012). Interactive and single effects of ectomycorrhiza formation and *Bacillus cereus* on metallothionein *MT1* expression and phytoextraction of Cd and Zn by willows. *Water, Air, and Soil Pollution*, *223*(3), 957–968. https://doi.org/10.1007/s11270-011-0915-5

Hura, T., Dziurka, M., Hura, K., Ostrowska, A., & Dziurka, K. (2016). Different allocation of carbohydrates and phenolics in dehydrated leaves of triticale. *Journal of Plant Physiology*, *202*, 1–9. https://doi.org/10.1016/J.JPLPH.2016.06.018

Hura, T., Hura, K., & Grzesiak, S. (2008). Contents of total phenolics and ferulic acid, and PAL activity during water potential changes in leaves of maize single-cross hybrids of different drought tolerance. *Journal of Agronomy and Crop Science*, *194*(2), 104–112. https://doi.org/10.1111/J.1439-037X.2008.00297.X

Islam, M. R., Xue, X., Mao, S., Ren, C., Eneji, A. E., & Hu, Y. (2011). Effects of water-saving superabsorbent polymer on antioxidant enzyme activities and lipid peroxidation in oat (*Avena sativa* L.) under drought stress. *Journal of the Science of Food and Agriculture*, *91*(4), 680–686. https://doi.org/10.1002/jsfa.4234

Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D., & Bohnert, H. J. (2001). Gene expression profiles during the initial phase of salt stress in rice. *The Plant Cell*, *13*(4), 889–905. https://doi.org/10.1105/TPC.13.4.889

Kawashima, I., Kennedy, T. D., Chino, M., & Lane, B. G. (1992). Wheat Ec metallothionein genes: Like mammalian Zn^{2+} metallothionein genes, wheat Zn^{2+} metallothionein genes are conspicuously expressed during embryogenesis. *European Journal of Biochemistry*, *209*(3), 971–976. https://doi.org/10.1111/j.1432-1033.1992.tb17370.x

Keunen, E., Peshev, D., Vangronsveld, J., van den Ende, W., & Cuypers, A. (2013). Plant sugars are crucial players in the oxidative challenge during abiotic stress: Extending the traditional concept. *Plant and Cell Environment*, *36*(7), 1242–1255. https://doi.org/10.1111/pce.12061

Koszucka, A. M., & Dąbrowska, G. (2006). Plant metallothioneins. *Advances in Cell Biology*, *33*(2), 285–302.

Król, A., Amarowicz, R., & Weidner, S. (2014). Changes in the composition of phenolic compounds and antioxidant properties of grapevine roots and leaves (*Vitis vinifera* L.) under continuous of long-term drought stress. *Acta Physiologiae Plantarum*, *36*(6), 1491–1499. https://doi.org/10.1007/s11738-014-1526-8

Kumar, G., Kushwaha, H. R., Panjabi-Sabharwal, V., Kumari, S., Joshi, R., Karan, R., Mittal, S., Pareek, S. L. S., & Pareek, A. (2012). Clustered metallothionein genes are co-regulated in rice and ectopic expression of OsMT1e-P confers multiple abiotic stress tolerance in tobacco via ROS scavenging. *BMC Plant Biology*, *12*(1), 1. https://doi.org/10.1186/1471-2229-12-107

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Kumari, M. V. R., Hiramatsu, M., & Ebadi, M. (1998). Free radical scavenging actions of metallothionein isoforms I and II. *Free Radical Research*, *29*(2), 93–101. https://doi.org/10.1080/10715769800300111

Lee, J., Shim, D., Song, W. Y., Hwang, I., & Lee, Y. (2004). *Arabidopsis* metallothioneins 2a and 3 enhance resistance to cadmium when expressed in *Vicia faba* guard cells. *Plant Molecular Biology*, *54*(6), 805–815. https://doi.org/10.1007/s11103-004-0190-6

Leszczyszyn, O. I., Imam, H. T., & Blindauer, C. A. (2013). Diversity and distribution of plant metallothioneins: A review of structure, properties and functions. *Metallomics*, *5*(9), 1146–1169. https://doi.org/10.1039/c3mt00072a

Li, Y., Chen, Y. Y., Yang, S. G., & Tian, W. M. (2015). Cloning and characterization of *HbMT2a*, a metallothionein gene from *Hevea brasiliensis* Muell. Arg differently responds to abiotic stress and heavy metals. *Biochemical and Biophysical Research Communications*, *461*(1), 95–101. https://doi.org/10.1016/j.bbrc.2015.03.175

Li, Z., Zhang, Y., Zhang, X., Peng, Y., Merewitz, E., Ma, X., Huang, L., & Yan, Y. (2016). The alterations of endogenous polyamines and phytohormones induced by exogenous application of spermidine regulate antioxidant metabolism, metallothionein and relevant genes conferring drought tolerance in white clover. *Environmental and Experimental Botany*, *124*, 22–38. https://doi.org/10.1016/j.envexpbot.2015.12.004

Lichtenthaler, H., & Wellburn, A. (1983). Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochemical Society Transactions*, *11*(5), 591–592. https://doi.org/10.1042/bst0110591

Liu, J., Shi, X., Qian, M., Zheng, L., Lian, C., Xia, Y., & Shen, Z. (2015). Copper-induced hydrogen peroxide upregulation of a metallothionein gene, OsMT2c, from *Oryza sativa* L. confers copper tolerance in *Arabidopsis thaliana*. *Journal of Hazardous Materials*, *294*, 99–108. https://doi.org/10.1016/j.jhazmat.2015.03.060

Llanes, A., Andrade, A., Masciarelli, O., Alemano, S., & Luna, V. (2016). Drought and salinity alter endogenous hormonal profiles at the seed germination phase. *Seed Science Research*, *26*, 1–13. https://doi.org/10.1017/S0960258515000331

Lűck, H. (1962). Methoden der enzymatischen analyse. In H. U. Bergmeyer (Ed.), *Verlag Chemie* (pp. 895–897). GmbH Weinheim.

Maghsoodi, M., & Razmjoo, J. (2015). Identify physiological markers for drought tolerance in alfalfa. *Agronomy Journal*, *107*(1), 149–157. https://doi.org/10.2134/AGRONJ14.0255

Maret, W. (2017). Zinc in cellular regulation: The nature and significance of "zinc signals". *International Journal of Molecular Sciences*, *18*(11), 2285. https://doi.org/10.3390/ijms18112285

McCord, J., & Fiodovich, I. (1969). Superoxide dismutase an enzimic function for erytrocuperein (hemocuperein). *The Journal of Biological Chemistry*, *244*, 6049–6055.

Mekawy, A. M. M., Assaha, D. V. M., & Ueda, A. (2020). Constitutive overexpression of rice metallothionein-like gene OsMT-3a enhances growth and tolerance of *Arabidopsis* plants to a combination of various abiotic stresses. *Journal of Plant Research*, *133*(3), 429–440. https://doi.org/10.1007/s10265-020-01187-y

Mierek-Adamska, A., Dąbrowska, G. B., & Blindauer, C. A. (2018). The type 4 metallothionein from *Brassica napus* seeds folds in a metal-dependent fashion and favours zinc over other metals. *Metallomics*, *10*(10), 1430–1443. https://doi.org/10.1039/c8mt00161h

Mierek-Adamska, A., Kotowicz, K., Goc, A., Boniecka, J., Berdychowska, J., & Dąbrowska, G. B. (2019). Potential involvement of rapeseed (*Brassica napus* L.) metallothioneins in the hydrogen peroxide-induced regulation of seed vigour. *Journal of Agronomy and Crop Science*, *205*(6), 598–607. https://doi.org/10.1111/jac.12361

Miller, G., Suzuki, N., Ciftci-Yilmaz, S., & Mittler, R. (2010). Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant, Cell and Environment*, *33*(4), 453–467. https://doi.org/10.1111/j.1365-3040.2009.02041.x

Mir, G., Domènech, J., Huguet, G., Guo, W. J., Goldsbrough, P., Atrian, S., & Molinas, M. (2004). A plant type 2 metallothionein (MT) from cork tissue responds to oxidative stress. *Journal of Experimental Botany*, *55*(408), 2483–2493. https://doi.org/10.1093/jxb/erh254

Mohammadkhani, N., & Heidari, R. (2008). Effects of drought stress on soluble proteins in two maize varieties. *Turkish Journal of Biology*, *32*, 23–30.

Mostajeran, A., & Rahimi-Eichi, V. (2009). Effects of drought stress on growth and yield of rice (*Oryza sativa* L.) cultivars and accumulation of proline and soluble sugars in sheath and blades of their different ages leaves. *American-Eurasian Journal of Agricultural and Environmental Sciences*, *5*(2), 264–272.

Murphy, A., & Taiz, L. (1995). Comparison of metallothionein gene expression and nonprotein thiols in ten *Arabidopsis thaliana* ecotypes. Correlation with copper tolerance. *Plant Physiology*, *109*(3), 945–954. https://doi.org/10.1104/pp.109.3.945

Nadarajah, K. K. (2020). ROS homeostasis in abiotic stress tolerance in plants. *International Journal of Molecular Sciences*, *21*(15), 1–29. https://doi.org/10.3390/IJMS21155208

Nayer, M., & Reza, H. (2008). Drought-induced accumulation of soluble sugars and Proline in two maize varieties. *World Applied Sciences Journal*, *3*(3), 448–453.

Ozturk, Z. N., Talamé, V., Deyholos, M., Michalowski, C. B., Galbraith, D. W., Gozukirmizi, N., Tuberosa, R., & Bohnert, H. J. (2002). Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. *Plant Molecular Biology*, *48*, 551–573. https://doi.org/10.1023/A:1014875215580

Pan, L., & Zhang, H. (2006). Metallothionein, antioxidant enzymes and DNA strand breaks as biomarkers of Cd exposure in a marine crab, *Charybdis japonica*. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *144*(1), 67–75. https://doi.org/10.1016/j.cbpc.2006.06.001

Pastuszak, J., Szczerba, A., Dziurka, M., Hornyák, M., Kopeć, P., Szklarczyk, M., & Płażek, A. (2021). Physiological and biochemical response to *Fusarium culmorum* infection in three durum wheat genotypes at seedling and full anthesis stage. *International Journal of Molecular Sciences*, *22*(14), 7433. https://doi.org/10.3390/ijms22147433

Patankar, H. V., Al-Harrasi, I., al Kharusi, L., Jana, G. A., Al-Yahyai, R., Sunkar, R., & Yaish, M. W. (2019). Overexpression of a Metallothionein 2A gene from date palm confers abiotic stress tolerance to yeast and *Arabidopsis thaliana*. *International Journal of Molecular Sciences*, *20*(12), 2871. https://doi.org/10.3390/ijms20122871

Perveen, S., Iqbal, M., Saeed, M., Iqbal, N., Zafar, S., & Mumtaz, T. (2019). Cysteine-induced alterations in physicochemical parameters of oat (*Avena sativa* L. var. Scott and F-411) under drought stress. *Biologia Futura*, *70*(1), 16–24. https://doi.org/10.1556/019.70.2019.03

Peterson, D. M. (2015). Composition and nutritional characteristics of oat grain and products. In H. G. Marshall & M. E. Sorrells (Eds.), *Oat science and technology* (pp. 265–292). John Wiley & Sons, Ltd. https://doi.org/10.2134/AGRONMONOGR33.C10

Pisulewska, E., Tobiasz-Salach, R., Witkowicz, R., Cieślik, E., & Bobrecka-Jamro, D. (2011). Effect of habitat conditions on content and quality of lipids in selected oat forms. *Żywność. Nauka. Technologia. Jakość*, *3*(76), 66–77.

Pluskota, W. E., Szablińska, J., Obendorf, R. L., Górecki, R. J., & Lahuta, L. B. (2015). Osmotic stress induces genes, enzymes and accumulation of galactinol, raffinose and stachyose in seedlings of pea (*Pisum sativum* L.). *Acta Physiologiae Plantarum*, *37*(8), 1–13. https://doi.org/10.1007/s11738-015-1905-9

Rabbani, M. A., Maruyama, K., Abe, H., Khan, M. A., Katsura, K., Ito, Y., Yoshiwara, K., Seki, M., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2003). Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. *Plant Physiology*, *133*(4), 1755–1767. https://doi.org/10.1104/PP.103.025742

Rahman, S.-U., Khalid, M., Hui, N., Kayani, S. I., & Tang, K. (2020). Diversity and versatile functions of metallothioneins produced by plants: A review. *Pedosphere*, *30*(5), 577–588. https://doi.org/10.1016/S1002-0160(20)60022-4

Reddy, A. R., Chaitanya, K. V., & Vivekanandan, M. (2004). Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. *Journal of Plant Physiology*, *161*(11), 1189–1202. https://doi.org/10.1016/j.jplph.2004.01.013

Ren, Y., Liu, Y., Chen, H., Li, G., Zhang, X., & Zhao, J. (2012). Type 4 metallothionein genes are involved in regulating Zn ion accumulation in late embryo and in controlling early seedling growth in *Arabidopsis*. *Plant, Cell and Environment*, *35*(4), 770–789. https://doi.org/10.1111/j.1365-3040.2011.02450.x

Rines, H. W., Molnar, S. J., Tinker, N. A., & Phillips, R. L. (2006). Oat. In C. Kole (Ed.), *Cereals and millets. Genome mapping and molecular Breeding in plants* (pp. 211–242). Springer. https://doi.org/10.1007/978-3-540-34389-9_5

Rosa, M., Prado, C., Podazza, G., Interdonato, R., González, J. A., Hilal, M., & Prado, F. E. (2009). Soluble sugars—Metabolism, sensing and abiotic stress. *Plant Signaling & Behavior*, *4*(5), 388–393. https://doi.org/10.4161/PSB.4.5.8294

RStudio Team. (2020). *RStudio: Integrated development for R*. RStudio.

Ruan, Y. L. (2014). Sucrose metabolism: Gateway to diverse carbon use and sugar signaling. In *Annual review of plant biology* (Vol. *65*, pp. 33–67). Annual Reviews Inc. https://doi.org/10.1146/annurev-arplant-050213-040251

Ruttkay-Nedecky, B., Nejdl, L., Gumulec, J., Zitka, O., Masarik, M., Eckschlager, T., Stiborova, M., Adam, V., & Kizek, R. (2013). The role of metallothionein in oxidative stress. *International Journal of Molecular Sciences*, *14*(3), 6044–6066. https://doi.org/10.3390/ijms14036044

Samardžić, J. T., Nikolić, D. B., Timotijević, G. S., Jovanović, Ž. S., Milisavljević, M. E., & Maksimović, V. R. (2010). Tissue expression analysis of *FeMT3*, a drought and oxidative stress related metallothionein gene from buckwheat (*Fagopyrum esculentum*). *Journal of Plant Physiology*, *167*(16), 1407–1411. https://doi.org/10.1016/j.jplph.2010.05.016

Sambrook, J., & Russell, D. W. (2001). *Molecular cloning a laboratory manual*. Cold Spring Harbor Laboratory Press.

Shao, H. B., Chu, L. Y., Jaleel, C. A., Manivannan, P., Panneerselvam, R., & Shao, M. A. (2009). Understanding water deficit stress-induced changes in the basic metabolism of higher plants-biotechnologically and sustainably improving agriculture and the ecoenvironment in arid regions of the globe. *Critical Reviews in Biotechnology*, *29*(2), 131–151. https://doi.org/10.1080/07388550902869792

Sikora, M., & Świeca, M. (2018). Effect of ascorbic acid postharvest treatment on enzymatic browning, phenolics and antioxidant capacity of stored mung bean sprouts. *Food Chemistry*, *239*, 1160–1166. https://doi.org/10.1016/j.foodchem.2017.07.067

Singh, R. K., Anandhan, S., Singh, S., Patade, V. Y., Ahmed, Z., & Pande, V. (2011). Metallothionein-like gene from *Cicer microphyllum* is regulated by multiple abiotic stresses. *Protoplasma*, *248*(4), 839–847. https://doi.org/10.1007/s00709-010-0249-y

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, *16*(3), 144–158.

Skrzypek, E., Szechyńska-Hebda, M., Dąbrowska, G. B., & Goc, A. (2008). The role of osmotic stress during in vitro regeneration of *Triticum aestivum* L. and *Vicia faba* ssp. minor. *Zeszyty Problemowe Postępów Nauk Rolniczych*, *524*, 221–230.

Soda, N., Sharan, A., Gupta, B. K., Singla-Pareek, S. L., & Pareek, A. (2016). Evidence for nuclear interaction of a cytoskeleton protein (OsIFL) with metallothionein and its role in salinity stress tolerance. *Scientific Reports*, *6*, 1–14. https://doi.org/10.1038/srep34762

Storey, K. B., & Storey, J. M. (2000). *Environmental stressors and gene responses*. Elsevier.

Swigonska, S., Amarowicz, R., Król, A., Mostek, A., Badowiec, A., & Weidner, S. (2014). Influence of abiotic stress during soybean germination followed by recovery on the phenolic compounds of radicles and their antioxidant capacity. *Acta Societatis Botanicorum Poloniae*, *83*(3), 209–218. https://doi.org/10.5586/asbp.2014.026

Szechyńska-Hebda, M., Skrzypek, E., Dąbrowska, G., Wędzony, M., & van Lammeren, A. (2012). The effect of endogenous hydrogen peroxide induced by cold treatment in the improvement of tissue

regeneration efficiency. *Acta Physiologiae Plantarum*, *34*(2), 547–560. https://doi.org/10.1007/s11738-011-0852-3

Szymańska, S., Dąbrowska, G. B., Tyburski, J., Niedojadło, K., Piernik, A., & Hrynkiewicz, K. (2019). Boosting the *Brassica napus* L. tolerance to salinity by the halotolerant strain *Pseudomonas stutzeri* ISE12. *Environmental and Experimental Botany*, *163*, 55–68. https://doi.org/10.1016/j.envexpbot.2019.04.007

Tarkowski, Ł. P., & Van den Ende, W. (2015). Cold tolerance triggered by soluble sugars: A multifaceted countermeasure. *Frontiers in Plant Science*, *6*, 203. https://doi.org/10.3389/FPLS.2015.00203/BIBTEX

Tomas, M., Pagani, M. A., Andreo, C. S., Capdevila, M., Bofill, R., & Atrian, S. (2014). His-containing plant metallothioneins: Comparative study of divalent metal-ion binding by plant MT3 and MT4 isoforms. *Journal of Biological Inorganic Chemistry*, *19*(7), 1149–1164. https://doi.org/10.1007/s00775-014-1170-1

Turchi, A., Tamantini, I., Camussi, A. M., & Racchi, M. L. (2012). Expression of a metallothionein A1 gene of *Pisum sativum* in white poplar enhances tolerance and accumulation of zinc and copper. *Plant Science*, *183*, 50–56. https://doi.org/10.1016/J.PLANTSCI.2011.11.008

Udawat, P., Mishra, A., & Jha, B. (2014). Heterologous expression of an uncharacterized universal stress protein gene (SbUSP) from the extreme halophyte, *Salicornia brachiata*, which confers salt and osmotic tolerance to *E. coli*. *Gene*, *536*(1), 163–170. https://doi.org/10.1016/j.gene.2013.11.020

Usha, B., Venkataraman, G., & Parida, A. (2009). Heavy metal and abiotic stress inducible metallothionein isoforms from *Prosopis juliflora* (SW) D.C. show differences in binding to heavy metals in vitro. *Molecular Genetics and Genomics*, *281*(1), 99–108. https://doi.org/10.1007/s00438-008-0398-2

Vishwakarma, K., Upadhyay, N., Kumar, N., Yadav, G., Singh, J., Mishra, R. K., Kumar, V., Verma, R., Upadhyay, R. G., Pandey, M., & Sharma, S. (2017). Abscisic acid signaling and abiotic stress tolerance in plants: A review on current knowledge and future prospects. *Frontiers in Plant Science*, *8*, 161. https://doi.org/10.3389/FPLS.2017.00161/BIBTEX

Vogt, T. (2010). Phenylpropanoid biosynthesis. *Molecular Plant*, *3*(1), 2–20. https://doi.org/10.1093/MP/SSP106

Weidner, S., Brosowska-Arendt, W., Szczechura, W., Karamać, M., Kosińska, A., & Amarowicz, R. (2011). Effect of osmotic stress and post-stress recovery on the content of phenolics and properties of antioxidants in germinating seeds of grapevine vitis californica. *Acta Societatis Botanicorum Poloniae*, *80*(1), 11–19.

Wilmer, L., Tränkner, M., Pawelzik, E., & Naumann, M. (2022). Sufficient potassium supply enhances tolerance of potato plants to PEG-induced osmotic stress. *Plant Stress*, *5*, 100102. https://doi.org/10.1016/J.STRESS.2022.100102

Woo, E. S., & Lazo, J. S. (1997). Nucleocytoplasmic functionality of metallothionein. *Cancer Research*, *57*, 4236–4241.

Xia, Y., Qi, Y., Yuan, Y., Wang, G., Cui, J., Chen, Y., Zhang, H., & Shen, Z. (2012). Overexpression of *Elsholtzia haichowensis* metallothionein 1 (EhMT1) in tobacco plants enhances copper tolerance and accumulation in root cytoplasm and decreases hydrogen peroxide production. *Journal of Hazardous Materials*, *233–234*, 65–71. https://doi.org/10.1016/j.jhazmat.2012.06.047

Xue, T., Li, X., Zhu, W., Wu, C., Yang, G., & Zheng, C. (2009). Cotton metallothionein GhMT3a, a reactive oxygen species scavenger, increased tolerance against abiotic stress in transgenic tobacco and yeast. *Journal of Experimental Botany*, *60*(1), 339–349. https://doi.org/10.1093/jxb/ern291

Yadav, N. S., Singh, V. K., Singh, D., & Jha, B. (2014). A novel gene SbSI-2 encoding nuclear protein from a halophyte confers abiotic stress tolerance in *E. coli* and tobacco. *PLoS One*, *9*(7), e101926. https://doi.org/10.1371/journal.pone.0101926

Yang, M., Zhang, F., Wang, F., Dong, Z., Cao, Q., & Chen, M. (2015). Characterization of a type 1 metallothionein gene from the stresses-tolerant plant *Ziziphus jujuba*. *International Journal of Molecular Sciences*, *16*(8), 16750–16762. https://doi.org/10.3390/ijms160816750

Yang, Z., Wang, K., Aziz, U., Zhao, C., & Zhang, M. (2020). Evaluation of duplicated reference genes for quantitative real-time PCR analysis in genome unknown hexaploid oat (*Avena sativa* L.). *Plant Methods*, *16*(1), 1–14. https://doi.org/10.1186/s13007-020-00679-1

Yang, Z., Wu, Y., Li, Y., Ling, H. Q., & Chu, C. (2009). OsMT1a, a type 1 metallothionein, plays the pivotal role in zinc homeostasis and drought tolerance in rice. *Plant Molecular Biology*, *70*(1–2), 219–229. https://doi.org/10.1007/s11103-009-9466-1

Yoshida, T., Mogami, J., & Yamaguchi-Shinozaki, K. (2014). ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. *Current Opinion in Plant Biology*, *21*, 133–139. https://doi.org/10.1016/j.pbi.2014.07.009

You, J., & Chan, Z. (2015). ROS regulation during abiotic stress responses in crop plants. *Frontiers in Plant Science*, *6*, 1092. https://doi.org/10.3389/FPLS.2015.01092/BIBTEX

Yuan, J., Chen, D., Ren, Y., Zhang, X., & Zhao, J. (2008). Characteristic and expression analysis of a metallothionein gene, OsMT2b, down-regulated by cytokinin suggests functions in root development and seed embryo germination of rice. *Plant Physiology*, *146*(4), 1637–1650. https://doi.org/10.1104/pp.107.110304

Zeitoun-Ghandour, S., Leszczyszyn, O. I., Blindauer, C. A., Geier, F. M., Bundy, J. G., & Stürzenbaum, S. R. (2011). *C. elegans* metallothioneins: Response to and defence against ROS toxicity. *Molecular BioSystems*, *7*(8), 2397–2406. https://doi.org/10.1039/c1mb05114h

Zhou, B., Yao, W., Wang, S., Wang, X., & Jiang, T. (2014). The metallothionein gene, *TaMT3*, from *Tamarix androssowii* confers Cd^{2+} tolerance in tobacco. *International Journal of Molecular Sciences*, *15*(6), 10398–10409. https://doi.org/10.3390/ijms150610398

Zhou, J., & Goldsbrough, P. B. (1994). Functional homologs of fungal metallothionein genes from *Arabidopsis*. *The Plant Cell*, *6*, 875–884. https://doi.org/10.1105/TPC.6.6.875

Zhou, Y., Li, H., Chen, H., Yang, X., Yu, T., Wang, Y., Wang, Y., Jiang, K., Wang, Y., Chen, Z., & Cui, X. (2022). Proteomic investigation of molecular mechanisms in response to PEG-induced drought stress in soybean roots. *Plants*, *11*(9), 1173. https://doi.org/10.3390/plants11091173

Zhou, Y., Liu, J., Liu, S., Jiang, L., & Hu, L. (2019). Identification of the metallothionein gene family from cucumber and functional characterization of CsMT4 in *Escherichia coli* under salinity and osmotic stress. *3 Biotech*, *9*(11), 1–11. https://doi.org/10.1007/s13205-019-1929-8

Zhu, Q., Zhang, J., Gao, X., Tong, J., Xiao, L., Li, W., & Zhang, H. (2010). The Arabidopsis AP2/ERF transcription factor RAP2.6 participates in ABA, salt and osmotic stress responses. *Gene*, *457*(1–2), 1–12. https://doi.org/10.1016/J.GENE.2010.02.011

Zhu, W., Zhao, D. X., Miao, Q., Xue, T. T., Li, X. Z., & Zheng, C. C. (2009). *Arabidopsis thaliana* metallothionein, AtMT2a, mediates ROS balance during oxidative stress. *Journal of Plant Biology*, *52*(6), 585–592. https://doi.org/10.1007/s12374-009-9076-0

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Changes in physio-biochemical parameters and expression of metallothioneins in *Avena sativa* L. in response to drought

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Drought is one of the major threats to food security. Among several mechanisms involved in plant stress tolerance, one protein family—the plant metallothioneins (MTs)—shows great promise for enhancing drought resistance. Plant metallothioneins in oat (*Avena sativa* L.) have not yet been deeply analysed, and the literature lacks a comprehensive study of the whole family of plant MTs in response to drought. In this study, we showed that the number and nature of *cis*-elements linked with stress response in promoters of *AsMTs1–3* differed depending on the MT type. Drought stress in oat plants caused an increase in the expression of *AsMT2* and *AsMT3* and a decrease in the expression of *AsMT1* compared to well-watered plants. Moreover, the low values of relative water content, water use efficiency, net photosynthesis (P_N), transpiration (E), stomatal conductance (g_s), chlorophyll *a*, and carotenoid were accompanied by high levels of electrolyte leakage, internal CO_2 concentration (C_i) and abscisic acid content, and high activity of antioxidants enzymes in plants under drought stress. The present study puts forward the idea that *AsMTs* are crucial for oat response to drought stress not only by regulating antioxidant activity but also by changing the plant water regime and photosynthesis. Our results support the hypothesis that structural differences among types of plant MTs reflect their diversified physiological roles.

Anthropogenic activities have raised the level of CO_2 and other greenhouse gasses in the atmosphere by 50% since the eighteenth century. As a result, Earth's temperature is rising and rainfall patterns are changing[1]. Studies show that the Earth's surface temperature is expected to exceed the limit of 2 °C above pre-industrial levels (1850–1900) by the end of the twenty-first century[2]. As Earth warms, incidents of drought will be longer and more severe[3].

Water deficit is one of the crucial factors limiting plant productivity and thus threatening food security. Agricultural or ecological drought occurs when water demand exceeds supply[1]. When subjected to drought stress, plants adopt one or more of the four main survival strategies, i.e. (1) drought avoidance, (2) drought escape, (3) drought resistance, and (4) drought recovery. To avoid drought, plants reduce water loss by partial stomatal closure, increased leaf wax accumulation, and leaf rolling[4]. Moreover, a well-developed root system enhances water uptake ability. Drought escape is the natural adjustment of the growth period and life cycle of a plant or artificial changes in planting time by farmers in order to decrease the possible harmful effects of drought. Drought resistance and recovery are the ability of plants to sustain a certain level of physiological activities under drought-stress conditions and then resume growth in non-drought conditions[4].

In response to drought, several mechanisms are activated in plant cells. To maintain cell turgor pressure, plants produce osmolytes such as proline, soluble sugars, spermine, and betaine[5–8]. In drought conditions, the production of abscisic acid (ABA) is induced. ABA is a stress-response phytohormone and functions as a crucial signal molecule in plant response to drought[9]. ABA triggers a range of physiological processes including induction of stomatal closure, modulation of root development, and inhibition of plant growth[10,11]. Several lines of evidence have shown that drought-responsive genes can be classified into two groups: ABA-dependent and

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ABA-independent[12,13]. Uno et al.[12] showed that many ABA-dependent drought-related genes possess an ABA-responsive *cis*-element (ABRE) in the promoter region. On the other hand, genes induced by drought that are not induced by ABA possess other *cis*-elements including drought-responsive elements (DRE) and C-repeat (CTR)[14].

Drought stress leads to increased production of reactive oxygen species (ROS). This causes oxidative damage to lipids, proteins, and DNA, which can lead to cell death[15]. Plants possess enzymatic and non-enzymatic antioxidant systems. The enzymatic antioxidant system consists of superoxide dismutase (SOD), which catalyses the dismutation of superoxide anion radical ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and oxygen (O_2). Hydrogen peroxide is converted to water and oxygen by ascorbate peroxidase (PX) in the presence of a reducing agent such as ascorbic acid or by catalase (CAT)[16]. The non-enzymatic antioxidants include various reducing compounds, such as tocopherols, glutathione, flavonoids, carotenoids, and ascorbic acid. Moreover, under drought, plants accumulate phenolic compounds, which can function as sources of electrons and protons for reactive oxygen species[17–19].

Metallothioneins (MTs) are a family of small cysteine-rich proteins present in eukaryotes[20] and some prokaryotes[21]. In plants, MTs (pMTs) are divided into four types depending on the amino-acid sequence, i.e. pMTs belonging to type 1 (MT1) have 12 cysteine residues, type-2 MTs (MT2) contain 14 cysteines, type-3 MTs (MT3) have ten cysteines, and type-4 pMTs (MT4) contain 17 cysteines[22,23]. MTs have been shown to bind a variety of heavy metal ions (in particular Cu^+, Zn^{2+}, and Cd^{2+}) via thiol groups of cysteine residues[24–30]. Some plant MTs have one or more histidine residues, which can also play a role in binding metal ions[31,32]. The expression of p*MT* genes is spatiotemporal and induced by various stimuli, including drought, which suggests that pMTs have a role that goes beyond the maintenance of micronutrient homeostasis and toxic metal detoxication[33,34]. Several lines of evidence have shown that thiol groups of pMTs are powerful antioxidants and can protect plants from oxidative stress[33]. Moreover, MTs can, by binding Cu^+ ions, stop the Fenton reaction[35–38].

One of the most cultivated cereals worldwide is oat (*Avena sativa* L.)[39]. This plant is mostly used as livestock feed, but every year it is increasing in popularity as human food. Oat has many nutritional benefits due to its high levels of calcium, soluble fibre, oil, and protein[40–42]. Oat is also a popular and proven-to-work ingredient in various skincare cosmetics. There are some clear indicators that pMTs play a role in drought tolerance, i.e. the increased expression of pMTs in water-limiting conditions has been observed for various plant species[43–46], and the expression of several pMTs is regulated by ABA[47–49]. We propound the hypothesis that certain pMTs might play essential roles in plant drought resistance. The literature information concerning pMTs and drought is rather scarce and usually limited to only one type of pMT. Therefore, we aimed to analyse and compare the possible roles that MTs of types 1–3 play in response to drought in single plant species. We chose economically important oat since the growth and yield of this crop plant are significantly limited by drought stress[15]. Moreover, we examined the physiological and biochemical parameters reflecting the water regime, photosynthesis efficiency, and antioxidant activity of oat plants subjected to drought stress. The knowledge generated in this study that allows us to gain deeper insight into the mechanisms of oat response to drought stress may enable us to obtain oat varieties more tolerant to drought stress and to reduce yield losses.

Results and discussion

A. sativa is a crop of increasing interest as it is well-adapted to a wide range of soil types. It can perform better than other small-grain cereals on marginal soils. However, oat is sensitive to hot, dry weather, and hence, in several regions of the world, drought is the main factor limiting the yield of oat[39]. To succeed in breeding programmes, the selection of plants with complex traits such as drought resistance should be based upon a comprehensive understanding of innate tolerance mechanisms[50]. In the face of global warming and a growing world population, an understanding of the cellular mechanisms underlying drought tolerance seems to be crucial for food security.

Since their discovery in wheat germs in 1987[51], plant metallothioneins have been linked with various physiological roles including micronutrient homeostasis[52], toxic metal detoxication[53], reactive oxygen species scavenging[54], senescence[55], and stress response[56]. Plant MTs have been investigated in various plants such as *Arabidopsis thaliana* (L.) Heynh.[57–60], *Nicotiana tabacum* L.[61], *Ipomoea nil* (L.) Roth[22], *Brassica napus* L.[24,38,62], *Cucumis sativus* L.[63], *Oryza sativa* L.[64], and *Zea mays* L.[65]. However, only one report on *A. sativa* metallothioneins has been published so far[66]. This may be because oat is an allohexaploid species ($2n = 6\times = 42$, AACCDD) with a large genome (12.5 Gb), which makes it difficult to work with on the gene level[67]. Beginning in 2016, attempts were made to sequence the oat genome[68], and in March 2022 the oat genome was published[69]. This will significantly accelerate the research on oat.

In silico analyses. Plant metallothioneins have been divided into four types based on the number and arrangement of cysteine residues. In all those angiosperm genomes analysed to date, all four types of pMTs are present. There is no clear picture of the possible physiological roles of each type of pMT. It is possible that there is no single unifying role of plant metallothioneins and that one type fulfils different functions depending on the stage of plant development, plant organ, and environmental conditions[70]. Type 4 pMTs are the best-known type of pMTs. This type of pMTs was excluded from this study because pMT4s are seed-specific proteins and in most analysed up-to-date species the expression of this type of pMTs is restricted to developing and mature seeds.

The putative amino acid sequences of oat metallothioneins analysed in this study have a high cysteine content. The number and arrangement of cysteines allow oat MTs to be classified into three types—AsMT1, AsMT2, and AsMT3 (Table 1). The predicted proteins were 64 (AsMT3) to 79 (AsMT2) amino acids long, and the molecular masses ranged from 6814.56 to 7594.64 kDa. The isoelectric point (pI) of AsMT1–3 was similar for all three proteins, and it ranged from 4.85 to 5.10 pI (Table 1). AsMT1 shares the highest homology with MT1 from *Festuca rubra* (87.93%, O24528.1) and MT1 from *Hordeum vulgare* (78.67%, CAD54078.1), AsMT2 is most similar to MT2a from *Lolium rigidum* (84.51%, XP_047054761.1) and MT2 from *Poa secunda* (84.72%,

Parameter	AsMT1	AsMT2	AsMT3
Length (aa)	72	79	64
Cys content (%)	16.7	17.7	15.6
pI	5.00	5.10	4.85
Mw (kD)	7289.20	7594.64	6814.56

Table 1. Characteristics of putative AsMT1–3 proteins.

AAK38824.1), and AsMT3 is most similar to MT3 from *Oryza coarctata* (73.44%, AAF68995.1) and MT3 from *Carica papaya* (70.77%, XP_021894753.1). Comparison of the oat MT sequences cloned by our group (Bingo cultivar) with the sequences deposited in the PanOat database revealed 100% identity for AsMT1, AsMT2 and AsMT3 (AVESA.00001b.r3.7Cg0001922, AVESA.00001b.r3.1Cg0000164 and AVESA.00001b.r1.3Ag0000786, respectively). Similarly to MTs from other plant species, AsMT1–3 had two Cys-rich domains separated by one Cys-free stretch. Two His residues were present in AsMT3 and one in AsMT1 (Fig. 1). His residues are involved in the binding of metal ions by MTs, which has been confirmed for bacterial metallothioneins[71] and type 4 plant MTs[32]. As in AsMT3, it is common for type-3 pMTs to have one or more His residues: one His residue located at the C-terminus of the protein, and a second His residue located in the spacer region of MT. In AsMT1, His residue is located in the middle of the Cys-free region, which is rather uncommon for this type of pMT[70]. The potential involvement of histidines in metal binding has been suggested also for type 3 pMTs[31].

Promoter analysis is a powerful tool that can provide an insight into the regulatory mechanisms of genes of interest. Moreover, studies on *cis*-regulatory elements (CREs) provide a foundation for future experiments[54,72,73]. Regulatory elements play a crucial role in plant responses to various stresses, including drought stress[74]. There are not many studies comparing promoters of different types of *MTs* in one plant species[64,65,72]. Our analyses of oat metallothionein promoters revealed the presence of several *cis*-acting elements involved in response to light, phytohormones, biotic and abiotic stress, and plant development (Fig. 2, Supplementary Table S1). CREs are not distributed equally among promoters of oat *MT*s. A similar observation was made for MT promoters of *B. napus*, *N. tabacum*, and *Z. mays*[61,65,72]. The highest number of CREs was found in *AsMT2* (104) and the lowest in *AsMT3* (92), whereas *AsMT1* promoter contains 97 CREs (Supplementary Table S2). Elements involved in abscisic acid (ABA), jasmonic acid (MeJA), gibberellin, and auxin response were found, of which the first two were the most numerous. ABA-responsive *cis*-elements, also called ABRE, were present in all oat metallothionein promoters: the promoter of *AsMT2* had seven ABRE elements, *AsMT1* had four and *AsMT3* had only one (Supplementary Table S1). In *N. tabacum*, ABRE elements were the most abundant regulatory motifs and were present in all 12 *NtMT* promoters[61]. The promoter of *AsMT2* had the highest number of elements involved in light

AsMT1 MSCSCGSSCGCGSNCKCGKMYPDLDEQASTTTQAQAVVVVGVAHENKAVQFEVASGEGCSCGPNCKCNPCNC

AsMT2 MSCCGGNCGCGSGCKCGTGCGGCKMYPEMEEGVTSSQTLVMGVAPSSKPSFEAAAGATGAENGGCKCGANCTCDPCTCK

AsMT3 MSNTCGNCDCADKTNCVKKGDSYGIVMVDTEKSHLEEVQQEVAENDGKCKCGTSCSCTNCTCGH

Figure 1. Sequences of putative AsMT1-3 proteins. Cysteines are highlighted in blue, histidines are highlighted in green.

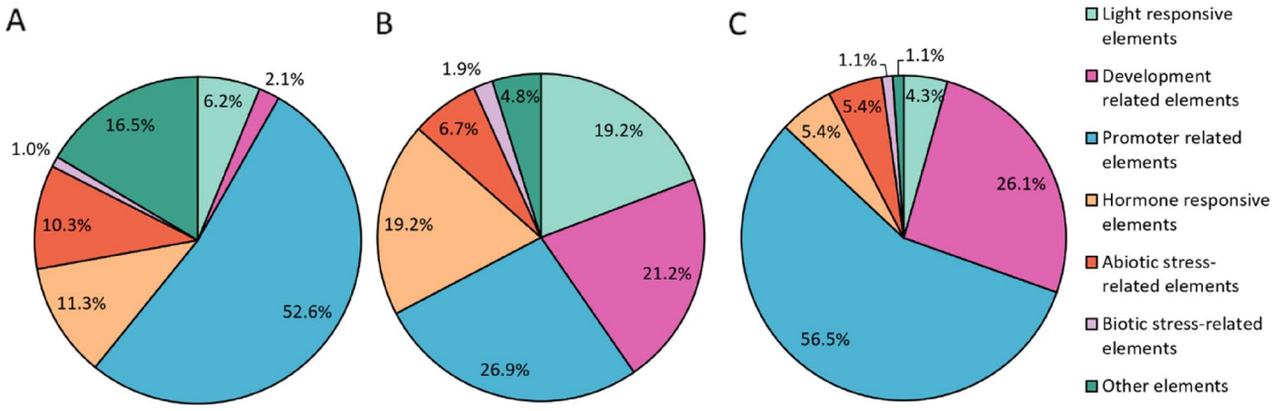

Figure 2. Pie charts depict frequencies of putative *cis*-regulatory elements in *A. sativa* L. *AsMT1* (**A**), *AsMT2* (**B**) and *AsMT3* (**C**) gene promoters. *Cis*-regulatory elements were categorised into seven types according to their predicted functions.

response (20), while *AsMT1* and *AsMT3* had six and four light-responsive elements, respectively (Supplementary Table S2). *Cis*-regulatory sequences related to the response to light have been identified in *MT* promoters in other plants, e.g. in *MT2* promoter of *L. esculentum*[75]. *AtMT1B* and *AtMT1C* in *A. thaliana*, *OsMT1F*, *OsMT2A*, and *OsMT2B* in *O. sativa*[72], *EgMT3A* and *EgMT3B* in *Elaeis guineensis*[76], *CgMT1* in *Casuarina glauca*[77], and *BrMT1* and *BrMT2* in *B. rapa*[78]. The analysed promoters also contained several development-related elements, i.e. seven elements in *AsMT2* and ten elements in *AsMT3*. Interestingly, *AsMT1* had only two development-related CREs (Supplementary Table S1).

Stress responsive elements were numerous in promoters of oat MTs, i.e. *AsMT1* (11.3%), *AsMT2* (8.6%), and *AsMT3* (6.5%) (Fig. 2). Crucially, drought-related CREs were the most common stress-responsive elements among promoters of *AsMT1–3*. *AsMT1* had nine drought-responsive elements, *AsMT2* had six and *AsMT3* had two (Supplementary Table S1). A regulatory element associated with drought response has been identified in the promoter of the rice *OsMT2b* gene[79]. Interestingly, elements involved in response to other stresses, such as fungal elicitor response, wounding response, and anaerobic conditions response were not distributed evenly among oat metallothioneins. In the promoter of *AsMT3*, there were no elements associated with wounding response, however; only in the promoter of this gene CREs involved in the response to fungal elicitors and anaerobic conditions were present (Supplementary Table S1).

Experimental research confirming the functionality of in silico identified CREs in plant MT promoters is scarce. The function of MT promoters has usually been studied using transgenic *A. thaliana* plants, where the promoter was fused with β-glucuronidase (GUS). For example, in the study by Ren and Zhao[79], a rice type 2 MT promoter was especially induced by wounding, ABA, gibberellin, cytokinin, PEG, cold, hot, NaCl and Zn treatment, and, in that promoter, respective CREs were found. In another, similar study, the promoter of another rice type 2 MT had ABA and metal-responsive CREs, and the application of ABA, Zn, and Cu caused an increase in GUS levels[80]. The promoter of rice type 1 metallothionein has been shown to be responsive to ABA, drought, dark, Zn, Cu, Pb, and Al, and respective CRE motifs have been found in the promoter sequence[81]. In an analysis of type 1 MT promoter from *C. glauca*, CREs involved in the response to metals and wounding were found. The researchers found that the promoter was indeed responsive to wounding, but did not find the responsiveness to metals that the promoter analysis suggested. In transgenic *A. thaliana*, levels of GUS did not increase significantly after Cu, Zn, and Cd treatment, whereas wounding and H_2O_2 treatments led to an increase in levels of the reporter gene activity[82].

***AsMT1–3* expression in response to drought stress.** In a limited number of studies, the upregulation of pMTs in response to drought has been shown, e.g. during drought stress, a higher expression of type 2 *MT* in watermelon[83] and a three-fold increase in the expression of *MT3* in leaves of buckwheat (*Fagopyrum esculentum* Moench) were observed[84]. Here, the exposure of oat seedlings to drought stress caused significant changes in *AsMT1–3* expression in oat shoots and roots. As mentioned before, in promoters of *AsMTs*, we found elements involved in ABA and drought response, but each *AsMT* promoter had a different number of those elements. In drought-stressed plants, the expression of *AsMT1* in the shoots did not change, and the *AsMT1* expression level in the roots was half that of control plants (Fig. 3A,B). The highest upregulation by drought was observed for *AsMT2*, i.e. it was 12-fold higher in the shoots and 27-fold higher in the roots in comparison to control plants (Fig. 3C,D). The expression of *AsMT3* in the shoots of drought-stressed plants was 2.6 times lower (Fig. 3E), but in the roots of drought-stressed plants a 2.6-fold increase was detected (Fig. 3F). Interestingly, the total number of ABA-responsive and drought-responsive CREs in the promoter regions of *AsMT1* and *AsMT2* is the same (13), but *AsMT2* had more ABA-responsive elements than drought-responsive elements, whereas the opposite is observed for *AsMT1* (Supplementary Table S1). *AsMT3* has the fewest drought-related and ABA-responsive *cis*-elements. These results indicate different roles of oat MT1–3 in drought response and suggest that the expression of oat *MT*s in drought-stressed plants is regulated via the ABA-dependent pathway. Previous studies have revealed that the expression levels of some *MT* genes, such as *OsMT1a*[44], *OsMT2b*[85], *GhMT3a*[43], and *BrMT1*[78], are increased by ABA treatment, while the transcription levels of *BrMT2* and *BrMT3* were downregulated[78].

Contrary to our results, Jaiswal et al.[86] showed that, under drought stress, the expression of genes of *MT*2 and *MT3* from guar (*Cyamopsis tetragonoloba* L.) was unchanged in roots or shoots, and the *MT1* gene was upregulated in both organs of the plant. Exposing *Citrullus lanatus* (Thunb.) Mansf. to drought stress resulted in increased expression of 32 genes, one of which was homologous to *Lycopersicon esculentum* L. *MT2*[83]. Overexpression of metallothioneins confers drought stress tolerance in plants. For example, drought-stressed *A. thaliana* L. plants overexpressing type 1 metallothionein from chickpea had longer roots, higher biomass, and higher levels of enzymatic and non-enzymatic antioxidants in comparison to WT[87]. Similar results were obtained for *A. thaliana* L. plants overexpressing the *MT2A* gene from date palm[88] and *OsMT3-a* from rice[89]. The regulation of *MT* genes expression in response to stress is multi-dimensional. Our results confirm the hypothesis that different types of MTs act differently and have different functions in plant cells. MTs exhibit a strong antioxidant property against oxidative damage via the neutralization of $O_2^{\cdot-}$ and enhanced H_2O_2 scavenging ability[83,90,91]. According to a study done by Li et al.[90], overexpression of *MT* genes can significantly improve drought tolerance and is accompanied by elevated antioxidant enzyme activities, supporting the view that the MTs are involved in the ROS scavenging pathway.

Water status and photosynthetic efficiency of oat seedlings under soil drought. Water deficiency is an important factor affecting the growth and yield of plants subjected to drought. During the drought period, disturbances of many metabolic processes such as photosynthesis are observed[92]. The ability to retain stability of cell membrane under drought stress is one of the key physiological indices widely used to evaluate the drought tolerance of plants[93]. Measurements of relative water content (RWC), water loss (WL), electrolyte

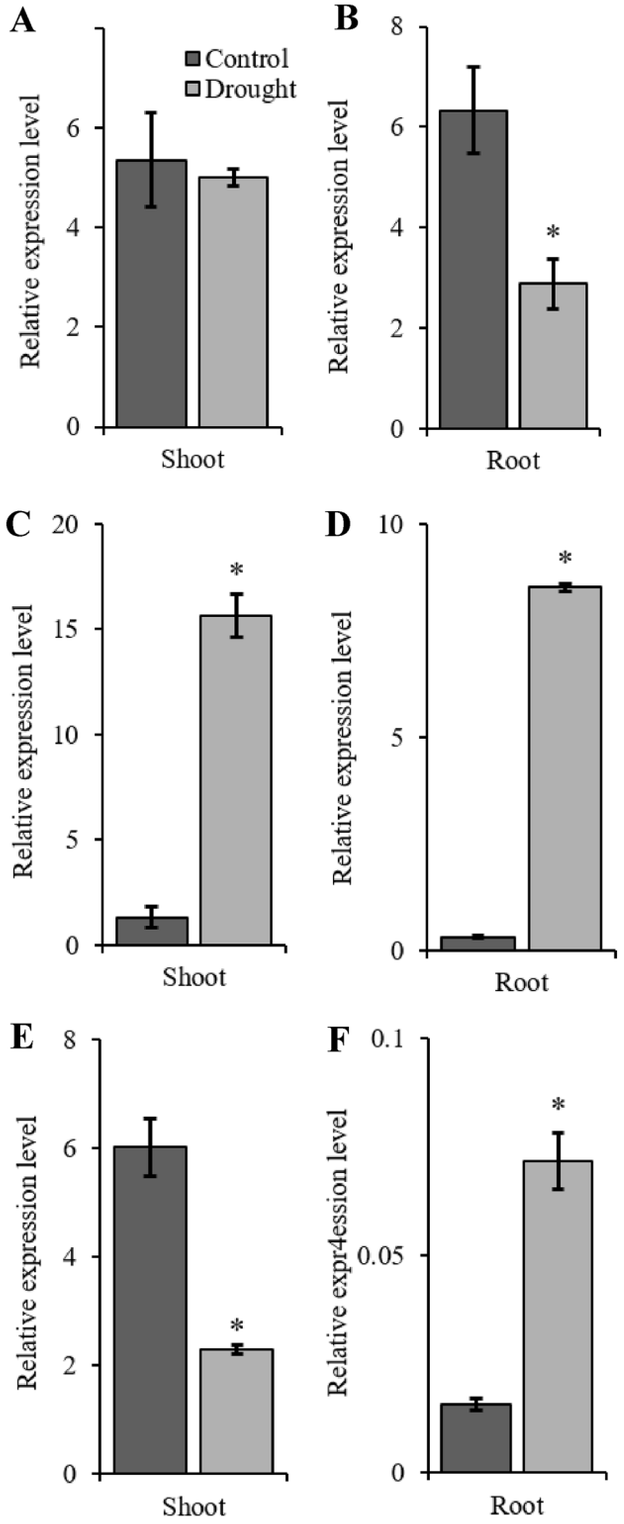

Figure 3. Relative gene expression of *AsMT1* (**A**, **B**), *AsMT2* (**C**, **D**) and *AsMT3* (**E**, **F**) in shoots and roots of oat seedlings in control and drought conditions. *AsMT1-3* genes were quantified with RT-qPCR and normalised using housekeeping gene *EIF4A*. Bars represent mean values ± SE of three replicates. Student's t-Test, $p \leq 0.05$.

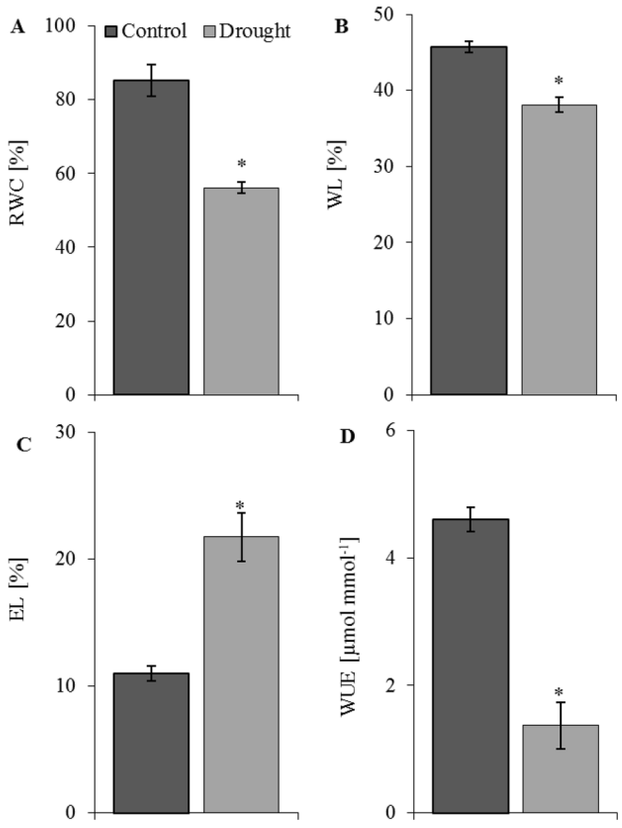

Figure 4. Water status: relative water content—RWC (**A**), water loss—WL (**B**), electrolyte leakage—EL (**C**), and photosynthetic water use efficiency—WUE (**D**) in shoots of oat seedlings in control and drought conditions. Bars represent mean values ± SE. Student's t-Test, $p \leq 0.05$.

leakage (EL), and photosynthetic water use efficiency (WUE) are parameters frequently used as a selection test for the assessment of plant cultivar tolerance to various stresses[94–97].

According to Hsiao[96], the level of RWC drop corresponds to the severity of the water stress. In our study, drought caused a significant decrease in leaf RWC (Fig. 4A), i.e. from 85.3% in well-watered plants to 56.1% in drought-treated plants. In response to drought, a significant decrease in WL (Fig. 4B) and a significant increase in EL (Fig. 4C) were observed. Both parameters show the loss of cell membrane permeability and are changed under many stresses[95]. The photosynthetic water use efficiency (WUE), defined as the ratio of carbon assimilation to transpiration, was considerably lower in drought-stressed plants (1.4 µmol $mmol^{-1}$) in comparison to well-watered plants (4.6 µmol $mmol^{-1}$) (Fig. 4D). WUE is controlled by synchronising the relation between carbon assimilation and water intake, which is a significant strategy used by plants to survive drought. In the study by Liang et al.[98], WUE and each of the gas exchange parameters of tomato leaves decreased in response to low levels of soil moisture. It is a common phenomenon that drought limits plant growth by reducing the photosynthetic rate. The key reasons for decreased photosynthesis are stomatal closure caused by decreased CO_2 levels and reduced photosynthetic activity in the mesophyll[98]. At the beginning of drought stress, the stomata close first to reduce water transpiration, and as a result, the level of CO_2 in the leaves decreases. When the decrease in net photosynthesis (P_N) as a result of drought is accompanied by increased (or unchanged) internal CO_2 concentration (*Ci*), non-stomatal factors are the main cause of reduced photosynthetic rate; meanwhile, when decreased P_N is accompanied by decreased *Ci*, stomatal factors are the main cause.

Usually, under moderate and severe drought stress, the *Ci* gradually increases as the P_N and stomatal conductance (g_s) decrease. This indicates that non-stomatal restriction is the main factor of the decrease in the photosynthetic rate as the drought stress extends, which could lead to damage to the chloroplast structure[99]. In our study, drought significantly reduced the content of chlorophyll *a* and carotenoids in oat leaves (Fig. 5). Moreover, the net photosynthesis (P_N), transpiration (E), and stomatal conductance (g_s) drastically decrease in drought-treated plants (Fig. 6A–C). These changes accompany a substantial increase in internal CO_2 concentration (C_i) (D) in oat leaves under drought (Fig. 6D). These observations suggest that nonstomatal restriction was accountable for reduced photosynthesis in oat leaves. As described by Zhao et al.[100] and Zhang et al.[99], P_N, E, g_s decreased significantly and were strictly associated with the degree and duration of drought stress in *Avena nuda* L. and *A. sativa*. A drought-prompted decrease in the photosynthetic activity of wheat leaves was also reported by Todorova et al.[101]. A large decrease in P_N and g_s has been observed in drought-stressed oat plants in comparison to control plants, while a lower decrease has been observed for E and WUE. Also, P_N rate has been closely related to chlorophyll loss[102] and all photosynthetic pigments, as well as the disruption or loss of thylakoid membranes[103].

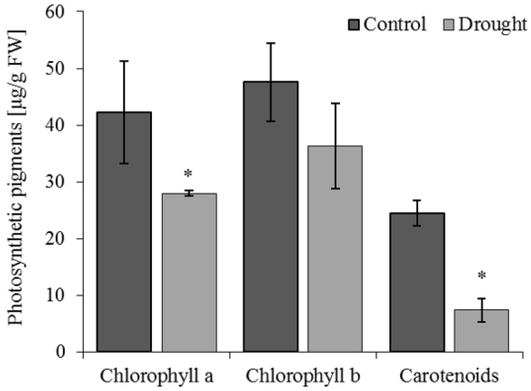

Figure 5. Photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and carotenoids) in shoots of oat seedlings in control and drought conditions. Bars represent mean values ± SE. Student's t-Test, $p \leq 0.05$.

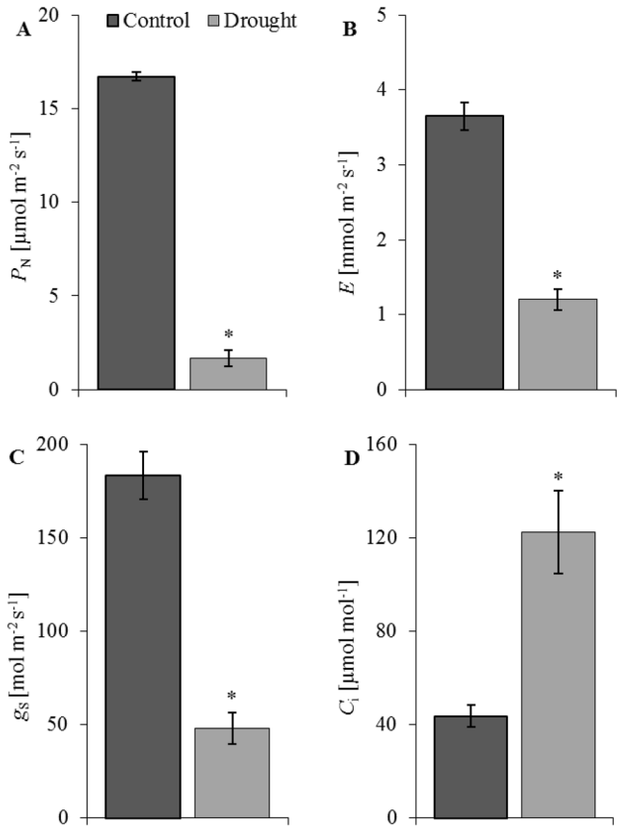

Figure 6. Leaf gas-exchange parameters: net photosynthesis—P_N (**A**), transpiration—E (**B**), stomatal conductance—g_s (**C**), and internal CO_2 concentration—C_i (**D**) in shoots of oat seedlings in control and drought conditions. Bars represent mean values ± SE. Student's t-Test, $p \leq 0.05$.

The activity of the antioxidant system of oat seedlings. Biochemical responses of crops associated with tolerance to drought are linked to changes in metabolic pathways, leading to the production of, e.g., sugars and phenolic compounds[104]. These metabolites mainly act as osmolytes, which reduce cellular dehydration and participate in the stabilization of enzymes and cellular membranes[105]. In our study, soil drought markedly increased the content of soluble sugars in roots and shoots of oat seedlings (Fig. 7A), whereas the amount of phenolic compounds was unaffected (Fig. 7B). As reported by Arabzadeh[106], the accumulation of sugars by plants enhances water-holding capacity in cells and can thus reduce drought stress via regulation of the plant's osmotic potential.

An oxidative burst commonly occurs in response to various stress conditions[107]. The question of whether plant MTs are general stress proteins because of their potential to scavenge radicals[108] or whether they are involved in response to some limited stress conditions is still open. Figure 8 shows the activities of antioxidant enzymes:

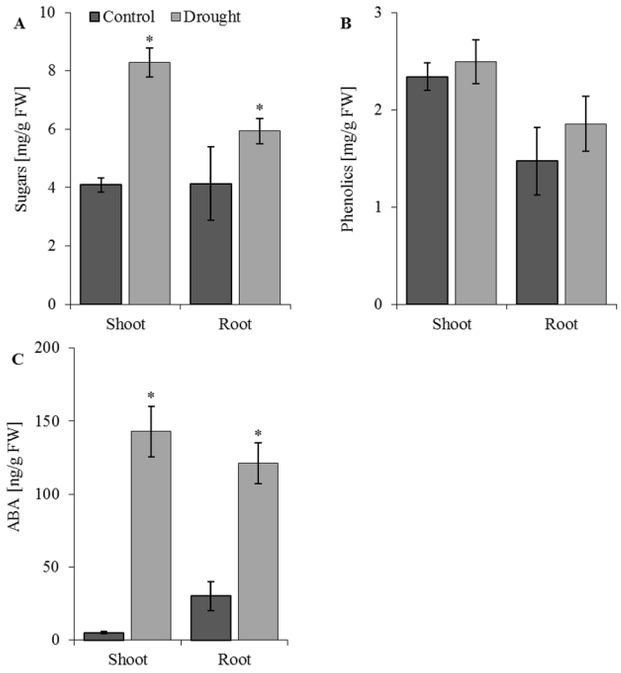

Figure 7. Concentration of sugars (**A**), phenolics (**B**) and abscisic acid (ABA) (**C**) in shoots and roots of oat seedlings in control and drought conditions. Bars represent mean values ± SE. Student's t-Test, $p \leq 0.05$.

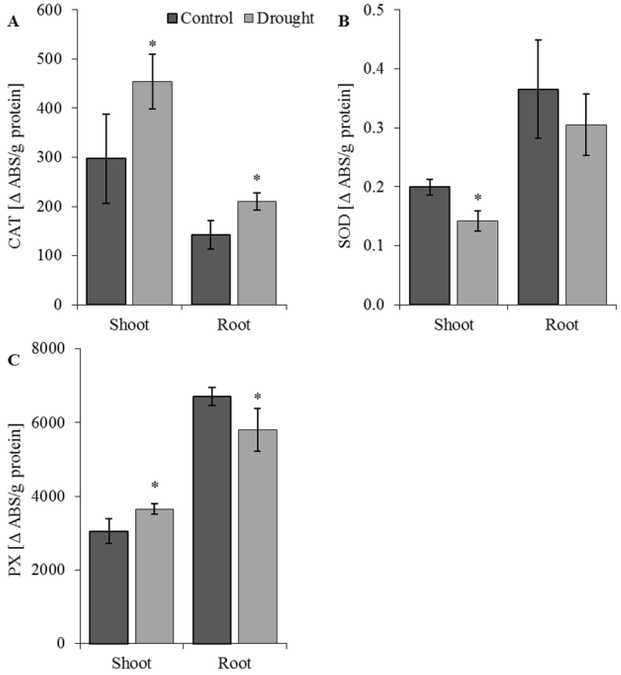

Figure 8. Activity of antioxidant enzymes: catalase—CAT (**A**), superoxide dismutase—SOD (**B**), and peroxidase—PX (**C**) in shoots and roots of oat seedlings in control and drought conditions. Bars represent mean values ± SE. Student's t-Test, $p \leq 0.05$.

catalase (CAT), superoxide dismutase (SOD), and peroxidase (PX) in shoots and roots of oat plants in response to drought. Compared to well-watered plants, CAT activity increases significantly after drought treatment in oat roots and shoots (Fig. 8A), while SOD activity decreases (Fig. 8B). PX activity was significantly higher in shoots and lower in roots of drought-treated plants in comparison to control plants (Fig. 8C). In our previous study[66], in oat seedlings subjected to osmotic stress, CAT and PX had the highest activity in the treated plants and SOD

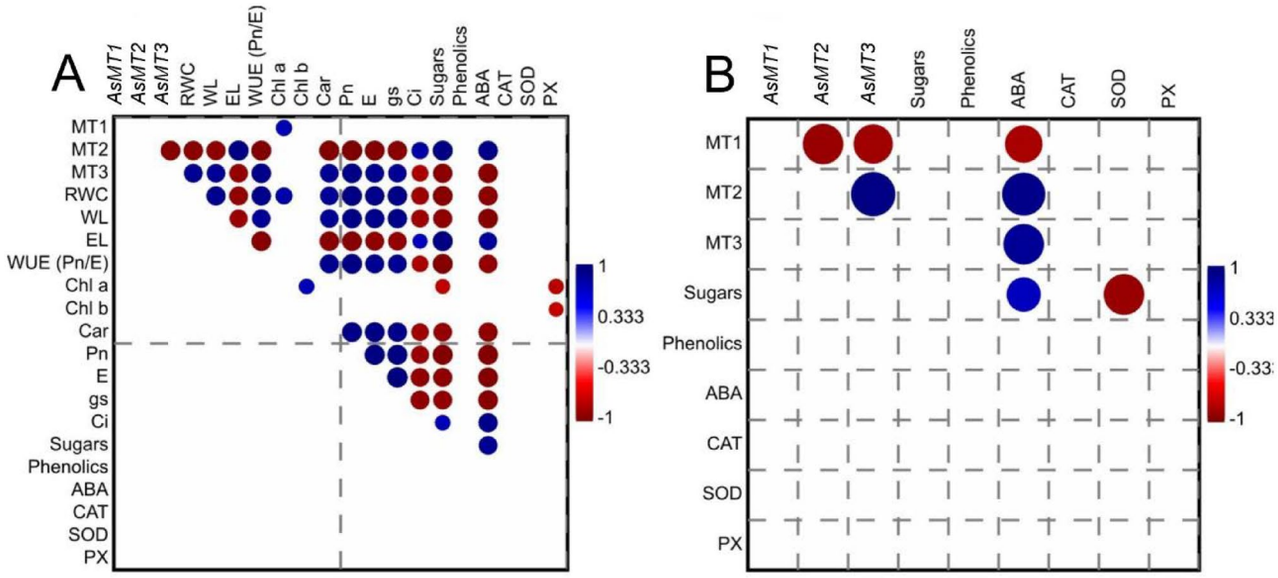

Figure 9. Pearson correlations between *AsMT* gene expressions and measured plant traits in shoots (**A**) and in roots (**B**). Only significant relations are demonstrated ($p < 0.05$). Abbreviations: *AsMT1*—oat metallothionein type 1, *AsMT2*—oat metallothionein type 2, *AsMT3*—oat metallothionein type 3, RWC—relative water content, WL—water loss, EL—electrolyte leakage, WUE—photosynthetic water use efficiency, *Chl* a—chlorophyll a, *Chl* b—chlorophyll b, Car—carotenoids, Pn—net photosynthesis, E—transpiration, gs—stomatal conductance, Ci—internal CO_2 concentration, ABA—abscisic acid, CAT—catalase activity, SOD—superoxide dismutase activity, PX—peroxidase activity.

had the lowest. A similar observation was reported by Chakraborty and Pradhan[109] in *Triticum aestivum* L., where SOD activity showed a general decline in activity and the activity of PX increased greatly during water deficit. This is in line with Gratão et al.[110], who hypothesised that SOD acts as the first line of defence against. H_2O_2 produced by SOD is then metabolized by the next enzyme, CAT. In our study we also observed a slight, but insignificant, increase in phenolic compounds in both roots and shoots of drought-treated plants (Fig. 7B). Soluble sugars and phenolics eliminate H_2O_2, and thus reduce the harmful effects of oxidative stress[111].

Many studies emphasize the well-established role of ABA in physiological processes and acclimation to abiotic stresses, thereby assigning it the role of a positive regulator of plant drought resistance[112,113]. Here, high levels of ABA were observed in shoots (142.8 ng/g FW) and roots (121.1 ng/g FW) of drought-treated plants comparing to control (5.0 ng/g FW and 30.2 ng/g FW, respectively) (Fig. 7C). In research by Peltonen-Sainio and Mäkelä[114] on 19 oat cultivars, it was determined that drought stress significantly increased the accumulation of ABA, whereas C_i and RWC decreased due to water deficit. It is known that ABA is a key regulator of abiotic stress resistance in plants. It mediates many stress-responsive genes, including genes regulating the efficiency of photosynthesis. ABA-induced stress tolerance is partly associated with the action of antioxidant systems, which protects plant cells from oxidative damage[14].

Correlations between gene expressions, water status and stress responses. In our study, the expressions of *AsMT2* and *AsMT3* were significantly negatively correlated to each other in shoots but positively correlated in roots. The expression of *AsMT1* was independent in shoots but negatively correlated with the expression of *AsMT2* and *AsMT3* in roots (Fig. 9). *AsMT2* expression was positively correlated with EL, Ci, sugars, and ABA in shoots, conversely to *AsMT3*, which was negatively correlated with these parameters. A negative correlation between the expression of *AsMT2* and RWC, WL, WUE, Car, P_N, E, and g_s was observed in shoots. Inverse correlations were observed for *AsMT3* expression and the mentioned parameters. In both shoots and roots, the expression of *AsMT2* was positively correlated with ABA. This might confirm that *AsMT2* is involved in oat response to drought and is regulated via an ABA-dependent pathway. The expression of *AsMT3* negatively correlated with ABA in shoots, but in roots the correlation was positive. Interestingly, *AsMT3* had significantly fewer ABA-responsive CREs than *AsMT1* and *AsMT2*. The expression level of *AsMT1* was positively correlated with chlorophyll *a* content in shoots and negatively correlated with ABA in roots (Fig. 9). Interestingly, no correlation between *AsMTs* expression and antioxidant enzymes was observed. A negative correlation was observed between PX activity and levels of chlorophyll in oat shoots. In oat roots, a negative correlation was observed between SOD activity and levels of sugars.

Drought stress is extensively investigated in plants of industrial importance, including oat. However, still little is known about the molecular mechanisms underlying oat's tolerance of or susceptibility to drought[50]. Thus, it is necessary to conduct further physiological and molecular research concerning the responses of oat to drought stresses. Plant MTs seem to participate in plant drought tolerance, but the exact pathways are still unclear, and more in-depth research is needed. Our results showed that oat metallothioneins type 1–3 have different roles

Figure 10. Oat (*Avena sativa* L.) cv. Bingo at day 14 of drought treatment.

in plant cells in response to drought stress. During drought stress in oat plants, the efficiency of photosynthesis decreased and the content of ABA significantly increased. We hypothesised that the expression of *AsMT2* was induced via ABA in drought-stressed plants. Metallothioneins together with sugars and antioxidant enzymes (CAT and PX) protect cells from a high level of ROS. We propound the hypothesis that a higher amount of MTs is necessary to provide elevated levels of zinc in cells. Zinc is a crucial cofactor of several enzymes and structural element of countless transcription factors. Moreover, prolonged stress leads to the activation of apoptosis, which is also regulated by Zn ions. Moreover, MTs are crucial for the translocation of zinc and possibly other metal ions to different parts of plants[115].

In conclusion, the conducted research provides important new information on the response of plants to stress mediated by metallothioneins. This knowledge about the role of AsMTs in drought stress response will enable the creation of plants via conventional or transgenic breeding that will be resistant to stresses, including drought. This will allow for greater yield from crops even in adverse environmental conditions.

Materials and methods

In silico analyses of promoters of *A. sativa* L. *MT* genes. We have previously cloned three oat metallothionein partial cDNA sequences[66]. Since then, the genome assembly of *A. sativa* has been published in the PanOat database (https://wheat.pw.usda.gov/GG3/PanOat)[116]. For each *AsMT* gene, a 1500-bp-long fragment of genomic DNA upstream of the start codon was retrieved from the PanOat database[116]. The promoters were analysed using the PlantCARE database[117]. Molecular masses and pI of putative oat MT proteins were calculated using the Compute pI/Mw tool (ExPaSy)[118].

Plant material. Grains of oat cv. Bingo, purchased from Plant Breeding Strzelce Ltd., PBAI Group, Strzelce, Łódź Voivodeship, Poland, were sown individually to 3-dm^3 pots filled with a mixture of soil and sand (3/1 v/v). Plants were grown at 25 °C under a 16-h photoperiod and 800 µmol (hv) m^2 s^{-1} PAR. Drought stress was induced by the cessation of watering the soil when the plants reached the four-leaf stage. The degree of soil moisture was determined by the gravimetric method and set at 70% field water capacity (FWC) for control conditions and 20% FWC for drought conditions. After 14 days, leaves and roots of control and drought-treated plants were collected (Fig. 10). The authors confirm that all methods used were performed in accordance with the relevant guidelines and legislation.

Isolation of nucleic acid and analysis of *AsMT1–3* expression in response to drought stress. The oat plants were washed several times in nuclease-free water. Shoots and roots were ground separately in liquid nitrogen. Total RNA was isolated using RNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. The quality and quantity of the extracted RNA were checked by agarose gel electrophoresis

Primer name	Sequence 5′→3′	Product size [bp]	Reference
AsMT1_qPCR_f AsMT1_qPCR_r	CAAACTGCAAGTGCGGGAAG TTGTTCTCATGAGCCACGCC	103	66
AsMT2_qPCR_f AsMT2_qPCR_r	CTGCGGAGGGTGCAAGATG AACGATGGCTTGGAAGAGGG	96	66
AsMT3_qPCR_f AsMT3_qPCR_r	TCCACCATGTCGAACACCTG TGGCTCTTCTCGGTGTCAAC	107	66
EIF4A_f EIF4A_r	TCTCGCAGGATACGGATGTCG TCCATCGCATTGGTCGCTCT	88	120
HNR_f HNR_r	ATTGGGTTTGTCACTTTCCGTAG CTTGGAGGGTGTCTCGCATCT	134	120

Table 2. Primers used for qPCR reactions.

and by spectrophotometric measurement using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, USA). To remove any DNA contamination from RNA samples, 1.5 µg of total RNA was treated with 1 U of DNase I (Thermo Fisher Scientific, US) and incubated at 37 °C for 30 min. The reaction was stopped by the addition of 1 µL 50 mM EDTA and incubation at 65 °C for 10 min. Reverse transcription reaction was performed using a RevertAid Reverse Transcriptase (Thermo Fisher Scientific, US) according to the manufacturer's protocol using 250 ng oligo $(dT)_{20}$ primer and 200 ng random hexamers.

RT-qPCR was performed in a total volume of 10 µL using a Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, US)[66]. The reaction mixture contained 4 µL of 10 × diluted cDNA and 0.3 µM gene-specific primers (Table 2). Three replicates were performed for each reaction. The qPCR reaction was conducted in a LightCycler 480 Instrument (Roche, Germany). The thermal cycling conditions were as follows: 95 °C for 10 min for initial denaturation, 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s[66,119]. Differences in the target gene expression were evaluated by a relative quantification method normalising the data to the reference genes for eukaryotic initiation factor 4A-3 (*EIF4A*) and heterogeneous nuclear ribonucleoprotein 27C (*HNR*)[120]. The fold-change in gene expression was calculated using LightCycler 480 Software (ver. 1.5.1.62).

Biochemical analyses. *Relative water content (RWC).* RWC was determined in leaves according to Ober et al.[121]. Samples were collected from the second fully developed leaf. RWC was calculated according to the equation: RWC (%) = (Wf – Wd)/(Wt – Wd) × 100, where Wf, Wd and Wt represent fresh weight, dry weight, and turgid weight, respectively. The experiment was repeated three times with five plants.

Water loss (WL) test. WL in leaves was determined using Clarke and McCaig's[122] method. Plants were grown in a greenhouse under well-watered conditions at 21 °C until the fourth leaf had fully emerged. This leaf was cut and placed in a growth chamber at 20 °C, 50% relative humidity, and continuous light of 250 µmol m^{-2} s^{-1}. The mass of leaf was recorded after cutting (0 h), 6 h later, and after drying at 70 °C for 48 h. WL was calculated as water loss per unit of initial water content according to the equation: WL (%) = (FW0 – FW6)/(FW0 – DW), where FW0 and FW6 are fresh weights after cutting and 6 h later, respectively, and DW is the dry weight after drying at 70 °C. The experiment was repeated three times with five plants.

Electrolyte leakage (EL). Three leaf discs (1 cm in diameter) were placed into a plastic tube containing 10 mL of redistilled water. They were shaken (100 rpm) at room temperature and the initial electrolyte leakage (EL0) was measured with a conductivity meter (CI 317, Elmetron, Poland) after 24 h. The tubes with leaves were stored at – 70 °C overnight, shaken after thawing, and then their conductivity, and total content of ions (EL1) were measured. The permeability of cell membranes was represented as a percentage of total electrolyte leakage according to the equation: EL = EL0 × 100/EL1. The experiment was repeated three times with five plants.

Leaf gas-exchange parameters. The rate of gas exchange was measured in the fully developed second leaf using a portable CIRAS-2 photosynthesis system (PP System, Hitchin, UK). The rate of net photosynthesis (P_N) and transpiration (E), stomatal conductance (g_s), and internal CO_2 concentration (C_i) were measured between 9:00 and 11:00 a.m. The photosynthetic water use efficiency (WUE) was also expressed as P_N/E. The experiment was repeated three times with five plants. The measurements included three replicates per plant.

Photosynthetic pigments content. The 100 mg of leaves was homogenized in 1 mL of 80% ethanol and then centrifuged at 2800 rpm for 10 min. The absorbance of the samples was measured at λ = 470 nm, λ = 648 nm, and λ = 664 nm on a micro-plate reader (Synergy 2, Bio-Tek, Winooski, VT, USA). Concentrations of photosynthetic pigments (chlorophylls *a*, *b* and carotenoids) were determined using a Lichtenthaler and the Wellburn method[123]. The experiment was repeated three times with five plants.

Soluble sugar content. The 100 mg of leaves was homogenized in 1 mL of 80% ethanol, then centrifuged at 2800 rpm for 10 min. The amounts of total soluble sugars were estimated by the phenol–sulphuric acid method[124]. Briefly, the supernatant was mixed with 5% phenol and 96% sulphuric acid. The absorbance of the samples was measured spectrophotometrically at λ = 490 nm on a micro-plate reader (Synergy 2, Bio-Tek, Winooski, VT,

USA). The amount of soluble sugars was expressed as milligrams of glucose per 100 g of fresh mass (FW) of plant tissue. The experiment was repeated three times with five plants.

Total phenolics content. The 100 mg of leaves was homogenized in 1 mL of 80% ethanol, then centrifuged at 2800 rpm for 10 min. To estimate the phenolics content, the supernatant was mixed with 20% Na_2CO_3 and Folin–Ciocalteu reagent[125]. The absorbance of samples was measured spectrophotometrically at $\lambda = 760$ nm on a micro-plate reader (Synergy 2, Bio-Tek, Winooski, VT, USA). The total phenolic content was calculated as milligrams of chlorogenic acid per gram of FW of plant tissue. The experiment was repeated three times with five plants.

Abscisic acid (ABA) content. The leaves were frozen in liquid nitrogen, lyophilised and homogenised. Then, 50 mg of plant material was extracted with a 1-mL mixture of methanol/water/formic acid (15/4/1; v/v/v) according to Dobrev and Kaminek[126]. An internal isotopic standard of ABA was added to each sample. The extract was then centrifuged, the supernatant was collected, and the extraction procedure was repeated. The combined supernatant was dried and reconstituted in 1 mL of 1 M formic acid. This extract was fractionated with SPE columns Oasis MCX 1 cc/30 mg (Waters, Milford, MA, USA). The acidic fraction was eluted from the SPE column with 1 mL methanol, evaporated to dryness, and reconstituted in 50 µL methanol. Samples prepared in this manner were analysed on a Supelco Ascentis RP-Amide HPLC column (Saint Louis, MO, USA) (7.5 cm, 4.6 mm, 2.7 µm). Mobile phases were 0.1% formic acid solution in water (solvent A) and acetonitrile/methanol (1/1) mixture. Gradient elution was applied under the flow rate of 0.5 mL/min. The HPLC apparatus was an Agilent Technologies 1260 equipped with an Agilent Technologies 6410 Triple Quad LC/ MS with ESI (Electrospray Interface, Agilent Technologies, Santa Clara, CA, USA). The two most abundant secondary ions were monitored: abscisic acid (ABA)—m/z 265.2 primary, m/z 229.1, 247.1 secondary; D-ABA (deuterium labelled ABA used as internal standard)—m/z 271.2 primary, m/z 167.1 secondary. Ten-point calibration curves were prepared for the analysed compounds. The experiment was repeated three times with five plants.

Antioxidant enzymes activities. The leaves were homogenised with 0.05 M phosphate buffer (pH 7.0) containing 0.1 mM EDTA at 4 °C and centrifuged at 2800 rpm for 10 min. Superoxide dismutase (SOD) activity was assayed according to McCord and Fridovich[127]. The reaction mixture consisted of 0.05 M phosphate buffer, 0.013 mM cytochrome c, 0.1 mM xanthine, 0.024 U per ml xanthine oxidase, and supernatant. Absorbance was measured at $\lambda = 550$ nm.

Catalase (CAT) activity was determined according to Aebi[128]. The reaction mixture contained 0.05 M phosphate buffer, 0.1 mM H_2O_2, and supernatant. The rate of H_2O_2 decomposition was measured at $\lambda = 240$ nm.

The activity of peroxidase (PX) was measured by the method of Lűck[129]. The amount of oxidation product of 1% p-phenylenediamine in the presence of 0.03 M H_2O_2 was measured at $\lambda = 485$ nm.

The reaction kinetics of all enzymes were measured spectrophotometrically using a micro-plate reader (Synergy 2, Bio-Tek, Winooski, VT, USA). Enzyme activities were calculated per milligram of protein measured by Bradford method with bovine serum albumin as a protein standard[130]. The experiment was repeated three times with five plants for each enzyme.

Statistical analysis

The results are expressed as mean values, and error bars represent standard error (SE). Before statistical assessment, data normality was tested by the Shapiro–Wilk test. The majority of parameters were normally distributed, and statistical analysis of the experimental data was done with the analysis of variance (ANOVA). Student's t-test (p value ≤ 0.05) was applied to determine differences between expression levels of control and drought-stressed plants. To demonstrate relations between measured traits, Pearson correlations were calculated[131]. The programs Past 4.0[132], STATISTICA 13.0 (Stat-soft, Inc., USA), and RStudio[133] were applied for calculations.

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

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References

1. Swann, A. L. S. Plants and drought in a changing climate. *Curr. Clim. Change Rep.* **4**, 192–201 (2018).
2. Mukherjee, S., Mishra, A. & Trenberth, K. E. Climate change and drought: a perspective on drought indices. *Curr. Clim. Change Rep.* **4**, 145–163 (2018).
3. Dai, A., Zhao, T. & Chen, J. Climate change and drought: a precipitation and evaporation perspective. *Curr. Clim. Change Rep.* **4**, 301–312 (2018).
4. Fang, Y. & Xiong, L. General mechanisms of drought response and their application in drought resistance improvement in plants. *Cell. Mol. Life Sci.* **72**, 673–689 (2015).
5. Hanson, A. D. *et al.* Osmoprotective compounds in the Plumbaginaceae: A natural experiment in metabolic engineering of stress tolerance. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 306–310 (1994).
6. Mohanty, A. K., Misra, M. & Drzal, L. T. Sustainable bio-composites from renewable resources: Opportunities and challenges in the green materials world. *J. Polym. Environ.* **10**, 19–26 (2002).
7. Gong, D. S. *et al.* Early activation of plasma membrane H+-ATPase and its relation to drought adaptation in two contrasting oat (*Avena sativa* L.) genotypes. *Environ. Exp. Bot.* **69**, 1–8 (2010).

8. Ashraf, M. & Foolad, M. R. Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environ. Exp. Bot.* **59**, 206–216 (2007).
9. Shinozaki, K. & Yamaguchi-Shinozaki, K. Gene networks involved in drought stress response and tolerance. *J. Exp. Bot.* **58**, 221–227 (2007).
10. Bhargava, S. & Sawant, K. Drought stress adaptation: metabolic adjustment and regulation of gene expression. *Plant Breed.* **132**, 21–32 (2013).
11. Mahmood, T. *et al.* Insights into drought stress signaling in plants and the molecular genetic basis of cotton drought tolerance. *Cells* **9**, 105 (2019).
12. Uno, Y. *et al. Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 11632–11637 (2000).
13. Yamaguchi-Shinozaki, K. & Shinozaki, K. Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends Plant Sci.* **10**, 88–94 (2005).
14. Liu, S., Lv, Z., Liu, Y., Li, L. & Zhang, L. Network analysis of ABA-dependent and ABA-independent drought responsive genes in *Arabidopsis thaliana*. *Genet. Mol. Biol.* **41**, 624–637 (2018).
15. Islam, M. R. *et al.* Effects of water-saving superabsorbent polymer on antioxidant enzyme activities and lipid peroxidation in oat (*Avena sativa* L.) under drought stress. *J. Sci. Food Agric.* **91**, 680–686 (2011).
16. Dąbrowska, G., Kata, A., Goc, A., Szechyńska-Hebda, M. & Skrzypek, E. Characteristics of the plant ascorbate peroxidase family. *Acta Biol. Crac. Ser. Bot.* **49**, 7–17 (2007).
17. Hura, T., Hura, K. & Grzesiak, S. Contents of total phenolics and ferulic acid, and pal activity during water potential changes in leaves of maize single-cross hybrids of different drought tolerance. *J. Agron. Crop Sci.* **194**, 104–112 (2008).
18. Latif, F. *et al.* Effects of salicylic acid on growth and accumulation of phenolics in *Zea mays* L. under drought stress. *Acta Agric. Scand. Soil Plant Sci.* **66**, 325–332 (2016).
19. Skrzypek, E., Szechyńska-Hebda, M., Dąbrowska, G. B. & Goc, A. The role of osmotic stress during in vitro regeneration of *Triticum aestivum* L. and *Vicia faba* ssp. minor. *Zesz. Prob. Post. Nauk Rol.* **524**, 221–230 (2008).
20. Blindauer, C. A. & Leszczyszyn, O. I. Metallothioneins: Unparalleled diversity in structures and functions for metal ion homeostasis and more. *Nat. Prod. Rep.* **27**, 720–741 (2010).
21. Blindauer, C. A. Bacterial metallothioneins: Past, present, and questions for the future. *J. Biol. Inorg. Chem.* **16**, 1011–1024 (2011).
22. Mierek-Adamska, A., Znajewska, Z., Goc, A. & Dąbrowska, G. B. Molecular cloning and characterization of *Ipomoea nil* metallothioneins. *Turk. J. Bot.* **42**, 247–256 (2018).
23. Koszucka, A. M. & Dąbrowska, G. Plant metallothioneins. *Adv. Cell Biol.* **33**, 285–302 (2006).
24. Mierek-Adamska, A., Dąbrowska, G. B. & Blindauer, C. A. The type 4 metallothionein from *Brassica napus* seeds folds in a metal-dependent fashion and favours zinc over other metals. *Metallomics* **10**, 1430–1443 (2018).
25. Zhang, H., Xu, W., Dai, W., He, Z. & Ma, M. Functional characterization of cadmium-responsive garlic gene *AsMT2b*: A new member of metallothionein family. *Chin. Sci. Bull.* **51**, 409–416 (2006).
26. Jin, S. *et al.* Functional characterization of a type 2 metallothionein gene, *SsMT2*, from alkaline-tolerant *Suaeda salsa*. *Sci. Rep.* **7**, 1–11 (2017).
27. Bellion, M. *et al.* Metal induction of a *Paxillus involutus* metallothionein and its heterologous expression in *Hebeloma cylindrosporum*. *New Phytol.* **174**, 151–158 (2007).
28. Evans, K. M. *et al.* Expression of the pea metallothionein-like gene *PsMTA* in *Escherichia coli* and *Arabidopsis thaliana* and analysis of trace metal ion accumulation: Implications for *PsMTA* function. *Plant Mol. Biol.* **20**, 1019–1028 (1992).
29. Cobbett, C. S. Phytochelatins and their roles in heavy metal detoxification. *Plant Physiol.* **123**, 825–832 (2000).
30. Freisinger, E. Structural features specific to plant metallothioneins. *J. Biol. Inorg. Chem.* **16**, 1035–1045 (2011).
31. Tomas, M. *et al.* His-containing plant metallothioneins: Comparative study of divalent metal-ion binding by plant MT3 and MT4 isoforms. *J. Biol. Inorg. Chem.* **19**, 1149–1164 (2014).
32. Leszczyszyn, O. I., Schmid, R. & Blindauer, C. A. Toward a property/function relationship for metallothioneins: Histidine coordination and unusual cluster composition in a zinc-metallothionein from plants. *Proteins* **68**, 922–935 (2007).
33. Mierek-Adamska, A. *et al.* Potential involvement of rapeseed (*Brassica napus* L.) metallothioneins in the hydrogen peroxide-induced regulation of seed vigour. *J. Agron Crop Sci.* **205**, 598–607 (2019).
34. Sekhar, S. *et al.* Comparative transcriptome profiling of low light tolerant and sensitive rice varieties induced by low light stress at active tillering stage. *Sci. Rep.* **9**, 1–14 (2019).
35. Mir, G. *et al.* A plant type 2 metallothionein (MT) from cork tissue responds to oxidative stress. *J. Exp. Bot.* **55**, 2483–2493 (2004).
36. Hrynkiewicz, K., Dąbrowska, G., Baum, C., Niedojadlo, K. & Leinweber, P. Interactive and single effects of ectomycorrhiza formation and *Bacillus cereus* on metallothionein MT1 expression and phytoextraction of Cd and Zn by willows. *Water Air Soil Pollut.* **223**, 957–968 (2012).
37. Lee, J., Shim, D., Song, W. Y., Hwang, I. & Lee, Y. *Arabidopsis* metallothioneins 2a and 3 enhance resistance to cadmium when expressed in *Vicia faba* guard cells. *Plant Mol. Biol.* **54**, 805–815 (2004).
38. Dąbrowska, G., Mierek-Adamska, A. & Goc, A. Characterisation of *Brassica napus* L. metallothionein genes (BnMTs) expression in organs and during seed germination. *Aust. J. Crop Sci.* **7**, 1324–1332 (2013).
39. Stevens, E. J., Armstrong, K. W., Bezar, H. J., Griffin, W. B. & Hampton, J. G. *Fodder Oats: A World Overview* (Food and Agriculture Organization of The United Nations, 2004).
40. Andon, M. B. & Anderson, J. W. The oatmeal-cholesterol connection: 10 years later. *Am. J. Lifestyle Med.* **2**, 51–57 (2008).
41. Jenkins, A. L., Jenkins, D. J. A., Zdravkovic, U., Würsch, P. & Vuksan, V. Depression of the glycemic index by high levels of β-glucan fiber in two functional foods tested in type 2 diabetes. *Eur. J. Clin. Nutr.* **56**, 622–628 (2002).
42. Peterson, D. M. Oat antioxidants. *J. Cereal Sci.* **33**, 115–129 (2001).
43. Xue, T. *et al.* Cotton metallothionein *GhMT3a*, a reactive oxygen species scavenger, increased tolerance against abiotic stress in transgenic tobacco and yeast. *J. Exp. Bot.* **60**, 339–349 (2009).
44. Yang, Z., Wu, Y., Li, Y., Ling, H. Q. & Chu, C. OsMT1a, a type 1 metallothionein, plays the pivotal role in zinc homeostasis and drought tolerance in rice. *Plant Mol. Biol.* **70**, 219–229 (2009).
45. Seki, M. *et al.* Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *Plant J.* **31**, 279–292 (2002).
46. Kumar, S. *et al.* Metallothionein (MT1): A molecular stress marker in chickpea enhances drought and heavy metal stress adaptive efficacy in transgenic plants. *Environ. Exp. Bot.* **199**, 104871 (2022).
47. Ren, Y. *et al.* Type 4 metallothionein genes are involved in regulating Zn ion accumulation in late embryo and in controlling early seedling growth in *Arabidopsis*. *Plant Cell Environ.* **35**, 770–789 (2012).
48. Cheng, M. *et al.* Genome-wide identification and analysis of the metallothionein genes in *Oryza* genus. *Int J. Mol Sci.* **22**, 9651 (2021).
49. Feng, M. *et al.* ScMT10, a metallothionein-like gene from sugarcane, enhances freezing tolerance in *Nicotiana tabacum* transgenic plants. *Environ. Exp. Bot.* **194**, 104750 (2022).
50. Sánchez-Martín, J. *et al.* A metabolomic study in oats (*Avena sativa*) highlights a drought tolerance mechanism based upon salicylate signalling pathways and the modulation of carbon, antioxidant and photo-oxidative metabolism. *Plant Cell Environ.* **38**, 1434–1452 (2015).

51. Lane, B., Kajioka, R. & Kennedy, T. The wheat-germ Ec protein is a zinc-containing metallothionein. *Biochem. Cell Biol.* **65**, 1001–1005 (1987).
52. Cobbett, C. & Goldsbrough, P. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annu. Rev. Plant Biol.* **53**, 159–182 (2002).
53. Saeed-Ur-Rahman, K. M., Hui, N., Kayani, S. I. & Tang, K. Diversity and versatile functions of metallothioneins produced by plants: A review. *Pedosphere* **30**, 577–588 (2020).
54. Hassinen, V. H., Tervahauta, A. I., Schat, H. & Kärenlampi, S. O. Plant metallothioneins - metal chelators with ROS scavenging activity?. *Plant Biol.* **13**, 225–232 (2011).
55. Buchanan-Wollaston, V. Isolation of cDNA clones for genes that are expressed during leaf senescence in *Brassica napus* (identification of a gene encoding a senescence-specific metallothionein-like protein). *Plant Physiol.* **105**, 839–846 (1994).
56. Kumar, S. *et al.* Plant metallothioneins as regulators of environmental stress responses. *Int. J. Plant Environ.* **7**, 27–38 (2021).
57. Guo, W.-J., Bundithya, W. & Goldsbrough, P. B. Characterization of the *Arabidopsis* metallothionein gene family: Tissue-specific expression and induction during senescence and in response to copper. *New Phytol.* **159**, 369–381 (2003).
58. Imam, H. T. & Blindauer, C. A. Differential reactivity of closely related zinc(II)-binding metallothioneins from the plant *Arabidopsis thaliana*. *J. Biol. Inorg. Chem.* **23**, 137–154 (2017).
59. Murphy, A., Zhou, J., Goldsbrough, P. B. & Taiz, L. Purification and immunological identification of metallothioneins 1 and 2 from *Arabidopsis thaliana*. *Plant Physiol.* **113**, 1293–1301 (1997).
60. Zhu, W. *et al.* Arabidopsis thaliana metallothionein, *AtMT2a*, Mediates ROS balance during oxidative stress. *J. Plant Biol.* **52**, 585–592 (2009).
61. Yu, Q. *et al.* Genome-wide identification and expression analysis of heavy metal stress–responsive metallothionein family genes in *Nicotiana tabacum*. *Plant Mol. Biol. Rep.* **39**, 443–454 (2020).
62. Pan, Y. *et al.* Genome-wide characterization and analysis of metallothionein family genes that function in metal stress tolerance in *Brassica napus* L.. *Int J. Mol. Sci.* **19**, 1–18 (2018).
63. Zhou, Y., Liu, J., Liu, S., Jiang, L. & Hu, L. Identification of the metallothionein gene family from cucumber and functional characterization of *CsMT4* in *Escherichia coli* under salinity and osmotic stress. *Biotech* **9**, 1–11 (2019).
64. Zhou, G., Xu, Y., Li, J., Yang, L. & Liu, J. Y. Molecular analyses of the metallothionein gene family in rice (*Oryza sativa* L.). *J. Biochem. Mol. Biol.* **39**, 595–606 (2006).
65. Gao, C. *et al.* Genome-wide analysis of metallothionein gene family in maize to reveal its role in development and stress resistance to heavy metal. *Biol. Res.* **55**, 1–13 (2022).
66. Konieczna, W., Mierek-Adamska, A., Warchoł, M., Skrzypek, E. & Dąbrowska, G. The involvement of metallothioneins and stress markers in response to osmotic stress in *Avena sativa* L. J. *Agron. Crop Sci.* (In press)
67. Yan, H. *et al.* Genome size variation in the genus *Avena*. *Genome* **59**, 209–220 (2016).
68. Chaffin, A. S. *et al.* A consensus map in cultivated hexaploid oat reveals conserved grass synteny with substantial subgenome rearrangement. *Plant Genome* **9**, 1–21 (2016).
69. Kamal, N. *et al.* The mosaic oat genome gives insights into a uniquely healthy cereal crop. *Nature* **606**, 113–119 (2022).
70. Leszczyszyn, O. I., Imam, H. T. & Blindauer, C. A. Diversity and distribution of plant metallothioneins: A review of structure, properties and functions. *Metallomics* **5**, 1146–1169 (2013).
71. Blindauer, C. A. *et al.* Multiple bacteria encode metallothioneins and SmtA-like zinc fingers. *Mol. Microbiol.* **45**, 1421–1432 (2002).
72. Dąbrowska, G., Mierek-Adamska, A. & Goc, A. Plant metallothioneins: Putative functions identified by promoter analysis in silico. *Acta Biol. Crac. Ser. Bot.* **54**, 109–120 (2012).
73. Dąbrowska, G. B., Turkan, S., Tylman-Mojżeszek, W. & Mierek-Adamska, A. In silico study of the rsh (Rela/spot homologs) gene family and expression analysis in response to PGPR bacteria and salinity in *Brassica napus*. *Int J. Mol. Sci.* **22**, 10666 (2021).
74. Javadi, S. M., Shobbar, Z. S., Ebrahimi, A. & Shahbazi, M. New insights on key genes involved in drought stress response of barley: Gene networks reconstruction, hub, and promoter analysis. *J. Genet. Eng. Biotechnol.* **19**, 1–12 (2021).
75. Whitelaw, C. A., le Huquet, J. A., Thurman, D. A. & Tomsett, A. B. The isolation and characterisation of type II metallothionein-like genes from tomato (*Lycopersicon esculentum* L.). *Plant Mol. Biol.* **33**, 503–511 (1997).
76. Omidvar, V., Abdullah, S. N. A., Izadfard, A., Ho, C. L. & Mahmood, M. The oil palm metallothionein promoter contains a novel AGTTAGG motif conferring its fruit-specific expression and is inducible by abiotic factors. *Planta* **232**, 925–936 (2010).
77. Laplaze, L. *et al.* Symbiotic and non-symbiotic expression of *cgMT1*, a metallothionein-like gene from the actinorhizal tree *Casuarina glauca*. *Plant Mol. Biol.* **49**, 81–92 (2002).
78. Ahn, Y. O. *et al.* Three *Brassica rapa* metallothionein genes are differentially regulated under various stress conditions. *Mol. Biol. Rep.* **39**, 2059–2067 (2012).
79. Ren, Y. & Zhao, J. Functional analysis of the rice metallothionein gene *OsMT2b* promoter in transgenic *Arabidopsis* plants and rice germinated embryos. *Plant Sci.* **176**, 528–538 (2009).
80. Lü, S. *et al.* The GUS reporter-aided analysis of the promoter activities of a rice metallothionein gene reveals different regulatory regions responsible for tissue-specific and inducible expression in transgenic *Arabidopsis*. *Transgenic Res.* **16**, 177–191 (2007).
81. Dong, C.-J.J., Wang, Y., Yu, S.-S.S. & Liu, J.-Y.Y. Characterization of a novel rice metallothionein gene promoter: Its tissue specificity and heavy metal responsiveness. *J. Integr. Plant Biol.* **52**, 914–924 (2010).
82. Obertello, M. *et al.* Functional analysis of the metallothionein gene *cgmt1* isolated from the actinorhizal tree *Casuarina glauca*. *Mol. Plant-Microbe Interact.* **20**, 1231–1240 (2007).
83. Akashi, K., Nishimura, N., Ishida, Y. & Yokota, A. Potent hydroxyl radical-scavenging activity of drought-induced type-2 metallothionein in wild watermelon. *Biochem. Biophys. Res. Commun.* **323**, 72–78 (2004).
84. Samardžić, J. T. *et al.* Tissue expression analysis of *FeMT3*, a drought and oxidative stress related metallothionein gene from buckwheat (*Fagopyrum esculentum*). *J. Plant Physiol.* **167**, 1407–1411 (2010).
85. Yuan, J., Chen, D., Ren, Y., Zhang, X. & Zhao, J. Characteristic and expression analysis of a metallothionein gene, *OsMT2b*, down-regulated by cytokinin suggests functions in root development and seed embryo germination of rice. *Plant Physiol.* **146**, 1637–1650 (2008).
86. Jaiswal, P. S., Mittal, N. & Randhawa, G. S. Cyamopsis tetragonoloba type 1 metallothionein (*CtMT1*) gene is upregulated under drought stress and its protein product has an additional C-X-C motif and unique metal binding pattern. *Int. J. Biol. Macromol.* **119**, 1324–1334 (2018).
87. Dubey, A. K. *et al.* Over-expression of *CarMT* gene modulates the physiological performance and antioxidant defense system to provide tolerance against drought stress in *Arabidopsis thaliana* L. *Ecotoxicol. Environ. Saf.* **171**, 54–65 (2019).
88. Patankar, H. V. *et al.* Overexpression of a metallothionein 2a gene from date palm confers abiotic stress tolerance to yeast and *Arabidopsis thaliana*. *Int. J. Mol. Sci.* **20**, 2871 (2019).
89. Mekawy, A. M. M., Assaha, D. V. M. & Ueda, A. Constitutive overexpression of rice metallothionein-like gene *OsMT-3a* enhances growth and tolerance of *Arabidopsis* plants to a combination of various abiotic stresses. *J. Plant Res.* **133**, 429–440 (2020).
90. Li, Z. *et al.* The alterations of endogenous polyamines and phytohormones induced by exogenous application of spermidine regulate antioxidant metabolism, metallothionein and relevant genes conferring drought tolerance in white clover. *Environ. Exp. Bot.* **124**, 22–38 (2016).
91. Ruttkay-Nedecky, B. *et al.* The role of metallothionein in oxidative stress. *Int. J. Mol. Sci.* **14**, 6044–6066 (2013).

92. Wang, Z. *et al.* Effects of drought stress on photosynthesis and photosynthetic electron transport chain in young apple tree leaves. *Biol. Open* **7**, bio035279 (2018).
93. Farooq, M., Wahid, A., Kobayashi, N., Fujita, D. & Basra, S. M. A. Plant drought stress: Effects, mechanisms and management. *Agron. Sustain. Dev.* **29**, 185–212 (2009).
94. Grzesiak, M. T. *et al.* Impact of soil compaction stress combined with drought or waterlogging on physiological and biochemical markers in two maize hybrids. *Acta Physiol. Plant* **38**, 1–15 (2016).
95. Bajji, M., Kinet, J. M. & Lutts, S. The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. *Plant Growth Reg.* **36**, 61–70 (2002).
96. Hsiao, T. C. Plant responses to water stress. *Annu. Rev. Plant Physiol.* **24**, 519–570 (1973).
97. Ostrowska, A. & Hura, T. Physiological comparison of wheat and maize seedlings responses to water stresses. *Sustainability* **14**, 7932 (2022).
98. Liang, G., Liu, J., Zhang, J. & Guo, J. Effects of drought stress on photosynthetic and physiological parameters of tomato. *J. Am. Soc. Hort. Sci.* **145**, 12–17 (2020).
99. Zhang, X. *et al.* Effects of drought stress during critical periods on the photosynthetic characteristics and production performance of naked oat (*Avena nuda* L.). *Sci. Rep.* **12**, 1–11 (2022).
100. Zhao, B., Ma, B. L., Hu, Y. & Liu, J. Source–sink adjustment: a mechanistic understanding of the timing and severity of drought stress on photosynthesis and grain yields of two contrasting oat (*Avena sativa* L.) genotypes. *J. Plant Growth Regul.* **40**, 263–276 (2021).
101. Todorova, D., Aleksandrov, V., Anev, S. & Sergiev, I. Photosynthesis alterations in wheat plants induced by herbicide, soil drought or flooding. *Agronomy* **12**, 390 (2022).
102. Marcińska, I. *et al.* Application of photochemical parameters and several indices based on phenotypical traits to assess intraspecific variation of oat (*Avena sativa* L.) tolerance to drought. *Acta Physiol. Plant* **39**, 1–13 (2017).
103. Esteban, R. *et al.* Internal and external factors affecting photosynthetic pigment composition in plants: A meta-analytical approach. *New Phytol.* **206**, 268–280 (2015).
104. Seki, M., Umezawa, T., Urano, K. & Shinozaki, K. Regulatory metabolic networks in drought stress responses. *Curr. Opin. Plant Biol.* **10**, 296–302 (2007).
105. Guy, C., Kaplan, F., Kopka, J., Selbig, J. & Hincha, D. K. Metabolomics of temperature stress. *Physiol. Plant* **132**, 220–235 (2008).
106. Arabzadeh, N. The effect of drought stress on soluble carbohydrates (sugars) in two species of *Haloxylon persicum* and *Haloxylon aphyllum*. *Asian J. Plant Sci.* **11**, 44–51 (2012).
107. Bhattacharjee, S. Reactive oxygen species and oxidative burst: Roles in stress, senescence and signal transducation in plants. *Curr. Sci.* **89** (2005).
108. Si, M. & Lang, J. The roles of metallothioneins in carcinogenesis. *J. Hematol. Oncol.* **11**, 1–20 (2018).
109. Chakraborty, U. & Pradhan, B. Oxidative stress in five wheat varieties (*Triticum aestivum* L.) exposed to water stress and study of their antioxidant enzyme defense system, water stress responsive metabolites and H2O2 accumulation. *Braz. J. Plant Physiol.* **24**, 117–130 (2012).
110. Gratão, P. L., Polle, A., Lea, P. J. & Azevedo, R. A. Making the life of heavy metal-stressed plants a little easier. *Funct. Plant Biol.* **32**, 481–494 (2005).
111. Bolouri-Moghaddam, M. R., le Roy, K., Xiang, L., Rolland, F. & van den Ende, W. Sugar signalling and antioxidant network connections in plant cells. *FEBS J.* **277**, 2022–2037 (2010).
112. Gietler, M., Fidler, J., Labudda, M. & Nykiel, M. Abscisic acid—Enemy or savior in the response of cereals to abiotic and biotic stresses?. *Int. J. Mol. Sci.* **21**, 4607 (2020).
113. Bharath, P., Gahir, S. & Raghavendra, A. S. Abscisic acid-induced stomatal closure: An important component of plant defense against abiotic and biotic stress. *Front. Plant Sci.* **12**, 324 (2021).
114. Peltonen-Sainio, P. & Mäkelä, P. Comparison of physiological methods to assess drought tolerance in oats. *Acta Agric. Scand. B Soil Plant Sci.* **45**, 32–38 (1995).
115. Formigari, A., Irato, P. & Santon, A. Zinc, antioxidant systems and metallothionein in metal mediated-apoptosis: Biochemical and cytochemical aspects. *Comp. Biochem. Physiol.* **146**, 443–459 (2007).
116. PepsiCo. *Avena sativa* - OT3098 v2, PepsiCo, https://wheat.pw.usda.gov/jb?data=/ggds/oat-ot3098v2-pepsico. (2021).
117. Lescot, M. *et al.* PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucl. Acids Res.* **30**, 325–327 (2002).
118. Gasteiger, E. *et al.* ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucl. Acids Res.* **31**, 3784–3788 (2003).
119. Cárdenas-Pérez, S. *et al.* Maternal salinity influences anatomical parameters, pectin content, biochemical and genetic modifications of two *Salicornia europaea* populations under salt stress. *Sci. Rep* **12**, 1–16 (2022).
120. Yang, Z., Wang, K., Aziz, U., Zhao, C. & Zhang, M. Evaluation of duplicated reference genes for quantitative real-time PCR analysis in genome unknown hexaploid oat (*Avena sativa* L.). *Plant Meth.* **16**, 1–14 (2020).
121. Ober, E. S. *et al.* Evaluation of physiological traits as indirect selection criteria for drought tolerance in sugar beet. *Field Crops Res.* **91**, 231–249 (2005).
122. Clarke, J. M. & McCaig, T. N. Excised-leaf water retention capability as an indicator of drought resistance of *Triticum* genotypes. *Can. J. Plant Sci.* **62**, 571–578 (2011).
123. Lichtenthaler, H. K. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. *Methods Enzymol.* **148**, 350–382 (1987).
124. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**, 350–356 (1956).
125. Singleton, V. L. & Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **16**, 144–158 (1965).
126. Dobrev, P. & Kamínek, M. Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. *J. Chromatogr.* **950**, 21–29 (2002).
127. McCord, J. & Fiodovich, I. Superoxide dismutase an enzimic function for erytrocuperein (hemocuperein). *J. Biol. Chem.* **244**, 6049–6055 (1969).
128. Aebi, H. Catalase in vitro. *Methods Enzymol.* **105**, 121–125 (1984).
129. Lűck, H. *Methoden der enzymatischen Analyse. Verlag Chemie* (GmbH Weinheim, 1962).
130. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254 (1976).
131. Zar, J. H. *Biostatistical Analysis* (Prentice-Hall/Pearson, 2010).
132. Hammer, D. A. T., Ryan, P. D., Hammer, Ø. & Harper, D. A. T. Past: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeon. Electron.* **4**, (2001).
133. RStudio Team. RStudio: integrated development for R. RStudio. http://www.rstudio.com/ (2020).

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Author contributions
G.B.D. and W.K. conceived and designed the experiment; G.B.D., W.K., A.M.-A., M.W., E.S., carried out the experiments; G.B.D., W.K., A.M.-A., M.W., E.S., P.W., A.P performed the analyses; G.B.D., W.K., A.M.-A., M.W., E.S., P.W., A.P analysed the data; W.K. writing—original draft preparation; G.B.D., W.K., A.M.-A., M.W., E.S., A.P. writing—revision; G.B.D., W.K., funding acquisition; G.B.D. Supervision. All authors read the manuscript and accepted it. All the authors read and approved the manuscript.

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Characterization of the Metallothionein Gene Family in *Avena sativa* L. and the Gene Expression during Seed Germination and Heavy Metal Stress

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Abstract: Metallothioneins (MTs) are a family of small proteins rich in cysteine residues. The sulfhydryl group of metallothioneins can bind to metal ions, maintaining metal homeostasis and protecting the cells from damage caused by toxic heavy metals. Moreover, MTs can function as reactive oxygen species scavengers since cysteine thiols undergo reversible and irreversible oxidation. Here, we identified 21 metallothionein genes (*AsMT*s) in the oat (*Avena sativa* L.) genome, which were divided into four types depending on the amino acid sequences of putative proteins encoded by identified genes. Analysis of promoter sequences showed that *MTs* might respond to a variety of stimuli, including biotic and abiotic stresses and phytohormones. The results of qRT-PCR showed that all four types of *AsMT*s are differentially expressed during the first 48 hours of seed germination. Moreover, stress induced by the application of zinc, cadmium, and a mixture of zinc and cadmium affects the expression of oat *MTs* variously depending on the MT type, indicating that *AsMT1-4* fulfil different roles in plant cells.

Keywords: oat; metallothioneins; promoter; germination; zinc; cadmium; antioxidants

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1. Introduction

Metallothioneins (MTs) are low-molecular-weight, cysteine-rich proteins present in microorganisms, animals, and plants [1–4]. Upon the discovery of metallothioneins, they were described as proteins that bind to cadmium ions [5], and further, it was shown that MTs can bind to a variety of heavy metal ions including zinc and copper [4]. The first plant metallothionein (pMT) was discovered in wheat embryos and was named an early cysteine-labelled protein (E_c protein) [6]. Its mRNA was present in dry wheat embryos but not in germinated embryos [7].

Cysteine (Cys, C) is a unique amino acid thanks to the presence of a thiol group. The sulfhydryl group (-SH) is the high-affinity binding site of several metals including zinc and cadmium [8]. MTs are found throughout all kingdoms of living organisms and are highly diversified. The most common feature of all MTs is the high content of cysteines, even up to 30% of all amino acids are cysteines [9]. Plant metallothioneins are more diversified than MTs from other groups of organisms, and they are divided into four types (MT1—MT4) based on the arrangement and number of Cys residues [10]. Type-1 pMTs have 12 Cys residues, type-2 pMTs have 14 Cys residues, type-3 pMTs have 10 Cys residues, and type-4 pMTs have 17 Cys residues. The Cys residues in type-1–3 pMTs are grouped into two domains, whereas the Cys of type-4 pMTs are grouped into three domains [11–13]. In

addition, some plant MTs have histidine (His, H) residues, which is not a common feature of MTs. It has been shown that those histidines participate in metal binding [14]. Histidines are also present in some bacterial MTs, e.g., His in bacterial SmtA from *Synechococcus* PCC7942 stabilize the protein folding and impact metal cluster charge [15]. Moreover, according to Pearson's theory of hard and soft acids and bases (HSAB theory) [16], His could allow metallothioneins to differentiate between structurally similar zinc and cadmium ions; i.e., MTs fold properly only in the presence of zinc but not in the presence of cadmium [17].

Cysteine thiols can undergo reversible and irreversible oxidation [8]. Thus, MTs can act as reactive oxygen species (ROS) scavengers [18]. At low concentrations, ROS are important signaling molecules. However, in excess, ROS cause extensive damage to proteins, DNA, and lipids, disturbing cellular functioning [19]. Plants have developed diversified mechanisms that allow them to maintain redox homeostasis. An enzymatic antioxidant system consists of enzymes like superoxide dismutase (SOD), peroxidase (PX), and catalase (CAT). Moreover, non-enzymatic compounds like ascorbic acid, reduced glutathione, phenolics, and proline serve as antioxidants [20–23]. Numerous lines of evidence show that pMTs could be a part of the plant non-enzymatic antioxidant system. We showed previously that *Brassica napus* L. MTs can diminish ROS damage when overexpressed in *E. coli* cells [24]. Subsequently, we found that the expression of oat *MTs* correlates positively with the increased level of antioxidant enzymes like SOD, CAT, and PX [25]. Moreover, transgenic *Arabidopsis thaliana* (L.) Heynh. overexpressing date palm *MT2* had improved ROS scavenging ability [26]. MT3a from *Gossypium hirsutum* L. was shown to scavenge superoxide and hydroxyl radical in vitro [27]. Plant *MT* expression can be induced by various stress conditions, including cold [28], drought [23,25], and biotic stress [29]. Furthermore, plants that are overexpressing MTs exhibit higher tolerance to cadmium [30,31], drought [32], freezing, and salt stress [33]. Several studies have shown that the expression of *pMTs* also changes during plant development [34,35], including seed germination [34,36,37]. These observations indicate that MTs are crucial in plants' development, growth, and survival in adverse environmental conditions.

Metallothionein gene families have been investigated in numerous plants such as *A. thaliana* [38], *Oryza sativa* L. [39,40], *Zea mays* L. [41] *Nicotiana tabacum* L. [13], and *Cucumis sativus* L. [13]. However, relatively little is known about the diversity of *MT* genes in polyploid plants, e.g., *B. napus* [37,42]. Oat (*Avena sativa* L.) is a cereal crop from the family *Poaceae*, widely known for its healthy and nutritious properties. The seeds of modern oat cultivars are rich in minerals, including Zn, Cu, Ca, Fe, and Mg [43]. Moreover, oat seeds have a high content of proteins [44], antioxidants [45], vitamins E and B [46], and β-glucan. In particular, oat β-glucan has been afforded more attention recently, thanks to its cholesterol-lowering properties [47]. Oat consumption has many positive health effects since it can help reduce hyperglycemia, hyperinsulinemia, hypercholesterolemia, and hypertension. It is recommended to eat oats to prevent cardiovascular diseases [48]. The genus *Avena* consists of diploid (AA or CC genomes), tetraploid (AABB, AACC, CCCC, or CCDD genomes), and hexaploid species (AACCDD genomes). It is believed that the hexaploid oat (AACCDD, $2n = 6x = 42$; ~12,500 Mb) arose from hybridization between a CCDD allotetraploid and an AA diploid [49,50]. The sequencing of the oat genome was difficult due to the big size of its genome (12.5 Gb) and the mosaic structure of the chromosomes [51]. In 2019, the first chromosome-scale assemblies of oat diploid species were published [52]; the first hexaploid oat reference genome was published in 2021 [53]; and in 2022, the *A. sativa* cv. Sang genome sequence was published [51]. With the oat genome sequence available, it is expected that the amount of research at the molecular level will increase significantly. Already, a comparison between the oat and wheat genomes showing a lower number of genes encoding gluten-like proteins in the oat genome has been published [54]. Understanding oat on a genetic level will help to improve the nutritional quality and agronomic traits of oat.

This study aimed to show the complexity of the metallothionein gene family in oat. We assume that these Cys-rich proteins with antioxidant properties are of key importance

during plant growth and development, not only in favorable conditions but also in stress conditions caused by the presence of heavy metals. We identified 21 *MT* genes in the oat genome and analyzed their structure, evolution, and chromosomal localization. Moreover, we investigated the presence of cis-regulatory elements in the promoters of the *AsMT* genes. To verify the potential role of AsMTs in oat development and stress response, we examined the expression of *AsMT* genes during germination and in seedlings grown in the presence of Zn, Cd, and a mixture of Zn and Cd in a hydroponic culture.

2. Materials and Methods

2.1. Oat Metallothionein Genome-Wide Identification and Analysis of Putative AsMT Proteins

To identify the oat *MT* genes (*AsMT*), the *MT* sequences from *Hordeum vulgare* L., *O. sativa*, and *Z. mays* (downloaded from NCBI's GeneBank) were used as queries to search against the hexaploid oat genome OT3098 v2 [53] via the Grain Genes database (https://wheat.pw.usda.gov/, accessed on 25 April 2022). Default parameters for BLAST search were used. The putative *AsMT* gene family members were downloaded and verified using the Pfam database (http://pfam.xfam.org/, accessed on 25 April 2022). The theoretical molecular weight (MW) and isoelectric point (pI) of putative AsMTs were calculated using the ProtParam program (http://web.expasy.org/protparam/ accessed on 26 April 2022). Subcellular localizations were predicted using the Plany4t-mPLoc server (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/#, accessed on 5 May 2022) [55]. The Gene Structure Display Server (GSDS, http://gsds.gao-lab.org/, accessed on 5 May 2022) was used to compare the coding sequences (CDS) with the corresponding genomic DNA (gDNA) sequences of oat AsMTs downloaded from the Grain Genes database [56]. Data regarding the chromosome localization of *AsMT*s were downloaded from the Grain Genes database and analyzed using MG2C (http://mg2c.iask.in/mg2c_v2.1/, accessed on 6 May 2022).

2.2. Phylogenetic Analysis and Conserved Motif Analysis

The amino acid sequences of 21 AsMTs and MTs from *A. thaliana* (AtMT), *Z. mays* (ZmMT), and *O. sativa* (OsMT) were aligned, and a phylogenetic tree was constructed using MEGA-11 software (version 11.0.13) with a bootstrap of 1000 replicates [57]. The sequences of MT protein from *A. thaliana*, *O. sativa*, and *Z. mays* were downloaded from the Ensembl database (https://plants.ensembl.org/, accessed on 28 April 2022).

Multiple sequence alignments of type-1–4 AsMTs and MTs from other plants were constructed using MEGA-11 software (version 11.0.13). MT sequences from other plants were obtained from the NCBI database. The online tool MEME (ver. 5.4.1, https://meme-suite.org/meme/tools/meme, accessed on 6 May 2022) was used to find conserved motifs in the 21 AsMT amino acid sequences, with the maximum motif number set to 5.

2.3. Prediction of Cis-Responsive Elements in AsMT Promoters

A 1500 bp fragment of the genomic region upstream of the start codon (ATG) was obtained from the Grain Genes database and used to search for the cis-acting regulatory elements (CREs) using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 25 May 2022). The positions of the CREs were marked in the diagram using TBtools (https://bio.tools/tbtools, accessed on 25 May 2022, version 1.123).

2.4. Plant Material

Seeds of oat cv. Bingo, purchased from Plant Breeding Strzelce Ltd., PBAI Group, Strzelce, Łódź Voivodeship, Poland, were used for experiments. The seeds were sterilized using a mixture of 96% ethanol and 30% H_2O_2 (1:1, *v*/*v*) for 1 min and rinsed five times in sterile distilled water.

For the analysis of *AsMT1-4* expression during germination, sterilized seeds were placed in Petri dishes on filter paper soaked in 3 mL of sterile distilled water. The Petri dishes were incubated in darkness at 23 °C. After 3, 6, 9, 12, 24, and 48 hours, 10 seeds

were collected and frozen in liquid nitrogen and kept at −80 °C for further analysis. Dry, non-germinating seeds were used as a reference sample (0 h). The experiment was repeated 3 times.

For the analysis of *AsMT1-4* expression under heavy metal stress, sterilized seeds were germinated for 4 days in Petri dishes lined with filter paper that was moistened with 3 mL of sterile distilled water. Four-day-old seedlings of similar size were placed in 1000 mL plastic vessels containing Hoagland medium [58] and maintained in a hydroponic culture in a growth chamber at 21 ± 2 °C under a light intensity of 100 µmol m^{-2} s^{-1} (16/8 h light/dark). After 3 days of acclimation, stress was induced by changing the medium to Hoagland's medium amended with 200 µM $ZnSO_4$, 100 µM $CdSO_4$, or a mixture of 200 µM $ZnSO_4$ and 100 µM $CdSO_4$. The medium was aerated consistently using an air pump to avoid hypoxia (Hailea ACO-2201, Happet, Poznań, Poland). After 3, 7, and 14 days of treatment, the length of oat shoots and roots and their fresh and dry biomass were measured. Moreover, roots and shoots were washed 3 times in sterile distilled water, frozen in liquid nitrogen, and kept at −80 °C for further analyses.

2.5. Total RNA Isolation

Plant tissues were ground in liquid nitrogen using a mortar and pestle. For isolating the RNA from shoots and roots, 0.1 g of tissue was used, and the RNA was isolated using an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). For the seeds, 0.2 g of ground tissue was used for RNA isolation according to the protocol described by Wang et al. [59], with some modifications. The changes to the protocol included a larger volume of RNA extraction buffer (600 µL), a larger volume of 20% sodium dodecyl sulfate (SDS, 30 µL), and longer RNA precipitation in ethanol (overnight at −20 °C). The quality and quantity of isolated RNA were checked via agarose gel electrophoresis and spectrophotometric measurement using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Quantitative Real-Time PCR (RT-qPCR) Analysis

To remove any DNA contamination from the RNA samples, 1 µg of total RNA was treated with 1 U of DNase I (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37 °C for 30 min. The cDNA was synthesized from 1 µg of total RNA using a mixture of 2.5 µM oligo$(dT)_{20}$ primer and 0.2 µg of random hexamers with an NG dART RT Kit (EURx, Gdańsk, Poland), according to the manufacturer's protocol. The reaction was performed at 25 °C for 10 min, followed by 50 min at 50 °C. The cDNA was stored at −20 °C. The quality of the cDNA was checked via RT-PCR. The PCR mixture contained 2 µL of 10x Pol Buffer B, 0.2 mM of dNTPs, 0.3 µM of forward and reverse AsACT primers (Table 1), 1.25 U of Opti*Taq* DNA Polymerase (EURx, Gdańsk, Poland), and 1 µl of cDNA for a total volume of 20 µL. The thermal cycling conditions were as follows: 94 °C for 5 min, 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 40 s, followed by 72 °C for 7 min.

The RT-qPCR reaction mixture included 4 µL of 1/5 (seeds) or 1/30 (shoots and roots) diluted cDNA, 0.5 µM of gene-specific primers (Table 1), and 5 µL of LightCycler 480 SYBR Green I Master (Roche, Penzberg, Germany) for a total volume of 10 µL. *EIF4A* (Eukaryotic Initiation Factor 4A-3) was used as a reference gene [60]. The reactions were performed in three technical replicates in a LightCycler 480 Instrument II (Roche, Penzberg, Germany). The thermal cycling conditions were as follows: 95 °C for 5 min, 95 °C for 10 s, 60 °C for 20 s, 72 °C for 20 s, over 45 cycles. The SYBR Green I fluorescence signal was recorded at the end of the extension step in each cycle. The specificity of the assay was confirmed by the melt curve analysis, i.e., increasing the temperature from 55 to 95 °C at a ramp rate of 0.11 °C/s. The fold change in gene expression was calculated using LightCycler 480 Software, release 1.5.1.62 (Roche, Penzberg, Germany).

Table 1. Sequences of the primers used in this study.

Primer Name	Sequence 5′→ 3′	Product Size [bp]	Target	Reference
AsMT1_qPCR_f AsMT1_qPCR_r	CAAACTGCAAGTGCGGGAAG TTGTTCTCATGAGCCACGCC	103	AsMT1 chr7C	[25]
AsMT2_qPCR_f AsMT2_qPCR_r	CTGCGGAGGGTGCAAGATG AACGATGGCTTGGAAGAGGG	96	AsMT2 chr1C	
AsMT3_qPCR_f AsMT3_qPCR_r	TCCACCATGTCGAACACCTG TGGCTCTTCTCGGTGTCAAC	107	AsMT3 chr3A	
AsMT4_qPCR_f AsMT4_qPCR_r	CACGTGCGGAGAGCACTG ACAGGAGGCGCAGTCACAG	121	AsMT4 chr1D	This study
EIF4A_f EIF4A_r	TCTCGCAGGATACGGATGTCG TCCATCGCATTGGTCGCTCT	88	Eukaryotic Initiation Factor 4A-3	[60]
AsACT_f AsACT_r	CTTCCCCAGTATCGTCGGAC AGGGCAATGTAGGACAGCTT	573	Actin (MH260250.1)	This study

2.7. Determination of Photosynthetic Pigments

The content of photosynthetic pigments (chlorophyll a and b, and carotenoids) in oat shoots exposed to heavy metal stress was measured via spectrophotometric measurement using the Epoch Take 3 microplate reader (Agilent BioTek, Santa Clara, CA, USA). One hundred milligrams of plant material was ground in liquid nitrogen, and pigments were extracted with 1 mL of 80% ethanol. The samples were shaken for 15 min (180 rpm) at room temperature and then centrifuged (13,000× *g*, 10 min, room temperature). The absorbance of the supernatant was measured at λ = 470 nm, λ = 648 nm, and λ = 664 nm (Epoch Take 3 microplate reader, Agilent BioTek, Santa Clara, CA, USA). Concentrations of chlorophyll a, chlorophyll b, and carotenoids were calculated according to Lichtenthaler and Wellburn [61].

2.8. Determination of Total Phenolic Content

The total phenolic content (TPC) was determined spectrophotometrically using the method described by Singleton and Rossi [62]. Phenolic compounds were extracted from 100 mg of plant material that had been ground in liquid nitrogen (shoots and roots separately) in 1 mL of 80% ethanol. The samples were shaken for 15 min (180 rpm) at room temperature and then centrifuged (13,000× *g*, 10 min, room temperature). The reaction mixture consisted of 500 µL of distilled water, 100 µL of ethanolic plant extract, 250 µL of 25% Na_2CO_3, and 125 µL of Folin–Ciocalteau reagent (diluted with distilled water 1:1, *v*/*v*, before use). The samples were incubated for 15 min at room temperature and briefly centrifuged, and the absorbance was measured at λ = 760 nm (Epoch Take 3 microplate reader, Agilent BioTek, Santa Clara, CA, USA). TPC was expressed as micrograms of gallic acid (GA) per gram of FW of plant tissue.

2.9. Determination of Antioxidant Capacity

To extract antioxidants from the shoots and roots of heavy-metal-stressed oat plants, 300 mg of ground tissue was mixed with 5 mL of 50% methanol (*v*/*v*). The samples were shaken for 20 min (250 rpm) at room temperature and then centrifuged (15 min, 5000× *g*, 4 °C). The supernatant was collected, and the extraction procedure was repeated twice. The extracts obtained from each extraction step were mixed and subjected to further analysis.

2.9.1. ABTS Assay

The mixed-mode ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) method for determining the quantity of hydrophilic and lipophilic antioxidants was performed according to Re et al. [63]. An ABTS radical cation ($ABTS^{\bullet+}$) was produced during the reaction of a 7 mM solution of ABTS with 2.45 mM of potassium persulfate at a ratio of

2:1 (*v*/*v*) overnight in darkness. Before use, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.7 (±0.02) at λ = 734 nm. Next, 100 µL of plant methanolic extract was added to 150 µL of $ABTS^{\bullet+}$ solution and the mixture was incubated at 30 °C for 5 min. The absorbance was measured at λ = 734 nm (Epoch Take 3 microplate reader, Agilent BioTek, Santa Clara, CA, USA), and the antioxidant capacity (AC) was expressed as a water-soluble analog of vitamin E Trolox (TE, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) equivalents (µmol TE per 1 g fresh weight of plant tissue). The calibration curve, $\%ABTS = (166.03 \pm 0.53)c_{TE} + (6.51 \pm 0.30)$, was prepared using working solutions of TE in methanol between 0.01 and 0.15 µmol/mL.

2.9.2. Ferric Reducing Antioxidant Power (FRAP) Assay

To quantify the hydrophilic antioxidants in the oat seedlings, the single electron transfer (SET) method, i.e., a FRAP assay, was performed according to the procedure originally developed by Benzie and Strain [64], with some modifications. The FRAP solution contained 100 mL of 0.1 M acetate buffer (pH 3.6), 10 mL of a 10 mM TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) solution in 40 mM HCl, and 10 mL of 20 mM $FeCl_3$. Before usage, it was incubated at 40 °C for 15 min. The reaction mixture contained 50–100 µL of plant methanolic extract, 100 µL of FRAP solution, and distilled water to a final volume of 250 µL. The samples were incubated for 20 min in darkness. The absorbance was measured at λ = 593 nm (Epoch Take 3 microplate reader, Agilent BioTek, Santa Clara, CA, USA), and the AC was expressed as TE equivalents (µmol TE per 1 g FW). Calibration curves were prepared using working solutions of TE in methanol between 1.00×10^{-3} and 1.70×10^{-2} µmol/mL. The least squares method was applied to calculate the line's equation: $A_{593} = (51.51 \pm 0.42)c_{TE} + (0.023 \pm 0.004)$ resulting in $R^2 = 0.9997$.

2.10. Statistical Analysis

The results are expressed as mean values, and error bars represent the standard deviation (SD). Before the statistical assessment, data normality was tested using the Shapiro–Wilk test. The statistical analysis of the experimental data was performed via one-way analysis of variance (ANOVA) followed by a post-hoc Tukey's test. Pearson correlations were calculated to demonstrate the relations among the measured traits. The programs Microsoft Excel, Past 4.0 [65], RStudio [66], and Phyton were used for calculations and the preparation of graphs.

3. Results

3.1. Identification and Chromosome Distribution of A. sativa Metallothionein (AsMT) Genes

BLAST screening of the oat genome revealed the presence of 21 genes that encode putative oat MTs. *AsMT* genes were located in 12 out of the 21 oat chromosomes (Figure 1). The type of putative protein encoded by the identified genes was at this stage determined by the homology to the MT sequence used as a query in the BLAST search, to be further confirmed by an in silico analysis of putative amino acid sequences and phylogenetic analysis. The identified genes were named according to the encoded MT type (MT1-4) and chromosome localization (chr1-7, A, C, D). The highest number of *MT* genes were located in the subgenome D, which accounted for eight genes, whereas seven genes were present in the subgenome C, and six genes in the subgenome A. With some exceptions, the number and chromosomal localization of *AsMT1-4* differed among groups of chromosomes. On chromosomes 3A and 3C, nearby genes encoding AsMT2 and AsMT3 were present, whereas on chromosome 3D, only the *AsMT3* gene was present. On chromosomes 1A and 1D, the *AsMT1* and *AsMT4* genes were located close to one another. On chromosome 1C, the *AsMT4* gene was present, although not in the same locus (Figure 1). The majority of *AsMTs* were located on the distal parts of chromosomes; only *AsMT1_chr1A*, *AsMT4_chr1A*, *AsMT1_chr7C*, *AsMT1_chr1D*, and *AsMT4_chr1D* were located in the central part of chromosomes. In all three subgenomes, only chromosomes 2 and 6 had no *MT* genes (Figure 1).

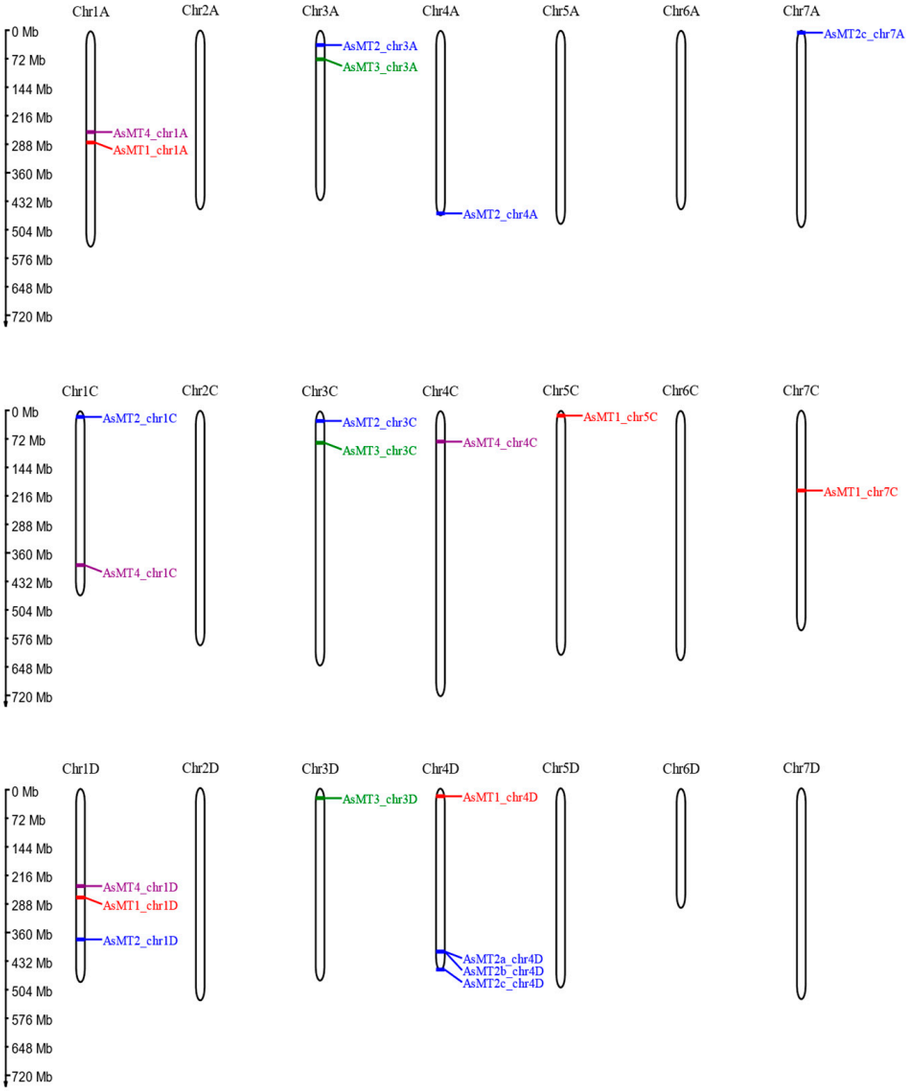

Figure 1. Chromosome localization of oat *MT* genes. Type-1 metallothioneins are labelled in red, type-2 *pMTs* in blue, type-3 *pMTs* in green, and type-4 *pMTs* in purple.

The length of the introns and exons of the *AsMT* nucleotide sequences was very variable and ranged from 243 bp (*AsMT4_chr1C*) to 910 bp (*AsMT3_chr3C*) in length (Figure 2, Table 2). Type-1 and -2 *AsMTs* had one intron, type-3 *AsMTs* had two introns, and type-4 *AsMTs* had no introns, except for *AsMT4_chr4C*, which had one intron (Figure 2).

Further in silico analysis of the putative amino acid sequences of AsMT1-4 was performed to confirm the type of pMTs based on the number and distribution of Cys residues (Table 2). It was shown that five AsMTs were type-1 pMTs, nine were type-2 pMTs, three were type-3 pMTs, and four were type-4 pMTs. Atypical numbers and distributions of cysteines were also revealed for some type-1 and -2 AsMTs. AsMT1_chr1A had 11 cysteines, and AsMT1_chr7 had 13 cysteines, instead of the typical 12 cysteines present in type-1 pMTs. AsMT2C_chr4A had 15 cysteines and AsMT2C_chr1D, AsMT2C1_chr4D, AsMT2C2_chr4D, AsMT2C3_chr4D, and AsMT2C_chr7A had 17 cysteines instead of the 14 Cys that is a typical number for type-2 pMTs. For AsMT3 and AsMT4, all analyzed sequences had a typical number of cysteines, i.e., 10 and 17, respectively. The distribution of cysteines was observed in pMTs in other plant species, i.e., two Cys-rich domains for pMT1-3 and three Cys-rich domains for pMT4 (Table 2). Cysteines in MTs are arranged in typical motifs, which were

also present in AsMT1–4 (Table 2). The length of the putative amino acid sequences of AsMT ranged from 63 (AsMT3_chr3C and AsMT3_chr3D) to 89 (AsMT4_chr4C), and the molecular weights ranged from 6.66 kDa for AsMT3_chr3C to 8.62 kDa for AsMT4_chr4C. The pI values of putative oat proteins ranged from 4.71 for AsMT2_chr3C to 7.36 for AsMT4_chr1A, AsMT4_chr1C, and AsMT4_chr1D (Table 2). The results of subcellular localization prediction showed that 11 AsMTs had a single subcellular localization in either cytoplasm or nuclei. For other AsMTs, multiple subcellular localizations were predicted, i.e., the cytoplasm, nuclei, cell membrane, and chloroplasts (Table 2).

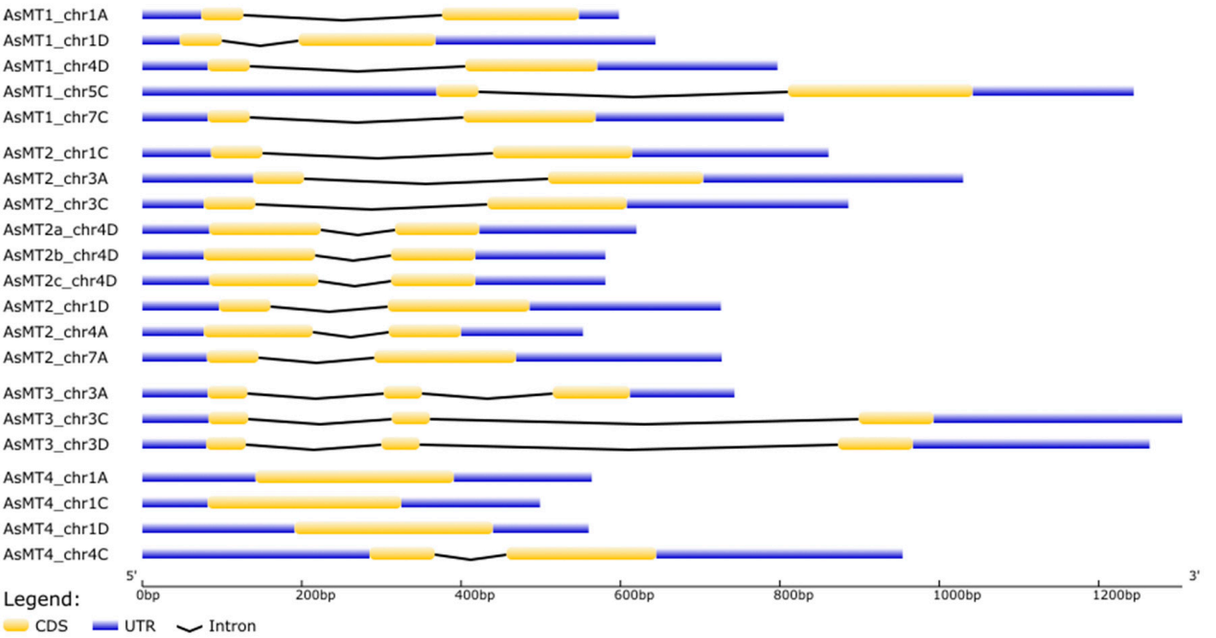

Figure 2. Intron–exon structure of oat *MT* genes. Coding sequences (CDS), untranslated regions (UTR), and introns are displayed as yellow boxes, blue boxes, and black lines, respectively.

3.2. Analysis of Conserved Motifs in the Amino Acid Sequences of AsMTs

The alignments of the amino acid sequences of the AsMT proteins showed that sequences belonging to the same type of pMT are highly conserved even in evolutionarily distant species (Figure 3). These alignments confirmed that identified AsMT proteins belong to respective types of pMTs. A comparison of AsMTs with MTs from other plant species showed that Cys-rich domains are the most conserved parts of pMT sequences in terms of the number and arrangement of cysteines. Moreover, in AsMT4, there were two highly conserved His residues, which were also present in other representatives of this type of pMTs. Interestingly, some of the AsMT4 proteins exhibited atypical features that were not present in pMTs from other plant species. For example, in AsMT1_chr1A, the last Cys is substituted with His, whereas in AsMT4_chr4C there is another, third His within the first Cys-rich domain. Moreover, in AsMT1_chr4D, AsMT1_chr5C, and AsMT1_chr7C, an additional His located within the Cys-free stretch is present. The additional His within this region is also present in six of the type-2 AsMTs and all of the type-3 AsMTs; however, for type-3 pMTs, this additional His is also present in OsMT3. All AsMT3 proteins contain histidine/s at the C-terminus of the amino acid sequence (Figure 3).

Table 2. Characterization of identified *AsMT* genes and putative AsMT proteins. The genes (*AsMT1*_chr7C, *AsMT2*_chr1C, *AsMT3*_chr3A, *AsMT4*_ch1D) that are marked in bold were further analyzed for their expression level (vide infra).

AsMT Type	Gene Name	Gene ID/Position	Strand	gDNA Size (bp)	Protein Length (aa)	MW (kDa)	pI	Cys Number	Cys Sequence Pattern	Length of Spacer between Cys Domains	Predicated Subcellular Localization
MT1	*AsMT1*_chr1A	AVESA.00001b.r3.1Ag0000996	–	474	74	7.45	5.11	11 (6 + 5)	CxC	43	Cyto/Nucl
	*AsMT1*_chr1D	AVESA.00001b.r3.1Dg0000988	+	321	74	7.32	6.50	12 (6 + 6)		43	Cyto/Nucl
	*AsMT1*_chr4D	AVESA.00001b.r3.4Dg0000092	–	489	72	7.26	5.05	12 (6 + 6)		41	Cell mem/ Cyto/Nucl
	*AsMT1*_chr5C	AVESA.00001b.r3.5Cg0000058	+	673	75	7.62	4.75	12 (6 + 6)		44	Cyto/Nucl
	***AsMT1*_chr7C**	AVESA.00001b.r3.7Cg0001922	–	487	72	7.29	5.00	13 (6 + 7)		41	Cell mem/ Cyto/Nucl
MT2	***AsMT2*_chr1C**	AVESA.00001b.r3.1Cg0000164	+	529	79	7.59	5.10	14 (8 + 6)	CC, CxC, CxxC, C	41	Cell mem/ Cyto/Nucl
	*AsMT2*_chr3A	AVESA.00001b.r3.3Ag0000375	–	565	79	7.56	5.05	14 (8 + 6)		41	Cell mem/ Chlo/Cyto/Nucl
	*AsMT2*_chr3C	AVESA.00001b.r3.3Cg0000320	+	531	79	7.61	4.71	14 (8 + 6)		41	Cell mem/ Chlo/Cyto/Nucl
	*AsMT2*_chr1D	AVESA.00001b.r3.1Dg0002311	–	390	80	7.59	6.47	17 (8 + 9)		37	Cyto
	*AsMT2*_chr4A	AVESA.00001b.r3.4Ag0003869	–	323	75	7.19	5.57	15 (8 + 7)		38	Cyto
	*AsMT2a*_chr4D	AVESA.00001b.r3.4Dg0003379	–	339	81	7.72	4.96	17 (8 + 9)		39	Cyto
	*AsMT2b*_chr4D	AVESA.00001b.r3.4Dg0003380	–	341	81	7.70	4.96	17 (8 + 9)		39	Cyto
	*AsMT2c*_chr4D	AVESA.00001b.r3.4Dg0003934	–	334	80	7.63	4.96	17 (8 + 9)		38	Cyto
	*AsMT2*_chr7A	AVESA.00001b.r3.7Ag0000076	+	388	80	7.53	6.47	17 (8 + 9)		37	Cyto
MT3	***AsMT3*_chr3A**	AVESA.00001b.r3.3Ag0000802	–	530	64	6.81	4.85	10 (4 + 6)		32	Nucl
	*AsMT3*_chr3C	AVESA.00001b.r3.3Cg0000779	–	910	63	6.66	5.07	10 (4 + 6)		31	Nucl
	*AsMT3*_chr3D	AVESA.00001b.r3.3Dg0000256	–	887	63	6.68	5.07	10 (4 + 6)		31	Nucl
MT4	*AsMT4*_chr1A	chr1A:252750400..252750656	+	249	82	7.91	7.36	17 (6 + 6 + 5)	C, CxC	16, 15	Cyto
	*AsMT4*_chr1C	AVESA.00001b.r3.1Cg0001463	+	243	80	7.83	7.36	17 (6 + 6 + 5)		14, 15	Cyto
	***AsMT4*_chr1D**	chr1D:243956192..243956442	+	249	82	7.94	7.36	17 (6 + 6 + 5)		16, 15	Cell mem/Cyto
	*AsMT4*_chr4C	chr4C:76127001..76127600	+	360	89	8.62	5.75	17 (6 + 6 + 5)		26, 13	Cell mem/Cyto

Cyto—cytoplasm, Nucl—nucleus, Cell mem—cell membrane, Chlo—chloroplast.

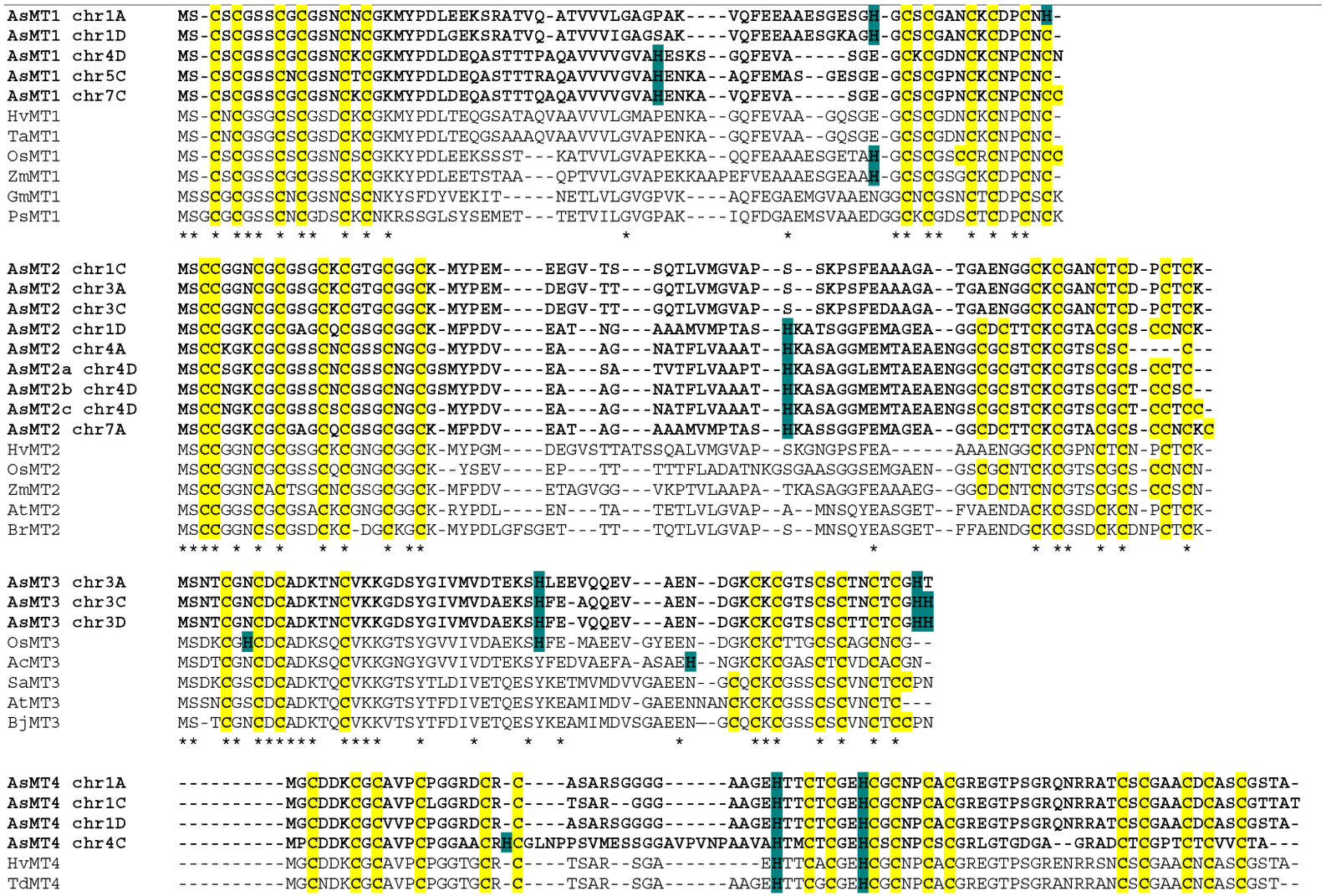

Figure 3. *Cont*.

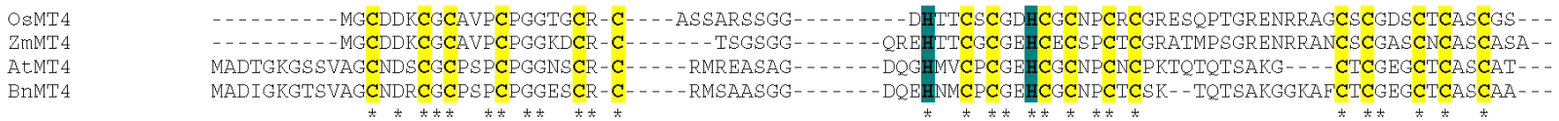

Figure 3. Amino acid alignments of the representative members of each type of pMT. Cysteine residues are highlighted in yellow, histidine residues are highlighted in blue, AsMTs are marked in bold, and asterisks (*) indicate identical amino acids. The GenBank accession numbers are as follows: HvMT1 (*Hordeum vulgare* MT1, XP_044981033.1), TaMT1 (*Triticum aestivum*, NP_001392631.1), OsMT1 (*Oryza sativa*, NP_001391526.1), ZmMT1 (*Zea mays*, PWZ25072.1), GmMT1 (*Glycine max*, NP_001359044.1), PsMT1 (*Pisum sativum*, BAD18382.1), HvMT2 (*H. vulgare*, XP_044974743.1), OsMT2 (*O. sativa*, NP_001384880.1), ZmMT2 (*Z. mays*, ACG26701.1), AtMT2 (*Arabidopsis thaliana*, NP_195858.1), BrMT2 (*Brassica rapa*, XP_009125444.1), OsMT3 (*O. sativa*, A2Y1D7.1), AcMT3 (*Ananas comosus*, OAY84410.1), SaMT3 (*Sinapis alba*, KAF8083573.1), AtMT3 (*A. thaliana*, NP_566509.1), BjMT3 (*Brassica juncea*, KAG2309813.1), HvMT4 (*H. vulgare*, KAI5017801.1), TdMT4 (*Triticum dicoccoides*, XP_037426553.1), OsMT4 (*O. sativa*, AAS78805.1), ZmMT4 (*Z. mays*, NP_001105499.1), AtMT4 (*A. thaliana*, NP_001189730.1), BnMT4 (*B. napus*, CAF1701889.1).

The MEME program was used to find in AsMT proteins de novo motifs that could be important for activity or the proper folding of proteins (Figure 4). Five different motifs were identified, and among them, only motifs 1 and 2 (containing Cys residues) were present in all 21 AsMT proteins. Motif 1 is present as the first motif on the N-terminus of the protein, whereas motif 2 is the last one at the C-terminus, except in the case of three type-4 AsMTs, where it was second-to-last. Motif 3 was present in all type-2 AsMTs, in three type-1 AsMTs, and in one type-4 AsMT. Motif 4 was only found in two type-1 MT sequences (AsMT1_chr7C and AsMT1_chr4D). Motif 5 was found in all sequences except for three AsMTs belonging to type-4 (Figure 4). The variation in the occurrence of motifs may be related to the functional divergence of AsMTs.

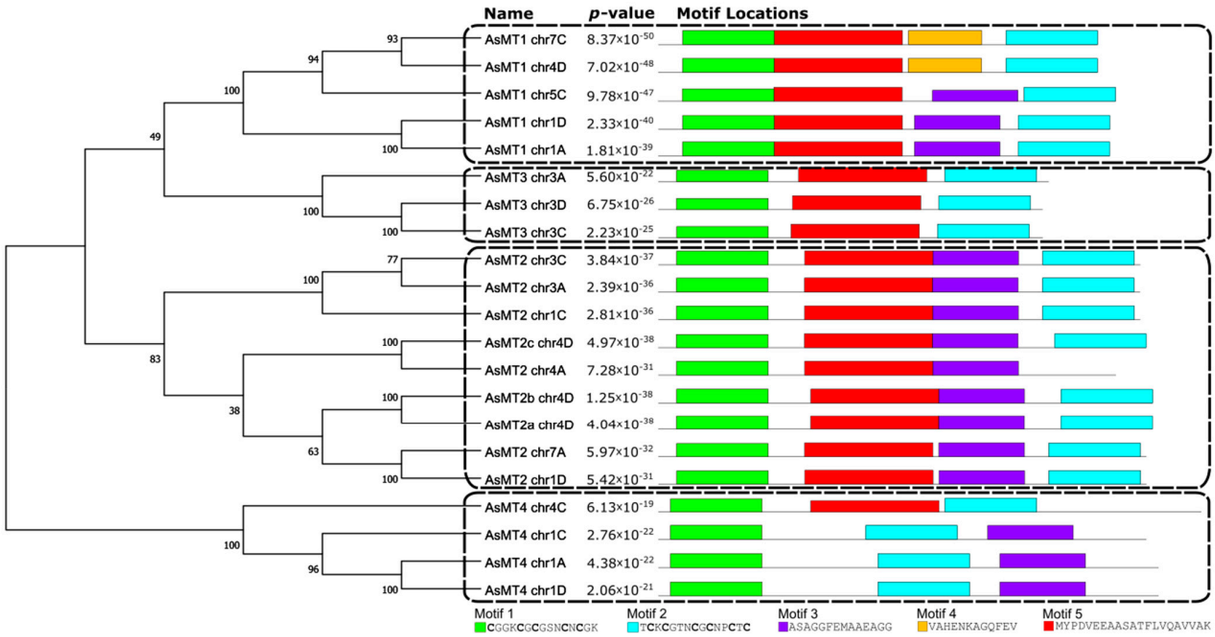

Figure 4. A neighbor-joining cladogram of AsMTs and five identified de novo motifs in oat MTs. Motifs were found using the MEME tool and are marked by different colors. The amino acid sequence of each motif is shown at the bottom of the figure.

A phylogenetic analysis of MT proteins from selected plant species showed the presence of four separated MT groups and confirmed that identified *AsMT* genes encode MTs belonging to the respective types of pMTs (Figure 5). AsMT2 proteins could be divided into three subgroups showing higher similarity to MT2 from other plant species than to each other. Moreover, one of the type-4 AsMTs is different from the other three AsMT4 proteins (Figure 5). These observations might reflect the polyploidy nature of *A. sativa* genome.

3.3. Prediction of Cis-Responsive Elements in AsMT Promoters

A 1500 bp region upstream of the ATG codon for all *AsMT* genes was analyzed using PlantCARE (Figure 6, Tables S1 and S2). Numerous cis-acting elements involved in phytohormone responses, stress reactions, pathogen defense, and development were found. The most common were elements involved in the response to phytohormones and abiotic stress (Table S2). Hormone-responsive elements, predominantly abscisic acid response elements, were found in all *AsMTs*. Regulatory elements related to the response to methyl jasmonate were found in 19 of the 21 analyzed promoters, whereas elements related to the response to gibberellins, salicylic acid, and auxins were less common (Table S1). Among abiotic-stress-responsive elements, the most common were drought response regulatory elements (Table S1). Other common elements were involved in light response and development, whereas the less common elements were those related to the response to biotic stress (Table S2).

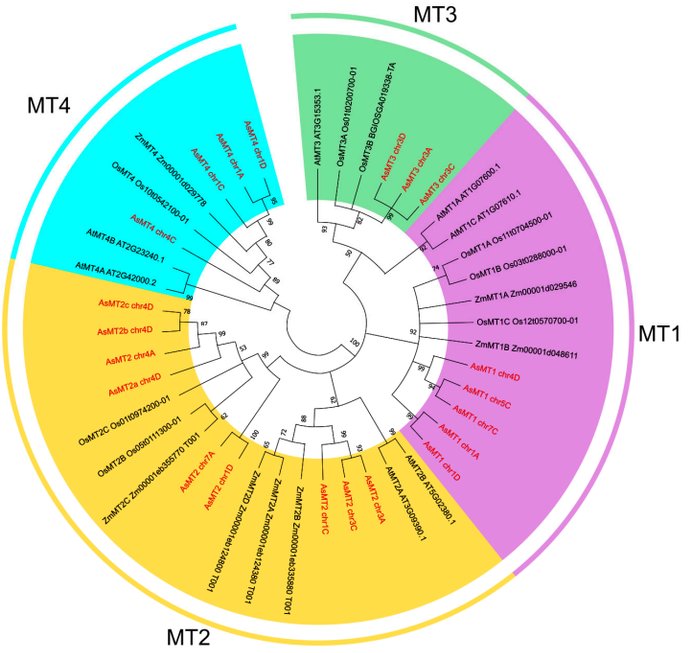

Figure 5. A phylogenetic tree based on the amino acid sequences of *Avena sativa*, *Arabidopsis thaliana*, *Zea mays*, and *Oryza sativa* MTs. The amino acid sequences were aligned by MEGA11 using the MUSCLE method, and the phylogenetic tree was built using the neighbor-joining method. The four MT types are highlighted in different colors and AsMTs are in red font.

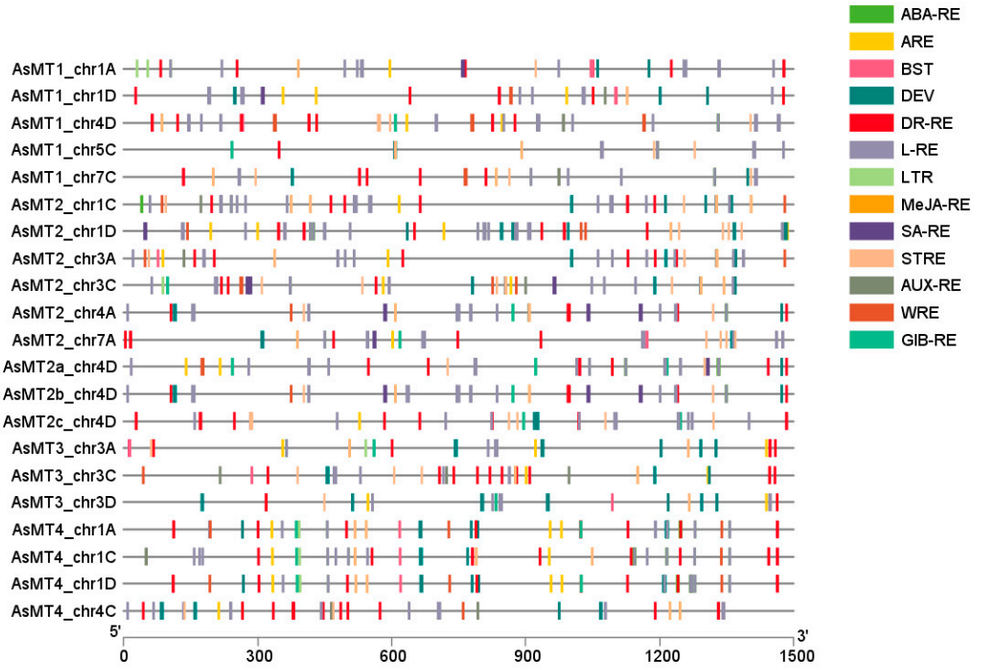

Figure 6. Schematic depiction of predicted cis-acting elements in *AsMT* promoters. The *cis*-acting elements are represented by different colored boxes. The scale at the bottom represents the length of the analyzed sequence. The abbreviations are as follows: ABA-RE—abscisic acid response, ARE —anaerobic induction, BST—biotic stress response, DEV—development-related, DR-RE—drought response, L-RE—light response, LTR—low-temperature response, MeJA-RE—methyl jasmonate response, SA-RE—salicylic acid response, STRE—stress response, AUX-RE—auxin response, WRE—wounding response, GIB-RE—gibberellin response.

3.4. AsMT1-4 Expression during Seed Germination

Seed germination is the first crucial step in plant growth and is significantly influenced by various environmental factors. Based on the promoter analysis, it might be predicted that AsMTs are involved in various developmental processes including germination. To evaluate the role of MTs during oat germination, the expression of four selected *AsMT* genes representing four types of pMT was determined (Figure 7). The expression of oat *MT* type 1 during the first hours of germination (3–9 h) decreased, but 12 h after the start of germination, *AsMT1* expression increased. Similarly, the expression of *AsMT3* was the highest in the dry seeds 48 h after the start of germination. An inverse trend was observed for *AsMT2*, where the expression peaked after the sixth and twelfth hour of germination, and its level was the lowest at the end of the experiment. The expression of *AsMT4* was the lowest after the forty-eighth hour of germination and the highest after the third and nineth hour (Figure 7A). The total number of *AsMT1-4* transcripts remained relatively constant throughout the analyzed period of germination, except during the 24th hour of germination. The relative amount of each AsMT in the dry and germinating oat seeds was variable throughout the analyzed period (Figure 7B).

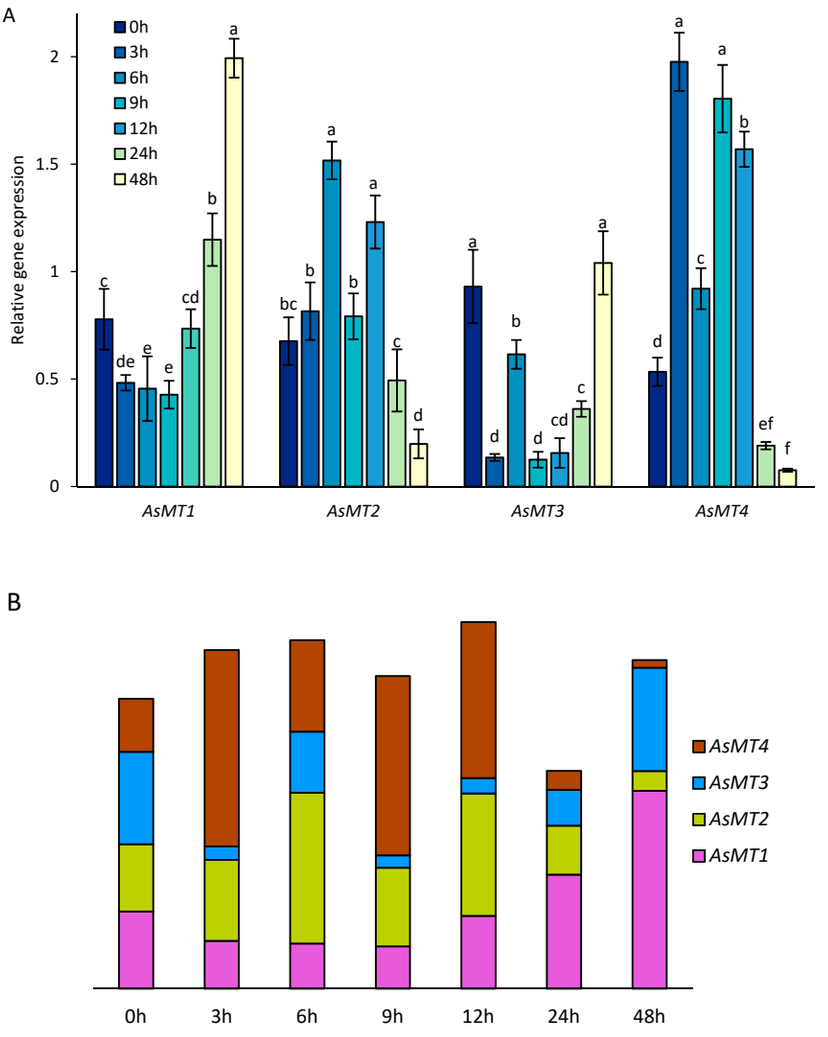

Figure 7. Levels of oat *AsMT1-4* transcripts in germinating seeds. (**A**) Relative expression of *AsMT1-4* in dry seeds (0 h) and during seed germination (3–48 h (hours after the start of germination)). Bars represent the means of three independent experiments ± SD. Values marked with different letters differ significantly (ANOVA, Tukey's test, $p < 0.05$). (**B**) A schematic representation of the number of *AsMT1-4* transcripts in dry (0 h) and germinating seeds (3–48 h).

3.5. Effect of Heavy Metal on Oat Seedling Growth

To assess the effect of heavy metals on oat seedling growth and development, the seedlings were treated with metal ions in hydroponic culture for 14 days (Figure 8). After 3 days of stress treatment, the length (Figure 8A) and biomass (Figure 8B,C) of oat shoots and roots were the same as for seedlings grown in the control conditions, with the only exception being the longer roots of Zn-treated plants; however, there were no differences in the root biomass. After 7 days of stress, there were no significant changes in either the fresh (Figure 8B) or dry (Figure 8C) biomass of the shoots and roots. However, the roots but not the shoots of Zn-treated plants were significantly longer than the roots of control seedlings, whereas the shoots but not the roots of Cd- and Zn + Cd-treated plants were shorter than the control (Figure 8A). After 14 days of heavy metal treatment, there were no differences between the root and shoot lengths of control and Zn-treated plants, but the fresh and dry biomass of the Zn-treated seedlings was higher. Treating plants with Cd and Zn + Cd for 14 days significantly shortened shoots and roots (Figure 8A,D) and reduced the biomass (Figure 8B,C).

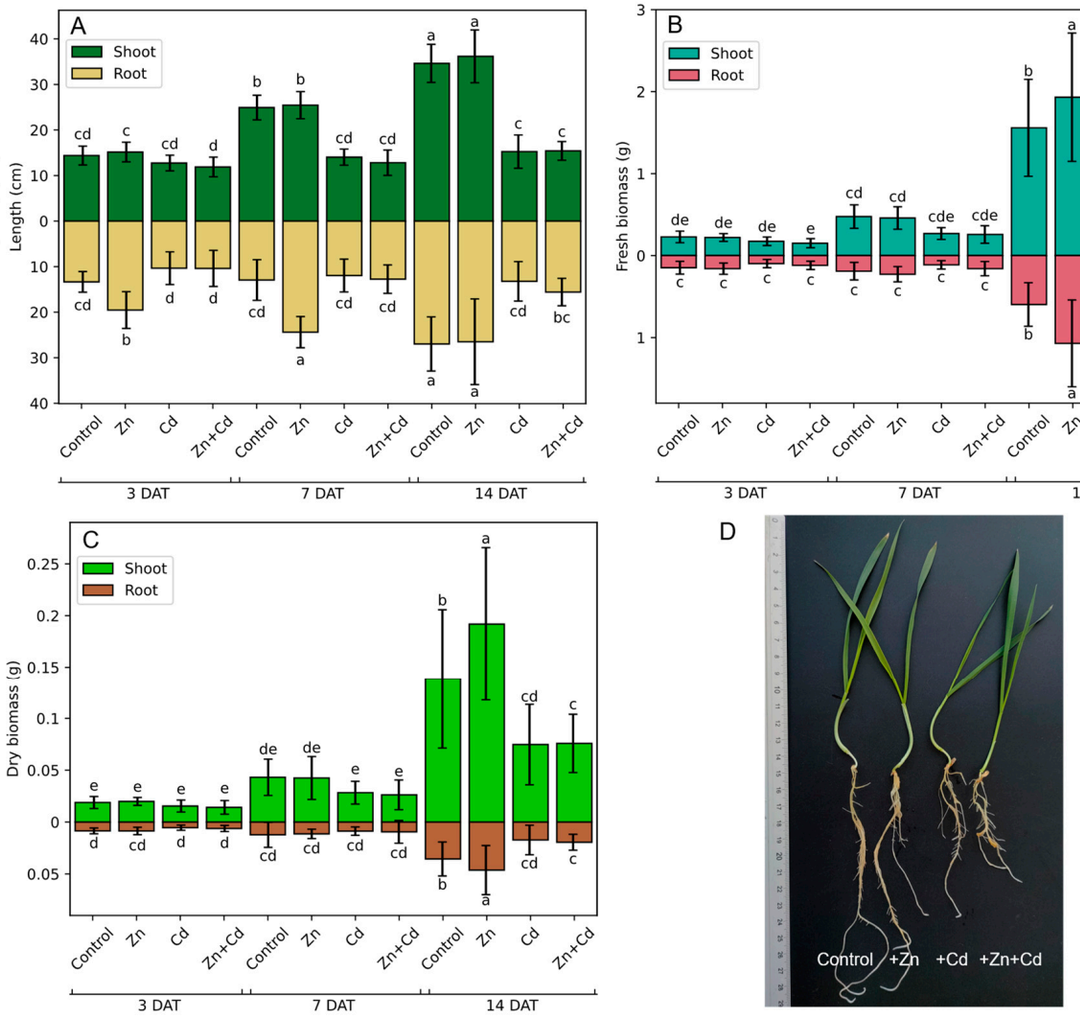

Figure 8. Shoot and root (**A**) length and the (**B**) fresh, and (**C**) dry biomass of oat seedlings subjected to heavy metal stress induced via the application of 200 µM $ZnSO_4$, 100 µM $CdSO_4$, and 200 µM $ZnSO_4$ + 100 µM $CdSO_4$. The control comprised non-stressed plants. Bars represent the mean values of measurements of 30 seedlings ± SD ($n = 3$). Values marked by different letters differ significantly (ANOVA, Tukey's test, $p < 0.05$). (**D**) Photo of oat seedlings after 14 days of stress. DAT—days after treatment.

3.6. Chlorophyll a and b and Carotenoid Content in Response to Heavy Metal

The level of chlorophyll a increased in response to Cd and Zn + Cd treatment after 3 and 14 days in comparison to control plants. The highest level of chlorophyll a was observed in Zn + Cd-treated plants after 3 days and was 1.6 times higher than in control plants. Interestingly, after 7 days of treatment, the content was similar across all experimental variants (Figure 9A). The highest level of chlorophyll b was detected in Zn + Cd-treated plants after 3 days of treatment and was around 1.5 times higher than in control plants. After 7 days of treatment, the content of chlorophyll b was similar across all experimental variants, whereas after 14 days of treatment, it increased in Zn- and Cd-treated plants in comparison to the control (Figure 9B). The level of carotenoids remained relatively consistent during the experiment, except that after 14 days of treatment the carotenoid level in the control was lower than that in Zn- and Cd-treated plants (Figure 9C). In the control and Zn-treated plants, the levels of chlorophyll a and b and carotenoids peaked on day 7. In Cd-treated plants, the levels of chlorophyll a and b remained the same throughout the experiment period, but the levels of carotenoids were slightly higher after 14 days of treatment. A similar situation was observed for Zn + Cd-treated plants, where neither the chlorophyll a nor the carotenoid content differed throughout the experiment, but the level of chlorophyll b increased over the treatment period, reaching its maximum after 14 days of treatment (Figure 9).

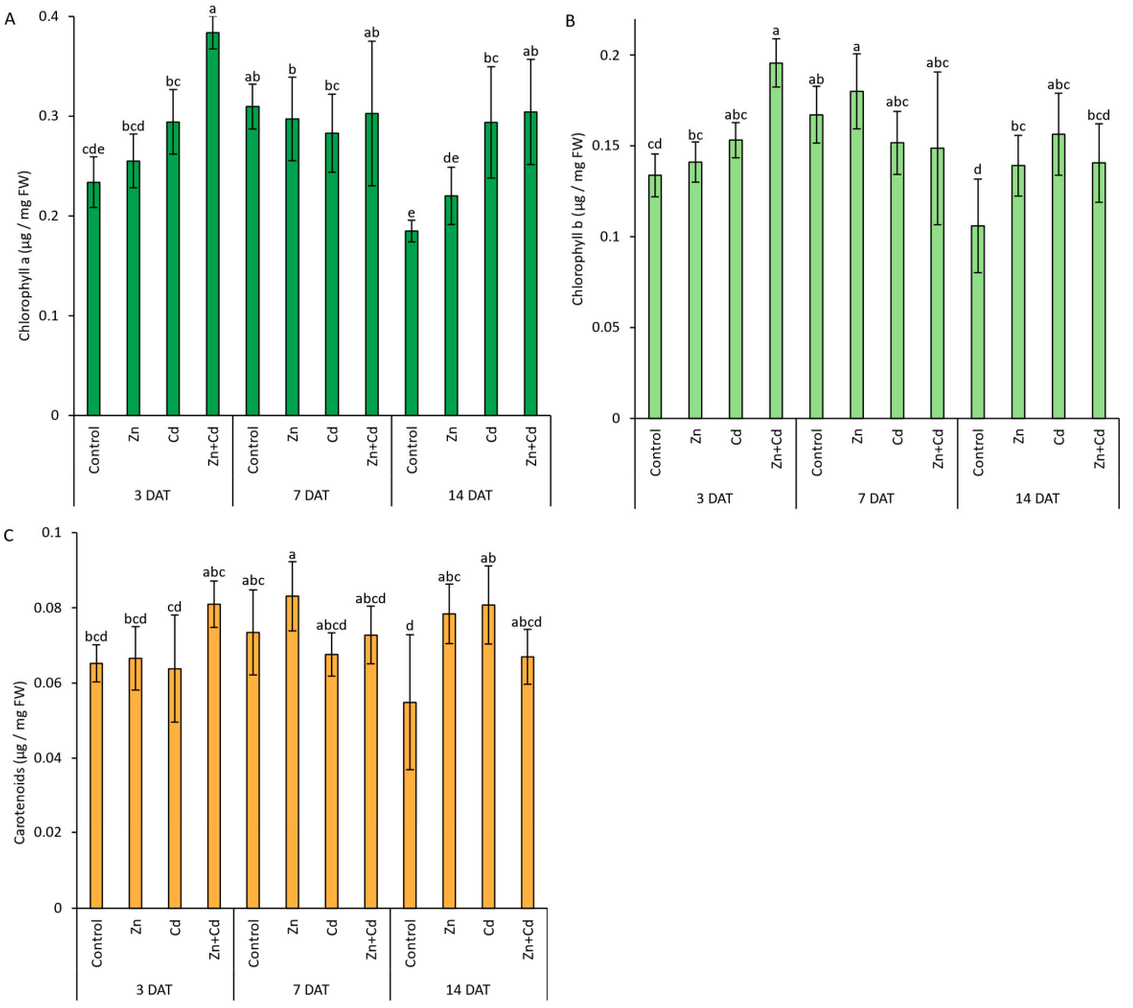

Figure 9. The content of photosynthetic pigments (**A**) chlorophyll a, (**B**) chlorophyll b, and (**C**) carotenoids in oat seedlings subjected to heavy metal stress induced via the application of 200 µM

$ZnSO_4$, 100 µM $CdSO_4$, and 200 µM $ZnSO_4$ + 100 µM $CdSO_4$. The control comprised non-stressed plants. Bars represent the mean values of three independent experiments ± SD (n = 3). Values marked by different letters differ significantly (ANOVA, Tukey's test, $p < 0.05$). DAT—days after treatment.

3.7. Antioxidant Capacity of Oat Seedlings in Response to Heavy Metal

To assess the effect of heavy metal stress on the antioxidant properties of oat seedlings, the TPC (Figure 10A) and AC (Figure 10B,C) were determined using the Folin–Ciocalteau, ABTS, and FRAP methods, respectively. The treatment of plants with Zn did not cause a significant difference in TPC in roots and shoots when compared to the control plants. However, on the seventh day of treatment, TPC was around 1.3 times higher in the roots of Zn-treated plants than in control plants (Figure 10A). Treating plants with Cd and Zn + Cd caused an increase in TPC in the shoots of oat seedlings that exceeded that of Cd-treated plants after 3 days of treatment. In the roots of the same plants, TPC increased in plants exposed to Zn + Cd after 3 days of treatment and to Zn and Zn + Cd after 7 days of treatment (Figure 10A).

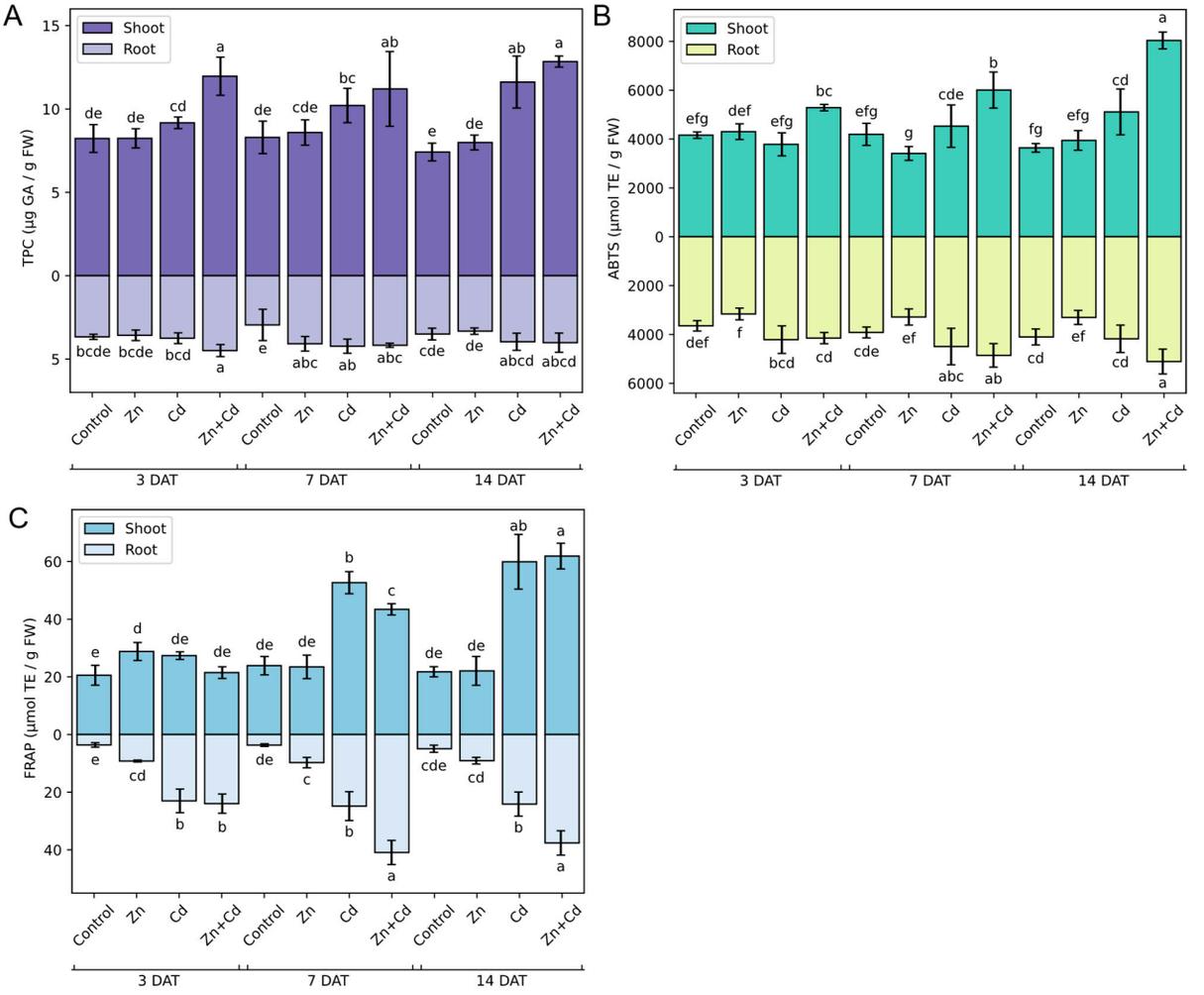

Figure 10. Effect of heavy metal stress induced via the application of 200 µM $ZnSO_4$, 100 µM $CdSO_4$, and 200 µM $ZnSO_4$ + 100 µM $CdSO_4$ on the (**A**) total phenolic content (TPC) and antioxidant capacity (**B**,**C**) of oat seedlings shoots and roots, evaluated using (**B**) ABTS and (**C**) FRAP assays. Bars represent the means of three independent experiments ± SD. The control comprised non-stressed plants. Values marked by different letters differ significantly (ANOVA, Tukey's test, $p < 0.05$). DAT—days after treatment.

The antioxidant potential of oat seedlings was measured using the ABTS (Figure 10B) and FRAP (Figure 10C) methods. In general, the AC was not affected by Zn treatment. In the shoots of Zn-treated seedlings after 3 days of treatment, an approximately 1.5 times higher FRAP value than in control seedlings was observed. In the roots of Zn-treated seedlings after 3 and 7 days of treatment, four times higher levels of total hydrophilic antioxidants analyzed by FRAP assay (Figure 10C) were detected compared to the control sample. However, the increase in AC in response to zinc was not observed when using ABTS assay. In fact, in the roots of Zn-treated seedlings, after 14 days of treatment, the ABTS result was lower than for control seedlings (Figure 10B). Treating plants with Cd caused an increase in total antioxidant levels in shoots and roots, but in roots, the high AC was observed on the seventh day after treatment, whereas in shoots, this was observed on the fourteenth day after treatment (Figure 10B). More significant differences between Cd-treated and control seedlings were detected via the FRAP method; i.e., in roots, the AC was higher throughout the experimental period and after 14 days of treatment was 4.9 times higher than in the roots of control seedlings. The FRAP values for shoots of Cd-treated seedlings were higher after 7 and 14 days of treatment, and after 14 days of treatment, the FRAP value was 2.7 times higher than for control seedlings (Figure 10C). Treating plants with the mixture of Zn and Cd had the biggest effect on the AC of oat seedlings. With time, the differences between the Zn + Cd-treated plants and the control plants increased, and on the 14th day, the ABTS values of the Zn + Cd-treated plants were 2.2 and 1.2 times higher for shoots and roots, respectively (Figure 10B). Similarly, the roots of seedlings subjected to Zn and Cd treatment after 3, 7, and 14 days of treatment showed higher FRAP results, and on the 14th day of treatment, the FRAP result was 7.6 times higher than for the control sample. The highest FRAP result was observed for shoots after 14 days of treatment with Zn and Cd, which was 2.8 times higher than in control seedlings (Figure 10C).

3.8. AsMT1-4 Expression during Heavy Metal Stress

To examine the potential role of oat MTs in the response to heavy metals, changes in *MTs* expression were determined after 3, 7, and 14 days of treatment (Figure 11). The expression of *AsMT1* in the roots of Cd- and Zn + Cd-stressed plants was lower than in control conditions; however, in shoots, *AsMT1* expression was generally higher. Zinc treatment did not affect the expression of *AsMT1* (Figure 11A). The expression of *AsMT2* in the shoots of Zn-stressed plants was lower than in the control plants after 7 days of treatment, but after 14 days of treatment, it was on the same level as the expression in the control. However, in roots after 7 days of Zn treatment, the expression of *AsMT2* increased over to three times more than that of the control, and the high expression level lasted until the 14th day of treatment. After 3 days of treatment, the expression of *AsMT2* was the lowest in both the shoots and roots of Zn + Cd-treated plants, but in the following days of treatment, its expression increased in oat shoots, reaching a transcript level over three times higher than in the control on the 14th day of stress (Figure 11B). The expression of *AsMT3* remained unchanged after 3 days of stress induction both in shoots and roots. The first differences in *AsMT3* expression between the control and the heavy-metal-treated plants were detected on the seventh day, where a twofold decrease in expression was observed in the shoots of Cd-treated plants. Moreover, an over sixfold increase in *AsMT3* expression in the roots of Zn-treated plants was observed on the seventh day after treatment, and on the last day of treatment, this high *AsMT3* expression in the roots of Zn-treated plants was accompanied by an increase in expression in shoots. On the 14th day of stress, *AsMT3* expression in the shoots and roots of Zn + Cd stressed plants was 2.0 and 2.5 times higher when compared to control plants (Figure 11C). Treating plants with Zn and Cd lowered *AsMT4* expression when compared to the control, and the transcript level of *AsMT4* in Zn- and Cd-treated plants remained at the same level over the course of treatment. Treating plants with a mixture of Zn + Cd caused a fourfold increase in *AsMT4* expression in shoots and an increase of 2.5 times in roots after just 3 days of stress. Over the following days of stress, the expression level in shoots decreased, and on the 14th day, it became 4.5 times

lower than the control plants. However, in the roots of the same plants, i.e., the Zn + Cd-treated plants, *AsMT4* expression increased and was 11 times higher when compared to the control (Figure 11D).

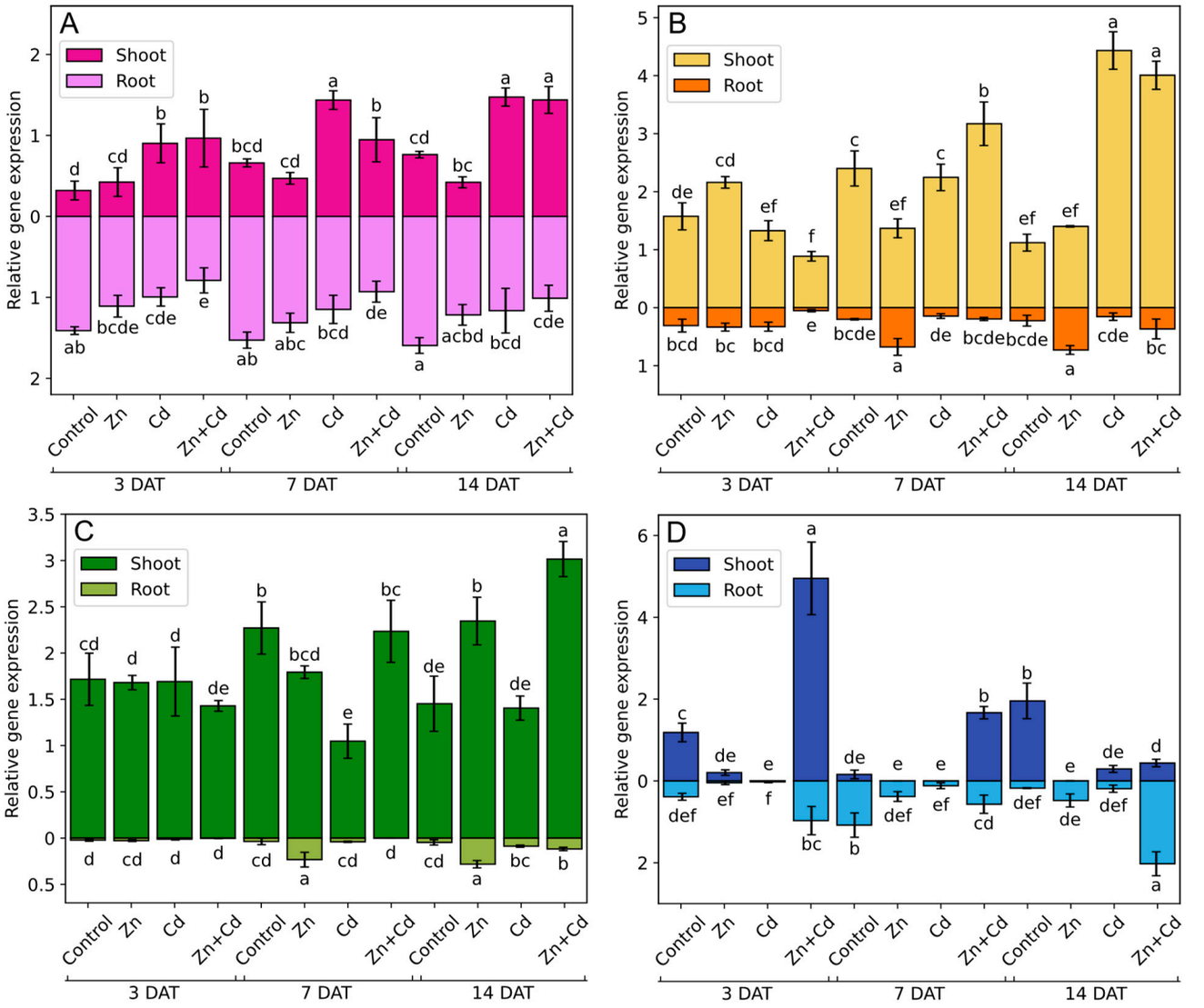

Figure 11. Relative gene expression of (**A**) *AsMT1*, (**B**) *AsMT2*, (**C**) *AsMT3*, and (**D**) *AsMT4* in the shoots and roots of oat seedlings subjected to 14 days of heavy metal stress induced via the application of 200 µM $ZnSO_4$, 100 µM $CdSO_4$, and 200 µM $ZnSO_4$ + 100 µM $CdSO_4$. Bars represent the means of three independent experiments ± SD. The control comprised non-stressed plants. Values marked by different letters differ significantly (ANOVA, Tukey's test, $p < 0.05$). DAT—days after treatment.

3.9. Correlations among AsMT1-4 Gene Expression, the Content of Photosynthetic Pigments, and Antioxidant Content

Pearson correlation analysis (Figure 12) showed high positive correlations between the expression of *AsMT1* and *AsMT2* and TPC and AC (detected using FRAP and ABTS methods) in shoots. The expression of *AsMT3* in shoots had a significant positive correlation with ABTS results. In contrast, negative correlations were noted between the *AsMT1*, *AsMT2*, and *AsMT3* expression and TPC and AC determined via FRAP and ABTS in roots. The expression of *AsMT4* in shoots showed a low but significant positive correlation with TPC, whereas a negative correlation was observed with AC measured via FRAP. In roots, the

expression of *AsMT4* correlated positively with the ABTS and FRAP values. In both shoots and roots, TPC and AC correlated positively with each other, and a positive correlation among those parameters was also observed between shoots and roots. The content of photosynthetic pigments correlated positively with TPC and ABTS values. Interestingly, the expression of *AsMT4* in both shoots and roots correlated positively with chlorophyll a and b but not with carotenoids. In contrast, the expression of *AsMT1*, *AsMT2*, and *AsMT3* in roots correlated negatively with photosynthetic pigments. In general, the expression of *AsMT1* and *AsMT2* in roots correlated negatively with parameters measured in shoots (i.e., TPC, AC, Chl a, and Chl b). Positive correlations were also observed between TPC and chlorophyll a, chlorophyll b, and carotenoids. Interestingly, a positive correlation was found between the levels of chlorophyll a and AC measured via ABTS in both shoots and roots. In shoots and roots, the expression of *AsMT2* correlated positively with the expression of *AsMT1* and *AsMT3*, whereas a negative correlation between the expression of *AsMT2* and *AsMT4* in roots was observed. The expression of *AsMT1* and *AsMT4* in shoots correlated negatively with the expression of *AsMT1*, *AsMT2*, and *AsMT3* in roots (Figure 12).

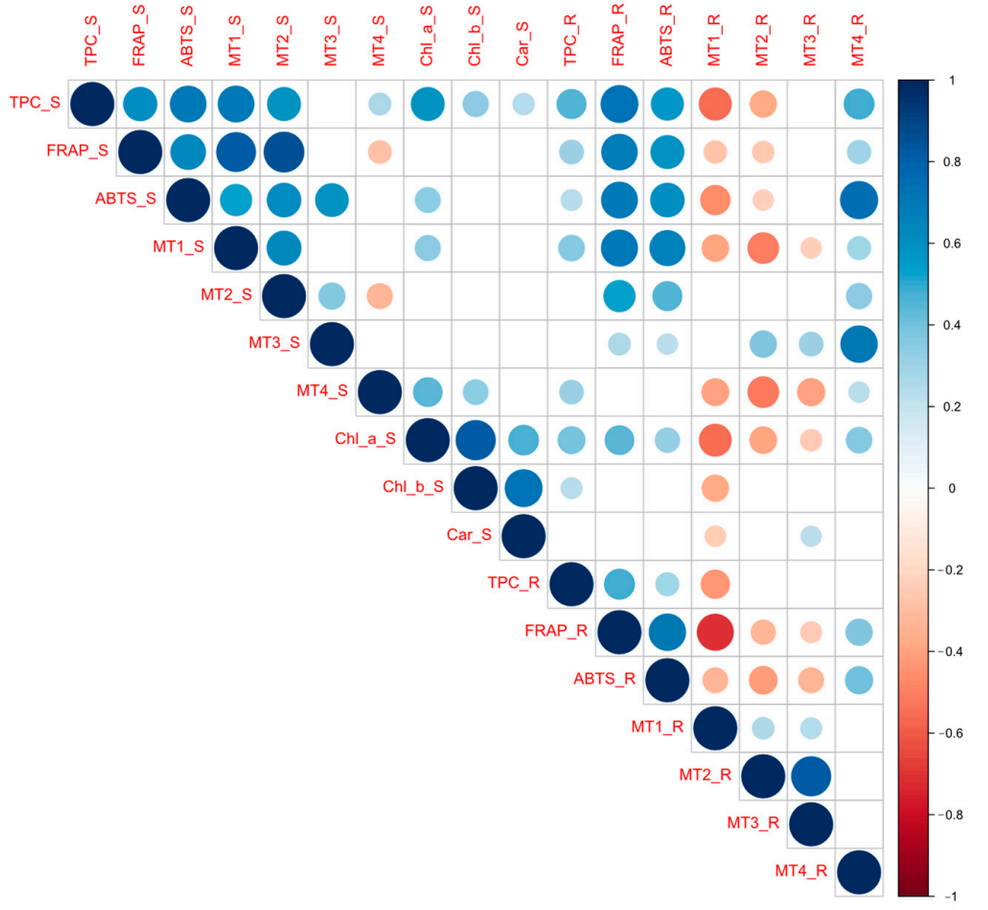

Figure 12. Pearson correlation between *AsMT* gene expression (MT1, MT2, MT3, and MT4), antioxidant capacity (measured using ABTS and FRAP methods), total phenolic content (TPC), and the levels of photosynthetic pigments (chlorophyll a-Chl_a, chlorophyll b-Chl_b, and carotenoids-Car) in shoots (S) and roots (R) of oat seedlings. Only significant correlations are demonstrated ($p < 0.05$).

4. Discussion

Oat has a complex evolutionary history, which reflects the high number of *MT* genes, of which there are 21. In comparison, *A. thaliana* has a 135 Mb genome and seven *MT* genes [38], *O. sativa* has a 420 Mb genome and eleven *MT* genes [40], and *Z. mays* has a

genome size of ~2500 Mb and nine *MT* genes [41]. Previous studies have shown that the number of *MT* genes does not correlate with genome size but with plant ploidy [41]. In the allotetraploid plant *B. napus*, 16 *MT* genes have been identified, and in *Brassica juncea*, 12 *MT* genes have been found. In comparison, in diploid *Brassica rapa*, *Brassica oleracea*, and *Brassica nigra*, eight, nine, and seven *MT* genes have been identified, respectively [42]. The number of introns found in *AsMT* genes is typical for *pMT* genes (data from NCBI's GenBank) and is rather low since the average number of introns per gene, based on the analysis of monocot rice and dicot *A. thaliana* genomes, is four [67]. In general, in plants, genes that have a less compact structure (i.e., more and longer introns) are expressed at higher levels than those that are more compact [68]. It would be interesting to verify whether the number and length of introns have an impact on the level of expression of oat *MT* genes.

The putative AsMT proteins encoded by the identified genes are very similar to pMTs from other plant species, as confirmed by the amino acid sequence alignments and phylogenetic analysis. However, some AsMTs contain additional His residues that might function in metal binding [14,69]. In general, among pMTs found in angiosperm species, some sequences are characterized by unusual topologies of cysteines and histidines. It is possible that at least some of these non-canonical pMTs are pseudogenes. In MT4B from soybean (*Glycine max* (L.) Merr.), second His is substituted by tyrosine. For this particular protein, it has been shown that the lack of metal-binding His residue results in a lower number of zinc ions (5 Zn^{2+} ions vs. 6 Zn^{2+} ions) that can be bound by this protein [70,71]. The motif-based sequence analysis tool (MEME) results showed that two Cys-rich motifs are conserved in each AsMT [1,72,73]. Moreover, we also identified some other motifs in AsMTs located outside of Cys-rich domains. Similar observations have been made for MTs from *Nicotiana tabacum* L. [13] and *B. napus* [42]. The role of the stretch/linker between Cys-rich domains is not well understood. It has been hypothesized that the linker either allows the protein to fold properly, and metal ions are bound in separate clusters, meaning each Cys-rich domain binds metal ions independently from the other Cys-rich domain, or it allows for the formation of a single metal-binding cluster [12]. Recent experiments on *Cicer arietinum* L. MT2 showed that the linker does not play an important role in protein folding [74]. The presence of conserved motifs outside of Cys-rich domains suggests that the linker region has some physiological functions; however, this needs to be further evaluated. Although MTs are typically viewed as cytosolic proteins, in silico prediction showed that AsMTs could also be localized in the nucleus. The subcellular localizations were shown experimentally for pMTs from different plant species, e.g., rice MT1e in the nucleus [75], *Ziziphus jujuba* Mill. MT1 in the cytosol and the nucleus [76], and type-2 MT from *B. napus* in cytosol when heterologously expressed in yeast cells [77]. Interestingly, proteins smaller than 10 kDa could be transported into the nucleus by passive diffusion, and this kind of transport to the nucleus has been observed for MTII in animals [78]. Moreover, in silico analysis has shown that AsMTs could be localized in the chloroplast and the cell membrane. The membrane localization has not yet been observed for any MTs, but for animal MT, the localization in the intermembrane space of mitochondria has been shown [79]. Therefore, it is possible that in plants, MTs also function in the nucleus and chloroplasts.

The accurate prediction of cis-regulatory elements in promoters remains a challenge for bioinformatics and computational biology; however, this analysis provides valuable insight into the probable functions of proteins encoded by analyzed genes [80]. Based on this analysis, it is highly plausible to hypothesize that oat MTs are involved in stress adaptation and growth and development. As shown previously, the *MT* promoters of other plants, including *Z. mays*, *O. sativa*, and *A. thaliana*, also have a large number of diversified regulatory elements [2,40,41]. Unfortunately, experimental studies confirming the functionality of in-silico-predicted regulatory elements in *pMTs* are rather scarce. It has been shown that the type-1 rice MT promoter can be induced by wounding, Cu, and PEG treatment [81]. In another study, promoter analysis of type-2 *O. sativa* MT in a transgenic *A. thaliana* plant showed that the promoter activity was affected by phytohormones, PEG,

cold, heat, H_2O_2, and metals. Although different deletion mutants of the full promoters were generated in this study, their activity under various stresses was not evaluated [82]. A more detailed analysis of heavy-metal-responsive elements has been provided for type-1 MT from rice showing which regions of the promoter are responsible for metal-inducible expression [83]. Most studies focus on the expression of *MTs* under specific stresses, which is only indirect proof of the promoter functionality [13,84–87].

ROS play dual roles in each living organism; i.e., on the one hand they serve as signaling molecules, and on the other they are toxic and might lead to oxidative stress [88]. Every stress ultimately leads to an increase in the number of ROS in the cells [89]. The expression of *pMTs* is upregulated by a myriad of stress stimuli, and one possible explanation is that pMTs are general stress proteins participating in plant adaptation to a variety of environmental stimuli via ROS scavenging [90]. The reaction of the metaled form of MT with ROS leads to the release of metal ions, which, depending on the type of metal, might have a positive/negative impact on the cell. Moreover, -SH groups of cysteines could be further oxidized, which will lead to the formation of disulfide bridges (the oxidized form of MT). To bind the metal-ion disulfide bridges in MTs need to be reduced [72] by enzymes such as protein-disulfide isomerase (PDI; EC 5.3.4.1) [91]. Seed dormancy and germination are complex processes controlled by ROS [92]. Some studies have demonstrated a clear link between ROS, germination, and pMTs. In magnetoprimed tomato seeds, the level of H_2O_2 increased significantly, and the expression of type-1 and type-4 *MTs* increased by around 15 times [93]. *A. thaliana* seeds overexpressing *Nelumbo nucifera* Gaernt. *MT2a* and *MT3* were more resistant to accelerated aging (caused by high-temperature treatment). Although the number of ROS was not determined in those seeds, it was shown that the SOD level was significantly downregulated by accelerated aging and was higher in transgenic seeds than in wild-type seeds, though only after accelerated aging treatment [36]. In our study, we observed that although the expression of oat *MT* genes changed at every investigated stage of germination, the total number of *AsMT* transcripts remained stable throughout the analyzed period. It is plausible to hypothesize that pMTs serve as ROS regulators during germination; however, the lack of detailed studies on pMTs and germination does not allow us to provide a comprehensive picture of the function/s of pMTs in this process.

Zinc is essential for all living organisms; it is a micronutrient involved in almost every conceivable metabolic process [94], whereas cadmium is highly toxic and does not play any physiological roles [95]. Although completely different in terms of function, these two metal ions share similar physiochemical properties, and therefore, cadmium can be uptaken from soil and transported within the plant via zinc transport proteins. The toxicity of cadmium is at least in part due to its interference with zinc homeostasis [96]. An excess of zinc is toxic to plants; however, plants significantly differ in their level of zinc sensitivity. The threshold for zinc toxicity depends on the plant species, the time of treatment, and the composition of the medium. We observed that zinc promoted the growth of oat seedlings, and a similar effect was observed for various plant species [97]. As expected, cadmium significantly reduced the growth of oat seedlings, as has been demonstrated in various plant species. In contrast to our study, one study showed that the application of a mixture of zinc and cadmium did not negatively affect the growth of tomato, whereas the same concentration of cadmium significantly decreased the growth of plants, possibly due to the limiting amount of cadmium that can enter plant roots when both metals are present in the medium [98]. In wheat, the foliar application of zinc also alleviated the negative effects of cadmium on plant growth and yield [99], which is possibly caused by the inability of cadmium to replace zinc in Zn-binding proteins when the number of zinc ions is high. This positive effect of zinc on Cd-treated plants is highly dependent on zinc concentration [100]; therefore, it could be concluded that in our study the concentration of zinc was too high to mitigate the negative effect of cadmium. In various plant species, a decrease in chlorophyll and carotenoid content in Cd-treated plants was observed, e.g., in tomato (*Lycopersicum esculentum* Mill.) [101], pea (*Pisum sativum* L.) [102], and *Salvia sclarea* L. plants [103]. The effect of zinc on the content of photosynthetic pigments is dose-dependent; i.e., a low

level of zinc increased the content of pigments, whereas a high level of zinc led to a decrease in pigment content [104,105]. However, here, we observed that the levels of photosynthetic pigments were unaffected by zinc, whereas cadmium elevated the level of pigments, especially chlorophyll a. It might be hypothesized that the zinc concentration was too high to induce the biosynthesis of photosynthetic pigments and too low to induce pigment degradation. The observed induction of pigment biosynthesis by cadmium could be supported by studies on the age-dependent response to Cd stress. For instance, in maize, the increase in chlorophyll a + b content in response to Cd was observed in young leaf segments but not in mature and old ones [106]. Moreover, most plants that are not hyperaccumulators sequester toxic metal ions into root vacuoles and do not translocate them into shoots [107]. Therefore, we hypothesized that in the oat tested in this study, cadmium ions were retained in the root. The observed increase in photosynthetic pigment content is a consequence of signal transduction about stress stimuli rather than the presence of Cd ions in the shoot.

The ABTS assay is suitable for the analysis of both hydrophilic and lipophilic antioxidants. It is a mixed-mode test based on single electron transfer (SET), hydrogen atom transfer (HAT), and proton-coupled electron transfer (PCET) mechanisms [108]. FRAP assay is a SET-based method and allows for the quantification of most hydrophilic antioxidants with a redox potential not lower than that of the redox pair Fe^{3+}/Fe^{2+} [109]. The different mechanisms of the used methods and varied affinities toward hydrophobic and hydrophilic antioxidants account for why the AC values measured by the ABTS and FRAP methods differ by two orders of magnitude. Moreover, the content of hydrophilic phenolics as well-known antioxidants [110] was determined. A significant positive correlation among the values obtained by these analyses was observed in this study and has been shown previously [111]. Plant extracts with high values obtained by ABTS assay probably contain more primary antioxidants, i.e., hydrogen or electron donors, whereas those with higher FRAP values might contribute to the higher content of secondary antioxidants (i.e., antioxidants that act indirectly by oxygen scavenging and the chelation of transition metal ions) [109]. Metallothioneins could act as primary antioxidants, because the direct reaction of MT with ROS has been shown [27]. On the other hand, MTs bind to transition metal ions including copper, thus limiting the Fenton and Haber Weiss reactions [112].

The regulation of *MT1-3* expression by heavy metal ions has been demonstrated for a wide range of plant species and different metals [38,85,86,113], although not for oat MTs. The comprehensive analysis of the expression of the *MT* genes representing each type of pMT in one plant species is rather rare in the literature. For example, Ahn et al. [91] showed the variable expression of *Brassica rapa MT1-3* in response to metals; however, in this study, analysis was performed on the whole seedlings. Similarly, in *A. thaliana* seedlings, different levels of regulation of the expression of *MT1-3* by copper was observed [38]. A more detailed analysis of the expression of *MT* genes in response to arsen was performed for *B. napus* [42] and *Z. mays MTs* in response to Cu, Cd, and Pb [41]. Similarly to our results, this research highlights the potential distinct role of each type of pMT depending on the metal ions, plant tissue, and stage of plant development. The expression of type-4 pMTs is restricted to developing and mature seeds and declines rapidly after the start of germination [7,37,41,114]. It has however been shown that in the resurrection plant, *Xerophyta humilis MT4* is upregulated during dehydration and downregulated during rehydration [115], indicating that the role of type-4 pMTs is not limited to seeds. Most studies analyzing the metal-responsiveness of *pMTs* are restricted to vegetative tissue, and thus, knowledge about the expression of *pMT4* in response to various metals is limited. We have previously shown that the expression of *B. napus MT4* is induced by Zn and more significantly by Cd but not by Cu in germinating seeds [69]. The expression of *B. napus MT4* was also regulated by arsen in 7-day-old seedlings [42]. In *A. thaliana* siliques, the expression of *MT4* genes is induced by Cd but not by Cu, Fe, Zn, or Hg [114]. Interestingly, here, we showed that the highest increase in the expression of *AsMT4* was observed when seedlings were treated with a mixture of Zn and Cd, whereas treatment with zinc

or cadmium separately did not change or even downregulate *AsMT4* expression. This phenomenon could be explained by the possible role of type-4 pMTs as specificity filters; i.e., due to the presence of His residues, pMT4 can discriminate between essential zinc and toxic cadmium [14,69].

5. Conclusions

The widely known hypothesis that there is no single unifying function for all types of pMTs and that each type of pMT might play a different role is supported by our comprehensive in silico and wet-lab analysis of the whole family of oat MTs. The expression of *AsMT1* in shoots was induced by Cd and Zn + Cd but not by Zn, which suggests that AsMT1 plays a role in cadmium detoxification. In roots, the expression of *AsMT2* and *AsMT3* is upregulated by Zn but not by Cd and Zn + Cd, which might implicate the role of these oat MTs in zinc homeostasis in roots. The opposite trend, especially 14 days after treatment, was observed for *AsMT2* expression in shoots, which implies that AsMT2 is responsible for Cd binding in roots. The expression of *AsMT3* in shoots 14 days after treatment was induced by Zn and Zn + Cd but not by Cd, which indicates the role of AsMT3 in zinc homeostasis in shoots, but at later developmental stages. For AsMT4, we propose the role of a zinc specificity filter. Moreover, based on the Pearson correlation analysis, we propose that AsMT1 and AsMT2 play a role in antioxidative response in shoots but not in roots, whereas AsMT4 plays this role in roots but not in shoots. AsMT3 is probably not involved in defense against ROS in oat seedlings, at least when the oxidative stress is induced by heavy metals.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox12101865/s1, Table S1: Cis-regulatory elements identified in *A. sativa* metallothionein genes using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 25 May 2022).; Table S2: The numbers of particular types of cis-regulatory motifs in each type of *A. sativa* metallothionein genes and the total number of particular types of regulatory motifs in all *AsMT* genes.

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References

1. Blindauer, C.A. Bacterial metallothioneins: Past, present, and questions for the future. *J. Biol. Inorg. Chem.* **2011**, *16*, 1011–1024. [CrossRef]
2. Dąbrowska, G.; Mierek-Adamska, A.; Goc, A. Plant metallothioneins: Putative functions identified by promoter analysis in silico. *Acta Biol. Crac. Ser. Bot.* **2012**, *54*, 109–120. [CrossRef]

3. Parameswari, E.; Ilakiya, T.; Davamani, V.; Kalaiselvi, P.; Sebastian, S.P. Metallothioneins: Diverse Protein Family to Bind Metallic Ions. In *Heavy Metals-Their Environmental Impacts and Mitigation*; IntechOpen: London, UK, 2021; ISBN 978-1-83968-122-6.
4. Blindauer, C.A.; Leszczyszyn, O.I. Metallothioneins: Unparalleled diversity in structures and functions for metal ion homeostasis and more. *Nat. Prod. Rep.* **2010**, *27*, 720–741. [CrossRef] [PubMed]
5. Margoshes, M.; Valiee, B.L. A cadmium protein from equine kidney cortex. *J. Am. Chem. Soc.* **1957**, *79*, 4813–4814. [CrossRef]
6. Lane, B.; Kajioka, R.; Kennedy, T. The wheat-germ Ec protein is a zinc-containing metallothionein. *Biochem. Cell Biol.* **1987**, *65*, 1001–1005. [CrossRef]
7. Kawashima, I.; Kennedy, T.D.; Chino, M.; Lane, B.G. Wheat Ec metallothionein genes: Like mammalian Zn^{2+} metallothionein genes, wheat Zn^{2+} metallothionein genes are conspicuously expressed during embryogenesis. *Eur. J. Biochem.* **1992**, *209*, 971–976. [CrossRef] [PubMed]
8. Ulrich, K.; Jakob, U. The role of thiols in antioxidant systems. *Free Radic. Biol. Med.* **2019**, *140*, 14. [CrossRef]
9. Ziller, A.; Fraissinet-Tachet, L. Metallothionein diversity and distribution in the tree of life: A multifunctional protein. *Metallomics* **2018**, *10*, 1549–1559. [CrossRef] [PubMed]
10. Freisinger, E. Plant MTs-Long neglected members of the metallothionein superfamily. *Dalton Trans.* **2008**, *47*, 6663–6675. [CrossRef] [PubMed]
11. Saeed-Ur-Rahman;Khalid, M.; Hui, N.; Kayani, S.I.; Tang, K. Diversity and versatile functions of metallothioneins produced by plants: A review. *Pedosphere* **2020**, *30*, 577–588. [CrossRef]
12. Freisinger, E. Structural features specific to plant metallothioneins. *J. Biol. Inorg. Chem.* **2011**, *16*, 1035–1045. [CrossRef] [PubMed]
13. Zhou, Y.; Liu, J.; Liu, S.; Jiang, L.; Hu, L. Identification of the metallothionein gene family from cucumber and functional characterization of CsMT4 in *Escherichia coli* under salinity and osmotic stress. *3 Biotech* **2019**, *9*, 394. [CrossRef] [PubMed]
14. Leszczyszyn, O.I.; Schmid, R.; Blindauer, C.A. Toward a property/function relationship for metallothioneins: Histidine coordination and unusual cluster composition in a zinc-metallothionein from plants. *Proteins Struct. Funct. Genet.* **2007**, *68*, 922–935. [CrossRef] [PubMed]
15. Blindauer, C.A.; Razi, M.T.; Campopiano, D.J.; Sadler, P.J. Histidine ligands in bacterial metallothionein enhance cluster stability. *J. Biol. Inorg. Chem.* **2007**, *12*, 393–405. [CrossRef] [PubMed]
16. Pearson, R.G. Hard and soft acids and bases. *J. Am. Chem. Soc.* **1963**, *85*, 3533–3539. [CrossRef]
17. Perinelli, M.; Tegoni, M.; Freisinger, E. Different behavior of the histidine residue toward cadmium and zinc in a cadmium-specific metallothionein from an aquatic fungus. *Inorg. Chem.* **2020**, *59*, 16988–16997. [CrossRef]
18. Mekawy, A.M.M.; Assaha, D.V.M.; Ueda, A. Constitutive overexpression of rice metallothionein-like gene OsMT-3a enhances growth and tolerance of Arabidopsis plants to a combination of various abiotic stresses. *J. Plant Res.* **2020**, *133*, 429–440. [CrossRef]
19. Das, K.; Roychoudhury, A. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Front. Environ. Sci.* **2014**, *2*, 53. [CrossRef]
20. Islam, M.R.; Xue, X.; Mao, S.; Ren, C.; Eneji, A.E.; Hu, Y. Effects of water-saving superabsorbent polymer on antioxidant enzyme activities and lipid peroxidation in oat (*Avena sativa* L.) under drought stress. *J. Sci. Food Agric.* **2011**, *91*, 680–686. [CrossRef]
21. Skrzypek, E.; Szechyńska-Hebda, M.; Dąbrowska, G.B.; Goc, A. The role of osmotic stress during in vitro regeneration of *Triticum aestivum* L. and *Vicia faba* ssp. minor. *Problemowe Postępów Nauk Rolniczych* **2008**, *524*, 221–230.
22. Dąbrowska, G.; Kata, A.; Goc, A.; Szechyńska-Hebda, M.; Skrzypek, E. Characteristics of the plant ascorbate peroxidase family. *Acta Biol. Crac. Ser. Bot.* **2007**, *49*, 7–17.
23. Konieczna, W.; Warchoł, M.; Mierek-Adamska, A.; Skrzypek, E.; Waligórski, P.; Piernik, A.; Dąbrowska, G.B. Changes in physiobiochemical parameters and expression of metallothioneins in *Avena sativa* L. in response to drought. *Sci. Rep.* **2023**, *13*, 2486. [CrossRef]
24. Mierek-Adamska, A.; Kotowicz, K.; Goc, A.; Boniecka, J.; Berdychowska, J.; Dąbrowska, G.B. Potential involvement of rapeseed (*Brassica napus* L.) metallothioneins in the hydrogen peroxide-induced regulation of seed vigour. *J. Agron. Crop Sci.* **2019**, *205*, 598–607. [CrossRef]
25. Konieczna, W.; Mierek-Adamska, A.; Warchoł, M.; Skrzypek, E.; Dąbrowska, G.B. The involvement of metallothioneins and stress markers in response to osmotic stress in *Avena sativa* L. *J. Agron. Crop Sci.* **2023**, *209*, 371–389. [CrossRef]
26. Patankar, H.V.; Al-Harrasi, I.; Al Kharusi, L.; Jana, G.A.; Al-Yahyai, R.; Sunkar, R.; Yaish, M.W. Overexpression of a Metallothionein 2A Gene from Date Palm Confers Abiotic Stress Tolerance to Yeast and *Arabidopsis thaliana*. *Int. J. Mol. Sci.* **2019**, *20*, 2871. [CrossRef] [PubMed]
27. Xue, T.; Li, X.; Zhu, W.; Wu, C.; Yang, G.; Zheng, C. Cotton metallothionein GhMT3a, a reactive oxygen species scavenger, increased tolerance against abiotic stress in transgenic tobacco and yeast. *J. Exp. Bot.* **2009**, *60*, 339–349. [CrossRef] [PubMed]
28. Singh, R.K.; Anandhan, S.; Singh, S.; Patade, V.Y.; Ahmed, Z.; Pande, V. Metallothionein-like gene from *Cicer microphyllum* is regulated by multiple abiotic stresses. *Protoplasma* **2011**, *248*, 839–847. [CrossRef] [PubMed]
29. Dąbrowska, G.; Baum, C.; Trejgell, A.; Hrynkiewicz, K. Impact of arbuscular mycorrhizal fungi on the growth and expression of gene encoding stress protein-metallothionein BnMT2 in the non-host crop *Brassica napus* L. *J. Plant Nutr. Soil Sci.* **2014**, *177*, 459–467. [CrossRef]
30. Zhi, J.; Liu, X.; Yin, P.; Yang, R.; Liu, J.; Xu, J. Overexpression of the metallothionein gene PaMT3-1 from *Phytolacca americana* enhances plant tolerance to cadmium. *Plant Cell Tissue Organ. Cult.* **2020**, *143*, 211–218. [CrossRef]

31. Gu, C.-S.; Liu, L.-Q.; Zhao, Y.-H.; Deng, Y.-M.; Zhu, X.-D.; Huang, S.-Z. Overexpression of Iris. lactea var. chinensis metallothionein llMT2a enhances cadmium tolerance in *Arabidopsis thaliana*. *Ecotoxicol. Environ. Saf.* **2014**, *105*, 22–28. [CrossRef]
32. Kumar, S.; Yadav, A.; Verma, R.; Dubey, A.K.; Narayan, S.; Pandey, A.; Sahu, A.; Srivastava, S.; Sanyal, I. Metallothionein (MT1): A molecular stress marker in chickpea enhances drought and heavy metal stress adaptive efficacy in transgenic plants. *Environ. Exp. Bot.* **2022**, *199*, 104871. [CrossRef]
33. Feng, M.; Yu, Q.; Chen, Y.; Fu, Z.; Xu, L.; Guo, J. ScMT10, a metallothionein-like gene from sugarcane, enhances freezing tolerance in *Nicotiana tabacum* transgenic plants. *Environ. Exp. Bot.* **2022**, *194*, 104750. [CrossRef]
34. Yuan, J.; Chen, D.; Ren, Y.; Zhang, X.; Zhao, J. Characteristic and expression analysis of a metallothionein gene, OsMT2b, down-regulated by cytokinin suggests functions in root development and seed embryo germination of rice. *Plant Physiol.* **2008**, *146*, 1637–1650. [CrossRef]
35. Moyle, R.; Fairbairn, D.J.; Ripi, J.; Crowe, M.; Botella, J.R. Developing pineapple fruit has a small transcriptome dominated by metallothionein. *J. Exp. Bot.* **2005**, *56*, 101–112. [CrossRef] [PubMed]
36. Zhou, Y.; Chu, P.; Chen, H.; Li, Y.; Liu, J.; Ding, Y.; Tsang, E.W.T.; Jiang, L.; Wu, K.; Huang, S. Overexpression of *Nelumbo nucifera* metallothioneins 2a and 3 enhances seed germination vigor in Arabidopsis. *Planta* **2012**, *235*, 523–537. [CrossRef] [PubMed]
37. Dąbrowska, G.; Mierek-Adamska, A.; Goc, A. Characterisation of *Brassica napus* L. metallothionein genes (BnMTs) expression in organs and during seed germination. *Aust. J. Crop Sci.* **2013**, *7*, 1324–1332.
38. Bundithya, W.; Goldsbrough, P.B.; Guo, W.-J. Characterization of the Arabidopsis metallothionein gene family: Tissue-specific expression and induction during senescence and in response to copper. *New Phytol.* **2003**, *159*, 369–381. [CrossRef]
39. Cheng, M.; Yuan, H.; Wang, R.; Zou, J.; Liang, T.; Yang, F.; Li, S. Genome-wide identification and analysis of the metallothionein genes in oryza genus. *Int. J. Mol. Sci.* **2021**, *22*, 9651. [CrossRef]
40. Zhou, G.; Xu, Y.; Li, J.; Yang, L.; Liu, J.Y. Molecular analyses of the metallothionein gene family in rice (*Oryza sativa* L.). *J. Biochem. Mol. Biol.* **2006**, *39*, 595–606. [CrossRef] [PubMed]
41. Yu, Q.; He, L.; Huo, C.; Jiang, X.; Chen, H.; Wang, R.; Tang, M.; Dong, L.; Chen, J.; Li, Y.; et al. Genome-Wide Identification and Expression Analysis of Heavy Metal Stress–Responsive Metallothionein Family Genes in *Nicotiana tabacum*. *Plant Mol. Biol. Report.* **2020**, *39*, 443–454. [CrossRef]
42. Pan, Y.; Zhu, M.; Wang, S.; Ma, G.; Huang, X.; Qiao, C.; Wang, R.; Xu, X.; Liang, Y.; Lu, K.; et al. Genome-wide characterization and analysis of metallothionein family genes that function in metal stress tolerance in *Brassica napus* L. *Int. J. Mol. Sci.* **2018**, *19*, 2181. [CrossRef] [PubMed]
43. Mahmoud, M.; Zhou, Z.; Kaur, R.; Bekele, W.; Tinker, N.A.; Singh, J. Toward the development of Ac/Ds transposon-mediated gene tagging system for functional genomics in oat (*Avena sativa* L.). *Funct. Integr. Genom.* **2022**, *22*, 669–681. [CrossRef]
44. Zwer, P. Oats: Characteristics and Quality Requirements. In *Cereal Grains: Assessing and Managing Quality*; Woodhead Publishing: Sawston, UK, 2010; pp. 163–182.
45. Alfieri, M.; Redaelli, R. Oat phenolic content and total antioxidant capacity during grain development. *J. Cereal Sci.* **2015**, *65*, 39–42. [CrossRef]
46. Butt, M.S.; Tahir-Nadeem, M.; Khan, M.K.I.; Shabir, R.; Butt, M.S. Oat: Unique among the cereals. *Eur. J. Nutr.* **2008**, *47*, 68–79. [CrossRef] [PubMed]
47. Othman, R.A.; Moghadasian, M.H.; Jones, P.J.H. Cholesterol-lowering effects of oat β-glucan. *Nutr. Rev.* **2011**, *69*, 299–309. [CrossRef]
48. Quiñones-Muñoz, T.A.; Villanueva-Rodríguez, S.J.; Torruco-Uco, J.G. Nutraceutical Properties of *Medicago sativa* L., *Agave* spp., *Zea mays* L. and *Avena sativa* L.: A Review of Metabolites and Mechanisms. *Metabolites* **2022**, *12*, 806. [CrossRef] [PubMed]
49. Jiang, W.; Jiang, C.; Yuan, W.; Zhang, M.; Fang, Z.; Li, Y.; Li, G.; Jia, J.; Yang, Z. A universal karyotypic system for hexaploid and diploid Avena species brings oat cytogenetics into the genomics era. *BMC Plant Biol.* **2021**, *21*, 213. [CrossRef] [PubMed]
50. Chaffin, A.S.; Huang, Y.; Smith, S.; Bekele, W.A.; Babiker, E.; Gnanesh, B.N.; Foresman, B.J.; Blanchard, S.G.; Jay, J.J.; Reid, R.W.; et al. A consensus map in cultivated hexaploid oat reveals conserved grass synteny with substantial subgenome rearrangement. *Plant Genome* **2016**, *9*. [CrossRef] [PubMed]
51. Kamal, N.; Tsardakas Renhuldt, N.; Bentzer, J.; Gundlach, H.; Haberer, G.; Juhász, A.; Lux, T.; Bose, U.; Tye-Din, J.A.; Lang, D.; et al. The mosaic oat genome gives insights into a uniquely healthy cereal crop. *Nature* **2022**, *606*, 113–119. [CrossRef] [PubMed]
52. Maughan, P.J.; Lee, R.; Walstead, R.; Vickerstaff, R.J.; Fogarty, M.C.; Brouwer, C.R.; Reid, R.R.; Jay, J.J.; Bekele, W.A.; Jackson, E.W.; et al. Genomic insights from the first chromosome-scale assemblies of oat (*Avena* spp.) diploid species. *BMC Biol.* **2019**, *17*, 92. [CrossRef]
53. PepsiCo Avena Sativa-OT3098 v2, PepsiCo. Available online: https://wheat.pw.usda.gov/jb/?data=%2Fggds%2Foat-ot3098v2-pepsico&loc=chr7A%3A5130802..5132383&tracks=genes&highlight= (accessed on 1 May 2022).
54. Tinker, N.A.; Wight, C.P.; Bekele, W.A.; Yan, W.; Jellen, E.N.; Renhuldt, N.T.; Sirijovski, N.; Lux, T.; Spannagl, M.; Mascher, M. Genome analysis in Avena sativa reveals hidden breeding barriers and opportunities for oat improvement. *Commun. Biol.* **2022**, *5*, 474. [CrossRef] [PubMed]
55. Chou, K.C.; Shen, H. bin Plant-mPLoc: A top-down strategy to augment the power for predicting plant protein subcellular localization. *PLoS ONE* **2010**, *5*, e11335. [CrossRef]
56. Hu, B.; Jin, J.; Guo, A.-Y.; Zhang, H.; Luo, J.; Gao, G. GSDS 2.0: An upgraded gene feature visualization server. *Bioinformatics* **2015**, *31*, 1296–1297. [CrossRef] [PubMed]

57. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]
58. Hoagland, D.; Arnon, D. The water-culture method for growing plants without soil. *Circ. Calif. Agric. Exp. Stn.* **1938**, *347*, 1–39.
59. Wang, G.G.; Wang, G.G.; Zhang, X.; Wang, F.; Song, R. Isolation of high quality RNA from cereal seeds containing high levels of starch. *Phytochem. Anal.* **2012**, *23*, 159–163. [CrossRef]
60. Yang, Z.; Wang, K.; Aziz, U.; Zhao, C.; Zhang, M. Evaluation of duplicated reference genes for quantitative real-time PCR analysis in genome unknown hexaploid oat (*Avena sativa* L.). *Plant Methods* **2020**, *16*, 138. [CrossRef] [PubMed]
61. Lichtenthaler, H.K.H.H.K.; Wellburn, A.R.A.A.R. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochem. Soc. Trans.* **1983**, *11*, 591–592. [CrossRef]
62. Singleton, V.L.; Rossi, J.A. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158. [CrossRef]
63. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237. [CrossRef]
64. Benzie, I.F.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76. [CrossRef] [PubMed]
65. Hammer, D.A.T.; Ryan, P.D.; Hammer, Ø.; Harper, D.A.T. Past: Paleontological statistics software package for education and data analysis. *Palaeontol. Electron.* **2001**, *4*, 1–9.
66. RStudio Team. *RStudio: Integrated Development for R*; RStudio, PBC: Boston, MA, USA, 2020.
67. Morello, L.; Breviario, D. Plant spliceosomal introns: Not only cut and paste. *Curr. Genom.* **2008**, *9*, 227–238. [CrossRef]
68. Ren, X.; Vorst, O.; Fiers, M.; Stiekema, W.; Nap, J. In plants, highly expressed genes are the least compact. *Trends Genet.* **2006**, *22*, 528–532. [CrossRef]
69. Mierek-Adamska, A.; Dąbrowska, G.B.; Blindauer, C.A. The type 4 metallothionein from *Brassica napus* seeds folds in a metal-dependent fashion and favours zinc over other metals. *Metallomics* **2018**, *10*, 1430–1443. [CrossRef]
70. Tomas, M.; Pagani, M.A.; Andreo, C.S.; Capdevila, M.; Bofill, R.; Atrian, S. His-containing plant metallothioneins: Comparative study of divalent metal-ion binding by plant MT3 and MT4 isoforms. *J. Biol. Inorg. Chem.* **2014**, *19*, 1149–1164. [CrossRef]
71. Pagani, M.A.; Tomas, M.; Carrillo, J.; Bofill, R.; Capdevila, M.; Atrian, S.; Andreo, C.S. The response of the different soybean metallothionein isoforms to cadmium intoxication. *J. Inorg. Biochem.* **2012**, *117*, 306–315. [CrossRef] [PubMed]
72. Krężel, A.; Maret, W. The bioinorganic chemistry of mammalian metallothioneins. *Chem. Rev.* **2021**, *121*, 14594–14684. [CrossRef]
73. Capdevila, M.; Bofill, R.; Palacios, O.; Atrian, S. State-of-the-art of metallothioneins at the beginning of the 21st century. *Coord. Chem. Rev.* **2012**, *256*, 46–62. [CrossRef]
74. Salim, A.; Chesnov, S.; Freisinger, E. Metallation pathway of a plant metallothionein: *Cicer arietinum* MT2. *J. Inorg. Biochem.* **2020**, *210*, 111157. [CrossRef]
75. Rono, J.K.; Le Wang, L.; Wu, X.C.; Cao, H.W.; Zhao, Y.N.; Khan, I.U.; Yang, Z.M. Identification of a new function of metallothionein-like gene OsMT1e for cadmium detoxification and potential phytoremediation. *Chemosphere* **2021**, *265*, 129136. [CrossRef]
76. Yang, M.; Zhang, F.; Wang, F.; Dong, Z.; Cao, Q.; Chen, M. Characterization of a type 1 metallothionein gene from the stresses-tolerant plant *Ziziphus jujuba*. *Int. J. Mol. Sci.* **2015**, *16*, 16750–16762. [CrossRef]
77. Zhang, P.-H.; Zhang, X.-J.; Tang, T.-W.; Hu, H.-L.; Bai, N.-N.; Zhang, D.-W.; Meng, S.; Peng, J.-S. Isolation of three metallothionein genes and their roles in mediating cadmium resistance. *Agronomy* **2022**, *12*, 2971. [CrossRef]
78. Woo, E.S.; Dellapiazza, D.; Wang, A.S.; Lazo, J.S. Energy-dependent nuclear binding dictates metallothionein localization. *J. Cell Physiol.* **2000**, *182*, 69–76. [CrossRef] [PubMed]
79. Ye, B.; Maret, W.; Vallee, B.L. Zinc metallothionein imported into liver mitochondria modulates respiration. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 2317–2322. [CrossRef]
80. Mariño-Ramírez, L.; Tharakaraman, K.; Spouge, J.L.; Landsman, D. Promoter analysis: Gene regulatory motif identification with A-GLAM. *Methods Mol. Biol.* **2009**, *537*, 263–276. [CrossRef]
81. Lü, S.; Gu, H.; Yuan, X.; Wang, X.; Wu, A.M.; Qu, L.; Liu, J.Y. The GUS reporter-aided analysis of the promoter activities of a rice metallothionein gene reveals different regulatory regions responsible for tissue-specific and inducible expression in transgenic Arabidopsis. *Transgenic Res.* **2007**, *16*, 177–191. [CrossRef] [PubMed]
82. Ren, Y.; Zhao, J. Functional analysis of the rice metallothionein gene OsMT2b promoter in transgenic *Arabidopsis* plants and rice germinated embryos. *Plant Sci.* **2009**, *176*, 528–538. [CrossRef] [PubMed]
83. Dong, C.-J.; Wang, Y.; Yu, S.-S.; Liu, J.-Y. Characterization of a novel rice metallothionein gene promoter: Its tissue specificity and heavy metal responsiveness. *J. Integr. Plant Biol.* **2010**, *52*, 914–924. [CrossRef] [PubMed]
84. Zhou, G.K.; Xu, Y.F.; Liu, J.Y. Characterization of a rice class II metallothionein gene: Tissue expression patterns and induction in response to abiotic factors. *J. Plant Physiol.* **2005**, *162*, 686–696. [CrossRef]
85. Ahn, Y.O.; Kim, S.H.; Lee, J.; Ran Kim, H.; Lee, H.-S.S.; Kwak, S.-S.S.; Kim, H.; Lee, H.-S.S.; Kwak, S.-S.S.; Ran Kim, H.; et al. Three Brassica rapa metallothionein genes are differentially regulated under various stress conditions. *Mol. Biol. Rep.* **2012**, *39*, 2059–2067. [CrossRef] [PubMed]
86. Usha, B.; Venkataraman, G.; Parida, A. Heavy metal and abiotic stress inducible metallothionein isoforms from Prosopis juliflora (SW) D.C. show differences in binding to heavy metals in vitro. *Mol. Genet. Genom.* **2009**, *281*, 99–108. [CrossRef] [PubMed]

87. Kim, Y.-O.; Kang, H. Comparative expression analysis of genes encoding metallothioneins in response to heavy metals and abiotic stresses in rice (*Oryza sativa*) and Arabidopsis thaliana. *Biosci. Biotechnol. Biochem.* **2018**, *82*, 1656–1665. [CrossRef]
88. Huang, H.; Ullah, F.; Zhou, D.-X.; Yi, M.; Zhao, Y. Mechanisms of ROS regulation of plant development and stress responses. *Front. Plant Sci.* **2019**, *10*, 800. [CrossRef]
89. Choudhury, F.K.; Rivero, R.M.; Blumwald, E.; Mittler, R. Reactive oxygen species, abiotic stress and stress combination. *Plant J.* **2017**, *90*, 856–867. [CrossRef]
90. Leszczyszyn, O.I.; Imam, H.T.; Blindauer, C.A. Diversity and distribution of plant metallothioneins: A review of structure, properties and functions. *Metallomics* **2013**, *5*, 1146–1169. [CrossRef]
91. Matsusaki, M.; Okuda, A.; Matsuo, K.; Gekko, K.; Masuda, T.; Naruo, Y.; Hirose, A.; Kono, K.; Tsuchi, Y.; Urade, R. Regulation of plant ER oxidoreductin 1 (ERO1) activity for efficient oxidative protein folding. *J. Biol. Chem.* **2019**, *294*, 18820–18835. [CrossRef] [PubMed]
92. Bailly, C. The signalling role of ROS in the regulation of seed germination and dormancy. *Biochem. J.* **2019**, *476*, 3019–3032. [CrossRef]
93. Anand, A.; Kumari, A.; Thakur, M.; Koul, A. Hydrogen peroxide signaling integrates with phytohormones during the germination of magnetoprimed tomato seeds. *Sci. Rep.* **2019**, *9*, 8814. [CrossRef]
94. King, J.C. Zinc: An essential but elusive nutrient. *Am. J. Clin. Nutr.* **2011**, *94*, 679S. [CrossRef]
95. Clemens, S.; Aarts, M.G.M.; Thomine, S.; Verbruggen, N. Plant science: The key to preventing slow cadmium poisoning. *Trends Plant Sci.* **2013**, *18*, 92–99. [CrossRef] [PubMed]
96. Palmgren, M.G.; Clemens, S.; Williams, L.E.; Krämer, U.; Borg, S.; Schjørring, J.K.; Sanders, D. Zinc biofortification of cereals: Problems and solutions. *Trends Plant Sci.* **2008**, *13*, 464–473. [CrossRef]
97. Shi, G.; Cai, Q. Zinc tolerance and accumulation in eight oil crops. *J. Plant Nutr.* **2010**, *33*, 982–997. [CrossRef]
98. Cherif, J.; Mediouni, C.; Ben Ammar, W.; Jemal, F. Interactions of zinc and cadmium toxicity in their effects on growth and in antioxidative systems in tomato plants (*Solarium lycopersicum*). *J. Environ. Sci.* **2011**, *23*, 837–844. [CrossRef] [PubMed]
99. Qian, L.; Dawar, K.; Ullah, I.; Irfan, M.; Zhang, Z.; Mian, I.A.; Khan, B.; Gul, N.; Fahad, S.; Jalal, A.; et al. Zinc foliar application mitigates cadmium-induced growth inhibition and enhances wheat growth, chlorophyll contents, and yield. *ACS Omega* **2023**, *8*, 32372–32381. [CrossRef] [PubMed]
100. Rizwan, M.; Ali, S.; Rehman, M.Z.U.; Maqbool, A. A critical review on the effects of zinc at toxic levels of cadmium in plants. *Environ. Sci. Pollut. Res.* **2019**, *26*, 6279–6289. [CrossRef] [PubMed]
101. Yadav, M.; Gupta, P.; Seth, C.S. Foliar application of α-lipoic acid attenuates cadmium toxicity on photosynthetic pigments and nitrogen metabolism in *Solanum lycopersicum* L. *Acta Physiol. Plant* **2022**, *44*, 112. [CrossRef]
102. Manzoor, H.; Mehwish; Bukhat, S.; Rasul, S.; Rehmani, M.I.A.; Noreen, S.; Athar, H.-R.; Zafar, Z.U.; Skalicky, M.; Soufan, W.; et al. Methyl jasmonate alleviated the adverse effects of cadmium stress in pea (*Pisum sativum* L.): A nexus of photosystem II activity and dynamics of redox balance. *Front. Plant Sci.* **2022**, *13*, 860664. [CrossRef]
103. Adamakis, I.-D.S.; Sperdouli, I.; Hanć, A.; Dobrikova, A.; Apostolova, E.; Moustakas, M. Rapid hormetic responses of photosystem II photochemistry of clary sage to cadmium exposure. *Int. J. Mol. Sci.* **2020**, *22*, 41. [CrossRef]
104. Jain, R.; Srivastava, S.; Solomon, S.; Shrivastava, A.K.; Chandra, A. Impact of excess zinc on growth parameters, cell division, nutrient accumulation, photosynthetic pigments and oxidative stress of sugarcane (*Saccharum* spp.). *Acta Physiol. Plant* **2010**, *32*, 979–986. [CrossRef]
105. Fatima, N.; Ahmad, N.; Anis, M. Enhanced in vitro regeneration and change in photosynthetic pigments, biomass and proline content in *Withania somnifera* L. (Dunal) induced by copper and zinc ions. *Plant Physiol. Biochem.* **2011**, *49*, 1465–1471. [CrossRef] [PubMed]
106. Drążkiewicz, M.; Baszyński, T. Growth parameters and photosynthetic pigments in leaf segments of Zea mays exposed to cadmium, as related to protection mechanisms. *J. Plant Physiol.* **2005**, *162*, 1013–1021. [CrossRef] [PubMed]
107. Xu, W.; Xiang, P.; Liu, X.; Ma, L.Q. Closely-related species of hyperaccumulating plants and their ability in accumulation of As, Cd, Cu, Mn, Ni, Pb and Zn. *Chemosphere* **2020**, *251*, 126334. [CrossRef]
108. Apak, R.; Özyürek, M.; Güçlü, K.; Çapanoğlu, E. Antioxidant activity/capacity measurement. 2. Hydrogen atom transfer (HAT)-based, mixed-mode (electron transfer (ET)/HAT), and lipid peroxidation assays. *J. Agric. Food Chem.* **2016**, *64*, 1028–1045. [CrossRef]
109. Apak, R.; Özyürek, M.; Güçlü, K.; Çapanoğlu, E. Antioxidant activity/capacity measurement. 1. Classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays. *J. Agric. Food Chem.* **2016**, *64*, 997–1027. [CrossRef] [PubMed]
110. Naikoo, M.I.; Dar, M.I.; Raghib, F.; Jaleel, H.; Ahmad, B.; Raina, A.; Khan, F.A.; Naushin, F. Role and Regulation of Plants Phenolics in Abiotic Stress Tolerance. In *Plant Signaling Molecules*; Khan, M.I.R., Reddy, P.S., Ferrante, A., Khan, N.A., Eds.; Elsevier: Amsterdam, The Netherlands, 2019; pp. 157–168.
111. Szydłowska-Czerniak, A.; Bartkowiak-Broda, I.; Karlović, I.; Karlovits, G.; Szłyk, E. Antioxidant capacity, total phenolics, glucosinolates and colour parameters of rapeseed cultivars. *Food Chem.* **2011**, *127*, 556–563. [CrossRef] [PubMed]
112. Vašák, M.; Meloni, G. Mammalian metallothionein-3: New functional and structural insights. *Int. J. Mol. Sci.* **2017**, *18*, 117. [CrossRef] [PubMed]
113. Kim, S.H.; Jeong, J.C.; Ahn, Y.O.; Lee, H.S.; Kwak, S.S. Differential responses of three sweetpotato metallothionein genes to abiotic stress and heavy metals. *Mol. Biol. Rep.* **2014**, *41*, 6957–6966. [CrossRef]

114. Ren, Y.; Liu, Y.; Chen, H.; Li, G.; Zhang, X.; Zhao, J. Type 4 metallothionein genes are involved in regulating Zn ion accumulation in late embryo and in controlling early seedling growth in *Arabidopsis*. *Plant Cell Environ.* **2012**, *35*, 770–789. [CrossRef]
115. Collett, H.; Shen, A.; Gardner, M.; Farrant, J.M.; Denby, K.J.; Illing, N. Towards transcript profiling of desiccation tolerance in Xerophyta humilis: Construction of a normalized 11 k X. humilis cDNA set and microarray expression analysis of 424 cDNAs in response to dehydration. *Physiol. Plant* **2004**, *122*, 39–53. [CrossRef]

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The Contribution of *Trichoderma viride* and Metallothioneins in Enhancing the Seed Quality of *Avena sativa* L. in Cd-Contaminated Soil

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Abstract: Pollution of arable land with heavy metals is a worldwide problem. Cadmium (Cd) is a toxic metal that poses a severe threat to humans' and animals' health and lives. Plants can easily absorb Cd from the soil, and plant-based food is the main means of exposure to this hazardous element for humans and animals. Phytoremediation is a promising plant-based approach to removing heavy metals from the soil, and plant growth-promoting micro-organisms such as the fungi *Trichoderma* can enhance the ability of plants to accumulate metals. Inoculation of *Avena sativa* L. (oat) with *Trichoderma viride* enhances germination and seedling growth in the presence of Cd and, in this study, the growth of 6-month-old oat plants in Cd-contaminated soil was not increased by inoculation with *T. viride*, but a 1.7-fold increase in yield was observed. The content of Cd in oat shoots depended on the Cd content in the soil. Still, it was unaffected by the inoculation with *T. viride*. *A. sativa* metallothioneins (AsMTs) participate in plant–fungi interaction, however, their role in this study depended on MT type and Cd concentration. The inoculation of *A. sativa* with *T. viride* could be a promising approach to obtaining a high yield in Cd-contaminated soil without increasing the Cd content in the plant.

Keywords: cadmium; heavy metals; metallothioneins; oat; phytoremediation; *Trichoderma*

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1. Introduction

Urbanisation, industrialisation, and agricultural activities increase heavy metal (HM) contamination in soil and water worldwide, resulting in increased accumulation of HM in plants and food [1]. Among various HMs, cadmium (Cd) is easily absorbed by plants. Due to the usage of phosphate fertilisers, sewage sludge, and atmospheric deposition, this toxic element is widely spread on agricultural land [2]. Cd causes plant cell damage through leaf rolling and chlorosis, and reduces root and shoot length and biomass [3–5]. This element has properties similar to the essential micronutrient zinc (Zn) and can compete with it in biological processes [6], leading to, e.g., oxidative stress and damage to photosystems [7]. The EU regulation 2023/915 sets the maximum levels of HM in food. For example, the maximum level of Cd for wheat germ is 0.2 mg kg^{-1}, 0.05 mg kg^{-1} for barley and rye, and 0.1 mg kg^{-1} for other cereals, including oat [8]. For non-smokers, plant-based food is the primary route of Cd exposure. Multiple studies have shown higher amounts of Cd than regulatory threshold levels in edible plant parts, e.g., durum wheat [9] and rice [10]. Heavy metals have been persistent in the environment for centuries or even millennia. They can disperse to distant areas and accumulate in biotic and abiotic components of ecosystems. This is a potential threat to human health because HM can enter the food chain through

bioaccumulation in the tissues of plants and animals [2]. Cadmium causes severe health problems, including congenital disabilities and osteomalacia, and affects the functioning of the kidneys, respiratory system, circulatory system, and central nervous system. The best-known example of Cd toxicity is Itai-Itai disease. This Cd poisoning occurs among inhabitants of the Jinzu River in Japan and is mainly characterised by severe pain as a result of osteomalacia [11]. Therefore, the remediation of Cd-contaminated arable land is essential for food security.

Plants have evolved several mechanisms to maintain the homeostasis of micronutrients and detoxify non-essential HMs, including metal transporters and metal-binding proteins, peptides, and low molecular weight ligands. Metallothioneins (MTs) are small proteins that maintain metal (Zn and Cu) homeostasis and detoxify hazardous metals (Cd) by binding metal ions via cysteine residues. In plants, four MT types (MT1-4) differ in the number and arrangement of the cysteines. Several lines of evidence suggest that different MT types fulfil different roles [12–14]. MTs can act as antioxidants due to the presence of the sulfhydryl groups of cysteine residues [3,15]. This could be one of the reasons why *MT* expression is activated in plants' responses to various stress-inducing factors [3,16–19]. In silico analyses of promoters of *MT* genes in canola (*Brassica napus* L.), *Arabidopsis thaliana* (L.) Heynh., rice (*Oryza sativa* L.), maize (*Zea mays* L.), and oat (*Avena sativa* L.) showed the presence of *cis*-regulatory elements (CREs) involved in the response to light, phytohormones, drought, and other abiotic stresses [3,17,20–22]. Moreover, the expression of *MTs* can be affected by microorganisms. For example, *MT* expression was determined in *Festuca arundinacea* (Schreb.) Darbysh inoculated or not inoculated with the fungus *Epichloë coenophiala* and subjected to nickel (Ni) stress. In non-inoculated plants, the *MT* expression was higher and increased with an increase in Ni concentration. In inoculated samples, the level of MT was similar in most of the tested Ni concentrations [23].

In soil, microorganisms maintain ecological balance. They are responsible for up to 90% of all processes in soils; without them, the soil becomes lifeless [24]. They can interact with each other and other living organisms, including plants [25]. HM-degraded areas often have low micro-organism activity, and to restore the degraded soil, it is crucial to re-establish microorganism populations [26]. Fungi belonging to the genus *Trichoderma* are fast-growing and omnipresent in the environment, and they are found in soil, water, and air. Several species belonging to *Trichoderma* can promote plant growth and development; some of them have been shown to increase plant growth by up to 300% [27]. They can inhibit the growth of some fungal plant pathogens, e.g., *Botrytis cinerea*, *Colletotrichum* sp., and *Fusarium culmorum* [28]. *Trichoderma* spp. produce many secondary metabolites, including indole-3-acetic acid and other auxin analogues that promote the growth of plant roots [29]. Moreover, they secrete organic acids like citric acid, gluconic acid, and fumaric acid, reducing soil pH, which increases the bioavailability of soil macroelements such as phosphorus for plants [30]. They can also increase the uptake of microelements by plants via solubilisation of, e.g., Cu, Zn, manganese (Mn), and iron (Fe) [31,32]. By lowering the soil pH, *Trichoderma* can also increase the bioavailability of hazardous HM [33]. Fungi belonging to *Trichoderma* were found to be highly tolerant to high concentrations of various elements, including micronutrients like nickel (Ni), copper (Cu), and zinc (Zn), but also non-essential elements like lead (Pb) and arsenic (As) [34–38]. Therefore, it was proposed that *Trichoderma* could be used to increase the phytoextraction of HM. For example, *Brassica juncea* (L.) Czern. plants treated with *Trichoderma atroviride* F6 accumulated more Cd and Ni than non-inoculated plants [33]. *Trichoderma* also increased the uptake of Cd, chromium (Cr), Cu, Zn, and Ni by plants like *Miscanthus x giganteus* J.M. Greef, *Salix* sp. L., *Phalaris arundinacea* L., and *Panicum virgatum* L. [26]. Moreover, fungi belonging to *Trichoderma* can accumulate HM. For example, *Trichoderma viride* bioaccumulated Cd and Pb, and the bioaccumulation efficacy increased with the increasing HM metal concentration in the medium [39]. Interestingly, the biomass production of *Trichoderma simmonsii* (UTFC 10063) increased by 46.1% when the fungus was cultured in a medium containing Cd. Still, the bioaccumulation efficacy of Cd decreased with increased Cd concentration [40].

The interactions of saprophytic fungi *Trichoderma* with plants are widely described in the literature [41–44]. Oat (*Avena sativa* L.) belongs to mycorrhizal plants, and most of the research has been conducted on mycorrhizal fungi and their effects on oat growth and yield [45]. There is little data on the interactions of saprophytic fungi with oat. Therefore, the potential role of saprophytic fungi *T. viride* in promoting the growth of *A. sativa* in Cd-contaminated soils and the possible molecular mechanisms underneath these interactions were evaluated in this study. Oat is the world's sixth most important food, feed, and industrial cereal [46]. The importance of oats in the human diet increases constantly [47]. Therefore, it is crucial to understand the mechanisms underlying the uptake, transport, and accumulation of HM in organs of this plant. In addition, the well-developed root system [48–50] and the ability to accumulate toxic HM, including Cd and Pb [51,52], make this plant potentially suitable for phytoremediation. This study aimed to assess the ability of *T. viride* to increase oat's tolerance to Cd and, at the same time, increase Cd accumulation. Moreover, based on data from the literature, we hypothesised that oat MTs are involved in Cd detoxification, accumulation, and interaction with *T. viride*. Therefore, the in vivo metal-binding ability of oat MT1-4 was verified via heterologous expression in bacteria cells and *AsMTs* expression was investigated in the early stages of oat growth in Cd-contaminated soil. Our results suggest that inoculating oat seeds with *T. viride* could increase oat yield in Cd-contaminated soils without increasing Cd-accumulation in above-ground parts of plants.

2. Materials and Methods

2.1. Microorganisms

Six previously identified *T. viride* strains of known plant-promoting properties were used in this study (NCBI GenBank accession numbers: T1—OL221590.1, T2—OL221591.1, T3—OL221592.1, T4—OL221593.1, T5—OL221594.1, T6—OL221595.1) [53]. The fungi were grown in liquid potato dextrose media or on potato dextrose agar (PDA) (Biocorp, Warsaw, Poland) at 23 °C. The fungi were kept on PDA slants at 4 °C for stock culture.

2.2. Metal Resistance of T. viride and Minimal Inhibitory Concentration

Cu, Zn, and Cd ions were added to the PDA medium separately at increasing concentrations from 0 to 29.8 mM for Zn, 2.6 mM for Cu, and 3.7 mM for Cd. The PDA plates were then inoculated with a mycelial disk of 7 mm diameter and grown for 7 days at 23 °C. The Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration of metal that wholly inhibited fungi growth [54]. The experiment was repeated three times.

2.3. Growth of A. sativa in the Presence of Fungi

Seeds of *Avena sativa* L. cultivar Bingo (Plant Breeding Strzelce Ltd., PBAI Group, Strzelce, Poland) were sterilised with a mixture of 30% hydrogen peroxide and 96% ethanol (1:1, *v:v*) for 1 min. The seeds were then rinsed six times with sterile distilled water. Sterile seeds were then suspended in *T. viride* T5 spore suspension. To obtain spore suspension, sterile distilled water was poured on the PDA plate with a one-week-old fungi culture, and spores were suspended using a cell spreader. The suspension was then filtered using sterile MiraCloth (Calbiochem, Merck, Darmstadt, Germany), and the number of spores in the filtrate was counted using a hemocytometer. The solution was diluted to the final concentrations of 10^6, 10^4, and 10^2 spores mL^{-1} and used on the same day. Sterile oat seeds were inoculated with spore-suspension by incubation for 15 min, with shaking at room temperature. The inoculated seeds were placed in glass Petri dishes lined with filter paper moistened with 3.5 mL of sterile distilled water. The control was non-inoculated seeds. The seeds were kept in darkness at 23 °C for six days. The germinated seeds were counted every day, and on the 6th day, the lengths and fresh and dry (moisture analyser MA 110.R, RADWAG, Radom, Poland) biomass of shoots and roots were measured. Germination parameters, i.e., germination percentage (G), germination index (GI), mean germination time (MGT), mean germination rate (MGR), and the coefficient velocity of germination

(CVG) [55] were calculated according to the formulas provided in the cited literature. The experiment was repeated three times.

2.4. Effect of Heavy Metals on the Germination and Growth of A. sativa Seedlings

Seeds were prepared as described above, then placed on Petri dishes lined with filter paper moistened with 3.5 mL of sterile distilled water (control) or solutions of 25, 80, 150, or 245 µM Cd (as $CdSO_4$ solution). The seeds were kept in darkness at 23 °C for six days. Every day, the number of germinated seeds was counted, and on the 6th day, the lengths and fresh and dry (Moisture analyser MA 110.R, RADWAG, Radom, Poland) biomass of shoots and roots were measured. The experiment was repeated three times.

2.5. Effect of T. viride on the Growth of A. sativa in the Presence of Heavy Metals

Seeds inoculated with *T. viride* T5 spore suspension (10^2 spores mL^{-1}) were placed on Petri dishes lined with filter papers moistened with 3.5 mL of sterile distilled water or solution of 25, 80, 150 or 245 µM Cd (as $CdSO_4$ solution). Non-inoculated seeds served as a control. The seeds were kept in darkness at 23 °C for six days. Every day, the number of germinated seeds was counted, and on the 6th day, the lengths and fresh and dry biomass of shoots and roots were measured. The experiment was repeated three times.

2.6. Pot Experiment

For the pot experiment, a mixture of autoclaved soil and sand (5:1, *v*:*v*), amended with $CdSO_4$ solution to the final concentration of 1, 5, 10, or 20 mg Cd kg^{-1} of soil, was used. To ensure the binding of Cd ions to the soil particles, the soil–Cd mixture was incubated for two weeks before seed sowing. Oat seeds were inoculated with 10^6 spores mL^{-1} solution of *T. viride* T5, as described above, and 5 seeds per pot were sown (for each condition, 4 pots were used). The control was non-inoculated seeds. The plants were watered with tap water twice a week, and once a month, they were watered with Hoagland solution. After two weeks, leaves were collected and frozen in liquid nitrogen for gene expression analyses. After 6 months, shoot and root length and fresh and dry (moisture analyser MA 110.R, RADWAG, Radom, Poland) biomass were measured, and the number of leaves, seeds, and panicles was counted.

2.7. Level of Heavy Metals in A. sativa L. Plants

Dry shoot biomass was ground using a mortar and pestle, and the content of Cu, Cd, and Zn was analysed by ICP-MS 7500 CX (Agilent Technologies, Santa Clara, CA, USA) in the Instrumental Analysis Laboratory, Department of Chemistry, Nicolaus Copernicus University in Toruń. The analyses were performed in three biological replicates.

2.8. Identification of Metal-Responsive Elements in the Promoters of A. sativa Metallothioneins

A 1500 bp region upstream of the ATG codon for all *AsMT* genes was downloaded from the GrainGenes database (https://wheat.pw.usda.gov/, accessed on 20 April 2023). Metal response elements (MRE) and copper response elements (CuRE) were identified in the promoters of *A. sativa MT*s using the following sequences as well as their reverse and complementary sequences: 5′-TGCAGGC-3′ [56], 5′-TGCRCNC-3′ [56,57], 5′-TGCAACC-3′, 5′-TGCACCCC-3′, 5′-GAGAGCA-3′ [58] and 5′GTAC-3′ [59].

2.9. Functional Analysis of AsMT1-4 in E. coli

Expression constructs of *AsMT1-3* were prepared as described previously [16]. The coding region of *AsMT4* was amplified with sequence-specific primers containing restriction sites for *Nde*I in the forward primer 5′-AAACATATGGGCTGCGACGACAAGTG-3′ and *Xho*I in the reverse primer 5′-AACTCGAGTCAGGCGGTGGAG-3′. The PCR products were digested with *Nde*I and *Xho*I, and ligated into a pET21a(+) expression vector (Novagen, Darmstadt, Germany) to be later transformed into *E. coli* DH5α. The plasmids were isolated (Gene MATRIX Plasmid Miniprep DNA Purification Kit; EURx, Gdańsk, Poland) and

sequenced to confirm the presence of the correct open reading frame (Genomed, Warsaw, Poland). The constructs were named pET-*AsMT1-4*.

For functional analysis, the *E. coli* Rosetta (DE3) cells (Novagen, Darmstadt, Germany) were transformed with an empty pET21a vector (control) or pET-*AsMT1-4* constructs using the heat shock method [53]. Overnight cultures of transformed bacterial cells were diluted (1:100, *v:v*) in LB medium with antibiotics (50 μg mL^{-1} ampicillin and 34 μg mL^{-1} chloramphenicol) to $OD_{600} \approx 0.2$. To induce heavy metal stress, the cultures were supplemented with solutions of $ZnSO_4$ or $CdSO_4$ to final concentrations of 0.25 mM or 0.5 mM $ZnSO_4$ and 0.1 mM or 0.25 mM $CdSO_4$. The expression of *MT*s was induced using isopropyl-β-D-1-thiogalacto-pyranoside (IPTG) at the final concentration of 0.1 mM, avoiding high transgene overexpression. The controls were cultures without HM. The bacteria were incubated for 7 h (37 °C, 180 rpm), and OD_{600} (Implen OD_{600} DiluPhotometer, München, Germany) was measured every hour. The analysis was performed in three technical replicates for each of the three biological replicates. The growth rate of *E. coli* cultures was expressed as the slope of a linear proportion of the growth curve and was calculated using Microsoft Excel.

2.10. Gene Expression

Plant tissues were ground in liquid nitrogen, and 100 mg of the ground tissue was used for RNA isolation using an RNeasy kit (QIAGEN, Hilden, Germany). The quality and quantity of the isolated total RNA were checked via spectrophotometric measurement using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis stained with EtBr. The RNA was then treated with 1 U of DNase (Thermo Fisher Scientific, Waltham, MA, USA) to remove DNA contamination. The cDNA was synthesised from 1 μg of RNA using a mixture of 2.5 μM oligo(dT)20 primer and 0.2 μg of random hexamers with an NG dART RT Kit (EURx, Gdańsk, Poland), according to the manufacturer's protocol. The reaction was performed at 25 °C for 10 min, followed by 50 min at 50 °C. The cDNA was stored at −20 °C.

The RT-qPCR reaction mixture included 4 μL of 1/30 diluted cDNA, 0.5 μM of gene-specific primers (Table 1), and 5 μL of LightCycler 480 SYBR Green I Master (Roche, Penzberg, Germany) for a total volume of 10 μL. Eukaryotic Initiation Factor 4A-3 (*EIF4A*) was a reference gene [60]. The reactions were performed in three technical replicates using LightCycler 480 Instrument II (Roche, Penzberg, Germany). The thermal cycling conditions were as follows: 95 °C for 5 min, 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s over 40 cycles. The SYBR Green I fluorescence signal was recorded at the end of the extension step in each cycle. The melt curve analysis confirmed the assay's specificity, i.e., increasing the temperature from 55 to 95 °C at a ramp rate of 0.11 °C/s. The fold change in gene expression was calculated using LightCycler 480 Software, release 1.5.1.62 (Roche, Penzberg, Germany) [3,16,17].

Table 1. Sequence of primers used in this study.

Primer Name	Sequence 5′→3′	Target	Reference
AsMT1_qPCR_f	CAAACTGCAAGTGCGGGAAG	*AsMT1*	[17]
AsMT1_qPCR_r	TTGTTCTCATGAGCCACGCC		
AsMT2_qPCR_f	CTGCGGAGGGTGCAAGATG	*AsMT2*	
AsMT2_qPCR_r	AACGATGGCTTGGAAGAGGG		
AsMT3_qPCR_f	TCCACCATGTCGAACACCTG	*AsMT3*	
AsMT3_qPCR_r	TGGCTCTTCTCGGTGTCAAC		
AsMT4_qPCR_f	CACGTGCGGAGAGCACTG	*AsMT4*	[3]
AsMT4_qPCR_r	ACAGGAGGCGCAGTCACAG		
EIF4A_f	TCTCGCAGGATACGGATGTCG	*EIF 4A*	[60]
EIF4A_r	TCCATCGCATTGGTCGCTCT		

2.11. Statistical Analysis

Statistical analyses were conducted using Microsoft Excel and RStudio [61]. The results are expressed as mean values with error bars representing standard error (SE). The one-way ANOVA (post hoc Tukey and Dunn's tests), or the Kruskal–Wallis (post hoc Mann–Whitney test), were conducted based on sample type, normality, and homogeneity. Correlations were calculated using the Pearson correlation coefficient.

3. Results

3.1. Tolerance of T. viride to Cd, Cu, and Zn

The tolerance of six *T. viride* fungi to Zn, Cu, and Cd was tested using the minimal inhibitory concentration (MIC) method. MIC is defined as the lowest concentration of metal that completely inhibits fungi growth [54]. All six fungi could survive in tested metal concentrations (Table 2). The highest tolerance to Cd was observed for *Trichoderma* strain T1 (3.6 mM), but the same fungi had the lowest tolerance to Zn (22.3 mM). Strain T6 could tolerate Cu in concentrations lower than 2.5 mM, but strain T5 could not grow in 1.6 mM Cu. Based on these results, *T. viride* T5 was chosen for further experiments because it had a high tolerance to both Cd and Zn.

Table 2. Minimal inhibitory concentration (MIC) of Zn, Cu, and Cd for *Trichoderma viride* strains T1–T6.

***T. viride* Strain**	**Minimal Inhibitory Concentration [mM]**		
	Zn	**Cu**	**Cd**
T1	22.3	1.7	3.6
T2	29.0	2.0	2.6
T3	28.5	2.4	2.6
T4	28.5	2.0	2.7
T5	29.2	1.6	2.9
T6	30.0	2.0	1.9

3.2. Seed Germination and Seedling Growth of A. sativa in the Presence of T. viride

To examine the effect of the inoculation of oat seeds with the spores of *T. viride* T5 on seed germination and early seedling growth, we inoculated oat seeds with fungal spores at concentrations 10^2, 10^4, and 10^6 spores mL^{-1} (Table 3). The highest germination percentage, which reflects the viability of the seed population, was observed for seeds inoculated with 10^4 spores. In contrast, treatment with 10^6 spore concentrations significantly decreased germination percentage (G) compared to non-inoculated seeds. Inoculation of seeds with 10^4 spores also increased the germination index (GI) (a measure of germination percentage and speed) and other tested parameters; however, the differences were not statistically significant. The highest GI was observed for 10^6 spore concentration, i.e., a 1.1-fold significant increase. Moreover, mean germination time (MGT), mean germination rate (MGR), i.e., a reciprocal of MGT that measures the time it takes for the seed to germinate, and coefficient velocity of germination (CVG), which is an indicator of the rapidity of germination, were increased by the inoculation with spores at the concentration of 10^6 (Table 3).

On the other hand, we observed that higher concentrations of *T. viride* spores (10^4 and 10^6) did not positively affect the growth of oat seedlings (Table 4). For the spore concentration 10^6, shoot length and biomass were the same as control plants, but roots had 1.4- and 2.0 times lower fresh and dry biomass, respectively. For plants inoculated with spores at a concentration of 10^4, the shoots were 1.2 times shorter, but the roots' growth was unaffected compared to the control (Table 4). On the other hand, slight growth stimulation of oat seedlings was observed only when seeds were inoculated with 10^2 spores, i.e., a 1.1-time increase in shoot dry biomass. Interestingly, a 1.4-time decrease in fresh root biomass after inoculation with spores at 10^2 was also noticed (Table 4).

Table 3. Germination parameters of *Avena sativa* seeds treated with increasing concentration of *Trichoderma viride* T5 spores (10^2, 10^4, and 10^6 spores mL^{-1}). Values are means (n = 100) ± SE. Values marked by distinct letters in a row differ significantly (one-way ANOVA, Tukey post-hoc test, $p < 0.05$).

T. viride T5 Spore Concentration (Spores mL^{-1})	G (%)	GI (days)	MGT (days)	MGR (1/days)	CVG
0 (Control)	93.45 ± 1.48 ^{a}	5.10 ± 0.08 ^{b}	1.90 ± 0.08 ^{a}	0.55 ± 0.02 ^{b}	54.98 ± 2.13 ^{b}
10^2	94.33 ± 1.11 ^{a}	5.16 ± 0.05 ^{ab}	1.84 ± 0.05 ^{ab}	0.55 ± 0.01 ^{ab}	55.42 ± 1.28 ^{b}
10^4	100.00 ± 0.20 ^{a}	5.38 ± 0.04 ^{ab}	1.62 ± 0.04 ^{ab}	0.62 ± 0.01 ^{ab}	61.76 ± 1.39 ^{ab}
10^6	80.00 ± 1.67 ^{b}	5.63 ± 0.06 ^{a}	1.38 ± 0.06 ^{b}	0.73 ± 0.03 ^{a}	73.33 ± 3.14 ^{a}

G—germination percentage, GI—germination index, MGT—mean germination time, MGR—mean germination rate, CVG—coefficient velocity of germination.

Table 4. Effect of *Trichoderma viride* T5 inoculation with different spore concentrations (10^2, 10^4, and 10^6 spores mL^{-1}) on the growth of 6-day-old *Avena sativa* seedlings. Values are means (n = 40) ± SE. Values marked by distinct letters in a column differ significantly (one-way ANOVA, Tukey post-hoc test, $p < 0.05$).

Treatment	Spore conc.	Shoot Length (cm)	Fresh Shoot Biomass (g)	Dry Shoot Biomass (g)	Root Length (cm)	Fresh Root Biomass (g)	Dry Root Biomass (g)
Control (non-inoculated)	0	5.73 ± 0.28 ^{a}	0.068 ± 0.004 ^{a}	0.0054 ± 0.0005 ^{ab}	8.26 ± 0.51 ^{a}	0.060 ± 0.003 ^{a}	0.0052 ± 0.0002 ^{a}
Inoculated with *T. viride* T5	10^2	5.24 ± 0.15 ^{ab}	0.063 ± 0.003 ^{a}	0.0062 ± 0.0002 ^{a}	8.18 ± 0.49 ^{a}	0.044 ± 0.003 ^{b}	0.0052 ± 0.0002 ^{a}
	10^4	4.92 ± 0.23 ^{b}	0.059 ± 0.004 ^{a}	0.0051 ± 0.0005 ^{ab}	7.74 ± 0.62 ^{a}	0.052 ± 0.004 ^{ab}	0.0048 ± 0.0003 ^{a}
	10^6	5.43 ± 0.13 ^{ab}	0.061 ± 0.002 ^{a}	0.0046 ± 0.0004 ^{b}	7.69 ± 0.28 ^{a}	0.043 ± 0.003 ^{b}	0.0026 ± 0.0002 ^{b}

3.3. Effect of Cadmium and T. viride on the Seed Germination and Seedling Growth of A. sativa

Further, the effect of Cd and simultaneous Cd and *T. viride* T5 treatment on oat seed germination (Table 5) and seedling growth (Table 5) was tested. Cd treatment did not only affect the total number of germinated seeds (G)—all other parameters, reflecting the germination speed, were negatively affected by Cd treatment (Table 5). For example, GI was 1.2-fold and MGR 1.4-fold lower for seeds germinated in the presence of 245 µM Cd than the control. The inhibitory effect of Cd on germination was dependent on Cd concentration. After inoculation with *T. viride* spores, the negative impact of Cd on germination was also observed; however, the differences were not statistically significant (Table 5). Moreover, non-inoculated seeds in the presence of 150 and 245 µM Cd germinated more slowly (as shown by higher GI, MGR, and CVG, and lower MGT) than inoculated seeds. Interestingly, inoculation with *T. viride* spores decreased the total number of germinated seeds (Table 5).

Table 6 shows the morphological parameters of 6-day-old oat seedlings that grew in the presence of Cd and/or *T. viride* spores. The increase in Cd concentration decreased the length of shoots and roots, and fresh and dry biomass, of both inoculated and non-inoculated samples. The most noticeable decrease in shoot length was observed for plants treated with 150 µM Cd—1.8 and 1.6 times shorter for non-inoculated and inoculated samples, respectively, than the control. Root length was the most affected by 245 µM Cd, i.e., a 3.2 and 2.7 times reduction for non-inoculated and inoculated samples, respectively, compared to the control. Seedlings grown in the highest Cd concentration had the lowest fresh and dry shoot biomass in both non-inoculated and inoculated samples. The exception was the dry shoot biomass of non-inoculated plants, which was 1.1 times higher than in seedlings grown in control conditions. Inoculation with *T. viride* spores increased the growth of roots in high Cd concentrations, i.e., the length of roots was 1.2-fold higher in inoculated seedlings than in non-inoculated ones, both with 150 and 245 µM Cd (Table 6).

Table 5. Germination parameters of *Avena sativa* seeds germinated in the presence of Cd (25–245 µM) or in water (0 µM). Seeds were inoculated with *Trichoderma viride* T5 spores at a concentration of 10^2 mL^{-1} before sowing; control seeds were not inoculated. Values are means (n = 100) ± SE. Values marked by distinct letters in a column differ significantly (one-way ANOVA, Tukey post-hoc test, $p < 0.05$).

	Cd conc. [µM]	G (%)	GI (days)	MGT (days)	MGR (1/day)	CVG
Control (non-inoculated)	0	93.45 ± 1.48 ^{a}	5.10 ± 0.08 ^{a}	1.90 ± 0.08 ^{b}	0.55 ± 0.02 ^{a}	54.98 ± 2.13 ^{a}
	25	88.75 ± 1.56 ^{a}	4.88 ± 0.04 ^{ab}	2.12 ± 0.04 ^{ab}	0.47 ± 0.01 ^{ab}	47.49 ± 0.74 ^{ab}
	80	90.47 ± 1.11 ^{a}	4.90 ± 0.05 ^{ab}	2.10 ± 0.05 ^{ab}	0.48 ± 0.01 ^{ab}	48.25 ± 1.15 ^{ab}
	150	92.38 ± 0.84 ^{a}	4.52 ± 0.04 ^{b}	2.48 ± 0.04 ^{a}	0.41 ± 0.01 ^{b}	40.71 ± 0.73 ^{b}
	245	89.43 ± 2.15 ^{a}	4.42 ± 0.05 ^{b}	2.58 ± 0.05 ^{a}	0.39 ± 0.01 ^{b}	39.16 ± 0.81 ^{b}
Inoculated with *T. viride* T5	0	94.33 ± 1.11 ^{a}	5.16 ± 0.05 ^{a}	1.84 ± 0.05 ^{b}	0.55 ± 0.01 ^{a}	55.42 ± 1.28 ^{a}
	25	83.81 ± 1.37 ^{a}	4.88 ± 0.03 ^{ab}	2.12 ± 0.03 ^{ab}	0.47 ± 0.01 ^{ab}	47.36 ± 0.66 ^{ab}
	80	89.05 ± 1.39 ^{a}	4.91 ± 0.04 ^{ab}	2.09 ± 0.04 ^{ab}	0.48 ± 0.01 ^{ab}	48.25 ± 0.98 ^{ab}
	150	87.73 ± 0.76 ^{a}	4.88 ± 0.05 ^{ab}	2.12 ± 0.05 ^{ab}	0.48 ± 0.01 ^{ab}	47.90 ± 1.25 ^{ab}
	245	86.85 ± 1.66 ^{a}	4.79 ± 0.09 ^{ab}	2.21 ± 0.09 ^{ab}	0.48 ± 0.02 ^{ab}	47.53 ± 2.09 ^{ab}

G—germination percentage, GI—germination index, MGT—mean germination time, MGR—mean germination rate, CVG—coefficient velocity of germination.

Table 6. Effect of Cd (25–245 µM) and *Trichoderma viride* T5 inoculation (10^2 spores mL^{-1}) on the growth of 6-day-old *Avena sativa* seedlings. Values are means (n = 40) ± SE. Values marked by distinct letters in a column differ significantly (one-way ANOVA, Tukey post-hoc test, $p < 0.05$).

	Cd conc. [µM]	Shoot Length (cm)	Fresh Shoot Biomass (g)	Dry Shoot Biomass (g)	Root Length (cm)	Fresh Root Biomass (g)	Dry Root Biomass (g)
Control (non-inoculated)	0	5.73 ± 0.28 ^{a}	0.068 ± 0.004 ^{a}	0.0054 ± 0.0005 ^{bc}	8.26 ± 0.51 ^{a}	0.060 ± 0.003 ^{a}	0.0052 ± 0.0002 ^{b}
	25	4.27 ± 0.34 ^{b}	0.052 ± 0.003 ^{b}	0.0060 ± 0.0004 ^{b}	6.47 ± 0.62 ^{b}	0.047 ± 0.005 ^{abc}	0.0052 ± 0.0004 ^{b}
	80	5.60 ± 0.22 ^{a}	0.066 ± 0.003 ^{a}	0.0073 ± 0.0003 ^{a}	6.46 ± 0.24 ^{b}	0.058 ± 0.003 ^{ab}	0.0065 ± 0.0003 ^{a}
	150	3.21 ± 0.20 ^{c}	0.047 ± 0.003 ^{bc}	0.0042 ± 0.0001 ^{d}	3.27 ± 0.19 ^{cd}	0.037 ± 0.003 ^{cd}	0.0030 ± 0.0000 ^{d}
	245	3.85 ± 0.18 ^{bc}	0.047 ± 0.003 ^{bc}	0.0060 ± 0.0004 ^{b}	2.57 ± 0.13 ^{d}	0.028 ± 0.002 ^{d}	0.0037 ± 0.0003 ^{cd}
Inoculated with *T. viride* T5	0	5.24 ± 0.15 ^{a}	0.063 ± 0.003 ^{a}	0.0062 ± 0.0002 ^{ab}	8.18 ± 0.49 ^{a}	0.044 ± 0.003 ^{bcd}	0.0052 ± 0.0002 ^{b}
	25	3.42 ± 0.17 ^{c}	0.045 ± 0.002 ^{c}	0.0042 ± 0.0002 ^{d}	4.59 ± 0.28 ^{c}	0.032 ± 0.002 ^{cd}	0.0037 ± 0.0001 ^{cd}
	80	3.45 ± 0.21 ^{c}	0.041 ± 0.003 ^{c}	0.0044 ± 0.0001 ^{cd}	4.32 ± 0.30 ^{c}	0.034 ± 0.002 ^{cd}	0.0041 ± 0.0002 ^{c}
	150	3.28 ± 0.19 ^{c}	0.042 ± 0.003 ^{c}	0.0044 ± 0.0002 ^{d}	3.75 ± 0.21 ^{cd}	0.033 ± 0.002 ^{cd}	0.0040 ± 0.0001 ^{c}
	245	3.45 ± 0.22 ^{c}	0.039 ± 0.003 ^{c}	0.0042 ± 0.0003 ^{d}	3.09 ± 0.20 ^{d}	0.034 ± 0.009 ^{cd}	0.0034 ± 0.0002 ^{cd}

3.4. Effect of T. viride on the Growth and Yield of A. sativa Plants Grown in the Presence of Cd and on the Level of Cd Phytoextraction

The following experiment was conducted to verify the effect of oat seed inoculation with *T. viride* T5 on mature plant growth and yield in Cd-contaminated soil. After six months of growth, the length of the oat roots was affected neither by inoculation with *T. viride* nor by cadmium treatment (Figure 1B). Fresh and dry root biomass in non-inoculated samples was not affected by cadmium. Inoculation caused an increase in fresh root biomass, i.e., in the presence of 1, 5, and 20 mg Cd kg^{-1} soil, fresh root biomass was respectively 1.6, 1.4, and 3.8 times higher when compared to non-inoculated samples (Figure 1D). Similarly, in inoculated plants grown in soil containing 1, 5, and 20 mg Cd kg^{-1}, dry root biomass was respectively 2, 2.5, and 3.3 times higher when compared to non-inoculated samples (Figure 1F). In non-inoculated plants, the shoot length decreased with increased Cd concentration in soil—shoots of plants treated with 20 mg Cd were 1.2 times shorter than those treated with only 1 mg Cd. However, when inoculated with *T. viride* T5, the shoot length remained the same across all tested Cd concentrations (Figure 1A). Shoot fresh and dry biomass of non-inoculated plants decreased with increased Cd concentrations, with plants treated with 20 mg Cd having their fresh and dry biomass 1.4 and 1.5 times lower, respectively, compared to plants treated with only 1 mg Cd (Figure 1C,E). In inoculated

samples, the fresh shoot biomass was similar across all Cd concentrations. Dry shoot biomass of inoculated plants treated with 1, 5, and 20 mg Cd was respectively 1.2, 1.5, and 1.4 times higher when compared to non-inoculated plants (Figure 1E).

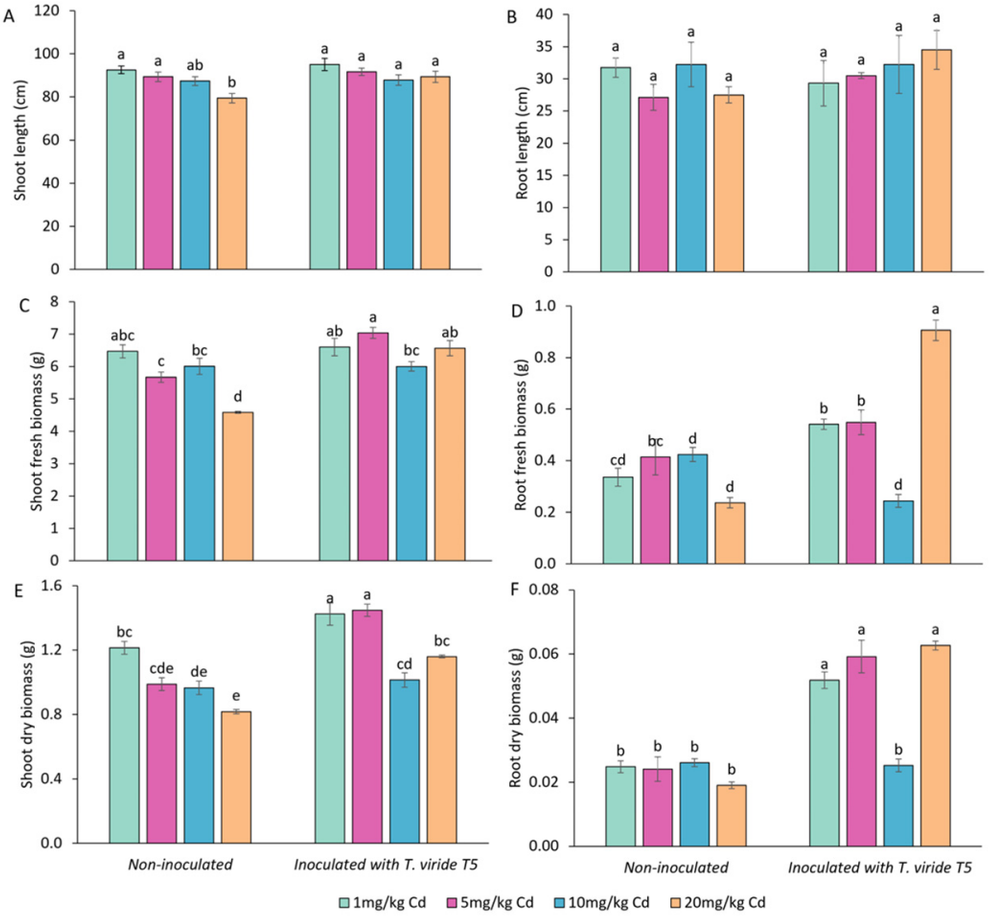

Figure 1. Effect of *Trichoderma viride* T5 inoculation on the growth of *Avena sativa* plants in soil containing 1 mg, 5 mg, 10 mg, and 20 mg of Cd per 1 kg of soil. Length, fresh and dry biomass of shoot (A, C, E, respectively) and root (B, D, F, respectively) were measured. Bars represent means (n = 40) ± SE. Means indicated with distinct letters are significantly different (Kruskal–Wallis, Dunn post hoc test, $p < 0.05$).

Treatment with cadmium significantly affected the yield, i.e., the number of panicles was 1.7-fold lower, and the number of seeds was 2.2-fold lower in plants grown in soil containing 20 mg kg^{-1} of Cd compared to plants grown in soil containing 1 mg kg^{-1} of Cd (Table 7). Similar observations were made for plants treated with *T. viride* T5; however, for Cd concentration, the number of panicles and seeds was higher in inoculated plants compared to in non-inoculated ones. For example, plants inoculated with *T. viride* grown in soil containing 20 mg kg^{-1} of Cd produced 1.7-fold more seeds and 1.3-fold more panicles than non-inoculated plants grown in the same Cd concentration (Table 7).

To assess the potential of *A. sativa* for Cd phytoextraction and the potential involvement of *T. viride* in this process, Cu, Cd, and Zn content was analysed in the above-ground parts of 6-month-old plants (Table 8). As expected, the concentration of Cd in the oat shoots increased with an increase in Cd concentration in the soil, i.e., plants grown in soil with 20 mg kg^{-1} of Cd had 6.6 times (non-inoculated) and 5.3 times (inoculated) higher Cd concentration in shoots than plants grown in soil with 1 mg kg^{-1} of Cd. Inoculation with *T. viride* T5 spores did not significantly increase the Cd uptake by oat. The content of copper in oat shoots was affected neither by Cd concentration in soil nor by inoculation with *T.*

viride T5 spores. Interestingly, the level of Zn in shoots of oat plants was the highest in plants growing in soil containing 20 mg Cd kg^{-1}, and it was 1.6 and 1.8 times higher in non-inoculated than in inoculated plants, respectively, compared to plants growing in soil contaminated with 1 mg Cd kg^{-1}. Inoculation did not increase the Zn uptake regardless of Cd concentration in the soil (Table 8).

Table 7. The number of *Avena sativa* leaves, panicles, and seeds per plant grown in soil contaminated with Cd and inoculated with *Trichoderma viride* T5. Values are means (n = 15) ± SE. Values indicated with distinct letters in a column differ significantly (Kruskal–Wallis, Dunn post hoc test, $p < 0.05$).

	Cd Content in the Soil [mg kg^{-1}]	Leaves Number	Panicles Number	Seeds Number
Non-inoculated	1	17.9 ± 1.1 ^{b}	3.2 ± 0.4 ^{bc}	11.9 ± 1.4 ^{abd}
	5	17.1 ± 1.0 ^{b}	2.7 ± 0.2 ^{c}	10.0 ± 0.9 ^{bd}
	10	15.4 ± 1.1 ^{b}	1.8 ± 0.2 ^{d}	6.5 ± 1.3 ^{c}
	20	17.1 ± 0.7 ^{b}	1.9 ± 0.4 ^{cd}	5.5 ± 1.1 ^{c}
Inoculated with *T. viride* T5	1	19.8 ± 0.8 ^{a}	4.1 ± 0.4 ^{b}	14.5 ± 1.3 ^{ab}
	5	17.9 ± 0.8 ^{ab}	4.2 ± 0.3 ^{ab}	16.0 ± 1.5 ^{a}
	10	16.5 ± 0.7 ^{b}	2.2 ± 0.4 ^{cd}	7.8 ± 1.4 ^{cd}
	20	16.7 ± 0.8 ^{b}	2.5 ± 0.5 ^{cd}	9.5 ± 1.9 ^{cd}

Table 8. Content of Cd, Cu, and Zn in shoots of *Avena sativa* plants inoculated or non-inoculated with *Trichoderma viride* T5 grown in soil contaminated with Cd. Values are means (n = 15) ± SE. Values indicated with distinct letters in a column are significantly different (one-way ANOVA, Tukey post hoc test, $p < 0.05$).

Treatment	Cd content in the Soil [mg kg^{-1}]	Content in Shoots		
		Cd [mg kg^{-1}]	Cu [mg kg^{-1}]	Zn [mg kg^{-1}]
Non-inoculated	1	0.143 ± 0.01 ^{c}	5.674 ± 0.84 ^{a}	29.625 ± 3.31 ^{bc}
	5	0.209 ± 0.04 ^{c}	5.751 ± 0.33 ^{a}	49.310 ± 1.97 ^{a}
	10	0.387 ± 0.07 ^{bc}	4.249 ± 0.27 ^{a}	39.665 ± 0.41 ^{ab}
	20	0.931 ± 0.11 ^{a}	4.048 ± 1.55 ^{a}	47.805 ± 6.13 ^{a}
Inoculated with *T. viride* T5	1	0.198 ± 0.03 ^{c}	4.134 ± 0.94 ^{a}	25.515 ± 4.16 ^{c}
	5	0.377 ± 0.07 ^{bc}	5.145 ± 1.60 ^{a}	36.770 ± 8.36 ^{abc}
	10	0.577 ± 0.08 ^{b}	3.618 ± 1.26 ^{a}	29.555 ± 2.86 ^{bc}
	20	1.050 ± 0.18 ^{a}	4.104 ± 1.16 ^{a}	47.080 ± 3.09 ^{a}

Positive correlations were observed between the amount of cadmium added to the soil and the level of Zn and Cd in oat plants. In contrast, the level of Cd in soil was negatively correlated with the level of Cu in oat shoots (Figure 2). Cadmium application was also negatively correlated with shoot length, fresh and dry biomass, and number of panicles and seeds (Supplementary Figure S3). A positive correlation was observed between inoculation with *T. viride* T5 spores and root length, fresh and dry biomass, and shoots' fresh and dry biomass (Supplementary Figure S3). Interestingly, a negative correlation between inoculation with *T. viride* T5 spores and the levels of Cu and Zn, and a positive between inoculation with *T. viride* spores and the level of Cd, were observed (Figure 2).

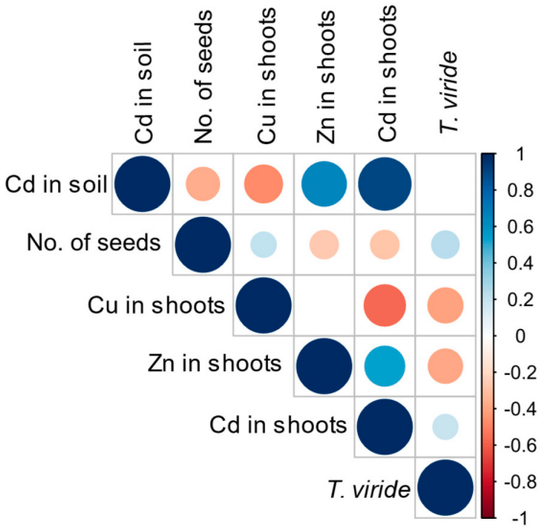

Figure 2. Pearson correlation between the amount of cadmium added to the soil, number of seeds, levels of Cu, Zn, and Cd in *Avena sativa* shoots, and the inoculation of oat seeds with *Trichoderma viride* T5 spores. Only significant correlations are shown.

3.5. Functional Analysis of A. sativa Metallothioneins (AsMT1-4) in Bacteria Cells

To verify the metal-binding ability of oat MT1-4, the proteins were expressed in *E. coli* cells in the presence of Zn and Cd (Figure 3). Bacteria transformed with plasmids carrying *AsMT3* and *AsMT4* grew faster under control and stress conditions caused by metal ions than bacteria transformed with an empty pET vector. The highest difference in growth rates between bacteria transformed with pET_AsMT3 and pET_AsMT4, and bacteria bearing an empty pET vector (6 and 5 times higher growth rates, respectively) was observed in medium supplemented with 0.25 mM Zn. The expression of *AsMT1* and *AsMT2* in bacterial cells did not increase bacteria growth. To verify the possible adverse effect of IPTG on bacteria growth, the experiment was also performed without the addition of IPTG (Supplementary Figure S1). Only bacteria transformed with pET_AsMT4 had a faster growth rate (both under control conditions and in the presence of Zn and Cd ions) than bacteria transformed with the empty pET vector.

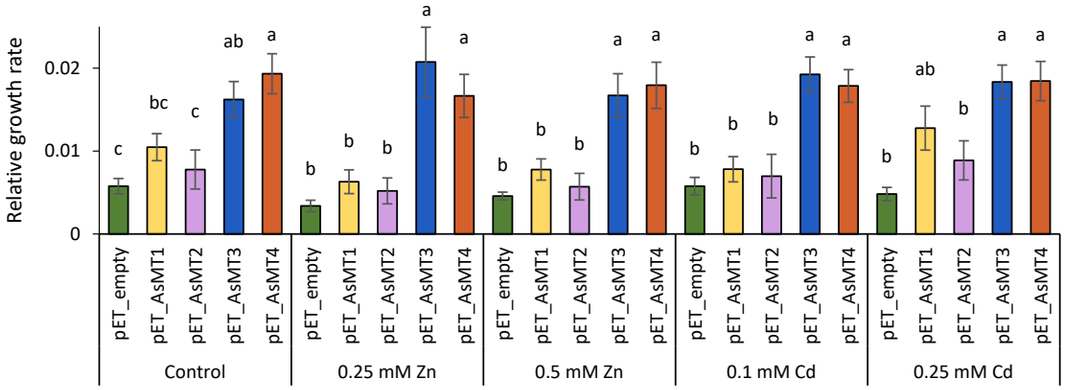

Figure 3. Comparison of the growth of *Escherichia coli* cells transformed with empty pET21a(+) vector and pET21a(+) vectors harbouring coding regions of *AsMT1-4* in LB medium (control) and LB medium supplemented with Zn or Cd ions. The expression of *AsMT1-4* was induced by 0.1 mM IPTG. The relative growth rate is expressed as a slope of bacterial growth curves obtained by plotting optical density against time. Bars represent means (n = 9) ± SE. The results obtained for a given condition were compared, and distinct letters indicate significant differences between *E. coli* carrying different plasmids (Kruskal–Wallis, Mann–Whitney; $p < 0.05$).

3.6. Expression of A. sativa AsMT1-4 in Plants Growing in Cd-Contaminated Soil

To give insight into molecular mechanisms underlying Cd detoxification and interaction with *T. viride* in oat plants, gene expression of *AsMT1-4* was analysed (Figure 4). The possibility that heavy metals induce the expression of oat MTs was verified by in silico analysis of promoter regions of *AsMT1-4* genes (Supplementary Table S1). In total, 4, 4, 8, and 11 MRE were found in *AsMT1*, *AsMT2*, *AsMT3*, and *AsMT4* promoters, respectively. The most common motif was the CuRE motif 5′-GTAC-3′, which appeared 20 times in the promoter sequences of *AsMT1-4.* The second most abundant motif was 5′-TGCRCNC-3′, found five times (Supplementary Table S1).

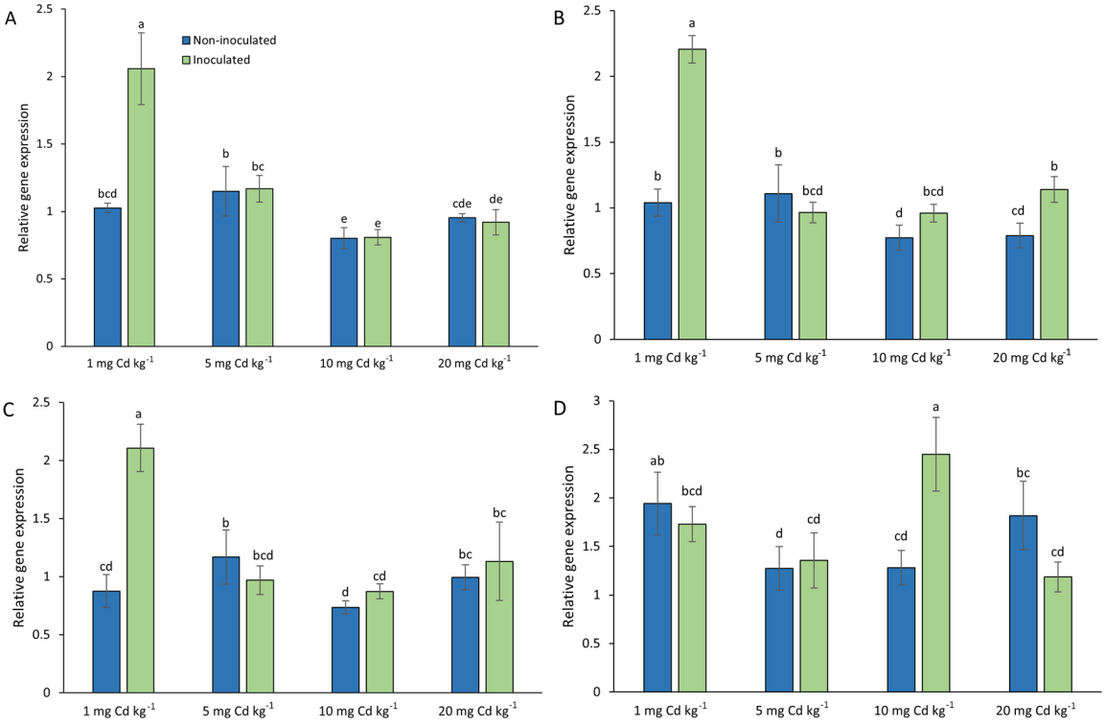

Figure 4. Relative gene expression of *Avena sativa* metallothioneins (**A**) *AsMT1*, (**B**) *AsMT2*, (**C**) *AsMT3*, and (**D**) *AsMT4* in two-week-old oat seedlings, inoculated (green bars) or non-inoculated (blue bars) with *Trichoderma viride* T5, grown in soil containing 1, 5, 10, and 20 mg Cd per kg of soil. Bars represent means (n = 2) ± SE. Distinct letters mark significant differences (one-way ANOVA, Tukey post hoc test, $p < 0.05$).

In inoculated plants, the expression of *AsMT1*, *AsMT2*, and *AsMT3* in the presence of 1 mg Cd was over two times higher than in non-inoculated samples. However, in plants growing in soil containing 5, 10, and 20 mg of Cd per kg of soil, the *AsMT1-3* expression in both variants, i.e., non-inoculated and inoculated, was on a similar level. The exception was *AsMT2*, where the expression in inoculated seedlings growing in 20 mg/kg of Cd was 1.5 times higher than in non-inoculated plants. The expression of *AsMT4* was the highest in samples inoculated with *T. viride* in seedlings grown in the presence of 10 mg/kg of Cd (almost 2-fold higher than in non-inoculated plants). In other variants, the *AsMT4* expression in both inoculated and non-inoculated samples was comparable (Figure 4).

Correlation analyses showed high positive correlations among *AsMT1-3* but not between *AsMT1-3* and *AsMT4* (Supplementary Figure S4). Positive correlations were also observed between *T. viride* inoculation and expression of *AsMT1-3*, but negative correlations were noted between *AsMT1-3* expression and the level of Cd in soil. Neither the inoculation with *T. viride* nor the level of Cd in the soil was correlated with the expression of *AsMT4* (Supplementary Figure S4).

4. Discussion

Anthropogenic activities, like mining, the metallurgic industry, fossil fuel extraction, global transport, and agriculture, contribute to the increasing concentration of heavy metals in soil [62,63]. Even low concentrations of HM can become hazardous since they accumulate in the food chain [64,65]. Thus, it is essential to ensure that HM levels in soils and crops meet regulatory standards [66,67]. Since soil worldwide is contaminated with HM, there is an urgent need for practical, eco-friendly, and cost-effective remediation methods [68]. Phytoremediation is an eco-friendly and cost-effective method of removing hazardous pollutants, including HM, by plants. The effectiveness of this method can be increased by applying microorganisms that can interact with plants to counteract stressful environmental conditions and improve the plant's capacity to absorb pollutants. Understanding how microorganisms and plants respond to HM in their environment is crucial for developing this remediation method [69,70]. Among several microorganisms, fungi belonging to *Trichoderma* are considered suitable for phytoremediation due to their ability to use various materials as a carbon source, including plastics [71,72], their ability to promote plant growth and development [18,73–75], and their resistance to xenobiotics [76]. Analysis of the *Trichoderma harzianum* transcriptome in response to Cd treatment revealed the up-regulation of cellular homeostasis, vesicle-mediated transport, and RNA processing. Moreover, sulfur-compound biosynthesis and glutathione metabolism were induced [77]. *T. viride* strains tested in this study differed in Cu, Cd, and Zn tolerance. Strain T1 exhibited the highest tolerance towards Cd and the lowest towards Zn, and the opposite was observed for strain T6. The tolerance of *Trichoderma* to HM, as reported in the literature, is variable. For example, for Zn, the concentration that inhibited the growth was reported to be four mM for unclassified *Trichoderma* strains [78] and 11.47 mM for *Trichoderma atrioviride* [35]. Those values range from 1.8 mM [79] to 2.67 mM [35] for Cd. This comparison showed that the strains of *T. viride* analysed in this study were highly tolerant to Zn, whereas tolerance to Cd was similar to other *Trichoderma* species and isolates. In contrast, the tolerance to Cu of *T. viride* T1-6 was relatively low since *T. harzianum* and *T. virens* tolerated Cu up to 12 mM [35,80]. Due to the similar physicochemical properties of Zn and Cd, the *T. viride* strain T5 was selected for further experiments because this stain had a high tolerance to both cadmium and zinc.

A seed coat is a rigid structure that protects the embryo from soil pollutants, including HM. During germination, it ruptures, and Cd content increases in seeds [81]. The negative impact of Cd on germination was shown for bean (*Phaseolus vulgaris* L.) [81], *Sorghum bicolor* (L.) Moench [82], and wheat (*Triticum aestivum* L.) [83]. The amount of Cd needed to inhibit germination differs from species to species and within one species from cultivar to cultivar [84]. Interestingly, it was also shown that low levels of Cd might positively affect seed germination and seedling growth [85]. In this study, Cd inhibited oat seed germination and further seedling growth, and the negative effect was more substantial for higher cadmium concentrations. In uncontaminated soil, the mean value of Cd is 0.36 mg/kg, although the Cd concentrations greatly depend on continent, country, and soil types [86]. The mean concentration of Cd in European agricultural soil is 0.15 mg/kg; in the wide-ranging analysis, croplands containing as much as 52.99 mg/kg of Cd were detected [87]. The EU risk assessment predicted no effective Cd concentration in soil of 1.1 mg per kg of dry soil based on the toxicity for plants, invertebrates, and animals [87]. Therefore, this study used soil containing 1 mg/kg Cd as a control. The yield (as shown by the number of panicles and seeds) of oat plants was significantly decreased by cadmium. For example, plants grown in soil containing 20 mg/kg Cd produced less than half of the seeds produced by plants grown in the presence of 1 mg/kg Cd. Crop plants significantly differ in their tolerance to Cd contamination, and there are also substantial differences in Cd tolerance among cultivars of the same species. A significant decrease in rice yield in soil contaminated with 1 mg/kg and 3 mg/kg of Cd was observed; however, the number of seeds produced also depends on the tested cultivar [88]. Compared to other grasses, oat is

relatively tolerant to Cd stress [89]. Similar to our observations, Cd in soil up to 25 mg/kg did not significantly affect the growth of oat plants, but the yield was reduced [90].

Fungi belonging to *Trichoderma* are known for their plant growth-promoting properties [28,42,44,74,75]. Our previous studies show that *T. viride* can promote the growth of *B. napus* [28,74]. Barley plants inoculated with *Trichoderma* have up to 20% higher dry biomass than non-inoculated plants [43]. Similar reports are available for rice [91], sunflower [92], and maize [93]. The positive effect of microbial inoculation is often visible only in stress conditions [94]. For example, in control conditions, inoculation with *Trichoderma* did not improve wheat growth. Still, under severe water stress, inoculated wheat plants had higher dry biomass, downregulated water stress-related genes, and lower levels of proline, hydrogen peroxide, and malondialdehyde compared to non-inoculated plants [95]. In this study, seed germination and seedling growth were not improved by inoculation with *T. viride* in control conditions. Still, in the presence of cadmium, inoculated seeds germinated quicker, and the growth of seedling roots was enhanced in the presence of 150 and 254 µM Cd. The growth of plants was improved in soil containing 20 mg/kg Cd by inoculation with *T. viride*. The most significant effect was the increase in yield by *T. viride* inoculation observed in all Cd concentrations. The improved growth of plants in the presence of cadmium by inoculation with *T. harzianum* [96] and *T. atrioviride* [33] was shown for *B. juncea*. Plant growth promotion by fungi in HM-contaminated environments may be caused by increasing root absorption area and nutrient uptake [33,96]. The growth of *Cicer arietinum* was enhanced by inoculation with *Trichoderma* sp.; however, the effect was more substantial when plants were co-inoculated with *Trichoderma* sp. and *Pseudomonas fluorescence* [97]. The authors further highlighted that mechanisms that allow micro-organisms to adapt to and survive in HM-contaminated environments include binding HM to the cell wall and using siderophores to stop the HM from entering the cell, metalloproteases that bind and sequester HM in the cell, efflux pumps that eliminate HM from the cells, and antioxidant systems that reduce the negative effect of HM [97]. Combined with the growth-promoting properties of fungi belonging to *Trichoderma* (i.e., production of auxin analogues, organic acids, and siderophores), an increased tolerance to HM stress in inoculated plants was observed [29,30,96,97]. Interestingly, the inoculation with *T. harzianum* did not improve the growth of barley [98], and inoculation with *Trichoderma* sp. did not increase the growth and yield [90] in Cd-contaminated soil. Our recent study demonstrated a significant increase in *B. napus* yield by inoculation with *T. viride* in a field experiment [73]. Those results indicate that the improvement of plant growth and yield depends on fungi species/strain, plant species/cultivar, and the condition of the experiments.

Some crop plants are considered Cd-hyperaccumulators, e.g., several species belonging to *Brassicaceae*, some legumes, and some cereals [99]. For example, *B. juncea* accumulated more than 400 µg/g dry weight in leaves [100] and wheat up to 18 mg/kg dry weight [101]. Interestingly, Cd tolerance and accumulation are not usually related [99]. For example, wheat Cd-sensitive cultivars accumulated more Cd than Cd-tolerant ones [102]. Also, for oat, low and high Cd-accumulating cultivars were described [103]. The amount of Cd accumulated in crops and the location of Cd within plants are crucial in terms of nutrition. The World Health Organization recommends consuming no more than 25 µg of Cd monthly per kg of body weight. Oat can survive in soil polluted with heavy metals by extracting the metals from it and transferring them to above-ground parts [52,89,90,104]. The increased concentration of Cd in the soil led to an increased concentration of Cd in oat shoots. It is a widely observed phenomenon that the application of fungi belonging to *Trichoderma* increases the amount of extracted heavy metals, and this effect was observed for Cd but not for Cu and Zn in this study. In a study by Cao et al. [33], *B. juncea* inoculated with *T. atroviride* extracted 24% more Ni and 8% more Cd from the soil than non-inoculated plants did. Applying *Trichoderma* increased Cd content in shoots of maize plants growing in a Cd-contaminated soil by 38%, compared to non-inoculated plants [38]. A study showed that applying *T. harzianum* positively affected Cd uptake in barley *(H. vulgare* L.) [98]. The application of *Trichoderma* improved the solubility of heavy metals and, as

a result, increased their uptake by *Miscanthus x giganteus* J.M. Greef, *Panicum virgatum* L., *Phalaris arundinacea* L., and *Salix* sp. [26]. *A. sativa* has excellent potential to be used in phytoremediation since it can grow on low-quality soil, can tolerate higher concentrations of toxic metal ions, and has higher biomass than hyperaccumulators, making the whole process more efficient [89]. Cadmium uptake influences the uptake of micronutrients since Cd enters the root using micronutrient transporters such as transporters belonging to ZIP (zinc-regulated, iron-regulated transporter-like protein) [105,106] and NRAMP (natural resistance-associated macrophage protein) [107]. In this study, the increased Cd content in the shoots was positively correlated with the Zn content in the shoots but negatively with the content of Cu. Zn and Cd have similar physicochemical properties, and usually, the higher the Cd concentration in the soil, the lower the Zn content in plants [108,109]. However, various external factors, such as pH, affect the interplay between Cd and micronutrients in soil [110]. Moreover, within plants, Cd interacts with micronutrients. For example, high zinc reduces Cd transport to shoots, whereas, in low Zn conditions, zinc translocation to shoots is increased by Cd [111].

Metallothioneins' primary and firmly documented role in all living organisms is the homeostasis of micronutrients, mainly zinc and copper, and the detoxification of toxic metals, mostly cadmium [112]. In mammals, the expression of *MT*s in response to heavy metals is regulated by transcription factor MTF-1 (MRE-binding transcription factor-1) that binds to conserved regulatory motif MRE (metal response element) 5′-TGCRCNC-3′ present in *MT* promoter sequences. MTF-1 contains zinc finger domains and recognises MRE upon Zn metallation [113]. In yeast, the expression of metallothionein *CUP1* is regulated by copper-sensing transcription factor ACE1 that binds to regulator element 5′-HTHNNGCTGD-3′ [114]. Relatively little is known about the molecular mechanisms underlying the induction of the expression of *MTs* by cadmium; however, it was demonstrated that Cd induces the expression of *MT* in animals [115], plants [116], and bacteria [117]. Databases used for the prediction of regulatory elements in plant-promoter sequences (e.g., PlantCARE [118] and New PLACE [119]) lack plant-specific MREs; therefore, the animal MRE consensus sequence was used. The potential role of the regulatory motifs similar to animal MREs in the induction of the expression of plant MTs in response to heavy metals has been confirmed [56,58,120]. In addition, plant-specific MRE [56] and CuRE (copper response element) identified in *Chlamydomonas reinhardtii* [121] were used. Multiple potential regulatory elements involved in response to biotic factors [3] and heavy metals present in analysed promoters support the hypothesis that AsMTs are engaged in plant interaction with *T. viride* and/or Cd response. Previously, we identified multiple abiotic stress response elements in *AsMT* promoters [3], and the role of AsMTs in response to drought [17] and osmotic [16] stress was demonstrated. Moreover, we suggested the role of AsMT1 and AsMT3 in Cd detoxification and the role of AsMT4 as a Zn specificity filter [3]. Heterologous expression of *AsMT3* and *AsMT4* in *E. coli* cells improved bacterial growth in the presence of Zn and Cd. Previously, it was shown that the expression of *Brassica rapa* L. *MT* types 1-3 increases the yeast tolerance to Zn, Cd, and Pb [122]. In contrast, the expression of *B. napus MT4* in *E. coli* cells improved the growth of bacteria in the presence of Zn, decreased it in the presence of Cu, and had no effect on its growth in the presence of Cd. The positive/negative impact on bacteria growth also depended on the concentration of metals and/or IPTG [123]. Metallothioneins were shown to be involved in HM hyperaccumulation. For example, in a model plant for hyperaccumulators *Thlaspi caerulescens* (J. Presl & C. Presl) F.K. Mey., the expression of *MT1* and *MT2* was higher than in closely related *Arabidopsis thaliana* (L.) Heynh. [124]. Moreover, the expression of *T. caerulescens MT3* was higher in a population with high Cd-accumulation and -tolerance [125,126]. A limited amount of evidence also suggests that MTs are involved in the interaction between plants and microorganisms. For instance, the expression of canola MTs types 1-3 was higher in seedlings inoculated with plant growth-promoting fungi *T. viride*. However, the enhanced expression was observed only for some *T. viride* strains, whereas for others, the *MT1-3* expression was unaffected [73]. On the other hand, inoculation of *B. napus* with spores of

arbuscular mycorrhizal (AM) fungi increased the expression of *BnMT2* when plants were grown in soil without indigenous micro-organisms. In contrast, when indigenous microbes were present, the inoculation with AM spores decreased the expression of *BnMT2* [18]. Inoculation of willow (*Salix viminalis* L.), growing in heavy metal-contaminated soil with rhizosphere bacteria *Bacillus cereus*, increased the expression of *MT1*. No increase in *MT1* expression was observed after inoculation with the fungi *Hebeloma mesophaeum* [127]. In this study, Cd did not affect *AsMT* expression, but the inoculation with *T. viride* increased the expression of *AsMT1-3* in soil containing 1 mg/kg Cd and *AsMT4* in soil containing 10 mg/kg Cd. The response of MTs to metals depends not only on metal, plant species, and type of MT but also on plant organs and the amount of metal [128].

5. Conclusions

Inoculating *A. sativa* with *T. viride* might be a promising approach to increase the yield in Cd-contaminated soil without increasing the Cd content in plant tissues. Using *Trichoderma* in agriculture may improve the quantity and quality of produced food. This could be due to the fungi's ability to produce auxin analogues, thus improving plant root growth. Moreover, the secretion of organic acids and siderophores affects the bioavailability of micronutrients, and toxic HM could be bound to fungi cell walls. This is of great importance because it could limit the inclusion of Cd in the food chain and thus improve the health of animals and humans. Meat consumption worldwide is declining for financial, environmental, and ethical reasons, thus, the importance of crops such as oats in nutrition is constantly increasing. There is an urgent need to develop strategies to enhance crop yields in contaminated soils without reducing food quality.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods13152469/s1, Table S1: *cis*-regulatory elements of *AsMT1-4* promoters; Figure S1: comparison of the growth of *E. coli* cells transformed with empty pET21a(+) vector and pET21a(+) vectors harbouring coding regions of *AsMT1-4* in the presence of Zn and Cd ions. The relative growth rate is expressed as a slope of bacterial growth curves obtained by plotting optical density against time. Media were supplemented with two concentrations of Zn and Cd ions without IPTG. The results obtained for a given condition were compared, and distinct letters indicate significant differences between *E. coli* carrying different plasmids (Kruskal–Wallis, Mann–Whitney; $p < 0.05$).; Figure S2: photographs of oat plants inoculated and not inoculated with *Trichoderma viride* T5 growing in soil containing Cd after 6 months of growth.; Figure S3: Pearson correlation between shoot and root length, fresh and dry biomass, number of leaves, panicles and seeds, levels of Cu, Zn, and Cd, the amount of cadmium added to the soil, and the inoculation of oat seeds with *Trichoderma viride* T5 spores. Only significant correlations are shown. Figure S4: Pearson correlation between *AsMT1-4* expression (MT1-4), *Trichoderma viride* T5 inoculation, and the level of Cd in soil. Only significant correlations are shown.

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References

1. Briffa, J.; Sinagra, E.; Blundell, R. Heavy Metal Pollution in the Environment and their Toxicological Effects on Humans. *Heliyon* **2020**, *6*, e04691. [CrossRef] [PubMed]
2. Roberts, T.L. Cadmium and Phosphorous Fertilizers: The Issues and the Science. *Procedia Eng.* **2014**, *83*, 52–59. [CrossRef]
3. Konieczna, W.; Mierek-Adamska, A.; Chojnacka, N.; Antoszewski, M.; Szydłowska-Czerniak, A.; Dąbrowska, G.B. Characterization of the Metallothionein Gene Family in *Avena sativa* L. and the Gene Expression During Seed Germination and Heavy Metal Stress. *Antioxidants* **2023**, *12*, 1865. [CrossRef] [PubMed]
4. Rizwan, M.; Ali, S.; Rehman, M.Z.; Maqbool, A. A Critical Review on the Effects of Zinc at Toxic Levels of Cadmium in Plants. *Environ. Sci. Pollut. Res.* **2019**, *26*, 6279–6289. [CrossRef] [PubMed]
5. Stafford, A.; Jeyakumar, P.; Hedley, M.; Anderson, C. Influence of Soil Moisture Status on Soil Cadmium Phytoavailability and Accumulation in Plantain (*Plantago lanceolata*). *Soil Syst.* **2018**, *2*, 9. [CrossRef]
6. Ellen, T.P.; Costa, M. Carcinogenic Inorganic Chemicals. In *Comprehensive Toxicology (Second Edition)*; McQueen, C.A., Ed.; Elsevier: Oxford, UK, 2010; pp. 139–160. ISBN 978-0-08-046884-6.
7. Zulfiqar, U.; Jiang, W.; Wang, X.; Hussain, S.; Ahmad, M.; Maqsood, M.F.; Ali, N.; Ishfaq, M.; Kaleem, M.; Haider, F.U.; et al. Cadmium Phytotoxicity, Tolerance, and Advanced Remediation Approaches in Agricultural Soils; a Comprehensive Review. *Front. Plant Sci.* **2022**, *13*, 773815. [CrossRef] [PubMed]
8. European Commission, Directorate-General for Health and Food Safety. Commission Regulation (EU) 2023/915 of 25 April 2023 on Maximum Levels for Certain Contaminants in Food and Repealing Regulation (EC) No 1881/2006. *Off. J. Eur. Union* **2023**, *119*, 103–157.
9. Zimmerl, S.; Lafferty, J.; Buerstmayr, H. Assessing Diversity in *Triticum durum* Cultivars and Breeding Lines for High versus Low Cadmium Content in Seeds Using the CAPS Marker Usw47. *Plant Breed.* **2014**, *133*, 712–717. [CrossRef]
10. Song, W.; Chen, S.; Liu, J.; Chen, L.; Song, N.; Li, N.; Liu, B. Variation of Cd Concentration in Various Rice Cultivars and Derivation of Cadmium Toxicity Thresholds for Paddy Soil by Species-Sensitivity Distribution. *J. Integr. Agric.* **2015**, *14*, 1845–1854. [CrossRef]
11. Charkiewicz, A.E.; Omeljaniuk, W.J.; Nowak, K.; Garley, M.; Nikliński, J. Cadmium Toxicity and Health Effects—A Brief Summary. *Molecules* **2023**, *28*, 6620. [CrossRef]
12. Freisinger, E. Structural Features Specific to Plant Metallothioneins. *J. Biol. Inorg. Chem.* **2011**, *16*, 1035–1045. [CrossRef] [PubMed]
13. Dąbrowska, G.; Mierek-Adamska, A.; Goc, A. Characterisation of *Brassica napus* L. Metallothionein Genes (*BnMT*s) Expression in Organs and during Seed Germination. *Aust. J. Crop Sci.* **2013**, *7*, 1324–1332.
14. Blindauer, C.A.; Schmid, R. Cytosolic Metal Handling in Plants: Determinants for Zinc Specificity in Metal Transporters and Metallothioneins. *Metallomics* **2010**, *2*, 510–529. [CrossRef] [PubMed]
15. Huang, S.S.; Deng, J.S.; Chen, H.J.; Lin, Y.H.; Huang, G.J. Antioxidant Activities of Two Metallothionein-like Proteins from Sweet Potato (*Ipomoea batatas* [L.] Lam. 'Tainong 57') Storage Roots and Their Synthesized Peptides. *Bot. Stud.* **2014**, *55*, 64. [CrossRef] [PubMed]
16. Konieczna, W.; Mierek-Adamska, A.; Warchoł, M.; Skrzypek, E.; Dąbrowska, G.B. The Involvement of Metallothioneins and Stress Markers in Response to Osmotic Stress in *Avena sativa* L. *J. Agron. Crop Sci.* **2023**, *209*, 371–389. [CrossRef]
17. Konieczna, W.; Warchoł, M.; Mierek-Adamska, A.; Skrzypek, E.; Waligórski, P.; Piernik, A.; Dąbrowska, G.B. Changes in Physio-Biochemical Parameters and Expression of Metallothioneins in *Avena sativa* L. in Response to Drought. *Sci. Rep.* **2023**, *13*, 2486. [CrossRef] [PubMed]
18. Dąbrowska, G.; Baum, C.; Trejgell, A.; Hrynkiewicz, K. Impact of Arbuscular Mycorrhizal Fungi on the Growth and Expression of Gene Encoding Stress Protein - Metallothionein BnMT2 in the Non-Host Crop *Brassica napus* L. *J. Plant Nutr. Soil Sci.* **2014**, *177*, 459–467. [CrossRef]
19. Dąbrowska, G.; Hrynkiewicz, K.; Trejgell, A. Do Arbuscular Mycorrhizal Fungi Affect Metallothionein *MT2* Expression in *Brassica napus* L. Roots? *Acta Biol. Cracoviensia Ser. Bot.* **2012**, *54*, 34–39. [CrossRef]
20. Dąbrowska, G.; Mierek-Adamska, A.; Goc, A. Plant Metallothioneins: Putative Functions Identified by Promoter Analysis in silico. *Acta Biol. Cracoviensia Ser. Bot.* **2012**, *54*, 109–120. [CrossRef]
21. Gao, C.; Gao, K.; Yang, H.; Ju, T.; Zhu, J.; Tang, Z.; Zhao, L.; Chen, Q. Genome-Wide Analysis of Metallothionein Gene Family in Maize to Reveal Its Role in Development and Stress Resistance to Heavy Metal. *Biol. Res.* **2022**, *55*, 1–13. [CrossRef]
22. Yu, Q.; He, L.; Huo, C.; Jiang, X.; Chen, H.; Wang, R.; Tang, M.; Dong, L.; Chen, J.; Li, Y.; et al. Genome-Wide Identification and Expression Analysis of Heavy Metal Stress–Responsive Metallothionein Family Genes in *Nicotiana tabacum*. *Plant Mol. Biol. Rep.* **2020**, *39*, 443–454. [CrossRef]
23. Mirzahossini, Z.; Shabani, L.; Sabzalian, M.R.; Sharifi-Tehrani, M. ABC Transporter and Metallothionein Expression Affected by Ni and *Epichloe* Endophyte Infection in Tall Fescue. *Ecotoxicol. Environ. Saf.* **2015**, *120*, 13–19. [CrossRef] [PubMed]

24. Gilbert, J.A.; Neufeld, J.D. Life in a World Without Microbes. *PLoS Biol.* **2014**, *12*, e1002020. [CrossRef] [PubMed]
25. Dąbrowska, G.B.; Garstecka, Z.; Trejgell, A.; Dąbrowski, H.P.; Konieczna, W.; Szyp-Borowska, I. The Impact of Forest Fungi on Promoting Growth and Development of *Brassica napus* L. *Agronomy* **2021**, *11*, 2475. [CrossRef]
26. Kacprzak, M.J.; Rosikon, K.; Fijalkowski, K.; Grobelak, A. The Effect of *Trichoderma* on Heavy Metal Mobility and Uptake by *Miscanthus giganteus*, *Salix* sp., *Phalaris arundinacea*, and *Panicum virgatum*. *Appl. Environ. Soil Sci.* **2014**, *2014*, 506142. [CrossRef]
27. Chacón, M.R.; Rodríguez-Galán, O.; Benítez, T.; Sousa, S.; Rey, M.; Llobell, A.; Delgado-Jarana, J. Microscopic and Transcriptome Analyses of Early Colonization of Tomato Roots by *Trichoderma harzianum*. *Int. Microbiol.* **2007**, *10*, 19–27. [CrossRef] [PubMed]
28. Turkan, S.; Mierek-Adamska, A.; Kulasek, M.; Konieczna, W.B.; Dąbrowska, G.B. New Seed Coating Containing *Trichoderma viride* with Anti-Pathogenic Properties. *PeerJ* **2023**, *11*, e15392. [CrossRef] [PubMed]
29. Vinale, F.; Sivasithamparam, K.; Ghisalberti, E.L.; Woo, S.L.; Nigro, M.; Marra, R.; Lombardi, N.; Pascale, A.; Ruocco, M.; Lanzuise, S.; et al. *Trichoderma* Secondary Metabolites Active on Plants and Fungal Pathogens. *Open Mycol. J.* **2014**, *8*, 127–139. [CrossRef]
30. Chagas, L.F.B.; De Castro, H.G.; Colonia, B.S.O.; De Carvalho Filho, M.R.; Miller, L.D.O.; Chagas, A.F.J. Efficiency of *Trichoderma* spp. as a Growth Promoter of Cowpea (*Vigna unguiculata*) and Analysis of Phosphate Solubilization and Indole Acetic Acid Synthesis. *Braz. J. Bot.* **2016**, *39*, 437–445. [CrossRef]
31. Aishwarya, S.; Viswanath, H.S.; Singh, A.; Singh, R. Biosolubilization of Different Nutrients by *Trichoderma* spp. and Their Mechanisms Involved: A Review. *Int. J. Adv. Agric. Sci. Technol.* **2020**, *7*, 34–39.
32. Li, R.-X.; Cai, F.; Pang, G.; Shen, Q.-R.; Li, R.; Chen, W. Solubilisation of Phosphate and Micronutrients by *Trichoderma harzianum* and Its Relationship with the Promotion of Tomato Plant Growth. *PLoS ONE* **2015**, *10*, e0130081. [CrossRef] [PubMed]
33. Cao, L.; Jiang, M.; Zeng, Z.; Du, A.; Tan, H.; Liu, Y. *Trichoderma atroviride* F6 Improves Phytoextraction Efficiency of Mustard (*Brassica juncea* (L.) Coss. Var. foliosa Bailey) in Cd, Ni Contaminated Soils. *Chemosphere* **2008**, *71*, 1769–1773. [CrossRef] [PubMed]
34. Govarthanan, M.; Mythili, R.; Selvankumar, T.; Kamala-Kannan, S.; Kim, H. Myco-Phytoremediation of Arsenic- and Lead-Contaminated Soils by *Helianthus annuus* and Wood Rot Fungi, *Trichoderma* sp. Isolated from Decayed Wood. *Ecotoxicol. Environ. Saf.* **2018**, *151*, 279–284. [CrossRef] [PubMed]
35. López Errasquín, E.; Vázquez, C. Tolerance and Uptake of Heavy Metals by *Trichoderma atroviride* Isolated from Sludge. *Chemosphere* **2003**, *50*, 137–143. [CrossRef] [PubMed]
36. Mei, J.; Wang, L.; Jiang, X.; Wu, B.; Li, M. Functions of the C2H2 Transcription Factor Gene Thmea1 in *Trichoderma harzianum* under Copper Stress Based on Transcriptome Analysis. *Biomed. Res. Int.* **2018**, *2018*, 8149682. [CrossRef]
37. Tansengco, M.; Tejano, J.; Coronado, F.; Gacho, C.; Barcelo, J. Heavy Metal Tolerance and Removal Capacity of *Trichoderma* Species Isolated from Mine Tailings in Itogon, Benguet. *Environ. Nat. Resour.* **2018**, *16*, 39–57. [CrossRef]
38. Pehlivan, N.; Gedik, K.; Eltem, R.; Terzi, E. Dynamic Interactions of *Trichoderma harzianum* TS 143 from an Old Mining Site in Turkey for Potent Metal(Oid)s Phytoextraction and Bioenergy Crop Farming. *J. Hazard. Mater.* **2021**, *403*, 123609. [CrossRef] [PubMed]
39. Sahu, A.; Mandal, A.; Thakur, J.; Manna, M.C.; Rao, A.S. Exploring Bioaccumulation Efficacy of *Trichoderma viride*: An Alternative Bioremediation of Cadmium and Lead. *Natl. Acad. Sci. Lett.* **2012**, *35*, 299–302. [CrossRef]
40. Yaghoubian, Y.; Siadat, S.A.; Moradi Telavat, M.R.; Pirdashti, H.; Yaghoubian, I. Bio-Removal of Cadmium from Aqueous Solutions by Filamentous Fungi: *Trichoderma* Spp. and Piriformospora Indica. *Environ. Sci. Pollut. Res.* **2019**, *26*, 7863–7872. [CrossRef] [PubMed]
41. Zhang, S.; Gan, Y.; Xu, B. Application of Plant-Growth-Promoting Fungi *Trichoderma longibrachiatum* T6 Enhances Tolerance of Wheat to Salt Stress through Improvement of Antioxidative Defense System and Gene Expression. *Front. Plant Sci.* **2016**, *7*, 1405. [CrossRef]
42. Harman, G.E.; Howell, C.R.; Viterbo, A.; Chet, I.; Lorito, M. *Trichoderma* Species—Opportunistic, Avirulent Plant Symbionts. *Nat. Rev. Microbiol.* **2004**, *2*, 43–56. [CrossRef] [PubMed]
43. Moya, P.; Barrera, V.; Cipollone, J.; Bedoya, C.; Kohan, L.; Toledo, A.; Sisterna, M. New Isolates of *Trichoderma* spp. as Biocontrol and Plant Growth–Promoting Agents in the Pathosystem *Pyrenophora teres* Barley in Argentina. *Biol. Control* **2020**, *141*, 104152. [CrossRef]
44. Tyśkiewicz, R.; Nowak, A.; Ozimek, E.; Jaroszuk-Ściseł, J. *Trichoderma*: The Current Status of Its Application in Agriculture for the Biocontrol of Fungal Phytopathogens and Stimulation of Plant Growth. *Int. J. Mol. Sci.* **2022**, *23*, 2329. [CrossRef] [PubMed]
45. Koide, R.; Li, M.; Lewis, J.; Irby, C. Role of Mycorrhizal Infection in the Growth and Reproduction of Wild vs. Cultivated Plants. *Oecologia* **1988**, *77*, 537–543. [CrossRef] [PubMed]
46. Mushtaq, A.; Gul-Zaffar, G.Z.; Dar, A.D.; Mehfuza Habib, M.H. Review on Oat (*Avena sativa* L.) as a Dual-Purpose Crop. *Sci. Res. Essays* **2014**, *9*, 52–59. [CrossRef]
47. Kim, I.-S.; Hwang, C.-W.; Yang, W.-S.; Kim, C.-H. Multiple Antioxidative and Bioactive Molecules of Oats (*Avena sativa* L.) in Human Health. *Antioxidants* **2021**, *10*, 1454. [CrossRef] [PubMed]
48. Ehlers, W.; Khosla, B.K.; Köpke, U.; Stülpnagel, R.; Böhm, W.; Baeumer, K. Tillage Effects on Root Development, Water Uptake and Growth of Oats. *Soil Tillage Res.* **1980**, *1*, 19–34. [CrossRef]
49. Hoad, S.; Russell, G.; Lucas, M.; Bingham, I. The Management of Wheat, Barley, and Oat Root Systems. *Adv. Agron.* **2001**, *74*, 193–246. [CrossRef]

50. Khan, T.A.; Nadeem, F.; Gao, Y.; Yang, Y.; Wang, X.; Zeng, Z.; Hu, Y. A Larger Root System in Oat (*Avena nuda* L.) Is Coupled with Enhanced Biomass Accumulation and Hormonal Alterations under Low Nitrogen. *Appl. Ecol. Environ. Res.* **2019**, *17*, 4631–4653. [CrossRef]
51. Bjerre, G.K.; Schierup, H.-H. Uptake of Six Heavy Metals by Oat as Influenced by Soil Type and Additions of Cadmium, Lead, Zinc and Copper. *Plant Soil* **1985**, *88*, 57–69. [CrossRef]
52. Tuma, J.; Skalicky, M.; Tumova, L.; Flidr, J. Influence of Cadmium Dose and Form on the Yield of Oat (*Avena sativa* L.) and the Metal Distribution in the Plant. *J. Elem.* **2014**, *19*, 795–810. [CrossRef]
53. Sambrook, J.; Russell, D.W. *Molecular Cloning a Laboratory Manual*; Cold Spring Harbor Laboratory Press: New York, NY, USA, 2001; ISBN 978-85-7811-079-6.
54. Yoshida, N.; Ikeda, R.; Okuno, T. Identification and Characterization of Heavy Metal-Resistant Unicellular Alga Isolated from Soil and Its Potential for Phytoremediation. *Bioresour. Technol.* **2006**, *97*, 1843–1849. [CrossRef] [PubMed]
55. Ranal, M.A.; de Santana, D.G. How and Why to Measure the Germination Process? *Braz. J. Bot.* **2006**, *29*, 1–11. [CrossRef]
56. Qi, X.; Zhang, Y.; Chai, T. Characterization of a Novel Plant Promoter Specifically Induced by Heavy Metal and Identification of the Promoter Regions Conferring Heavy Metal Responsiveness. *Plant Physiol.* **2007**, *143*, 50–59. [CrossRef] [PubMed]
57. Stuart, G.W.; Searle, P.F.; Palmiter, R.D. Identification of Multiple Metal Regulatory Elements in Mouse Metallothionein-I Promoter by Assaying Synthetic Sequences. *Nature* **1985**, *317*, 828–831. [CrossRef] [PubMed]
58. Dong, C.-J.J.; Wang, Y.; Yu, S.-S.S.; Liu, J.-Y.Y. Characterization of a Novel Rice Metallothionein Gene Promoter: Its Tissue Specificity and Heavy Metal Responsiveness. *J. Integr. Plant Biol.* **2010**, *52*, 914–924. [CrossRef]
59. Quinn, J.M.; Merchant, S. Two Copper-Responsive Elements Associated with the *Chlamydomonas Cyc6* Gene Function as Targets for Transcriptional Activators. *Plant Cell* **1995**, *7*, 623–638. [PubMed]
60. Yang, Z.; Wang, K.; Aziz, U.; Zhao, C.; Zhang, M. Evaluation of duplicated reference genes for quantitative real-time PCR analysis in genome unknown hexaploid oat (*Avena sativa* L.). *Plant Methods* **2020**, *16*, 138. [CrossRef]
61. RStudio Team. *RStudio: Integrated Development for R*; RStudio, Inc.: Boston, MA, USA, 2020.
62. Zbieralski, K.; Staszewski, J.; Konczak, J.; Lazarewicz, N.; Nowicka-Kazmierczak, M.; Wawrzycka, D.; Maciaszczyk-Dziubinska, E. Multilevel Regulation of Membrane Proteins in Response to Metal and Metalloid Stress: A Lesson from Yeast. *Int. J. Mol. Sci.* **2024**, *25*, 4450. [CrossRef] [PubMed]
63. Khan, Z.; Elahi, A.; Bukhari, D.A.; Rehman, A. Cadmium Sources, Toxicity, Resistance and Removal by Microorganisms—A Potential Strategy for Cadmium Eradication. *J. Saudi Chem. Soc.* **2022**, *26*, 101569. [CrossRef]
64. Guo, L.; Li, Z.; Xu, J. Effects of Cadmium Stress on Bacterial and Fungal Communities in the Whitefly *Bemisia tabaci*. *Int. J. Mol. Sci.* **2023**, *24*, 13588. [CrossRef]
65. Peralta-Videa, J.R.; Lopez, M.L.; Narayan, M.; Saupe, G.; Gardea-Torresdey, J. The Biochemistry of Environmental Heavy Metal Uptake by Plants: Implications for the Food Chain. *Int. J. Biochem. Cell Biol.* **2009**, *41*, 1665–1677. [CrossRef]
66. Khan, M.A.; Khan, S.; Khan, A.; Alam, M. Soil Contamination with Cadmium, Consequences and Remediation Using Organic Amendments. *Sci. Total Environ.* **2017**, *601–602*, 1591–1605. [CrossRef] [PubMed]
67. Arao, T.; Ishikawa, S.; Murakami, M.; Abe, K.; Maejima, Y.; Makino, T. Heavy Metal Contamination of Agricultural Soil and Countermeasures in Japan. *Paddy Water Environ.* **2010**, *8*, 247–257. [CrossRef]
68. Mierek-Adamska, A.; Dąbrowska, G.B.; Goc, A. Genetically modified plants and strategies of soil remediation from haevy metals. *Adv. Cell Biol.* **2009**, *36*, 649–662.
69. Dąbrowska, G.; Hrynkiewicz, K.; Trejgell, A.; Baum, C. The Effect of Plant-Growth-Promoting Rhizobacteria on the Phytoextraction of Cd and Zn by *Brassica napus* L. *Int. J. Phytoremediat.* **2017**, *19*, 597–604. [CrossRef] [PubMed]
70. Khan, A.R.; Ullah, I.; Waqas, M.; Shahzad, R.; Hong, S.J.; Park, G.S.; Jung, B.K.; Lee, I.J.; Shin, J.H. Plant Growth-Promoting Potential of Endophytic Fungi Isolated from *Solanum nigrum* Leaves. *World J. Microbiol. Biotechnol.* **2015**, *31*, 1461–1466. [CrossRef]
71. Znajewska, Z.; Dąbrowska, G.B.; Hrynkiewicz, K.; Janczak, K. Biodegradation of Polycaprolactone by *Trichoderma viride* Fungi. *Przem. Chem.* **2018**, *97*, 1676–1679. [CrossRef]
72. Dąbrowska, G.B.; Garstecka, Z.; Olewnik-Kruszkowska, E.; Szczepańska, G.; Ostrowski, M.; Mierek-Adamska, A. Comparative Study of Structural Changes of Polylactide and Poly(Ethylene Terephthalate) in the Presence of *Trichoderma viride*. *Int. J. Mol. Sci.* **2021**, *22*, 3491. [CrossRef]
73. Garstecka, Z.; Antoszewski, M.; Mierek-Adamska, A.; Krauklis, D.; Niedojadło, K.; Kaliska, B.; Hrynkiewicz, K.; Dąbrowska, G.B. *Trichoderma viride* Colonizes the Roots of *Brassica napus* L., Alters the Expression of Stress-Responsive Genes, and Increases the Yield of Canola under Field Conditions during Drought. *Int. J. Mol. Sci.* **2023**, *24*, 15349. [CrossRef]
74. Znajewska, Z.; Narbutt, O.; Dąbrowska, G.B.; Narbutt, O. *Trichoderma viride* Strains Stimulating the Growth and Development of Winter Rapeseed (*Brassica napus* L.). *Prog. Plant Prot.* **2018**, *58*, 264–269. [CrossRef]
75. Antoszewski, M.; Mierek-Adamska, A.; Dąbrowska, G.B. The Importance of Microorganisms for Sustainable Agriculture—A Review. *Metabolites* **2022**, *12*, 1100. [CrossRef] [PubMed]
76. Balcázar-López, E.; Méndez-Lorenzo, L.H.; Batista-García, R.A.; Esquivel-Naranjo, U.; Ayala, M.; Kumar, V.V.; Savary, O.; Cabana, H.; Herrera-Estrella, A.; Folch-Mallol, J.L. Xenobiotic Compounds Degradation by Heterologous Expression of a *Trametes sanguineus* Laccase in *Trichoderma atroviride*. *PLoS ONE* **2016**, *11*, e0147997. [CrossRef] [PubMed]

77. Oshiquiri, L.H.; dos Santos, K.R.A.; Ferreira Junior, S.A.; Steindorff, A.S.; Barbosa Filho, J.R.; Mota, T.M.; Ulhoa, C.J.; Georg, R.C. *Trichoderma harzianum* Transcriptome in Response to Cadmium Exposure. *Fungal Genet. Biol.* **2020**, *134*, 103281. [CrossRef] [PubMed]
78. Tripathi, P.; Singh, P.C.; Mishra, A.; Chauhan, P.S.; Dwivedi, S.; Bais, R.T.; Tripathi, R.D. *Trichoderma*: A Potential Bioremediator for Environmental Clean Up. *Clean Technol. Environ. Policy* **2013**, *15*, 541–550. [CrossRef]
79. Nongmaithem, N.; Roy, A.; Bhattacharya, P.M. Screening of *Trichoderma* Isolates for Their Potential of Biosorption of Nickel and Cadmium. *Braz. J. Microbiol.* **2016**, *47*, 305–313. [CrossRef] [PubMed]
80. Siddiquee, S.; Aishah, S.N.; Azad, S.A.; Shafawati, S.N.; Naher, L. Tolerance and Biosorption Capacity of Zn^{2+}, Pb^{2+}, Ni^{3+} and Cu^{2+} by Filamentous Fungi (*Trichoderma harzianum*, *T. aureoviride* and *T. virens*). *Adv. Biosci. Biotechnol.* **2013**, *4*, 570–583. [CrossRef]
81. Sfaxi-Bousbih, A.; Chaoui, A.; El Ferjani, E. Unsuitable Availability of Nutrients in Germinating Bean Embryos Exposed to Copper Excess. *Biol. Trace Elem. Res.* **2010**, *135*, 295–303. [CrossRef] [PubMed]
82. Kuriakose, S.V.; Prasad, M.N.V. Cadmium Stress Affects Seed Germination and Seedling Growth in *Sorghum bicolor* (L.) Moench by Changing the Activities of Hydrolyzing Enzymes. *Plant Growth Regul.* **2008**, *54*, 143–156. [CrossRef]
83. Ahmad, I.; Akhtar, M.; Zahir, Z.; Jamil, A. Effect of Cadmium on Seed Germination and Seedling Growth of Four Wheat (*Triticum aestivum* L.) Cultivars. *Pak. J. Bot.* **2012**, *44*, 1569–1574.
84. Huybrechts, M.; Cuypers, A.; Deckers, J.; Iven, V.; Vandionant, S.; Jozefczak, M.; Hendrix, S. Cadmium and Plant Development: An Agony from Seed to Seed. *Int. J. Mol. Sci.* **2019**, *20*, 3971. [CrossRef] [PubMed]
85. Carvalho, M.E.A.; Agathokleous, E.; Nogueira, M.L.; Brunetto, G.; Brown, P.H.; Azevedo, R.A. Neutral-to-Positive Cadmium Effects on Germination and Seedling Vigor, with and without Seed Priming. *J. Hazard. Mater.* **2023**, *448*, 130813. [CrossRef] [PubMed]
86. Kubier, A.; Wilkin, R.T.; Pichler, T. Cadmium in Soils and Groundwater: A Review. *Appl. Geochem.* **2019**, *108*, 104388. [CrossRef] [PubMed]
87. Joint Research Centre (European Commission); Ballabio, C.; Jones, A.; Montanarella, L.; Toth, G. *Cadmium in the Soils of the EU: Analysis of LUCAS Soils Data for the Review of Fertilizer Directive*; Publications Office of the European Union: Luxembourg, 2023; ISBN 978-92-79-66931-6.
88. Siddique, A.B.; Rahman, M.M.; Islam, M.R.; Mondal, D.; Naidu, R. Response of Iron and Cadmium on Yield and Yield Components of Rice and Translocation in Grain: Health Risk Estimation. *Front. Environ. Sci.* **2021**, *9*, 716770. [CrossRef]
89. Ebbs, S.D.; Kochian, L.V. Phytoextraction of Zinc by Oat *Avena sativa*, Barley *Hordeum vulgare*, and Indian Mustard *Brassica juncea*. *Environ. Sci. Technol.* **1998**, *32*, 802–806. [CrossRef]
90. Marchel, M.; Kaniuczak, J.; Hajduk, E.; Właśniewski, S. Response of Oat (*Avena sativa*) to the Addition Cadmium to Soil Inoculation with the Genus *Trichoderma* Fungi. *J. Elem.* **2018**, *23*, 471–482. [CrossRef]
91. Doni, F.; Isahak, A.; Che Mohd Zain, C.R.; Wan Yusoff, W.M. Physiological and Growth Response of Rice Plants (*Oryza sativa* L.) to *Trichoderma* spp. Inoculants. *AMB Express* **2014**, *4*, 45. [CrossRef] [PubMed]
92. Singh, B.N.; Singh, A.; Singh, B.R.; Singh, H.B. *Trichoderma harzianum* Elicits Induced Resistance in Sunflower Challenged by *Rhizoctonia solani*. *J. Appl. Microbiol.* **2014**, *116*, 654–666. [CrossRef] [PubMed]
93. Akladious, S.A.; Abbas, S.M. Application of *Trichoderma harzianum* T22 as a Biofertilizer Potential in Maize Growth. *J. Plant Nutr.* **2014**, *37*, 30–49. [CrossRef]
94. Xun, F.; Xie, B.; Liu, S.; Guo, C. Effect of Plant Growth-Promoting Bacteria (PGPR) and Arbuscular Mycorrhizal Fungi (AMF) Inoculation on Oats in Saline-Alkali Soil Contaminated by Petroleum to Enhance Phytoremediation. *Environ. Sci. Pollut. Res.* **2015**, *22*, 598–608. [CrossRef]
95. Illescas, M.; Morán-Diez, M.E.; Martínez de Alba, Á.E.; Hermosa, R.; Monte, E. Effect of *Trichoderma* Asperellum on Wheat Plants' Biochemical and Molecular Responses, and Yield under Different Water Stress Conditions. *Int. J. Mol. Sci.* **2022**, *23*, 6782. [CrossRef] [PubMed]
96. Chauhan, J.S.; Rai, J.P.N. Phytoextraction of Soil Cadmium and Zinc by Microbes-Inoculated Indian Mustard (*Brassica juncea*). *J. Plant Interact.* **2009**, *4*, 279–287. [CrossRef]
97. Syed, A.; Elgorban, A.M.; Bahkali, A.H.; Eswaramoorthy, R.; Iqbal, R.K.; Danish, S. Metal-Tolerant and Siderophore Producing Pseudomonas Fluorescence and *Trichoderma* spp. Improved the Growth, Biochemical Features and Yield Attributes of Chickpea by Lowering Cd Uptake. *Sci. Rep.* **2023**, *13*, 4471. [CrossRef] [PubMed]
98. Taghavi Ghasemkheili, F.; Ekelund, F.; Johansen, J.L.; Pirdashti, H.; Ghadirnezhad Shiade, S.R.; Fathi, A.; Kjøller, R. Ameliorative Effects of *Trichoderma harzianum* and Rhizosphere Soil Microbes on Cadmium Biosorption of Barley (*Hordeum vulgare* L.) in Cd-Polluted Soil. *J. Plant Nutr. Soil Sci.* **2022**, *22*, 527–539. [CrossRef]
99. Niu, L.; Li, C.; Wang, W.; Zhang, J.; Scali, M.; Li, W.; Liu, H.; Tai, F.; Hu, X.; Wu, X. Cadmium Tolerance and Hyperaccumulation in Plants—A Proteomic Perspective of Phytoremediation. *Ecotoxicol. Environ. Saf.* **2023**, *256*, 114882. [CrossRef] [PubMed]
100. Haag-Kerwer, A.; Schäfer, H.J.; Heiss, S.; Walter, C.; Rausch, T. Cadmium Exposure in *Brassica juncea* Causes a Decline in Transpiration Rate and Leaf Expansion without Effect on Photosynthesis. *J. Exp. Bot.* **1999**, *50*, 1827–1835. [CrossRef]
101. Zhang, Q.; Li, R.-Y.; Xu, X.-H.; Xie, X.-J.; Chambe, E.A. Effects of cadmium pollution in soil on growth and cadmium uptake of wheat. *J. Agric. Resour. Environ.* **2019**, *36*, 522–527. [CrossRef]

102. Jian, M.; Zhang, D.; Wang, X.; Wei, S.; Zhao, Y.; Ding, Q.; Han, Y.; Ma, L. Differential Expression Pattern of the Proteome in Response to Cadmium Stress Based on Proteomics Analysis of Wheat Roots. *BMC Genom.* **2020**, *21*, 343. [CrossRef] [PubMed]
103. Tanhuanpää, P.; Kalendar, R.; Schulman, A.H.; Kiviharju, E. A Major Gene for Grain Cadmium Accumulation in Oat (*Avena sativa* L.). *Genome* **2007**, *50*, 588–594. [CrossRef]
104. Gutiérrez-Ginés, M.J.; Pastor, J.; Hernández, A.J. Effect of Heavy Metals from Mine Soils on *Avena sativa* L. and Education Strategies. *Fresenius Environ. Bull.* **2010**, *19*, 2083–2086.
105. Fan, S.K.; Fang, X.Z.; Guan, M.Y.; Ye, Y.Q.; Lin, X.Y.; Du, S.T.; Jin, C.W. Exogenous Abscisic Acid Application Decreases Cadmium Accumulation in *Arabidopsis* Plants, Which Is Associated with the Inhibition of IRT1-Mediated Cadmium Uptake. *Front. Plant Sci.* **2014**, *5*, 721. [CrossRef]
106. Tan, L.; Qu, M.; Zhu, Y.; Peng, C.; Wang, J.; Gao, D.; Chen, C. ZINC TRANSPORTER5 and ZINC TRANSPORTER9 Function Synergistically in Zinc/Cadmium Uptake. *Plant Physiol.* **2020**, *183*, 1235–1249. [CrossRef]
107. Tang, L.; Dong, J.; Qu, M.; Lv, Q.; Zhang, L.; Peng, C.; Hu, Y.; Li, Y.; Ji, Z.; Mao, B.; et al. Knockout of *OsNRAMP5* Enhances Rice Tolerance to Cadmium Toxicity in Response to Varying External Cadmium Concentrations via Distinct Mechanisms. *Sci. Total Environ.* **2022**, *832*, 155006. [CrossRef]
108. Vasiliadou, S.; Dordas, C. Increased Concentration Of Soil Cadmium Affects On Plant Growth, Dry Matter Accumulation, Cd, And Zn Uptake Of Different Tobacco Cultivars (*Nicotiana tabacum* L.). *Int. J. Phytoremediat.* **2009**, *11*, 115–130. [CrossRef]
109. Murtaza, G.; Javed, W.; Hussain, A.; Qadir, M.; Aslam, M. Soil-Applied Zinc and Copper Suppress Cadmium Uptake and Improve the Performance of Cereals and Legumes. *Int. J. Phytoremediat.* **2017**, *19*, 199–206. [CrossRef]
110. Yang, Y.; Li, Y.; Wang, M.; Chen, W.; Dai, Y. Limestone Dosage Response of Cadmium Phytoavailability Minimization in Rice: A Trade-off Relationship between Soil pH and Amorphous Manganese Content. *J. Hazard. Mater.* **2021**, *403*, 123664. [CrossRef]
111. Palusińska, M.; Barabasz, A.; Kozak, K.; Papierniak, A.; Maślińska, K.; Antosiewicz, D.M. Zn/Cd Status-Dependent Accumulation of Zn and Cd in Root Parts in Tobacco Is Accompanied by Specific Expression of *ZIP* Genes. *BMC Plant Biol.* **2020**, *20*, 37. [CrossRef]
112. Ziller, A.; Fraissinet-Tachet, L. Metallothionein Diversity and Distribution in the Tree of Life: A Multifunctional Protein. *Metallomics* **2018**, *10*, 1549–1559. [CrossRef]
113. Bulathge, A.W.; Villones, R.L.E.; Herbert, F.C.; Gassensmith, J.J.; Meloni, G. Comparative Cisplatin Reactivity towards Human Zn7-Metallothionein-2 and MTF-1 Zinc Fingers: Potential Implications in Anticancer Drug Resistance. *Metallomics* **2022**, *14*, mfac061. [CrossRef]
114. Shi, H.; Jiang, Y.; Yang, Y.; Peng, Y.; Li, C. Copper Metabolism in *Saccharomyces cerevisiae*: An Update. *Biometals* **2021**, *34*, 3–14. [CrossRef]
115. Priante, E.; Pietropoli, E.; Piva, E.; Santovito, G.; Schumann, S.; Irato, P. Cadmium–Zinc Interaction in *Mus musculus* Fibroblasts. *Int. J. Mol. Sci.* **2022**, *23*, 12001. [CrossRef]
116. Rono, J.K.; Le Wang, L.; Wu, X.C.; Cao, H.W.; Zhao, Y.N.; Khan, I.U.; Yang, Z.M. Identification of a New Function of Metallothionein-like Gene *OsMT1e* for Cadmium Detoxification and Potential Phytoremediation. *Chemosphere* **2021**, *265*, 129136. [CrossRef]
117. Enshaei, M.; Khanafari, A.; Sepahey, A.A. Metallothionein Induction in Two Species of *Pseudomonas* Exposed to Cadmium and Copper Contamination. *Iran. J. Environ. Health Sci. Eng.* **2010**, *7*, 287–298.
118. Lescot, M.; Déhais, P.; Thijs, G.; Marchal, K.; Moreau, Y.; Van De Peer, Y.; Rouzé, P.; Rombauts, S. PlantCARE, a Database of Plant Cis-Acting Regulatory Elements and a Portal to Tools for in Silico Analysis of Promoter Sequences. *Nucleic Acids Res.* **2002**, *30*, 325–327. [CrossRef]
119. Higo, K.; Ugawa, Y.; Iwamoto, M.; Korenaga, T. Plant Cis-Acting Regulatory DNA Elements (PLACE) Database: 1999. *Nucleic Acids Res.* **1999**, *27*, 297–300. [CrossRef]
120. Lü, S.; Gu, H.; Yuan, X.; Wang, X.; Wu, A.M.; Qu, L.; Liu, J.Y. The GUS Reporter-Aided Analysis of the Promoter Activities of a Rice Metallothionein Gene Reveals Different Regulatory Regions Responsible for Tissue-Specific and Inducible Expression in Transgenic *Arabidopsis*. *Transgenic Res.* **2007**, *16*, 177–191. [CrossRef]
121. Quinn, J.M.; Kropat, J.; Merchant, S. Copper Response Element and Crr1-Dependent Ni^{2+}-Responsive Promoter for Induced, Reversible Gene Expression in *Chlamydomonas reinhardtii*. *Eukaryot. Cell* **2003**, *2*, 995–1002. [CrossRef]
122. Liu, J.; Zhang, J.; Kim, S.H.; Lee, H.S.; Marinoia, E.; Song, W.Y. Characterization of *Brassica rapa* Metallothionein and Phytochelatin Synthase Genes Potentially Involved in Heavy Metal Detoxification. *PLoS ONE* **2021**, *16*, e0252899. [CrossRef]
123. Mierek-Adamska, A.; Dąbrowska, G.B.; Blindauer, C.A. The Type 4 Metallothionein from *Brassica napus* Seeds Folds in a Metal-Dependent Fashion and Favours Zinc over Other Metals. *Metallomics* **2018**, *10*, 1430–1443. [CrossRef]
124. Roosens, N.H.; Leplae, R.; Bernard, C.; Verbruggen, N. Variations in Plant Metallothioneins: The Heavy Metal Hyperaccumulator *Thlaspi caerulescens* as a Study Case. *Planta* **2005**, *222*, 716–729. [CrossRef]
125. Roosens, N.H.; Bernard, C.; Leplae, R.; Verbruggen, N. Evidence for Copper Homeostasis Function of Metallothionein (MT3) in the Hyperaccumulator *Thlaspi caerulescens*. *FEBS Lett.* **2004**, *577*, 9–16. [CrossRef] [PubMed]
126. Hassinen, V.H.; Tuomainen, M.; Peräniemi, S.; Schat, H.; Kärenlampi, S.O.; Tervahauta, A.I. Metallothioneins 2 and 3 Contribute to the Metal-Adapted Phenotype but Are Not Directly Linked to Zn Accumulation in the Metal Hyperaccumulator, *Thlaspi caerulescens*. *J. Exp. Bot.* **2009**, *60*, 187–196. [CrossRef] [PubMed]

127. Hrynkiewicz, K.; Dąbrowska, G.; Baum, C.; Niedojadło, K.; Leinweber, P. Interactive and single effects of ectomycorrhiza formation and *Bacillus cereus* on metallothionein *MT1* expression and phytoextraction of Cd and Zn by willows. *Water Air Soil Pollut.* **2012**, *223*, 957–968. [CrossRef] [PubMed]
128. Pagani, M.A.; Tomas, M.; Cattillo, J.; Bofill, R.; Capdevila, M.; Atrian, S.; Andreo, C.S. The Response of the Different Soybean Metallothionein Isoforms to Cadmium Intoxication. *J. Inorg. Biochem.* **2012**, *117*, 306–315. [CrossRef]

Dorobek naukowy

1. Wykaz publikacji niebędących podstawą rozprawy doktorskiej

1.	Turkan S., Mierek-Adamska A., Kulasek M., Konieczna W. B. i Dąbrowska G. B. New seed coating containing *Trichoderma viride* with anti-pathogenic properties. *PeerJ,* 2023, 11, e15392. 10.7717/peerj.15392	IF: 2,7 $IF_{5\text{-year}}$: 2,8 MNiSW: 100
2.	Dąbrowska G. B., Garstecka Z., Trejgell A., Dąbrowski H. P., Konieczna W. i Szyp-Borowska I. The Impact of Forest Fungi on Promoting Growth and Development of *Brassica napus* L. *Agronomy*, 2021, 11(12), 2475. 10.3390/agronomy11122475	IF: 3,3 $IF_{5\text{-year}}$: 3,7 MNiSW: 100

2. Inne osiągnięcia naukowe

2.1. Uzyskane granty

Lp.	Tytuł konkursu	Rok	Opis
1.	Minigranty AST 2021[1]	2021	Pokrycie kosztów wyjazdu na staż krajowy do Instytutu Fizjologii Roślin im. Franciszka Górskiego Państwowej Akademii Nauk w Krakowie w dniach 09.05-28.05.2021
2.	Grants 4NCU Students I edycja[2]	2021	Realizacja projektu badawczego pt. „Biological protection of crops to reduce the accumulation of heavy metals in seeds used in human nutrition”
3.	Mobility for Doctoral Students[2]	2022	Pokrycie kosztów wyjazdu na staż międzynarodowy do Warwick University, Department of Chemistry, Coventry, Wielka Brytania w dniach 17.01-16.02.2023
4.	Minigranty AST 2022	2022	Pokycie kosztów udziału w międzynarodowej konferencji „Mendel Genetics Conference” w Brnie, Czechy
5.	Publikacje - konkurs dla doktorantów[2]	2023	Pokrycie kosztów publikacji „Changes in physio-biochemical parameters and expression of metallothioneins in *Avena sativa* L. in response to drought.” Scientific Reports, 2023, 13(1), 2486
6.	Minigranty AST I/2023[1]	2023	Pokrycie kosztów udziału w międzynarodowej konferencji „Plant Biology Europe” Marsylia, Francja
7.	Grants 4NCU Students VI edycja[2]	2023	Realizacja projektu badawczego pt. „Understanding the role of zinc in oat seed germination during drought”

[1] sfinansowane w ramach działalności statutowej Szkoły Doktorskiej Nauk Ścisłych i Przyrodniczych Uniwersytetu Mikołaja Kopernika

[2] sfinansowane przez Uniwersytet Mikołaja Kopernika w ramach projektu Uczelnia Badawcza - Inicjatywa Doskonałości (IDUB)

2.2. Udział w realizacji projektów

1.	Wykonawca w projekcie przedwdrożeniowym „*Żywność zwiększająca odporność na wirusy*”, finansowanym w ramach programu MNiSW pn. Inkubator Innowacyjności UMK 4.0.
2.	Wykonawca w projekcie „*Innowacyjne, płynne nawozy azotowe z krzemem i mikroelementami wzbogacone w mikroorganizmy oraz dodatki funkcjonalne”* 09.2021-02.2023, projekt nr 5301.00000190 – WNBiW / 321_21 Grupa Azoty Zakłady Chemiczne SA., Police, nr 23/2021/GR, badania prowadzone w ramach projektu badawczo - rozwojowego nr POIR.01.01.01.-00-1313/20
3.	Wykonawca w projekcie „*Więcej wiedzy na hektar, mniej chemii na hektar – badania wstępne nad biologicznymi rozwiązaniami dla rolnictwa”* 06.2021-11.2021, badania finansowane z funduszy Urzędu Marszałkowskiego Województwa Kujawsko- Pomorskiego

2.3. Konferencje naukowe

Lp.	Nazwa i miejsce konferencji	Miesiąc i rok	Tytuł i forma wystąpienia	Autorzy

1.	Międzynarodowa konferencja online „Plant productivity and food safety: Soil science, Microbiology, Agricultural Genetics and Food quality”, Toruń	IX 2021	The expression of oat metallothioneins increases under osmotic stress. (referat)	Konieczna W., Mierek-Adamska A., Warchoł M., Skrzypek E., Dąbrowska G. B.
2.			Seed germination, antioxidant enzymes activity, proteins, and sugars content during *Brassica napus* L. development (referat)	Turkan S., Konieczna W., Mierek-Adamska A., Skrzypek E., Warchoł M., Dąbrowska G. B.
3.	Międzynarodowa Multidyscyplinarna Konferencja Doktorantów Uniwersytetu Szczecińskiego MKDUS 2.0, online, Szczecin	VI 2022	Does *Trichoderma* promote the growth of oilseed rape (*Brassica napus* L.) and oats (*Avena sativa* L.) in the presence of heavy metals? (referat)	Konieczna W., Turkan S., Mierek-Adamska A., Dąbrowska G. B.
4.	Międzynarodowa konferencja Mendel Genetics Conference, Brno, Czechy	VII 2022	Expression of *Avena sativa* L. metallothioneins under soil drought (poster)	Konieczna W., Mierek-Adamska A., Warchoł M., Skrzypek E., Dąbrowska G. B.
5.	Międzynarodowa konferencja online „Plant productivity and food safety: Soil science, Microbiology, Agricultural Genetics and Food quality”, Toruń	IX 2022	Characterization of *Avena sativa* L. metallothionein gene family and their expression during seed germination (poster)	Chojnacka N., Konieczna W., Mierek-Adamska A., Dąbrowska G. B.
6.	Międyznarodowa konferencja „Plant Biology Europe” Marsylia, Francja	VII 2023	Heavy metal-induced expression of oat (*Avena sativa* L.) metallothionein genes (poster)	Konieczna W., Chojnacka N., Antoszewski M., Mierek-Adamska A., Dąbrowska G. B.
7.	Międzynarodowa konferencja online "Plant security and food safety: Microbiology, Soil Science, Food Quality and Agricultural Genetics", Toruń	IX 2023	Protective role of metallothioneins during the germination of oat (*Avena sativa* L.) seed under drought stress (poster)	Konieczna W., Głowacka K., Mierek-Adamska A., Dąbrowska G. B.

2.4. Staże naukowe

1.	Staż międzynarodowy w Warwick University, Department of Chemistry, Coventry, Wielka Brytania w dniach 17.01-16.02.2023
2.	Staż krajowy do Instytutu Fizjologii Roślin im. Franciszka Górskiego Państwowej Akademii Nauk w Krakowie w dniach 09.05-28.05.2021