

Formation and function of stress granules in hypoxia stress in plants with special emphasis on the role of m6A (N6 - methyladenosine)

Due to their sessile lifestyle, plants must dynamically adapt to changing environmental conditions to survive abiotic stresses. This adaptation, among other mechanisms, is achieved through the spatial regulation of gene expression involving stress granules. Stress granules (SGs) are non-membranous biocondensates that form in the cytoplasm of cells exposed to biotic or abiotic stress. These structures, rich in proteins and poly(A) RNA, serve as reservoirs for untranslated transcripts. The dissociation of mRNA from ribosomes and its sequestration in SGs may represent a mechanism that prioritizes the synthesis of proteins essential for survival under stress conditions. Despite their significance, the exact roles of SGs and their connection to stress tolerance remain unclear. The mechanisms governing the selective sequestration of transcripts into SGs and the fate of stored mRNAs are also poorly understood. It has been hypothesized that epitranscriptomic modifications, such as N6-methyladenosine (m6A), may influence the cellular localization of mRNA. However, no studies to date have explored the role of m6A in the organization of the SGs transcriptome in plants.

This study aimed to investigate the nature and function of SGs in root cells of *Lupinus angustifolius* (narrow-leaved lupin) and *Arabidopsis thaliana* under hypoxic (low oxygen) stress. Specific objectives included:

- identifying and analyzing the stages of SGs formation and exploring their structure and the distribution of molecules within them.
- examining transcriptomic changes in *Lupinus* in response to hypoxic stress and during recovery to normal physiological conditions.
- investigating alterations in m6A levels at the epitranscriptomic level in *Lupinus*.
- determining the role of m6A in the formation of SGs.

The research revealed that SGs enriched with poly(A) RNA and PAB2 proteins form in the root cells of *L. angustifolius* during hypoxia. It was observed that SGs develop in the cytoplasm as a response to stress, initially appearing as small biocondensates that gradually merge into larger structures. Quantitative analyses of poly(A) RNA throughout the hypoxic period showed that cytoplasmic transcripts relocate to SGs during stress.

Analyses using high-resolution microscopy technique STORM (Stochastic Optical Reconstruction Microscopy) and Transmission Electron Microscopy (TEM) revealed a previously unreported two-zoned SGs structure. SGs consist of a ring rich in poly(A) RNA and PAB2, surrounding a central zone devoid of these molecules but containing ribosomes.

It was demonstrated that upon cessation of hypoxic stress, during plant reoxygenation, SGs disassembled. By comparing the levels of poly(A) RNA in root cells and blocking the nuclear-cytoplasmic transport of transcripts during both stress and reoxygenation, it was revealed that

SGs act as the primary source of increasing cytoplasmic mRNA levels. This pool of transcripts is sufficient to support cellular functions in the initial hours following reoxygenation.

RNA-seq analysis revealed a strong transcriptomic response to both the induction and cessation of hypoxic stress in the roots of *Lupinus*. A total of 21,036 differentially expressed genes were identified during hypoxia. For further analysis, transcripts associated with stress response genes and those showing reduced expression during hypoxia were selected. In the subsequent research phase, MeRIP-Seq analysis was performed, which enabled the identification of changes in the distribution and levels of m6A modifications across transcripts. It was found that m6A predominantly localized at the 3' end of all transcripts. The level of this modification increased in this part of transcripts during the initial phase of hypoxia but subsequently decreased during prolonged hypoxic conditions, suggesting a potential role in regulating mRNA stability.

Using fluorescence in situ hybridization (FISH) and MeRIP-qPCR, it was shown that hypoxia-related transcripts, such as *ADH1* and *HUP7*, exhibit low levels of m6A. Notably, *ADH1* localized exclusively in the central SGs zone, where ribosomes were observed. In contrast, mRNA of non-hypoxia-related genes was highly enriched in m6A and present in both SGs zones.

Further investigations into the role of m6A modifications in the formation and function of SGs were conducted using *Arabidopsis thaliana* mutants with significantly reduced levels of m6A. The absence of adenosine methylation resulted in a decreased number of SGs and a lower poly(A) RNA content within them.

The findings highlight the significant role of m6A in hypoxic stress responses. m6A contributes to both the regulation of mRNA levels and the storage of transcripts in SGs for post-stress recovery. These two processes may occur simultaneously but in different regions of the cell. The observed decrease in certain mRNA levels in the cytoplasm outside SGs during hypoxia was accompanied by an increase in m6A levels. Conversely, within SGs, m6A is essential for recruiting specific transcripts. To protect mRNA from degradation in SGs during the later stages of the hypoxic stress response, ALKBH9B may demethylate transcripts. This preserved mRNA can then be rapidly translated once the stress ends. These findings suggest that the role of m6A in the plant hypoxia response is highly complex. In addition to the transcriptional mechanisms that methylate adenosine at position 6 and the actions of demethylases, the cellular localization of individual mRNA transcripts is a crucial aspect of the function of this modification.

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