

ABIOTIC STRESS RESPONSE OF *MEDICAGO TRUNCATULA* PLANTS, MODIFIED WITH GAIN AND LOSS-OF-FUNCTION OF *GRAS7* TRANSCRIPTION FACTOR

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Abstract

Abiotic stresses, among which salinity, are substantial limiting factors for crop yield in contemporary agriculture. Abiotic stress response in plants is governed by a plethora of hormonal signalling, which in turn causes a fine-tuning of gene expression, regulated by stress-responsive transcription factors. The GRAS family of transcription factors mediates the gibberellin signalling and plays a major role in growth and development. In the present study, the role of *MtGRAS7* transcription factor in abiotic stress response was explored in the model legume *Medicago truncatula*. Three major biochemical markers, L-proline, malondialdehyde, and hydrogen peroxide, were measured under salinity, drought, and cold temperature stress in *MtGRAS7* overexpressing and loss-of-function lines. Under drought and salinity, while not directly related to osmoprotection and antioxidant response, the overexpression of *GRAS7* showed a positive effect on the plant stress response, possibly through improved nodulation. The same transcription factor may have a more direct role in response to cold stress, as suggested by our results and literature data.

Key words: abiotic stress, legumes, GRAS transcription factors

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Introduction. Gibberellins are key plant hormones, involved in a variety of developmental processes, as well as stress response. Among the proteins, involved in mediation of the gibberelin action is the GRAS family of transcription factors. It consists of widely distributed, yet insufficiently studied proteins, sharing a conserved C-terminal domain and a highly variable N-region [1, 2]. Their name was derived from three different members of the family, the GIBBERELLIC-ACID INSENSITIVE (GAI), REPRESSOR of GAI (RGA), and SCARECROW (SCR). The number of GRAS genes can vary significantly among plant species, from as little as 20 to as many as 188 [1]. Their roles in plant development are extremely diverse, ranging from phytochrome signal transduction to flowering and starch synthesis, both in negative and positive regulation of gibberellin signalling [2]. Among the proposed roles is also the regulation of nodulation and symbiotic arbuscular mycorrhiza interactions in members of Fabaceae, as well as a variety of abiotic stress responses, mainly drought and salinity [2]. They are also often potential targets for genetically engineered crops with improved agronomic traits and stress tolerance.

Legumes (family Fabaceae) are among the most important agricultural plants, as a source of oils and protein-rich seeds (pulses). Probably their most unique feature is their ability to establish a highly effective plant-microbe symbiosis, dedicated to biological nitrogen fixation, probably the most important pathway for nitrogen incorporation in the biosphere. Apart from important crop plants, such as soybean (*Glycine max*) and alfalfa (*Medicago sativa*), one of the most popular experimental legumes is *Medicago truncatula*. It was proposed as a model plant in the early 90s and grows popular in this role [3], sharing many of the preferred features of *Arabidopsis thaliana*. Unlike it, however, *M. truncatula* is able to establish both nodules and arbuscular mycorrhiza, thus being an attractive model plant for studying these interactions.

In terms of GRAS transcription factors, a total of 68, distributed into 16 groups were found in silico in *M. truncatula* [4]. Because of their extremely diverse functions, more data are needed to unravel the individual functions of every member of the family. In our recent work, we aimed to establish stable transgenic lines of *Medicago truncatula* with overexpression and repression of a single member of the GRAS family in this species, *MtGRAS7* [4, 5] to elucidate its function in nodulation and abiotic stress response. We reported significantly increased transcription levels under salinity and drought, especially in the early stages of the applied stress, as well as better growth and seed production in overexpressing lines compared to wild-type plants. There is a link between abiotic stress and *MtGRAS7* transcription factor. However, further experiments are needed to confirm that its overexpression is directly implicated in a more effective response to abiotic stress. In the present study, we further examined some of the most well-known and important biochemical markers of abiotic stress response and tolerance in *MtGRAS7* overexpressing and inhibited transgenic lines of *Medicago truncatula*.

Material and methods. Plant material and abiotic stress treatment. Wild type *M. truncatula* “Jemalong 2HA”, designated WT (wild type), along with two transgenic lines, *MtGRAS7^{OE}* (over-expressing, gain-of-function) and *MtGRAS7^{RNAi}* (loss-of-function) were used in the experiments. For detailed descriptions refer to REVALSKA [4]. Sixty days old plants, grown in hydroponics were subjected to salt (150 mM NaCl) or drought (350 mM mannitol) stress for 24, 48, and 72 h, or to cold stress at 4 °C for 72 h, 7 days, or 7 days stress plus 7 days recovery at 24 °C. Samples from leaves and nodules were collected at each of the indicated time intervals, immediately frozen in liquid nitrogen, and stored at –80 °C until further analyses.

Spectrophotometric determination of stress markers. All analyses were performed in triplicates, every repetition representing a separate plant. Each analysis started with 100 mg of plant fresh mass, ground in liquid nitrogen, and dissolved in specific reagents.

For hydrogen peroxide measurement the following reagents were successively added in a ratio of 1:2:1 to 1 mL final volume: 0.1% (w/v) trichloroacetic acid (TCA): 1M KI: 10 mM potassium phosphate buffer pH 7. After centrifugation at $12\,000 \times g$ for 15 min at 4 °C, the supernatant was transferred into a new tube and incubated at RT, 20 min in the dark, and afterward absorption was measured at $\lambda = 350$ nm [6].

Malondialdehyde (MDA) was measured according to [7]. The ground plant material was dissolved in 0.1% TCA and 0.5% (w/v) thiobarbituric acid (TBA), centrifuged at $12\,000 \times g$ for 15 min and the supernatants were incubated for 30 min at 95 °C, followed by cooling on ice. The specific absorption was measured at 532 nm, while the non-specific, which was subtracted was measured at 600 nm. The concentration of MDA was calculated in $\mu\text{mol g}^{-1}$ fresh weight using a molar absorption coefficient of $155\text{ mM}^{-1}\text{ cm}^{-1}$.

For proline concentration, the plant material was dissolved in 3% (w/v) sulphosalicylic acid (SSA) and centrifuged for 5 min at $10\,000 \times g$. The supernatant was mixed with acid ninhydrin solution and glacial acetic acid (in a 1:2:2 ratio), followed by 1-hour incubation at 95 °C, and the reaction was stopped on ice. Following extraction with toluene absorbance of the toluene fraction was measured at 520 nm [8] and concentration was calculated according to a standard curve, pre-made with L-proline.

Results and discussion. The concentrations of all three abiotic stress markers were substantially affected by both salinity and drought stress (Fig. 1, 2, 3). The concentration increased with the duration of treatment, suggesting the accumulating effect of the stress. The overall tendency is more pronounced changes in leaves, compared to nodules, and in NaCl-stressed, compared to mannitol-stressed plants, especially considering L-proline and hydrogen peroxide (Fig. 1, 3). However, the peak concentration of MDA was established at the 48 h, followed by a decline (Fig. 2), while the other two markers peaked at the 72 h. The highest

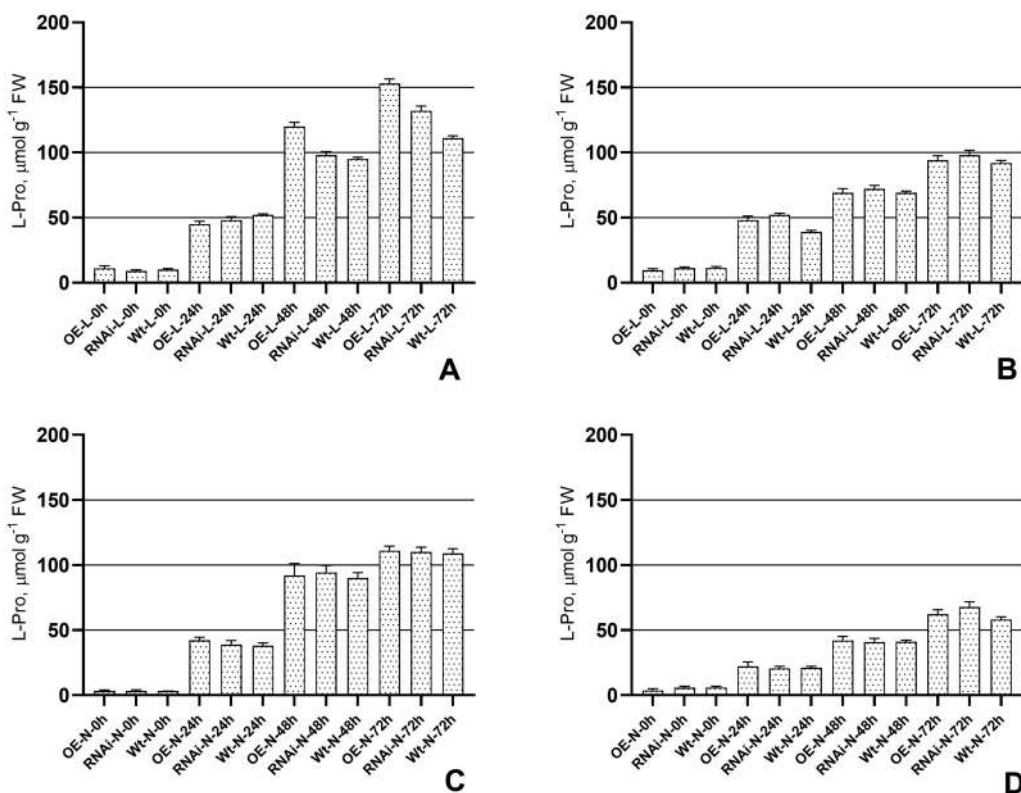


Fig. 1. The concentration of L-proline in leaves (A and B) and nodules (C and D) of wild type (Wt), *MtGRAS7*^{OE}, and *MtGRAS7*^{RNAi} plants after 24, 48, and 72 h of treatment with 150 mM NaCl (A and C) and 350 mM mannitol (B and D). Columns represent mean values ($n = 3$) $\pm$ SEM

concentrations were established in the *MtGRAS7*^{OE} plants, compared to Wt and *MtGRAS7*^{RNAi}.

Salinity and drought are among the most devastating abiotic stresses, affecting all plants and limiting agricultural yield globally [9]. In terms of physiological influence, they share remarkable similarities, due to the osmotic effect, limiting water availability to plant roots, often accompanied by abscisic acid-governed stomatal closure, inhibition of photosynthesis, and growth retardation. This is often accompanied by increased production of reactive oxygen species – ROS (e.g., oxidative stress) [9]. In addition, salinity also causes ion toxicity and mineral imbalance. Numerous research studies over the years established several biochemical markers for abiotic stress response, and stress tolerance, such as compatible solutes (also osmoprotectants, or osmolytes), among which L-proline is one of the most prominent, hydrogen peroxide, being the only stable ROS to be detected easily and malondialdehyde (MDA), an indirect evidence of lipid peroxidation in membranes. Although abiotic stress response is almost uniformly associated with an increase of all three markers, there is a common misconception, that the ability

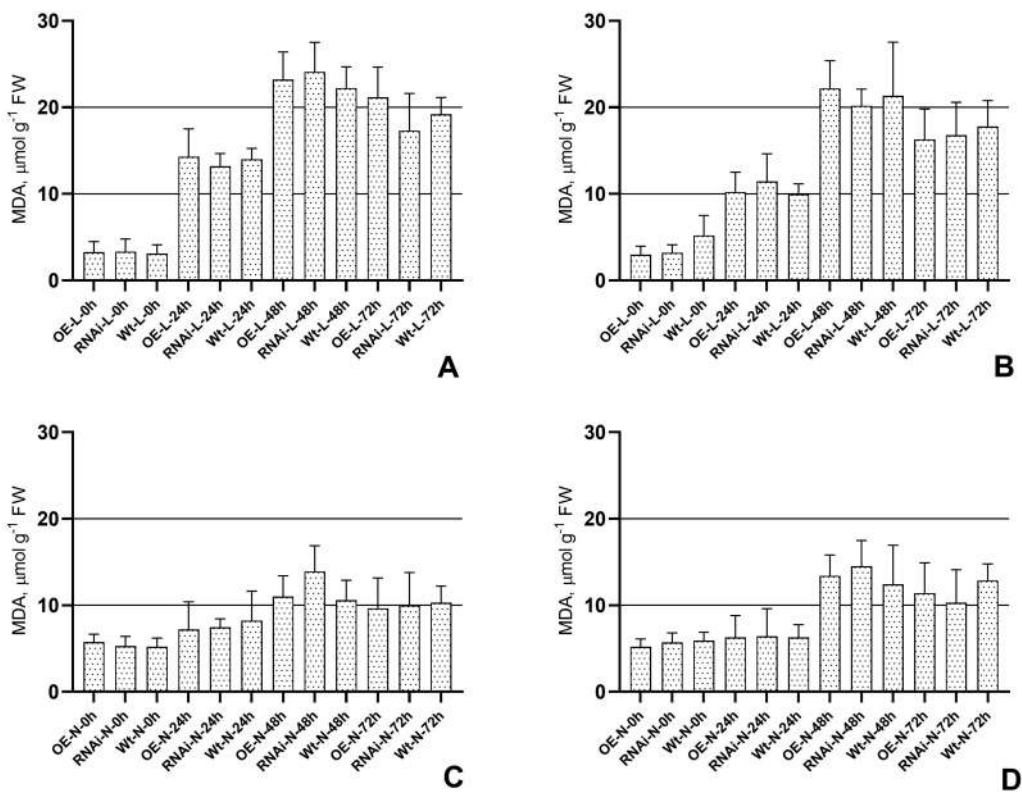


Fig. 2. The concentration of MDA in leaves (A and B) and nodules (C and D) of wild type (Wt), *MtGRAS7*^{OE}, and *MtGRAS7*^{RNAi} plants after 24, 48, and 72 h of treatment with 150 mM NaCl (A and C) and 350 mM mannitol (B and D). Columns represent mean values ($n = 3$) $\pm$ SEM

to accumulate more compatible solutes and keep the ROS and MDA levels low is associated with tolerance, but this was challenged both by experimental evidence and fundamental knowledge [10].

Our present results conform to general knowledge, showing an increase of all three biochemical markers under both salinity and drought. However, we aimed to establish whether these changes are related to the expression of *MtGRAS7*. The peak of transcripts levels was found on the 24th hour [4], while peak concentrations of L-Pro, MDA, and H₂O₂ were delayed toward the 48–72 h. This was expected for L-proline as generally accumulation of organic molecules takes time compared to an immediate increase in gene expression in response to stress, but is somehow puzzling for the markers for oxidative stress, as stomatal closure and ROS production is often an immediate result of abiotic stress [11]. It should be also noted, that *MtGRAS7* had much higher transcript levels in nodules, compared to leaves where the peak L-proline concentration (Fig. 1) was found, probably related to the important role of *GRAS* transcription factors in nodulation in legumes [1].

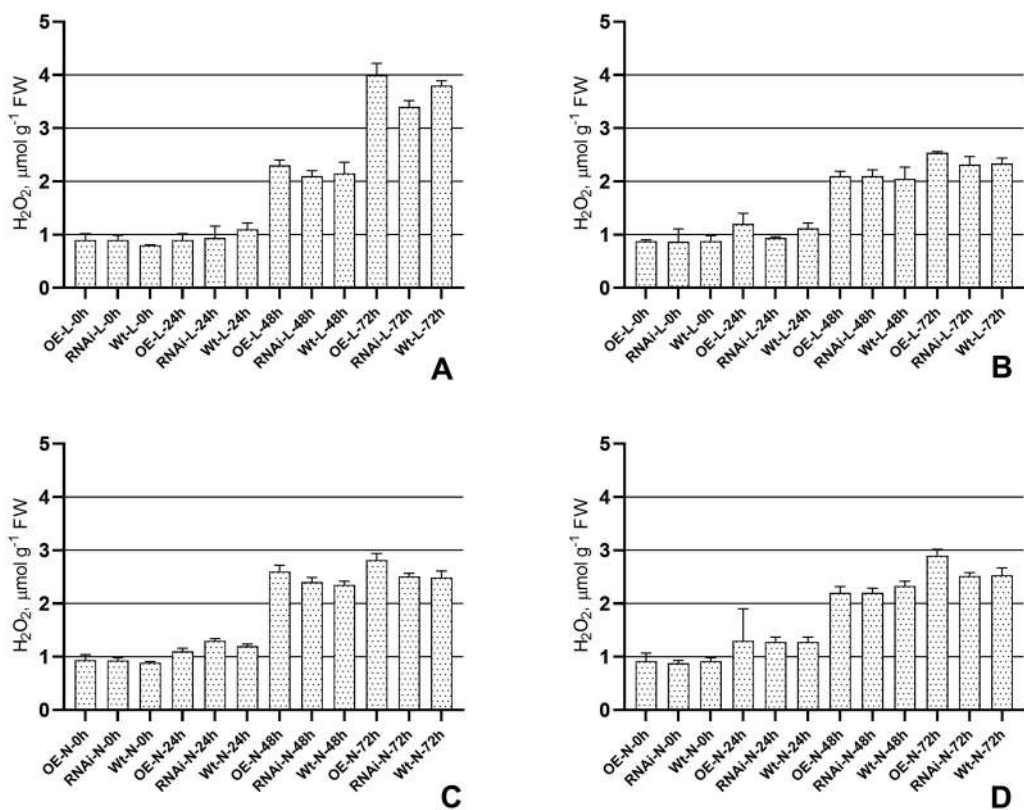


Fig. 3. The concentration of H_2O_2 in leaves (A and B) and nodules (C and D) of wild type (Wt), *MtGRAS7*^{OE}, and *MtGRAS7*^{RNAi} plants after 24, 48, and 72 h of treatment with 150 mM NaCl (A and C) and 350 mM mannitol (B and D). Columns represent mean values ($n = 3$) $\pm$ SEM

Looking at the overall tendencies, *GRAS7* overexpressing line showed higher L-Pro levels (Fig. 1) and lower MDA concentrations (Fig. 2), compared to Wt and RNAi-line in leaves, under salinity, which correlates with the highest transcript levels in nodules and preserved fecundity results [4]. However, we suggest this effect is an indirect benefit of better nodulation in *MtGRAS7*^{OE} plants, rather than a direct link between *GRAS7* overexpression and compatible solute synthesis and accumulation. Not surprisingly, better root symbiosis in legumes means higher tolerance to abiotic stress [12].

However, a different mechanism may be true for the cold-stressed plants (Fig. 4). The concentration of L-proline was much higher in leaves of *MtGRAS7*^{OE} plants, accompanied by lower MDA concentrations, which correlates with a remarkable increase in *GRAS7* transcript levels [4]. In comparison, the Wt plants showed little to no effect on L-Pro accumulation and increased MDA concentration. In the RNAi line, these markers were similarly affected as in the overexpressing line, though at a lower rate, and in both the 7-day recovery period resulted in a reversion to the initial levels (Fig. 4).

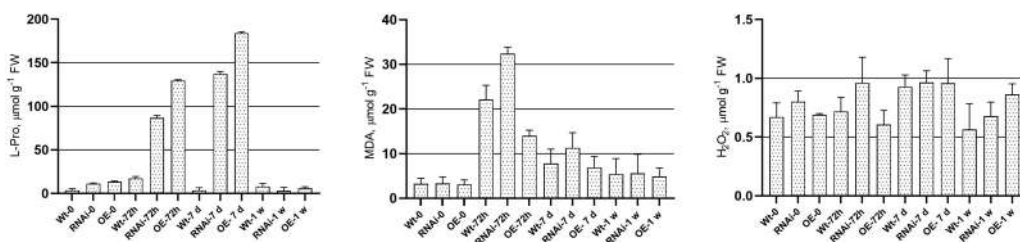


Fig. 4. Concentrations of L-proline, MDA, and H₂O₂ in leaves of cold-treated (4 °C) plants after 72 h, 7 days, and recovery for one week. Columns represent mean values ($n = 3$) $\pm$ SEM

Although the response to low-temperature stress shares multiple similarities with salinity and drought, it is also unique in terms of perception and signal transduction [13]. Therefore, it is not unexpected that Wt and *GRAS7* overexpressing plants performed differently under cold stress (Fig. 4), compared to osmotic stress (Fig. 1–3) and a possible differential role of the *GRAS7* transcription factor itself. Bioinformatic approaches suggested an important role of *GRAS* family TFs in cold stress response in *Trifolium repens* [14] and a very recent study also showed a direct link between *GRAS* overexpression and glycine-betaine (another important osmoprotectant) accumulation in *Poncirus trifoliata* [15]. Therefore, it is not unreasonable to suggest, that *MtGRAS7* has a similar effect in *Medicago truncatula*, with an emphasis on proline. However, the puzzling similarity between over-expressing and RNA-inhibited lines, both differing from Wt plants is to be elucidated in the future. Possibly, the inhibition of *GRAS7* transcription causes a compensatory mechanism under abiotic stress, but currently, there is no supporting evidence.

Conclusions. The presented results, along with previously published work on the *MtGRAS7* over-expression/inhibition in *Medicago truncatula* strongly suggested its important role in abiotic stress response. While under osmotic/salt stress this role may be mostly indirect, through the promotion of nodulation, its role under low-temperature stress may be more direct. Although not definitively involved in abiotic stress tolerance, this transcription factor is related to the stimulation of the defensive mechanisms, which ultimately results in preserved development and fecundity.

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