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Involvement of *Medicago truncatula* glutamate receptor-like channels in nitric oxide production under short-term water deficit stress

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ABSTRACT

Early stages of plant development are highly susceptible to environmental cues, and seedlings have to develop sophisticated mechanisms to sense and respond to abiotic stresses. We have previously identified that abscisic acid (ABA), nitric oxide (NO) and modulation of nitrogen metabolism are involved in adaptive responses in *Medicago truncatula* seedlings under water deficit stress. Here, we investigated whether glutamate receptor-like channels (GLRs) played a role in the developmental physiological processes of *Medicago* seedlings during post-germination after a short-term water deficit stress. Twenty-nine independent *MtGLR* genes have been identified and then divided into four clades following a phylogenetic analysis; seventeen of them exhibited specific domains which are characteristic of animal ionotropic glutamate receptors. Under drought stress, ABA-induced NO accumulation was significantly reduced in presence of a GLR competitive antagonist, suggesting that this water deficit-induced endogenous NO production was mediated through a *MtGLR*-dependent pathway. Water deficit-induced inhibition of embryo axis elongation was strongly reduced whereas loss of water content was alleviated when *MtGLRs* were inhibited. These results suggest that glutamate receptors-like channels are required, through their involvement in NO production, in adaptive responses under short-term water-deficit stress during *Medicago* seedling establishment.

1. Introduction

As a signaling molecule, glutamate has been reported to play a role in excitatory neurotransmission in vertebrates (Dingledine et al., 1999) and growth processes in plants (Forde, 2014). In animals, glutamate may interact with two main groups of receptors namely ionotropic glutamate receptors (iGluRs), such as *N*-methyl-D-aspartate (NMDA), kainate and α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, which are heterotetramers non-selective cation channels transporting Na^+ , K^+ , and Ca^{2+} , and metabotropic glutamate receptors (mGluRs) which belong to the superfamily of G-protein coupled proteins (Dingledine et al., 1999). iGluRs are ligand-gated permeable channels which possess a large N-terminal extracellular domain, three transmembrane regions, a hydrophobic loop defining the pore region and a C-terminal domain. Unlike to cyclic nucleotide-gated channels (CNGCs), iGluRs have their ligand-binding domain which is extracellular. Plants possess a family of glutamate receptor-like channels (GLRs), which are homologs of mammalian iGluRs (Lam et al.,

1998; Price et al., 2012). These plant GLRs have been mainly located in the plasma membrane and tonoplast (Davenport, 2002) and have been identified as potential candidates to mediate calcium influx at plasma membrane level (Lacombe et al., 2001; Mäser et al., 2001; Swarbreck et al., 2013) and are hypothesized to be potential amino acid sensor in plants (Tapken et al., 2013; Vincill et al., 2012). In Arabidopsis, 20 homologs of animal iGluRs have been identified and assigned as glutamate receptor-like genes (*AtGLRs*) (Chiu et al., 2002; Davenport, 2002; Lacombe et al., 2001). Phylogenetic analysis demonstrated that these 20 *AtGLR* genes were separated into three distinct clades (Chiu et al., 2002; Lacombe et al., 2001) and have similar organ level expression patterns depending on the specific clade. Similar to Arabidopsis, a new family of 13 *GLR* genes has been also identified in *Solanum lycopersicum* (Aouini et al., 2012).

Increasing evidences using genetic and/or pharmacological approaches indicated that GLRs play important roles in diverse developmental and physiological procedures in plants such as carbon and nitrogen sensing (Kang and Turano, 2003), light signal transduction (Lam

Abbreviations: ABA, abscisic acid; AP-5, D-2-amino-5-phosphono pentanoic acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; GLR, glutamate receptor-like channel; iGluR, ionotropic glutamate receptor; NO, nitric oxide; PEG, polyethylene glycol

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et al., 1998), seed germination (Cheng et al., 2018; Kang et al., 2004; Kong et al., 2015), root development/morphogenesis/gravitropism (Li et al., 2006; Miller et al., 2010; Singh et al., 2016), hypocotyl elongation (Brenner et al., 2000; Lam et al., 1998) and pollen tube growth (Michard et al., 2011). The involvement of GLRs in adaptation to environmental stresses has also been reported. During plant-pathogen interactions, plant GLRs have been shown to be involved in innate immune responses via calcium influx in *Arabidopsis* seedlings (Kwaaitaal et al., 2011) and in tobacco cell suspensions (Vasta et al., 2011). Genetic evidences have also shown more precisely that AtGLR3.3 is a key component of resistance against *Hyaloperonospora arabidopsidis* (Manzoor et al., 2013) and *Pseudomonas syringae* pv tomato DC3000 (Li et al., 2013) via the activation of salicylic acid plant defenses. Whereas AtGLR3.2, AtGLR3.3 and AtGLR3.6 are implicated in the defense response to mechanical wounding (Mousavi et al., 2013), AtGLR1.1 and AtGLR3.1 are involved in stomatal closure under drought stress (Cho et al., 2009; Kang et al., 2004). As for AtGLR3.4, it has been demonstrated to mediate a calcium influx involved in the regulation of seed germination under salt stress (Cheng et al., 2016, 2018) and its gene expression was induced after touch and cold stimulation in an abscisic acid (ABA)-independent, but calcium-dependent manner (Meyerhoff et al., 2005).

ABA plays crucial roles in developmental growth but also in the perception and transduction of drought-induced signals in order to confer plant tolerance to water-deficit stress (Shinozaki and Yamaguchi-Shinozaki, 2007). In this context of responses of water stress, strong evidences have been shown that the putative plant glutamate receptor 1.1 in *Arabidopsis* (AtGLR1.1) functions as a molecular and biochemical link between ABA metabolism and sensitivity, carbon/nitrogen status and metabolism (Kang and Turano, 2003). Moreover, AtGLR3.5 has been recently reported to modulate cytosolic calcium level to alleviate the inhibitory effects of ABA in seed germination (Kong et al., 2015). Nitric oxide (NO), a product of nitrogen metabolism (Kaiser et al., 2002; Planchet et al., 2005), is known to be a stress regulator involved in signal transduction pathways and to play multiple roles in growth developmental processes and in adaptive responses to various environmental constraints (Fancy et al., 2017). Under water deficit stress, NO has been identified as a key component in the signaling cascade of ABA-induced stomatal closure (García-Mata and Lamattina, 2003). NO has also emerged as major player of plant resistance responses to pathogens influencing both basal defense and hypersensitive responses (Mur et al., 2006). Interestingly, Vasta et al. (2011) have shown that putative plant glutamate receptors participated in fungal elicitor cryptogein-induced calcium influx and NO production. In our knowledge, no study about GLRs in *Medicago truncatula* and their involvement has been reported during abiotic stress. Since we have already shown that water deficit stress induced an ABA-dependent NO production in the model legume *M. truncatula* during post-germinative growth (Planchet et al., 2014), we investigated the study of the involvement of GLRs on NO production and some physiological parameters in *M. truncatula* seedlings after a short-term water deficit stress.

2. Materials and methods

2.1. Phylogenetic analysis and sequence alignment

In silico MtGLR sequence search was performed using the BLAST algorithm of the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the specific *Medicago* JCVI database Mt4.0 version (<http://www.jcvi.org/medicago/>) (Tang et al., 2014). The phylogenetic tree was constructed using the MEGA4 program via the neighbor-joining method (with *p*-distance correction) as described by Aouini et al. (2012). Bootstrap analysis (bootstrap method NJ, 1000 replicates) was performed to measure the robustness of all nodes. The amino acid sequences of the *M. truncatula* GLR family and the *A. thaliana* GLR family were aligned by Clustal W (Thompson et al., 1994) using three separate

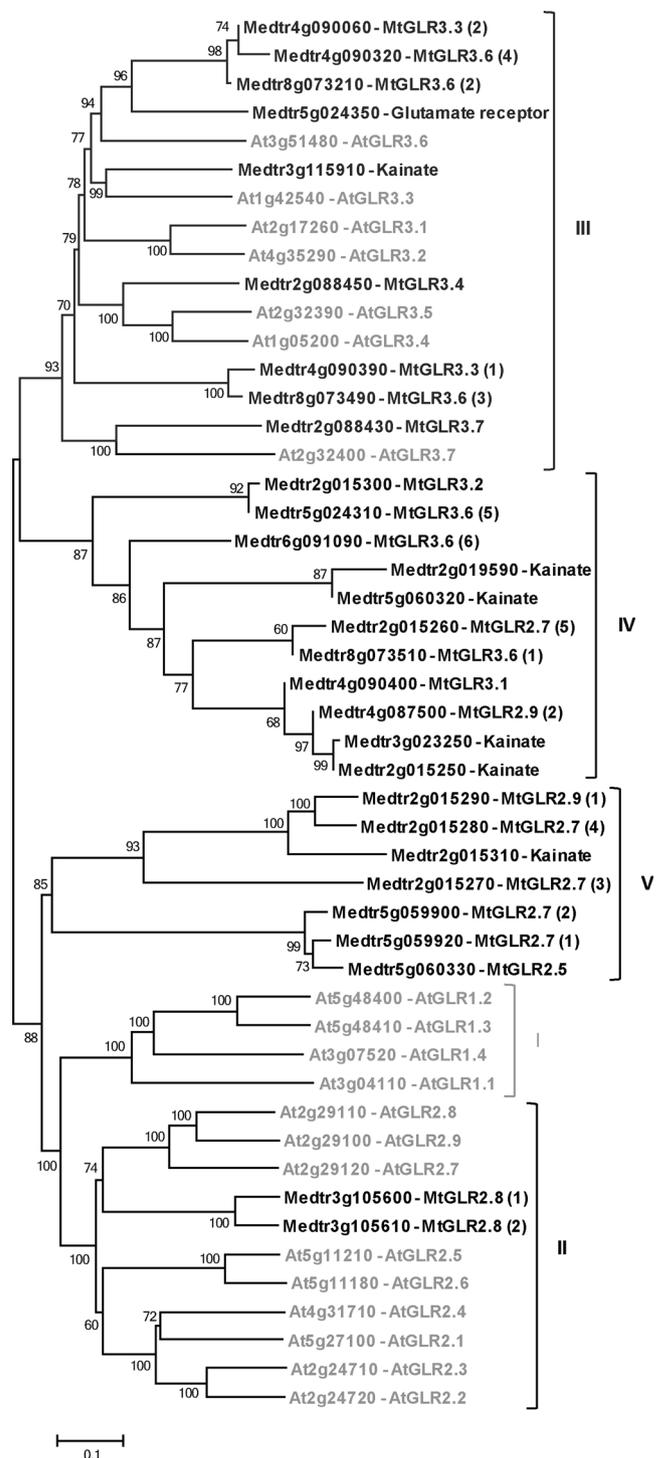


Fig. 1. Phylogenetic tree based on alignments of deduced amino acid sequences of MtGLRs and AtGLRs. Phylogenetic relationships of the sequences were examined using the neighbor-joining method with *p*-distance correction. The bootstrap values from 1000 replicates are shown at each node. GenBank accession and nomenclature numbers of the MtGLRs are represented in dark colour and those of AtGLRs in light colour.

sets of alignment parameters that produced preferable alignments (gap opening cost $\frac{1}{4}$ 10 ; gap extension cost $\frac{1}{4}$ 1.0; amino acid substitution matrix $\frac{1}{4}$ Blosum 30).

2.2. Germination and growth conditions

Seeds of *M. truncatula* cv. Jemalong were germinated in darkness at

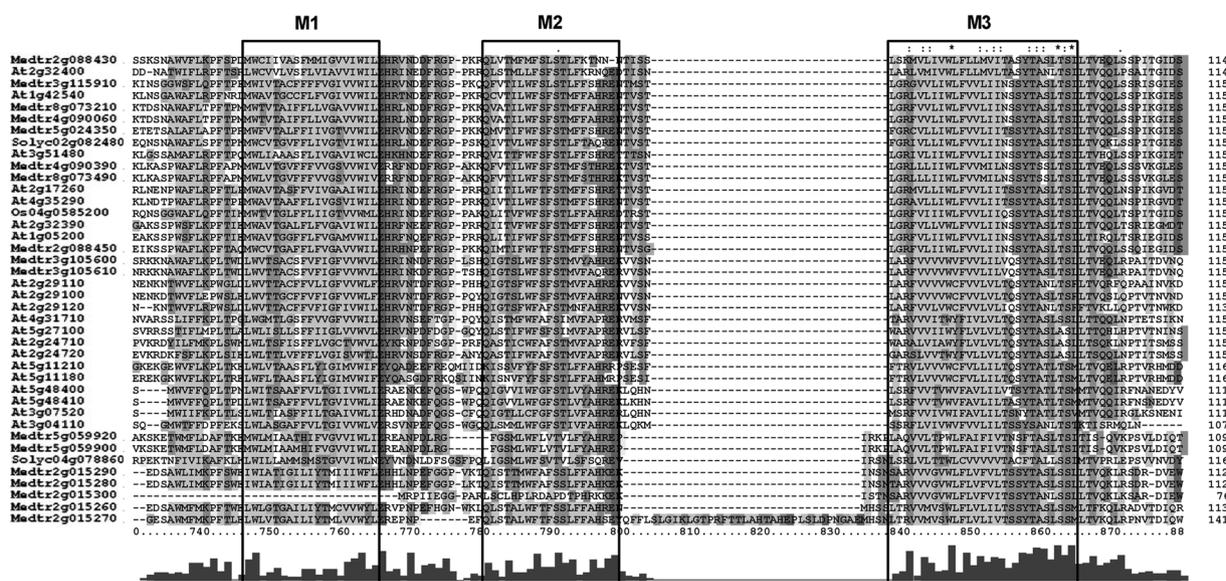


Fig. 2. Protein sequence alignments of MtGLRs with GLRs from various plant species. Seventeen MtGLR sequences were compared with twenty AtGLR sequences, two GLR sequences of *S. lycopersicum* (*Solyc02g082480* and *Solyc04g078860*) and one GLR sequence of *O. sativa* (*Os04g0585200*) containing the three conserved domains (M1, M2 and M3) of animal ionotropic GluRs.

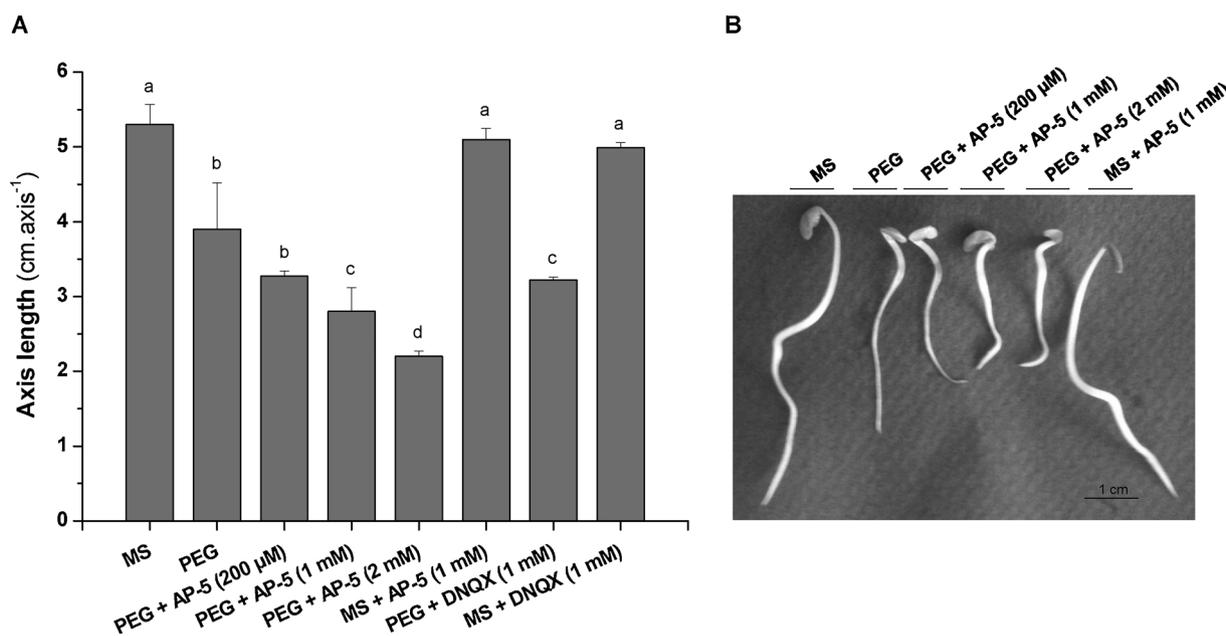


Fig. 3. Effects of a GLR antagonist on elongation of embryo axis of *M. truncatula* seedlings under water deficit stress. (A) Axis elongation after 24 h under normal condition (MS), PEG (-0.75 MPa) ± AP-5 (200 μM, 1 mM or 2 mM) or DNQX (1 mM). Each value represents the mean ± SE of three to five independent experiments. For statistical analysis, all the treatments were compared to MS condition. Different letters are used to indicate means that differ significantly ($P < 0.05$) according to a one-way ANOVA test. (B) Picture of *M. truncatula* seedlings under different treatments is a representative data.

21 °C in Petri dishes (diameter 9 cm) on Whatman paper soaked with 4 ml of modified Murashige and Skoog medium (MS medium containing macro- and micronutrients, buffered with MES) for 40 h. Afterwards ($T = 0$ h), seedlings were transferred in Petri dishes onto MS or in presence of polyethylene glycol 8000 solution (PEG; Sigma-Aldrich) at -0.75 MPa (prepared in MS medium) ± glutamate receptor antagonists: AP-5 (D-2-amino-5-phosphono pentanoic acid; Sigma-Aldrich, St Quentin Fallavier, France) and DNQX (6,7-dinitroquinoxaline-2,3-dione), inhibitors used with plant cells (Iwano et al., 2015; Lam et al., 1998; Manzoor et al., 2013; Rincon-Zachary, 2010). Into each Petri dish, thirty seedlings were grown in darkness (21 °C) and were, thereafter, harvested at 24 h of treatment. Cotyledons were discarded and embryo axes (hypocotyl plus radicle) were immediately frozen in liquid

nitrogen before being stored at -80 °C. Water content and elongation in embryo axes were measured after 24 h of growth. The axis elongation was determined using the ImageJ software.

2.3. Endogenous NO detection by fluorescence

For fluorometric NO determination, the fluorophore 4,5-diamino-fluorescein diacetate (DAF-2DA; Sigma-Aldrich) was used. Roots of intact seedlings were pre-incubated with 10 μM DAF-2DA in MES buffer (5 mM, pH 6) for 30 min at 21 °C in darkness and then rinsed twice with MES buffer during 10 min to remove excess fluorophore. DAF-2DA fluorescence (495 nm excitation and 515 nm emission wavelength) was observed by microscopy (LEICA DM4500B) connected to a fluorescence

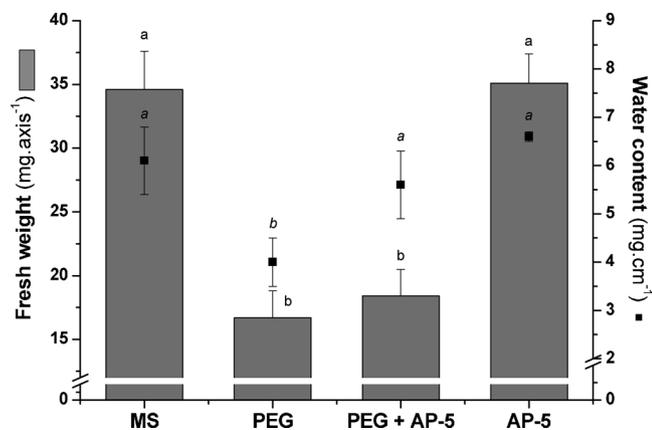


Fig. 4. Effects of a GLR antagonist on fresh weight and water content of embryo axis of *M. truncatula* seedlings under short-term water stress. Fresh weight (bar) and water content (point) measured after 24 h according to the different growth conditions (MS, PEG ± AP-5 (1 mM) and AP-5 (1 mM)). Each value represents the mean ± SE of three to eight independent experiments. For statistical analysis, all the treatments were compared to MS condition. Italic letters and normal letters (for water content and fresh weight, respectively) are used to indicate means that differ significantly ($P < 0.05$) according to a one-way ANOVA test.

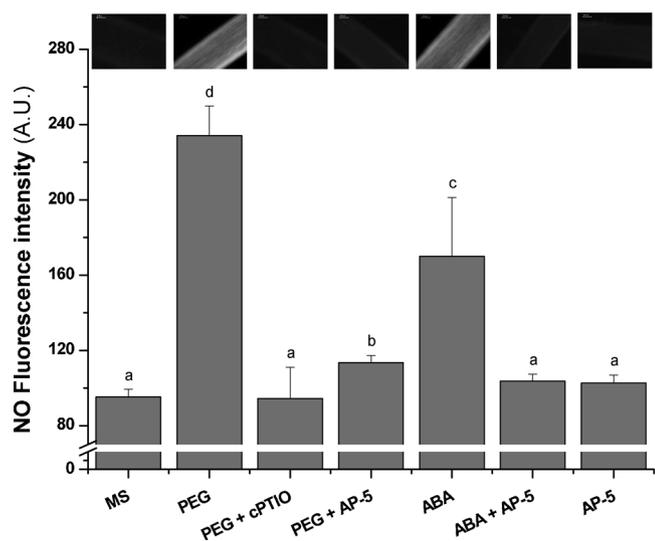


Fig. 5. PEG-induced NO production via GLRs. NO fluorescence intensity (A.U.) was measured in embryo axes from seedlings grown after 24 h under normal condition (MS) ± AP-5 (1 mM) or with ABA (100 μM) ± AP-5, and grown on MS in presence of PEG (-0.75 MPa) ± AP-5 or cPTIO (250 μM). Each datum point is the mean of four independent experiments ± SE. Different letters are used to indicate means that differ significantly ($P < 0.05$) according to a one-way ANOVA test. For statistical analysis, all the treatments were compared to MS condition. Pictures are representative data.

detection system L5. Fluorescence quantification was made using the ImageJ software. Before DAF-2DA treatment, seedlings were incubated during 24 h onto MS medium with or without ABA (100 μM; Sigma-Aldrich) solution or in presence of PEG (-0.75 MPa prepared in MS medium) ± cPTIO (2-(4-carboxy-phenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide; 250 μM) for NO scavenging. The inhibition of GLRs was performed by adding AP-5 (1 mM) in MS medium, PEG or ABA treatments.

2.4. Statistical analysis of data

All data are presented as mean ± SE of values from at least two independent experiments. A one-way ANOVA (analysis of variance) test

was also performed. Different letters are used to indicate means that differ significantly ($P < 0.05$).

3. Results

3.1. Identification of GLR members and motifs of *M. truncatula*

Through a comprehensive search conducted in Medicago databases, 29 independent MtGLR genes were identified. In order to infer phylogeny and to analyze the relationships between the MtGLR and AtGLR families, a phylogenetic tree was generated using protein sequences from these two species and referring to the denomination of Arabidopsis clades already known (Chiu et al., 2002; Lacombe et al., 2001). Multiple alignments indicated that the MtGLR proteins were classified into four phylogenetically distinct clades whereas the 20 AtGLR proteins were distributed in three clades as expected (Fig. 1). Two of these clades (clade IV and V) emerged distinctly and were specific to Medicago, whilst two other clades (clade II and III) exhibited homologies between GLRs from Arabidopsis and Medicago. Only the clade I was specific to Arabidopsis.

Among these 29 MtGLRs, 17 exhibited specific domains, M1 and M2, which are two characteristic and conserved transmembrane domains of animal ionotropic GluRs (Fig. 2). The amino acid sequences of these 17 members of the Medicago GLR family were aligned with other GLRs from different species (*Arabidopsis thaliana*, *Oriza sativa* and *S. lycopersicum*) containing also these M1 and M2 signature domains of animal ionotropic GluRs (Fig. 2). This alignment analysis highlighted a strong sequence similarity between these different GLRs, in particular the presence of a similar third transmembrane domain (M3) characterized by the sequence “SYTANLAA” which is a specific pattern of animal NMDA-type iGluRs (Sobolevsky et al., 2009).

3.2. Involvement of MtGLRs on growth physiological processes in response to water deficit

To gain insight into the role of GLRs on some physiological parameters in Medicago seedlings, we investigated whether MtGLRs modulated water content, fresh weight and elongation of axes (radicle + hypocotyl) in response to water deficit (PEG treatment). While PEG inhibited the axis length compared to the well-watered condition (MS medium), the application of a specific antagonist of glutamate receptor channels, AP-5, increased significantly the sensitivity to PEG in seedlings for the inhibition of axis length in a dose-dependent way (Fig. 3A, 3B). Whereas AP-5 at 200 μM in presence of PEG reduced in a non-significant way the axis length, AP-5 at 1 mM and 2 mM induced a significant decrease of axis length (reduction of 28% and 42% compared to PEG treatment, respectively). It is important to note that AP-5 at 1 mM applied alone did not induce any effect on axis length compared to control conditions. Similar result on axis length, in response to PEG treatment, was obtained with another glutamate receptor antagonist DNQX at 1 mM (Fig. 3A), which induced a significant reduction of axis length (a decrease of 17% compared to PEG treatment). DNQX applied alone did not affect the axis elongation of the seedlings (Fig. 3A). As expected, a strong loss of fresh weight and water content in embryo axes (around 52% and 35%, respectively) was observed after 24 h of water deficit stress (Fig. 4). The application of AP-5 alleviated this PEG-induced reduction in water content (gain of 40% compared to PEG treatment), whereas AP-5 did not impact the PEG-induced reduction of fresh weight.

3.3. Involvement of MtGLRs in water deficit-induced NO production in seedlings

Previously, we have reported that water deficit stress (mimicked by PEG) induced an ABA accumulation (Planchet et al., 2011a, 2011b) and a PEG-induced NO production, which was dependent of nitrate

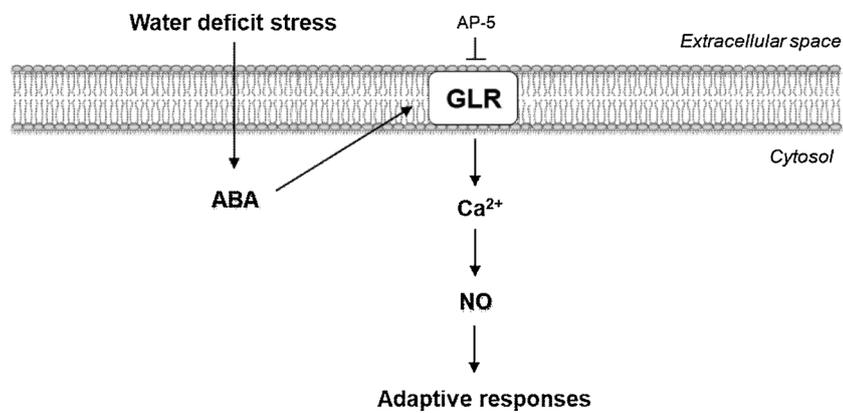


Fig. 6. Model of ABA, GLR and NO signaling pathways in *M. truncatula* seedlings submitted to a water deficit stress. Drought stress has been reported to induce ABA accumulation through *NCED* gene induction, a gene involved in the ABA synthesis (Planchet et al., 2011a). Adaptive responses to water deficit stress can be initiated by an ABA-induced MtGLR activation, resulting in a calcium influx (Vatsa et al., 2011). ABA-induced NO production is mediated by MtGLRs. (ABA, abscisic acid; AP-5, D-2-amino-5-phosphono pentanoic acid; GLR, glutamate receptor-like channel; NO, nitric oxide).

reductase, was mediated through ABA (Planchet et al., 2014) in embryo axes of *M. truncatula* seedlings. To investigate whether this production of NO was induced by ABA through the involvement of GLRs, the detection of endogenous NO from *Medicago* seedling axes has been achieved using DAF-2DA fluorescence microscopy (Fig. 5). After 24 h, NO accumulation in response to PEG and exogenous ABA was around 2.5- and 1.8-fold more than under normal conditions, respectively. As negative control, the NO scavenger cPTIO reduced strongly NO fluorescence, showing the specificity of NO detection. Interestingly, AP-5 applied with PEG or ABA induced a strong decrease of NO accumulation, showing a NO fluorescence intensity substantially similar to that under normal condition.

4. Discussion

The sensing of various environmental stimuli induces downstream intra- and intercellular signaling networks, including the activation of some ion channels. In this context, the plant glutamate receptor-like channels appear to be interesting signaling players to detect environmental cues. Arabidopsis GLRs have been reported to be significantly similar to animal iGluRs by inducing the glutamate-mediated calcium signaling (Dennison and Spalding, 2000). Interestingly, the high similarity in the deduced amino acid sequences of the M3 segment, the narrowest portion of the channel, between the 17 members (out of 29) of the *Medicago* GLR family and all 20 AtGLRs (Chiu et al., 2002; Lacombe et al., 2001) suggests that these MtGLRs could be implicated in calcium (Ca²⁺) network signal function as Ca²⁺ channels. More specifically, this hypothesis is supported by the common presence of three transmembrane domains (M1, M2 and M3) both in *Medicago* and Arabidopsis GLR sequences. These specific domains, which are characteristic of animal iGluRs, have been shown to be involved in ion selectivity, permeability and conductance of channel pores (Dingledine et al., 1999). Recently, treatments of wild-type Arabidopsis seedlings under salt stress evoked a marked elevation in cytosolic Ca²⁺ activity and this elevation was inhibited by antagonists of GLRs, while the NaCl-induced elevation in cytosolic Ca²⁺ activity was impaired in *atglr3.4-1* and *atglr3.4-2* mutants (Cheng et al., 2018). According to that, a calcium influx and a rise of cytosolic [Ca²⁺]_{cyt} were reported to be triggered after activation of GLRs in tobacco cells elicited with the fungal elicitor cryptogein and this GLR activation was dependent of a release of glutamate in the apoplast through exocytosis due to a previous increase of [Ca²⁺]_{cyt} (Vatsa et al., 2011). Interestingly, these putative GLRs have been also shown to be involved in the cryptogein-induced NO production (Vatsa et al., 2011). The interplay between Ca²⁺ and NO in plant cells is essential and complex in plant signaling. Whereas some evidences indicate that NO mediates Ca²⁺ signaling, it is also recognized that Ca²⁺ fluxes participate for NO production. Extracellular Ca²⁺ uptake has been notably shown to promote NO accumulation in guard cells of Arabidopsis epidermis leading to the calcium sensing receptor-regulated stomatal closure (Wang et al., 2012). In a context of water

deficit stress in which ABA has been reported to induce NO production (Desikan et al., 2002; Planchet et al., 2014), we demonstrated for the first time that ABA-induced NO production is mediated by putative GLRs in *M. truncatula* seedling axes during a short-term water deficit stress since GLR antagonists strongly affected ABA-induced NO production. A model, in which ABA-dependent MtGLRs could modulate NO production through Ca²⁺ influx, is proposed for adaptive responses during short-term water deficit stress (Fig. 6). Previously, our research group has also demonstrated that (i) ABA-induced inhibition of cell expansion in the radicles of *M. truncatula* seeds during post-germination (Gimeno-Gilles et al., 2009) and that (ii) this primary root inhibition was mainly mediated through a ABA-induced nitrate reductase-dependent NO pathway under water deficit stress (Planchet et al., 2014). In this current study, it appears that AP-5 accentuated the inhibition of *Medicago* axis growth in response to PEG. These data show that MtGLRs, through NO production, are required for primary root development under drought conditions, showing the important role of glutamate receptors in the earliest phase of the *Medicago* life cycle. This conclusion is strengthened by the fact that (i) *OsGLR3.1* is essential for the maintenance of cell division and individual cell survival in the root apical meristem at the early seedling stage (Li et al., 2006), (ii) *AtGLR3.6* is required for primary and lateral root development by stimulating cellular division (Singh et al., 2016), and (iii) *atglr3.4* mutants presented greater inhibitory effects on seed germination and root elongation than wild type in presence of NaCl (Cheng et al., 2018). Interestingly, Sivaguru et al. (2003) have shown that AtGLRs were involved in the root growth inhibition and microtubule depolymerisation after aluminium toxicity in seedlings. This inhibitory effect on Arabidopsis primary root growth could be attributed to the glutamate effect which is known to be strongly influenced by environmental conditions and to have similar effects to nitrate to inhibit primary root tip (Walch-Liu and Forde, 2008). To date, the only known relationship between nitrate transporters and glutamate receptor homologs in plants has been reported by Kang et al. (2004) who observed that the transcript abundance for the nitrate transporter CHL1 was lower (30% decrease) in *antiAtGLR1.1* plants compared to wild-type plants. These *antiAtGLR1.1* plants have increased tolerance to drought due to a decreased water loss compared to well-watered plants. This reduced water loss, due to limited transpiration, observed in *antiAtGLR1.1* plants was attributed to the involvement of AtGLR1.1 in the control of stomatal closure in guard cells (Kang et al., 2004). The stomatal apertures of *antiGLR1.1* lines were smaller than those of control plants, and this physiological change was explained by an increased endogenous ABA level in these *antiGLR1.1* lines. Interestingly, these observations by Kang et al. (2004) were consistent with our results that showed that the inhibition of MtGLRs by a glutamate receptor antagonist alleviated the PEG-induced water loss in *Medicago* embryo axis. Since our research group has demonstrated that the nitrate transporter MtNPF6.8, able to transport ABA, mediated nitrate inhibitory effects on primary root growth in *M. truncatula* (Pellizzaro et al., 2014, 2017), further work will be needed to

address the role of MtGLRs in nitrate signaling under water deficit stress.

5. Conclusion

Considering the involvement of MtGLRs on ABA-dependent NO production and adaptive responses on Medicago seedlings submitted to short-term water deficit stress, it will be important to define the distinct expression patterns of MtGLRs and to elucidate the physiological functions of MtGLRs, specifically those from the clade III in which numerous AtGLRs have been shown to be involved in response to environmental constraints.

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