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Abstract

Drought is a limiting factor for crop plant production, especially in arid and semi-arid climates. In this study, sorghum (*Sorghum bicolor*) was inoculated with two arbuscular mycorrhizal fungi, either the standard *Rhizophagus irregularis* or the desert-adapted *Rhizophagus arabicus*, and grown in experimental microcosms under well-watered or drought conditions. We investigated gene expression of selected major intrinsic proteins (MIPs) of sorghum in these mycorrhizal plants in comparison to non-inoculated, well-watered controls. Colonization with *R. irregularis* resulted in the induction of the MIPs *SbPIP2.2* and *SbPIP2.5*, regardless of whether sorghum plants were well watered or not. Root colonization with *R. arabicus*, however, caused an exclusive, strong reduction in the transcript levels of three MIP genes (*SbTIP2.*1, *SbNIP1.*2, *SbNIP2.*2) under drought conditions. . We also studied water transport properties of mycorrhiza-regulated MIPs. One particular MIP, *SbPIP2.8*, was found to mediate particularly high water permeability. Expression of this gene was strongly repressed upon drought, irrespectively on whether plants were mycorrhized or not.

Main text

Major intrinsic proteins (MIPs), also termed aquaporins (AQP), represent a phylogenetically rather old superfamily of channel proteins that facilitate the transport of water and small solutes (Abascal et al. 2014). Currently, MIPs are functionally grouped into three categories: aquaporins (AQPs) that are selective for water, aquaglyceroporins that confer permeability preferentially to solutes (*e.g.* glycerol, urea, boric acid, salicylic acid) or gases (*e.g.* ammonia, carbon dioxide), and bi-functional aquaglyceroporins that can facilitate efficient water and solute transfer. In plants, large gene families are found, with 55 members in poplar (Cohen et al. 2013), 41 members in sorghum (Reddy et al. 2015), 35 members in Arabidopsis (Johanson et al. 2001), and 33 members in maize (Chaumont et al. 2001) or rice (Sakurai et al. 2005). Based on their amino-acid sequence, plant MIPs can be divided into five subfamilies (Maurel et al. 2008; Lopez et al. 2012), namely plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), *NOD26*-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs) and intrinsic proteins characteristic for dicots (XIPs).

Among these groups, PIPs and TIPs are thought to be involved in the regulation of root water uptake (Ruiz-Lozano et al. 2012). Under drought conditions, various patterns of gene expressions have been reported: (Sarda et al. 1999; Cohen et al. 2013; Perez-Martin et al. 2014): PIPs and TIPs can be either down-regulated (*i.e. OePIP1.1* and *OePIP2.1* in olive; *PttPIP2.10* in poplar; *HaTIP18* in sunflower) or up-regulated (*i.e. PttTIP1.2*, *PttTIP2.2*, *PttTIP2.3*, *PttTIP2.4* and *PttPIP2.8* in poplar; *HaTIP7* in sunflower. Similarly to drought, MIPs are also regulated upon waterlogging stress in roots: Kadam et al. (2017) observed gene-specific regulation patterns with regard to *S. bicolor* genotype, root tissue, and duration of the stress. Furthermore, expression and/or activity of plant root MIPs is modulated by arbuscular mycorrhizal (AM) symbiosis, as shown in parsley (TIP: Roussel et al. 1997), *Medicago truncatula* (TIP: Krajinski et al. 2000; PIP and NIP: Uehlein et al. 2007), tomato (NIP, TIP and PIP: Chitarra et al. 2016) and *Lotus japonicus* (NIP and XIP: Giovannetti et al. 2012). Several studies dealing with the combined influence of AM symbiosis and abiotic stresses on MIP gene expression were performed. In AM

maize plants, where the expression profile of the entire MIP gene family was analyzed under short term and sustained drought conditions, six different gene expression patterns were observed, suggesting distinct physiological responses (Barzana et al. 2014). Similarly, drought-induced changes in PIP gene expression were also observed in mycorrhizal roots of *Phaseolus vulgaris* roots (Aroca et al. 2006), or of *Glycine max* and *Lactuca sativa* (Porcel et al. 2006).

Sorghum (*Sorghum bicolor* L.) is an important global crop grown for food, feed, fiber and fuel, and is particularly well adapted to hot and dry conditions (Paterson et al. 2009). Thus, we studied the effect of AM fungal species on MIP expression in sorghum roots under drought conditions. We also functionally characterized water permeability of selected sorghum MIP proteins by expression in *Xenopus* oocytes. Our data provide insight into the role of AMF (arbuscular mycorrhizal fungi) in plant water uptake and regulation.

Forty-one potential MIP encoding genes are found in the sorghum genome (Reddy et al. 2015). Based on the phylogenetic relationship of sorghum MIPs to MIPs known to be regulated by drought or mycorrhizal symbiosis in other plant species (highlighted in **Figure 1**), we selected 14 MIP genes (four PIPs, five TIPs, and five NIPs) for a detailed analysis. We measured the transcript levels of these genes in sorghum roots in the presence or absence of AM fungi under two water regimes. Plants were grown in microcosms (Koegel et al. 2013a), as described in Symanczik et al. (2018), in the presence of the desert-adapted *R. arabicus* MB804360, isolated from a hyper-arid sand plain in Oman (Symanczik et al. 2014), the standard *R. irregularis* BEG-75 (isolated from an agricultural field in Switzerland) or autoclaved *R. irregularis* BEG-75 inoculum (-AMF). During the first four weeks, plants were watered twice a week with distilled water to reach field capacity in order to allow the establishment of the mycorrhizal symbiosis. Then, two water regimes were applied: well-watered condition (80-100% field capacity) and drought condition (35-55% field capacity). Soil water content was monitored by weighing the pots periodically twice per week, and adjusted to the desired water content by adding the appropriate amount of distilled water. In addition, the pots received 10 mL of a modified Hoagland solution (Gamborg and Wetter 1975) weekly.

Plants were harvested after 16 weeks of growth, fine roots were isolated and used for gene expression analysis (**supplementary methods**). Mycorrhizal root colonization ranged from 58 to 75% for all treatments, independently of the water regime. However, the percentage of arbuscules in inoculated sorghum-plants significantly decreased under drought conditions (Symanczik et al. 2018). In addition, total plant dry weight was always lower under drought compared to well-water conditions, independently of the AMF treatment (Symanczik et al. 2018).

Under such experimental conditions, how do drought and mycorrhizal symbiosis with different AMF strains affect MIP expression? We report here changes in expression of the 14 selected MIPs in **Table 1** in detail, in comparison to the control (-AMF/well-watered conditions).

SbNIP1.3 and *SbTIP2.2* expression was not detectable in roots of *S. bicolor*, while only very low transcript levels were observed for *SbTIP1.2*. *SbPIP2.8* expression was exclusively and significantly repressed under drought treatment in mycorrhizal and non-mycorrhizal sorghum roots ($F= 27.7$, $p<0.001$). *SbTIP 1.1* expression was upregulated upon drought treatment in uninoculated roots and in roots inoculated with *R. irregularis*. This behavior was, however, not observed when roots were inoculated with *R. arabicus*.

For well-watered plants, the presence of *R. arabicus* had no or only a minor impact on MIP gene expression. However, transcript levels of *SbTIP2.1*, *SbNIP1.2*, and *SbNIP2.2* were significantly reduced upon drought treatment.

The strong suppression of MIP genes observed after *R. arabicus* inoculation under drought was not seen in the presence of *R. irregularis*. Instead, inoculation with *R. irregularis* led to elevated *SbPIP2.2 and SbPIP2.5* transcript levels in well-watered sorghum roots, an effect also seen in non-mycorrhized plant roots upon drought treatment, indicating corresponding adaptations upon *R. irregularis* colonization and drought response. This is in accordance with findings from Li et al. (2012), showing an up-regulation of several PIP genes under drought in *R. irregularis*-colonized maize plants. Differential expression of PIP genes has also been reported between upland- and lowland-rice roots when subjected to water stress (Lian et al. 2006). Surprisingly, in Sorghum, the two MIPs mentioned, *SbPIP2.2 and*

SbPIP2.5, were not upregulated in the presence of *R. arabicus*, neither under well-watered nor under drought conditions.

To get some clues on sorghum MIP water transport properties, the TIPs and PIPs regulated by AM symbiosis were analyzed by heterologous expression in *Xenopus laevis* oocytes. The permeability coefficients (Pf) of the controls were between 10.3 (± 2.1) µm/s (buffer-injected oocytes; negative control) and 60.7 (± 7) µm/s (oocytes injected with Lacbi1:39209, *Laccaria bicolor*; positive control), respectively, and thus in the range of previously published results (Dietz et al. 2011) (**Table 2**). All sorghum MIPs analyzed conferred enhanced water permeability in *Xenopus* oocytes. For SbPIP2.8, the effect was even larger (Pf value 75.2 ± 8.5 µm/s) than for the positive control. Interestingly, the expression of this particular gene was strongly down-regulated upon drought, irrespective of mycorrhizal colonization (see **Table 1**). We hypothesize that reduced plasma membrane permeability, based on downregulation of *SbPIP2.8* is functionally important to reduce drought stress. It is worth noting that Li et al. (2013) observed that the mycorrhizal MIPs *GintAQP1* and *GintAQP2*, which equally confer high water permeability, were up-regulated in arbuscule-rich cortical cells of maize roots under drought, potentially to guarantee an efficient water translocation from extraradical hyphae to the root cells. However, these scenarios are speculative at the moment since that *GintAQP1* and *GintAQP2* as well as *SbPIP2.8* may also be post-transcriptionally regulated, in addition to transcriptional control.

Overall, our data indicate that the regulation of MIPs in sorghum is differentially affected in response to the mycorrhizal partner. This may be explained by the different ecological niches of the two fungi. While *R. arabicus* is highly adapted to severe drought conditions, *R. irregularis* is not, possibly influencing the adaptation strategies of the plant upon mycorrhiza formation. Similar observations were made by Porcel et al. (2006) observed differential expression of *LsPIP2* after colonization of lettuce (*Lactuca sativa*) with *R. irregularis* or *Funneliformis mosseae*. While colonization of the lettuce roots with *R. irregularis* did not change *LsPIP2* expression, the presence of *F. mosseae* resulted in *LsPIP2* suppression (similar to the result observed with *SbPIP2.2* after colonization with *R. arabicus*).

AM symbiosis is extensively reported as being capable in improvement of plant drought resistance, as reflected by better growth and water status (Ruiz-Lozano, 2003). Two working models for MIP function upon drought stress are currently discussed. The first model supports an increased water transport capacity, which require up-regulation of MIPs (Jang et al. 2004; Yu et al. 2005). A second model, however, favors cellular water conservation which implies down-regulation of MIP function (and expression if post-translational gating is not feasible) to prevent cellular water loss (Smart et al. 2001; Aharon et al. 2003). Transcriptional MIP regulation as consequence of mycorrhiza formation and drought is commonly observed.

In this context, it is interesting that *R. irregularis*-inoculated tomato and maize plants appear to be able to switch between mainly apoplastic and mainly MIP-mediated transcellular water transport pathways; this implies a higher flexibility to changing environmental conditions, *e.g.* to improve plant response to drought (Bárzana et al. 2012). In addition, Recchia et al. (2018) revealed a further potential pathway of mycorrhizal plants to efficiently adapt to water deficit: Plant MIPs in arbuscule-containing cortical cells were immediately and specifically regulated upon drought as compared to cortical cells of non-mycorrhizal plants, followed by secondary signaling events throughout the whole plant, mediating a fast adaptation of mycorrhizal plants upon drought events.

The possible impact of post-translational modification of MIPs (e.g. by phosphorylation) on their function is in most cases still open and needs to be addressed in future (Chaumont et al., 2014). Individual plant/fungus genotype combinations might favor different adaptation strategies. In regard to climate change, with its foreseeable shifts towards longer drought periods and heavy flooding events (IPCC, 2014), the role of AM fungi in alleviating plant drought or flooding stress will become more important in the future to strengthen crop performance in order to safeguard crop yields and plant nutrition.

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Table 1. Mean fold changes in MIP gene expression in *Sorghum bicolor* roots inoculated with the arbuscular mycorrhizal (AM) fungal species *Rhizophagus irregularis*, *Rhizophagus arabicus* or non-inoculated (-AMF) exposed to well-watered and drought conditions.

Significant values for fold change in MIP expression are given. Fold change expression was calculated relative to -AMF well-watered conditions (control conditions). Ubiquitin was used as reference gene (Koegel et al. 2013b). PCR primers designed for qRT PCR are given in **table S1**. Data were analysed using independent samples t-test for fold change expression with a significant level of 0.05% compared to control conditions. 2-way ANOVA including the factors water regime (W) and AM treatment (followed by LSD's multiple range test with a significance level of 0.05%) was performed over all expression values within each MIP gene; F_{ANOVA} is given; *, $p<0.05$; **, $0.001<p<0.01$; ***, $p>0.001$. Grey cells correspond to significant values. Ns and — mean not significant and low or not expressed in roots, respectively. Changes in expression were considered significant when > than 2 fold-changes (up-regulated) or < than 0.5 (down-regulated) fold-changes.

Gene	Fold change expression					F_{ANOVA}		
	Well-watered		Drought					
	Rhizophagus irregularis	*Rhizophagus arabicus*	- AMF	*Rhizophagus irregularis*	*Rhizophagus arabicus*	Water	Mycorrhizal	Water x Mycorrhizal
SbTIP1.1	1.1	0.9	2.6 **	3.1 **	0.6	38.3 ***	20.5 ***	14.1 ***
SbTIP1.2	—	—	—	—	—	—	—	—
SbTIP2.1	1.2	0.8	1.1	1,0	0.2 ***	ns	4.3 *	ns
SbTIP2.2	—	—	—	—	—	—	—	—
SbTIP2.3	1.9	0.7	2.0 *	2.0 *	1.1	ns	5.5 *	ns
SbNIP1.1	1.3	0.9	0.8	1,0	0.8	ns	ns	ns
SbNIP1.2	0.7	1.1	1.3	0.8	0.5 **	ns	ns	ns
SbNIP1.3	—	—	—	—	—	—	—	—
SbNIP2.1	1.1	1.3	1,0	0.8	0.9	ns	ns	ns
SbNIP2.2	0.8	0.5	0.9	0.7	0.3 **	ns	5.3 *	ns
SbPIP1.1	2.0 *	1.3	1.2	1.3	1.3	ns	2.7 *	ns
SbPIP2.2	2.5 *	1.1	2.0 **	2.2 *	0.8	ns	22.1 ***	6.3 **
SbPIP2.5	7.5 *	0.7	5.7 *	6.9 **	2,0	4.7 *	17.7 ***	3.5 *
SbPIP2.8	1.1	1.4	0.2 ***	0.1 ***	0.1 ***	27.7 ***	ns	ns

Table 2. Water permeability of *X. laevis* oocytes expressing *Sorghum bicolor* MIPs. Water permeability (Pf) of ND96-buffer-injected oocytes (negative control) and those expressing aquaporins from either *S. bicolor* (Sb01g047140, SbTIP1.1; Sb06g024590, SbTIP2.3; Sb06g025150, SbPIP1.1; Sb02g010760, SbPIP2.2; Sb06g022840, PIP2.5; Sb02g031390, SbPIP2.8) or *Laccaria bicolor* (Lacbi1:392091, (Dietz et al. 2011)) as positive control). Pf values were calculated from swelling rates obtained after oocyte transfer into hypotonic medium.

	Pf-value	Standard deviation	No. Of replicates
Buffer control	10.3	2,1	37
Lacbi1:392091	60.7	7.0	25
SbTIP 1.1	34.4	4.9	38
SbTIP 2.3	45.2	5.0	42
SbPIP 1.1	39.3	5.5	27
SbPIP 2.2	22.7	2.8	37
SbPIP 2.5	40.2	4.7	48
SbPIP 2.8	75.2	8.5	27

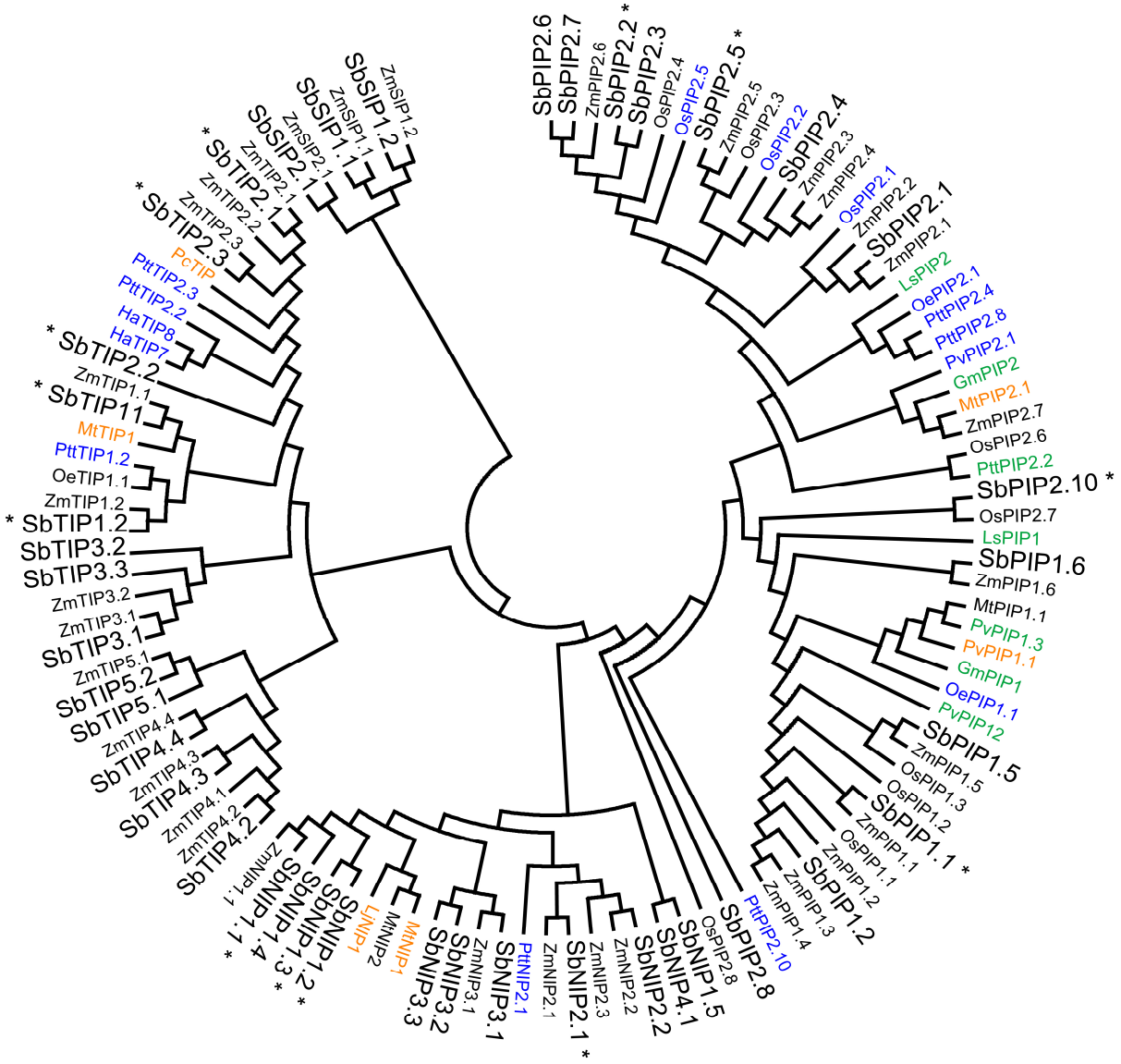

Fig. 1 Neighbor-joining tree for MIPs based on the full open reading frames. Sequence names consist of species code (first letter of genus and first letter of species name) and gene name. Species codes: Lj, *Lotus japonicus*; Ls, *Lactuca sativa*; Mt, *Medicago truncatula* Oe, *Olea europea*; Os, *Oryza sativa;* Ptt, *Populus trichocarpa*; Pc, *Petroselinum crispum*; Pv, *Phaseolus vulgaris*; Sb, *Sorghum bicolor;* Zm, *Zea mays*. *S. bicolor* MIPs are enlarged in font size. Selected sorghum MIPs are marked with an asterix. Homologs regulated by drought (blue), mycorrhizal symbiosis (orange) or both factors (green) are highlighted.

The MIP amino acid sequences were aligned with MEGA5 (Tamura et al. 2011) using the following multiple alignment parameters: gap opening penalty 15, gap extension

penalty 0.3, and delay divergent sequences set to 25%; and the Gonnet series was selected as the protein weight matrix. Neighbor joining trees were constructed using Poisson correction model for distance computation in MEGA5. Bootstrap analysis was carried out with 1000 replicates. Branch lengths are proportional to phylogenetic distances. Gene accession numbers of amino acids sequences from sorghum are given in **table S1**, accessions of homologs from the other plant species are given in **methods S1**.