

Aquaporins Regulation by Arbuscular Mycorrhizae

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Despite an intensive research in the field of aquaporins, the relationship between aquaporins and plant responses to water deficit remains still unresolved. However, several authors have recognized the importance of aquaporins for both nutrient and water exchanges during mycorrhizal symbiosis. We have conducted investigations showing that the AM symbiosis regulates the expression of a wide number of aquaporin genes in maize, including members of the different aquaporin subfamilies. Several of these AM-regulated aquaporins were functionally characterized in heterologous systems with *Xenopus laevis* oocytes and by yeast complementation. It was shown that they can transport water, but also other molecules of physiological importance for plant performance under both normal and stress conditions (glycerol, urea, ammonia, boric acid, silicon or hydrogen peroxide). AM plants grew and developed better than non-AM plants under the different conditions assayed. Thus, the investigation suggests that the well-known better performance of AM plants under drought stress may be due not only to the improved water movement in their tissues, but also to the mobilization of compounds with a role in abiotic stress tolerance such as glycerol, N compounds, signalling molecules or metalloids.

Keywords: aquaporins ; arbuscular mycorrhizal symbiosis ; drought ; plant performance

1. Introduction

Aquaporins are a group of membrane intrinsic proteins that facilitate and regulate the passive movement of water molecules down a water potential gradient (Maurel et al. 2008). In plants, aquaporins comprise a large and diverse protein family composed by 31 to 71 different genes, depending on the plant species and are subdivided in five subgroups based on their amino acid sequence similarity. These are plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-like intrinsic proteins (NIPs), small and basic intrinsic proteins (SIPs) and X intrinsic proteins (XIPs), a recently described group (Gupta and Sankararamakrishnan 2009), which has been shown to transport a variety of uncharged substrates (Bienert et al. 2011), including water (Lopez et al. 2012).

In plants, aquaporins discovery has caused a significant change in the understanding of plant water relations. High levels of aquaporin expression have been shown in tissues with high water fluxes across membranes, but also in roots where water uptake occurs (Otto and Kaldenhoff 2000). Thus aquaporins seem to play an important role in controlling transcellular water transport in plant tissues (Javot and Maurel 2002; Zhao et al. 2008). However, the relationship that exists between aquaporins and plant responses to water deficit still remains elusive and with contradictory results (Aharon et al. 2003; Lian et al. 2004).

Although many aquaporins are highly selective for water, the selectivity filters of plants aquaporins show a high divergence (Sui et al. 2001), suggesting a great functional diversity for these proteins (Bansal and Sankararamakrishnan 2007). Indeed, it is clear that some aquaporins do not exhibit a strict specificity for water and can transport other small neutral molecules such as ammonia (Loque et al. 2005), urea (Liu et al. 2003), carbon dioxide (CO₂) (Uehlein et al. 2003), hydrogen peroxide (H₂O₂) (Bienert et al. 2007), silicic acid (Ma and Yamaji 2006), boric acid (Mitani et al. 2008) and some other molecules with physiological significance (Bienert et al. 2008), highlighting the great relevance of aquaporins for many other processes of plant physiology (Chaumont and Tyerman 2014; Li et al. 2014). The ability of aquaporins to transport molecules such as urea and NH₃ points to important roles for aquaporins in nitrogen metabolism. The membrane diffusion through aquaporins of the gas molecule CO₂ suggests their involvement in carbon fixation. The ability of aquaporins to transport H₂O₂ points to potential roles in stress signalling and responses. Silicon uptake and metabolism seem to be crucial for plant responses to biotic and abiotic stresses and boron is closely related with nutrition and structural development (Maurel 2007).

Under natural conditions, most plants establish a symbiosis with arbuscular mycorrhizal (AM) fungi (Varma 2008). By the AM symbiosis, plants get nutrients and water resources less available to the plant roots, while the fungus receives carbon compounds from the plant and finds an ecological niche to complete its life cycle (Koide and Mosse 2004). At the same

time, AM symbiosis enhances plant tolerance to different biotic and abiotic stresses (Ruiz-Lozano et al. 2006; Ruiz-Lozano et al. 2012; Selosse et al. 2014).

During the AM symbiosis plant root cells must undergo extensive morphological alterations in order to accommodate the presence of an endophytic symbiont and most of these changes concern vacuolar or cytoplasmic membrane systems. Thus, Krajinski et al. (2000) hypothesized a variation of expression affecting genes that encode membrane-associated proteins such as the aquaporins. In addition, AM symbiosis has been shown to alter root hydraulic properties (Khalvati et al. 2005; Bárzana et al. 2012). Thus, it is not surprising that AM fungi can also change plant aquaporin gene expression and protein accumulation.

The first report on the modulation of aquaporin genes by AM symbiosis was provided by Roussel et al. (1997) followed by Krajinski et al. (2000), who found mycorrhiza-induced expression of TIP aquaporins in parsley and *Medicago truncatula*, respectively. Krajinski et al. (2000) related the changes in aquaporin gene expression to the changes in plant roots due to fungal colonization. In fact, during AM formation the plant plasma membrane extends to form a novel periarbuscular membrane, which closely surrounds the fungal hyphae resulting in an estimated 3- to 10-fold increase in the outer plant cell surface (Bonfante and Perotto 1995; Gianinazzi-Pearson 1996). It was proposed that the up-regulation of aquaporins by the AM symbiosis probably optimizes water and nutrient exchange between both symbiotic partners (Krajinski et al. 2000).

In the last years, our research group has been investigating the alteration by the AM symbiosis of aquaporin genes under conditions of drought stress. The results obtained show that a given aquaporin gene can respond differently depending on the specific characteristics of the applied osmotic stress and the presence or absence of the AM fungus in the roots of the host plant (Aroca et al. 2007). However, these studies were limited to a reduced number of aquaporin genes and in different host plants (two in soybean, two in lettuce, four in bean, four in tomato and seven in maize). Thus, there was still an important lack of knowledge about the modulation of expression for most aquaporin genes by the AM symbiosis, as well as about the relationship among this modulation and changes in root hydraulic conductance, plant water status and plant tolerance to water deficit (Ruiz-Lozano et al. 2006). In addition, the plant stress responses vary greatly depending upon the intensity and duration of the water deficit exposure. For such reasons, we conducted an investigation aimed at elucidating in which way the AM symbiosis modulates the expression of the whole set of aquaporin genes present in a plant under both optimal and drought stress conditions. For that, maize plants were subjected to drought stress treatments of different intensity and duration. A second objective aimed to test the hypothesis whether the aquaporins regulated by the AM symbiosis transport only water or have the capacity to transport other molecules of physiological interest for the plant. Thus, we characterized some of these aquaporins in order to shed further light on the molecules that could be involved in the mycorrhizal responses to drought.

2. Regulation of Aquaporins by the AM Symbiosis and Influence on Plant Water Relations

The AM symbiosis results in altered rates of water movement into, through and out of the host plants (Augé 2001) and also modifies the sap flow rate (J_v) and osmotic root hydraulic conductance (L_o) (Aroca et al. 2007; Ruiz-Lozano et al. 2009; Bárzana et al. 2012; 2014). Aquaporins provide a low resistance pathway for the movement of water across a membrane and PIPs and TIPs isoforms have been recognized as central pathways for intracellular and transcellular water transport (Maurel et al. 2008). Furthermore, since aquaporins can be gated, this provides a mechanism to control the movement of water along plant tissues. Thus, it seems likely that mycorrhizal symbiosis causes significant changes in aquaporin activity of host plants (Uehlein et al. 2007; Bárzana et al. 2014) and some of the plant aquaporins might be important for the mycorrhizal responses.

In the study conducted with maize, AM plants maintained higher J_v and L_o values than non-AM plants under short-term drought stress conditions. Under sustained drought stress, the hydrostatic root hydraulic conductance (L_h) was also maintained high in AM plants (Bárzana et al. 2014). These effects were related with the increased absorbing surface caused by fungal hyphae growing in the soil, combined with the fungal ability to take up water from soil pores inaccessible to roots, as AM hyphae represent a low-resistance way for water movement until root cells (Allen 2007, 2009) and maintain liquid continuity in the soil, limiting the loss of soil hydraulic conductance caused by air gaps (Smith et al. 2010). Thus, under such conditions water movement through AM fungal hyphae can be critical to improve the water supply to the plant, increasing cell-to-cell and apoplastic pathways (Bárzana et al. 2012). On the other hand, AM fungal aquaporins were related to water transport in the extraradical mycelium and in the periarbuscular membrane (Li et al. 2013) and aquaporins from the ectomycorrhizal fungus *Laccaria bicolor* are involved in root water transport by white spruce plants (Xu et al. 2015). Thus, in AM plants, the enhanced L_o values under short-term drought stress and L_h values under sustained drought could be due to the activity of the fungal aquaporins.

All the PIPs analyzed in the study with maize plants had typical features that allow water transport. Indeed, all PIP2s transported water, specially ZmPIP2;2 (Bárcana et al. 2014). Under short-term drought stress, almost all PIPs were down-regulated in non-AM plants, while in AM plants the expression of most PIPs (*ZmPIP1;1*, *ZmPIP1;2*, *ZmPIP1;3*, *ZmPIP1;4*, *ZmPIP1;6*, *ZmPIP2;2* and *ZmPIP2;4*) was kept high or even increased. Such an effect correlated with the measured Jv and Lo. On the contrary, the high values of Jv and Lo under well water conditions contrast with changes in the expression or protein accumulation of the different analyzed aquaporins (Bárcana et al. 2014). This lacking correlation has already been observed (Boursiac et al. 2005; Aroca et al. 2007; Ruiz-Lozano et al. 2009) and is likely due to the fact that aquaporin regulation is not only restricted to the transcriptional level but is also subjected to post-transcriptional modifications. Besides, aquaporins are not the unique way to control Lo. Depending on the specific environmental circumstances, symplastic movement of water via plasmodesmata may contribute significantly to Lo (Galmés et al. 2007). Moreover, a compensatory mechanism among AM fungal aquaporins and host plant aquaporins has been proposed (Aroca et al. 2009). Thus, the down-regulation of host plant aquaporins could be compensated with the activity of fungal aquaporins to maintain high Jv and Lo values in AM roots under well-watered conditions. Sustained drought decreased Jv and Lo in maize roots but did not reduce *PIP* gene expression or *PIP* protein accumulation. Under drought the posttranscriptional regulation may have an important role. In fact, there are evidences of a relationship between the reactive oxygen species produced under abiotic stress and the internalization and changes in the subcellular localization of PIPs (Boursiac et al. 2005, 2008) or with a direct blockage of aquaporins (Ye and Steudle, 2006). This would lead to a decrease in Lo without changing the expression or accumulation of the aquaporins (Benabdellah et al. 2009), as was observed in maize (Bárcana et al. 2014).

TIPs are the most abundant aquaporins in plants and are mainly associated to the tonoplast (Hunter et al. 2007). It has been proposed that TIPs may provide a quick way for cellular osmotic balance by controlling the exchange of water between vacuole and cytosol (Forrest and Bhavé 2007), playing an important role under osmotic stress conditions (Katsuhara et al. 2008). Thus, TIPs may also have an influence on Jv and Lo by affecting exchanges of water between transcellular and symplastic water pathways. In the study with maize, *ZmTIP1;1* and *ZmTIP1;2* were highly expressed in all treatments and in the oocyte system both exhibited a high capacity for water transport. Mycorrhization up-regulated their expression under short-term drought stress, but under sustained drought they were down-regulated, correlating with Jv and Lo parameters.

3. Regulation of Aquaporins by the AM Symbiosis and Possible Influence on the Transport of Solutes of Physiological Importance for the Plant

Uehlein et al. (2007) described the induction by the AM symbiosis of specific PIP and NIP isoforms exhibiting permeability to water and to ammonia, respectively. Authors suggested that these aquaporins could be involved in the symbiotic exchange processes between the fungus and the plant. Thus, Maurel and Plassard (2011) proposed that the role of aquaporins in the AM symbiosis could be more complex than simply regulating plant water status. The results obtained with maize plants point in the same direction, since many of the aquaporins regulated by the AM symbiosis can transport a variety of compounds of physiological importance for the plant (Bárcana et al. 2014).

3.1. Transport of Glycerol

Despite the number of aquaporins that has been described as glycerol transporters in plants, the physiological implication of this molecule remains unclear since the utilization of glycerol is well known in fungi and bacteria but not in plants (Dietz et al. 2011). As other compatible solutes, glycerol must be able to come into the cells and move through the lipids bilayer very fast to respond to osmotic imbalances. In diverse organisms aquaporins have been demonstrated to fulfil this role. Some aquaporins from plants have been characterized as functional glycerol transporters. Most of them belong to the NIP subgroup (Cabello-Hurtado et al. 2004). NIPs are supposed to originally come from bacteria by horizontal gene transfer (Zardoya et al. 2002) and were later adapted for glycerol transport, suggesting that this was a selective advantage for plants (Gustavsson et al. 2005). We demonstrate in oocytes that *ZmNIP1;1* and *ZmNIP2;1* can transport glycerol. The transport of glycerol has also been suggested for TIP4 isoforms, since their selectivity filter contains amino acids of small size that increase the pore diameter (Bansal and Sankararamkrishnan 2007), allowing the possible transport of big molecules such as glycerol. Both *ZmTIP4;1* and *ZmTIP4;2* were regulated in a different way as most of the other aquaporins, increasing their expression under sustained drought stress in non-AM plants and enhancing further its expression in AM plants. In addition, their selectivity filter is not adequate for water transport (Wallace and Roberts 2004; Bansal and Sankararamkrishnan 2007), which suggests that aquaporins grouped in pattern 6 may fulfil specific functions in the transport of solutes, including glycerol, that may be important for the AM symbiosis or for the plant-fungus interaction under sustained drought stress conditions. Indeed, a study has shown a transfer of glycerol from host plant to pathogenic fungi (Wei et al. 2004), and Gustavsson et al. (2005) suggested that exporting plant glycerol may be also important for symbiotic fungi and that this could be the reason why glycerol transporters were fixed in plant genomes.

3.2. Transport of Nitrogen Compounds

The ammonium ion (NH_4^+) and its conjugated base ammonia (NH_3) are the potential primary sources of N in plant nitrogen nutrition. Moreover, in the AM symbiosis, ammonium is suggested to be the major nitrogen compound transferred to the host plant, with urea playing a role as an intermediate solute (Govindarajulu et al. 2005; Tian et al. 2010; Perez-Tienda et al. 2011), which could be also involved in fungus-based nitrogen nutrition of plant hosts (Dietz et al. 2011). However, the mechanisms for a sustained efflux of $\text{NH}_4^+/\text{NH}_3$ into the apoplastic space at the AM symbiotic interface remain elusive. Maurel and Plassard (2011) have suggested fungal aquaporins among the alternative candidates proposed for N export from the fungal cytoplasm into the common apoplastic space, where it will become protonated due to acidic pH conditions. Thus, aquaporins have been pointed as low-affinity channels for N absorption, mobilization and detoxification in the nitrogen metabolism in plants (Liu et al. 2003) and in the AM symbiosis (Uehlein et al. 2007; Pérez-Tienda et al. 2011; Maurel and Plassard 2011).

Several TIP isoforms have been shown to transport ammonia and urea (Liu et al. 2003; Loque et al. 2005), and in maize, most of the TIPs possess an ar/R (aromatic/arginine) region which enables the protein to transport these compounds, including ZmTIP1;1 and ZmTIP1;2 (Sui et al. 2001; Jahn et al. 2004; Gu et al. 2012). Transport of urea and $\text{NH}_3/\text{NH}_4^+$ into the vacuole would avoid their toxicity in the cytoplasm and/or allow storage of N (Wang et al. 2008) and whenever required as an N-source, these compounds could be remobilized by a passive, low-affinity transport pathway, such as that provided by TIPs (Liu et al. 2003). Several TIP2 homologs were found to have a high permeability to NH_3 , and may participate in the compartmentalization of this compound in vacuoles (Loque et al. 2005). In the study with maize, the expression of most of the aquaporins decreased in non-AM plants under short-term drought stress conditions, while in AM plants the expression of several aquaporin genes was maintained under such conditions or even up-regulated for five genes. Such up-regulation would promote not only the transport of water, but also of nitrogen compounds (Uehlein et al. 2007). These nitrogen compounds may then need to be translocated to the vacuole and stored. This would avoid a possible toxicity in the cytosol, reason why ZmTIP1;1 and ZmTIP1;2 were up-regulated in AM plants as compared to non-AM ones (Bárzana et al. 2014). Under sustained drought stress, the expression of most aquaporins and their protein accumulation decreased in non-AM plants, probably because of a lower availability of mineral nutrients for the plant. In contrast, in these plants the levels of ZmTIP1;1 and ZmTIP1;2 were kept high. Under the limiting conditions originated by the sustained drought and by the general down-regulation of other aquaporins in non-AM plants, a remobilization of N stored in the vacuole may be necessary in these plants, reason why the levels of ZmTIP1;1 and ZmTIP1;2 would be kept high. In the case of AM plants, a down-regulation of these two genes was observed under sustained drought stress. In this case, the ability of AM fungi to get N compounds from a highly dry soil is likely to be strongly reduced. Thus, plants have no capacity to store N compounds in the vacuole and the expression of these TIP genes may be kept low.

3.3. Transport of Metalloids

Boron (B) and silicon (Si) are present in soil solution mainly as non-charged molecules at neutral pH and are not metabolized by plants. They are transported as boric and silicic acids, respectively (Miwa et al. 2009). Both metalloids have key structural functions in plant cells: boron cross-links with the pectin fraction of cell walls and polymers of hydrated silica-gel are important for the physical strength of plant cells, especially in monocots like maize (Miwa et al. 2009). Both metalloids are present in all plants tissues, although in different amounts depending on the species. For instance, boron is an essential element for plant growth, but it can be toxic when present at high concentrations, while silicon can account for 10% of shoot dry weight in cereals such as rice, where it enhances resistance to biotic and abiotic stresses (Li et al. 2014).

Among the aquaporins characterized in the study with maize, all except PIPs were able to transport boron. Maize has a low B requirement to maintain normal vegetative growth because graminaceous plants have the lowest cell wall pectin content. However, at the reproductive stage maize needs as much B as other species (Blevins and Lukaszewski 1998). At high concentrations B is toxic, reason why maize must control tightly the B distribution to maintain adequate B levels in their cells (Miwa et al. 2009). Thus, a complex regulation of B transport should exist and aquaporins and some other B transporters can be involved.

Under sustained drought, reduced plant transpiration can lead to B deficiencies, which can affect negatively plant growth. In maize, the B requirements in non-AM plants may be guaranteed by the aquaporins that can transport B (ZmTIP1;1, ZmTIP1;2, ZmNIP1;1 and ZmNIP2;2), which were highly expressed. However, we observed that the mycorrhization decreased the expression of almost all the aquaporins that can transport B. This can be due to the fact that the AM fungus may provide directly B to the host plant, and plant aquaporins involved in B transport are down regulated in order to avoid toxicity due to an excess of B.

Silicon is actively absorbed and accumulates in high amount in maize tissues where it functions in tolerance to abiotic stresses (Ma and Yamaji 2006). Thus, the existence of several mechanisms of transport and regulation of Si uptake is expected in maize. Both, ZmNIP2;1 and ZmNIP2;2 were characterized by Mitani et al. (2009) as Si transporters.

In the study with maize, the mycorrhization reduced the expression of ZmNIP2;1 and ZmNIP2;2 under well-watered conditions and the expression was kept low under drought stress conditions. The most direct explanation for this effect is that Si deposited beneath the cuticle can mechanically impede penetration by fungi and, thereby, a diminution of Si uptake in mycorrhizal plants can be expected. Even more, soluble Si acts as a modulator of host resistance to fungal infection and colonization (Fauteux et al. 2005), suggesting that soluble Si should be also reduced in mycorrhizal plants.

It is remarkable that in non-AM plants, ZmNIP2;2 increased its expression under sustained drought conditions. Si taken up by the crown roots, where ZmNIP2;2 is localized, might increase their mechanical resistance and protects the plants from lodging (Mitani et al. 2009). This could be the function of ZmNIP2;2 in non-AM plants subjected to sustained drought. In contrast, AM plants did not up-regulate this gene, probably because they were not at risk from lodging.

3.4. Transport of Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is continuously produced in the metabolism of aerobic organisms. Thus, it is one of the most abundant reactive oxygen species (ROS). At low concentrations, it acts as a signal molecule controlling different essential processes in plants during normal growth and development (Bienert et al. 2006; Quan et al. 2008), but it also functions as a defensive signal molecule against various abiotic and biotic stresses (Miller et al. 2010; Bienert et al. 2006). On the other hand, as oxidant molecule, it react with various cellular targets causing cell damage and, at high concentrations, it orchestrates programmed cell death (Bienert et al. 2006; Quan et al. 2008). Because of these opposing functions, an appropriate intracellular balance between H_2O_2 generation and scavenging exists in all cells (Miller et al. 2010) and maintaining the level of H_2O_2 in a normal dynamic balance is used by plants to activate stress responses that help the plant cope with environmental changes (Quan et al. 2008). H_2O_2 has almost the same size, electro-chemical properties and capacity to form hydrogen bonds as does water (Bienert et al. 2007). Indeed, some plant aquaporins were shown to transport H_2O_2 (Bienert et al. 2007; Dynowski et al. 2008) and in the study with maize, all aquaporins able to transport water could also transport H_2O_2 , specially ZmTIP1;1. Bienert et al. (2006, 2007) proposed that TIP1s could play a key role in the detoxification of excess H_2O_2 generated under stress conditions. This idea fits with the high gene expression and protein content maintained for ZmTIP1;1 under both short-term and sustained drought stress conditions. Additionally, the mobilization of H_2O_2 via aquaporins could serve as a regulatory mechanism for membrane internalization of plant PIPs (Boursiac et al. 2008), with subsequent effects on water transport.

4. Conclusion and Perspectives

The AM symbiosis regulates the expression of a wide number of aquaporin genes in the host plant, comprising members of the different aquaporin subfamilies. Most of these aquaporins can transport water, but also other molecules of physiological importance for plant performance under both normal and stressful conditions. The regulation of these genes depends on the watering conditions and on the severity of the drought stress imposed. Thus, we propose that the AM symbiosis acts on the host plant aquaporins in a concerted manner to alter both plant water relations and plant physiology and to cope better with the stressful environmental conditions. The future studies should be devoted to elucidate the specific function *in planta* of each aquaporin isoform regulated by the AM symbiosis in order to decipher which compounds can be transported *in vivo* by these aquaporins and to shed further light on how the symbiosis alters the plant fitness under stressful conditions.

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