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Expression Analysis of the First Arbuscular Mycorrhizal Fungi Aquaporin Described Reveals Concerted Gene Expression Between Salt-Stressed and Nonstressed Mycelium

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Roots of most plants in nature are colonized by arbuscular mycorrhizal (AM) fungi. Among the beneficial effects of this symbiosis to the host plant is the transport of water by the AM mycelium from inaccessible soil water resources to host roots. Here, an aquaporin (water channel) gene from an AM fungus (*Glomus intraradices*), which was named *GintAQP1*, is reported for the first time. From experiments in different colonized host roots growing under several environmental conditions, it seems that *GintAQP1* gene expression is regulated in a compensatory way regarding host root aquaporin expression. At the same time, from in vitro experiments, it was shown that a signaling communication between NaCl-treated mycelium and untreated mycelium took place in order to regulate gene expression of both *GintAQP1* and host root aquaporins. This communication could be involved in the transport of water from osmotically favorable growing mycelium or host roots to salt-stressed tissues.

The colonization of dry land by aquatic plants approximately 400 million years ago was possible thanks to the establishment of a symbiosis between plants and fungi (Pirozynski and Malloch 1975), including arbuscular mycorrhizal (AM) fungi (Bonfante and Genre 2008). Thanks to this symbiosis, plants were able to colonize a stressful environment such as the dry land. Thus, both partners of the symbiosis co-evolved successfully to colonize almost all dry-land ecosystems, from the tropics to subpolar latitudes (Rosendahl 2008). In the AM symbiosis, plants get nutrients and water resources that are less available to the plant roots from the fungi, while the fungi receive carbon compounds from the plant and find a niche to complete their life cycle (Koide and Mosse 2004). At the same time, AM symbiosis enhances plant tolerance to different abiotic stresses with osmotic components such as drought and salinity (Augé 2001; Jahromi et al. 2008; Ruiz-Lozano 2003; Ruiz-Lozano et al. 2006).

Under osmotic stress conditions, plants need to finely regulate their water relations. Thus, when plants are exposed to an osmotic stress, the first symptoms are a diminution of their root water permeability and a decrease of their leaf transpiration rate (Aroca et al. 2006; Muhsin and Zwiazek 2002). By both mechanisms, plants try to save as much water as possible. However, the relationship between diminutions of leaf transpiration or water uptake capacity and tolerance to osmotic stresses is not well established. Under certain osmotic stress conditions, it can be helpful to the plant to increase its root hydraulic conductivity and then enhance its water uptake capacity. However, under other circumstances, it can be more efficient to reduce root hydraulic conductivity in order to avoid water flow from roots to the soil when the water potential of the soil decreases. These apparent contradictory behaviors are still a matter of debate (Maurel et al. 2008). However, most recently, Matsuo and associates (2009) found a positive correlation between shoot dry weight and root hydraulic conductivity under water-limited conditions in different rice genotypes.

It is commonly accepted that plant water relations are ultimately regulated by aquaporins activity (Forrest and Bhave 2007; Maurel et al. 2008). Aquaporins are proteinaceous pores present in the membranes of all living organisms that facilitate the transport of water and other small and neutral solutes, always following an osmotic gradient (Chrispeels and Agre 1994; Forrest and Bhave 2007; Maurel et al. 2008; Pettersson et al. 2005). In plants, aquaporins are part of a large and diverse protein family consisting of 31 to 37 different genes (Maurel et al. 2008; Sade et al. 2009) depending on the plant species. Therefore, plant aquaporins are subdivided into four groups based on their amino acid sequence homology: plasma membrane intrinsic proteins (PIP), tonoplast intrinsic proteins (TIP), nodulin-like intrinsic proteins (NIP), and small and basic intrinsic proteins (SIP). Most recently, a fifth plant aquaporin group has been described in tomato, and it was called XIP (Sade et al. 2009). The role of aquaporins in plant drought and salt tolerance is still uncertain, due to contradictory results. Thus, constitutive overexpression of PIP genes in tobacco and rice enhanced plant sensitivity to drought and salinity (Aharon et al. 2003; Katsuhara et al. 2003). However, when a PIP gene was overexpressed in rice under the control of an inducible stress promoter, an enhancement of drought avoidance was achieved (Lian et al. 2004). Most recently, Yu and associates (2005) found that tobacco plants overexpressing or silencing some PIP aquaporin genes were more sensitive and tolerant, respectively, to drought than the untransformed plants. At the vacuolar level (tonoplast), Peng and associates (2007) found that *Arabidopsis* plants overexpressing a *Panax ginseng* TIP gene were more tolerant to salt stress but less tolerant to

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drought. On the other hand, tomato plants overexpressing a TIP gene were more drought tolerant than wild-type plants (Sade et al. 2009). Moreover, the manipulation of the expression of one aquaporin gene also alters the expression pattern of the whole aquaporin gene family (Jang et al. 2007). All these data highlight the specific role of each aquaporin gene under different environmental stress conditions.

Roussel and associates (1997) and Krajinski and associates (2000) were the first authors reporting an alteration of aquaporin gene expression by AM symbiosis in parsley and *Medicago truncatula* roots, respectively. In both works, AM symbiosis caused an upregulation of a TIP gene. Most recently, a review dealing with this topic has been published (Uehlein et al. 2007). The above reports were carried out in the absence of any stress. However, during the last few years, evidence has arisen showing the regulation of plant aquaporins by AM symbiosis under osmotic stress conditions (Ruiz-Lozano and Aroca 2008; Ruiz-Lozano et al. 2006). Thus, Porcel and associates (2006a) found an anticipated downregulation of two PIP genes in soybean and lettuce roots inoculated with an AM fungus during a drought treatment compared with noninoculated plants. These results were interpreted as a mechanism of avoiding water flow from roots to soil while soil was drying progressively. Ouziad and associates (2006) also found similar results analyzing one PIP and one TIP gene in tomato roots subjected to salt stress. However, in *Phaseolus vulgaris* plants inoculated with the AM fungus *Glomus intraradices* subjected to salt stress, three of four PIP genes analyzed increased their root expression in a higher proportion than in noninoculated plants (Aroca et al. 2007). When the same plants were subjected to drought, the expression of each PIP gene responded in a different way (up- or downregulated) depending on the presence or absence of the AM fungus. The above data support the idea that each plant aquaporin has its specific function under each environmental stress condition (Jang et al. 2007). By contrast, although it is largely known that AM mycelium transports water from the soil to the host roots (Hardie 1985; Khalvati et al. 2005; Marualanda et al. 2003; Ruiz-Lozano and Azcón 1995), no information concerning the regulation of fungal aquaporins during different osmotic stresses is available.

In the fungal kingdom, five kinds of aquaporins have been described (Pettersson et al. 2005). They are subdivided into orthodox aquaporins (two groups), initially described in *Saccharomyces cerevisiae* (Laizé et al. 2000); three different groups of aquaglyceroporins, described in *S. cerevisiae* (Oliveira et al. 2003; Van Aelst et al. 1991); and filamentous fungi (Pettersson et al. 2005). Aquaglyceroporins are able to transport small solutes as glycerol, urea, and other small molecules, instead of or co-transported with water (Rojek et al. 2008; Wallace et al. 2006). However, no aquaporins have been described so far from any AM fungus. Here, we cloned for the first time a putative aquaporin gene from the AM fungus *G. intraradices* (*GintAQP1*) and studied its regulation under a range of osmotic stress conditions, hosts plants, and growing media (soil or in vitro). The results from the in vitro studies highlight the interplay regulation of *GintAQP1* and plant host aquaporin genes during an osmotic stress episode.

RESULTS

Cloning of *G. intraradices* aquaporin cDNA (*GintAQP1*).

A cDNA was synthesized from total RNA extracted from *G. intraradices* hyphae growing in the hyphal compartment (HC) of a monoxenic culture (Porcel et al. 2006b; St.-Arnaud et al. 1996). By using degenerate primers, based on the NPA conserved motifs of several plants, yeasts, and filamentous fungi aquaporins, a partial clone of a putative aquaporin from *G. intraradices* was obtained. Later, by the rapid amplification of cDNA ends (RACE) technique, we cloned the full-length cDNA sequence of the *G. intraradices* aquaporin and called it *GintAQP1* (accession number FJ861239). The coding region comprised 762 bp corresponding to 253 amino acids (Fig. 1), with an expected molecular weight of 27.2 kDa. The *GintAQP1* has the two NPA motifs present in almost all aquaporins, and it also has the six transmembrane domains common to all aquaporins (Fig. 1) (Pettersson et al. 2005). Because the original biological material could be contaminated by carrot (*Daucus carota* L.) root residues (Recorbet et al. 2009), we extracted genomic DNA from *G. intraradices* spores, hyphae, and carrot roots and amplified the full open reading frame of *GintAQP1* by polymerase chain reaction (PCR) with specific primers (Fig. 2A). PCR amplified a fragment of approximately 1,200 bp only in the spore and hyphae DNA but not in that from carrot roots. Then, we transferred the DNA from the agarose gel to a Hybond-N membrane and hybridized it with the *GintAQP1* full open reading frame DNA as a probe. The signal was only detected at the same position as in the previous PCR (Fig. 2B). These data confirmed the fungal origin of the *GintAQP1* gene.

After a phylogenetic analysis, *GintAQP1* was found to not be integrated in any specific cluster, indicating specific features among fungal aquaporins (Fig. 2C). However, *GintAQP1* shares with endoplasmatic reticulum (ER) aquaporins a short N terminal without any diacid motif (Fig 1; Maeshima and Ishikawa 2008). At the same time, we carried out experiments with oocytes from *Xenopus laevis* in order to test the capacity of *GintAQP1* protein to transport water, glycerol, or urea. The results showed no transport activity for any of the three compounds tested (data not shown). In contrast, the positive control used showed water, glycerol, or urea transport capacity, discarding any problem during the experimental procedures.

GintAQP1 expression under different stress conditions and host plants.

We tested the regulation of *GintAQP1* gene expression by quantitative real-time PCR in roots of different host plants, under different stress conditions, and under hormonal treatment (Porcel et al. 2006b). First, we evaluated the regulation of *GintAQP1* expression in *P. vulgaris* colonized roots under drought, cold, or salt stresses (Aroca et al. 2007). The expression of the *GintAQP1* gene increased under drought and cold stresses but remained constant under salt stress (Fig. 3A). No significant differences in the percentage of mycorrhizal root length (percentage of root length that containing fungal structures) were observed among different stress treatments (Aroca et al. 2007). Later, we analyzed the expression of the *GintAQP1* gene in colonized roots of lettuce (*Lactuca sativa* L.) under well-watered conditions or subjected to drought and treated or not with abscisic acid (ABA) (Aroca et al. 2008), a plant hormone that regulates stomatal closure and root hydraulic properties (Hartung et al. 2005). *GintAQP1* gene expression was decreased dramatically by both drought and ABA treatments, being undetectable when both treatments were combined (Fig. 3B), even if the percentage of mycorrhizal root length was increased (Aroca et al. 2008). Finally, we evaluated *GintAQP1* expression in colonized roots of two different lines of tobacco (*Nicotiana tabacum* L.) subjected to drought stress (Porcel et al. 2005): a wild-type and antisense line where a PIP1 gene and its related aquaporin genes have reduced their expression by approximately 80% (Siefritz et al. 2002). The only significant difference observed between the two lines on the expression of *GintAQP1* was found under drought conditions, where the antisense line showed higher expression levels than the wild-type one (Fig. 3C).

***GintAQP1* expression in monoxenic culture.**

One of the most extended methods to study the regulation of AM fungi genes in the extraradical mycelium is the use of the monoxenic culture (Allen and Shachar-Hill 2009; Porcel et al. 2006b; Recobert et al. 2009). Here, we intended to ascertain how the *GintAQP1* gene was regulated in the extraradical mycelium under environmental conditions known to regulate plant aquaporins expression. Therefore, we added to the HC ABA polyethylene glycol as an osmotic agent or NaCl. We also incubated other plates at 5°C for 3 days. No changes in *GintAQP1* gene expression levels in the extraradical mycelium were observed for any of these treatments (data not shown).

A

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gcg ggg aaa gtt gat tga ttt ttg taa act cac ttt ttt cgt taa ata ata ctc gct ttc
A   G   K   V   D   -   F   L   -   T   H   F   F   R   -   I   I   L   A   F
cct ttt ttt ttt cat ttt gca cta aca ctt aaa aaa aat tta tac cac ttt caa aat aaa
P   F   F   F   H   F   A   L   T   L   K   K   N   L   Y   H   F   Q   N   K
ttt gaa ata att aag Atg gga tta aag gat gac ttt gtg act tca cta gca gaa ttt att
F   E   I   I   K   M   G   L   K   D   D   F   V   T   S   L   A   E   F   I
gga act aca tat ttc att ttc ata gga ctc gga ggt agt gat gca ata gct gct ttt tct
G   T   T   Y   F   I   F   I   G   L   G   G   S   D   A   I   A   A   F   S
gga aaa tct tta ggt gac ata aaa ctc ttt gct act gca ttc tca ttt gga tgg tca tta
G   K   S   L   G   D   I   K   L   F   A   T   A   F   S   F   G   W   S   L
atg ata aat gta tgg ctt tgg agt gat ata tcc ggc gga gta ttg aat cct gcc att act
M   I   N   V   W   L   W   S   D   I   S   G   G   V   L   N   P   A   I   T
ata gca tta atg ttt aca gat gat caa gaa tta aga ata agg aga gga att ttt tat atc
I   A   L   M   F   T   D   D   Q   E   L   R   I   R   R   G   I   F   Y   I
atc gca caa ttc gca gga gct ata ctc gga tca tta ctt gta aaa tta ttt tta cca gct
I   A   Q   F   A   G   A   I   L   G   S   L   L   V   K   L   F   L   P   A
cca att gca gct tta aca acc ctt tcg gac gga act aca att ctt caa gga ttg gta att
P   I   A   A   L   T   T   L   S   D   G   T   T   I   L   Q   G   L   V   I
gaa att att act aca tct tta tta aca tta act gtg tat aca tta gcc gtt aat gaa aga
E   I   I   T   T   S   L   L   T   L   T   V   Y   T   L   A   V   N   E   R
ggt gga ttt atg aaa tct ttt gga atg gga act tct gta tta att tct gta ctt gtg gct
G   G   F   M   K   S   F   G   M   G   T   S   V   L   I   S   V   L   V   A
gga cca tat aca gga gca agc ttg aat cca gcg aga act ctt ggt cca gcc att gta tca
G   P   Y   T   G   A   S   L   N   P   A   R   T   L   G   P   A   I   V   S
gga aaa atc tcc ggt gat att tgg att tat ttc att ggt cct att ata gga agt tta tta
G   K   I   S   G   D   I   W   I   Y   F   I   G   P   I   I   G   S   L   L
gcg gca tca ttt cat act tat ttt aaa aaa aat ttt gga gct cta cat ctt gat agg gat
A   A   S   F   H   T   Y   F   K   K   N   F   G   A   L   H   L   D   R   D
agg gat gat ctt gat agg gat ctt gat ctt gat agg gaa aat ctt ggt aag gat taa atg
R   D   D   L   D   R   D   L   D   L   D   R   E   N   L   G   K   D   -
aagtttaaggattaaaggatgtttaaaatattttaagacctttttttctatacctaaatattatatatattgtacaag
tatatattgtataactaataataaaggagaattgttgatctgttgtttgaaaaccaaaaaaaaaaaaaaaaaaaaa
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B

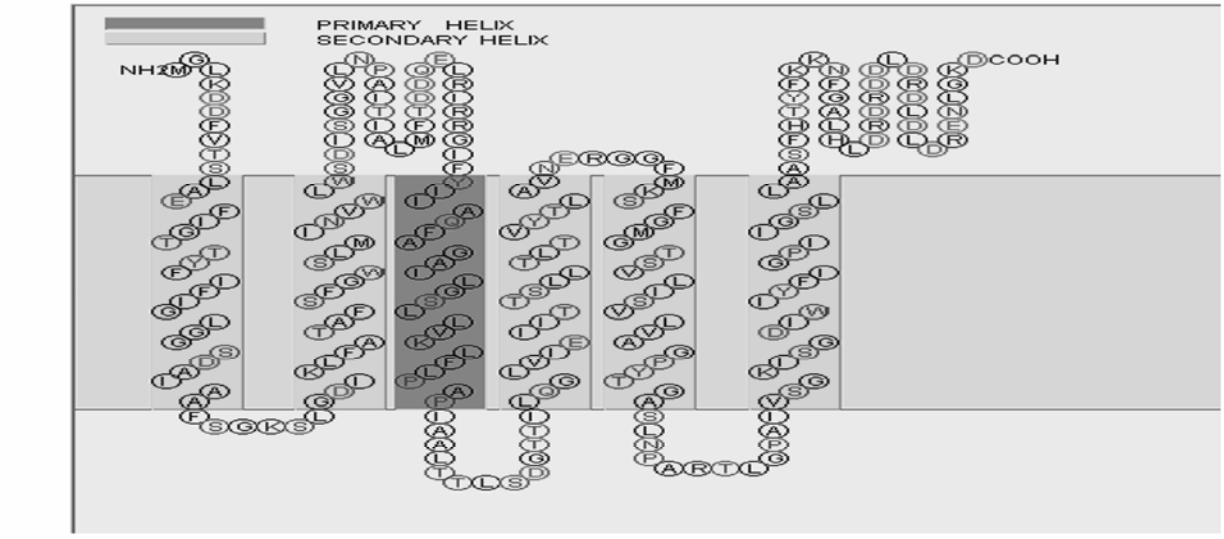

Fig. 1. A, Nucleotide and amino acid sequence of the *GintAQP1* gene, including 5′ and 3′ untranslated regions. The first methionine is marked in bold, as it is the two NPA motifs characteristic of aquaporin genes. The six transmembrane domains are in bold and underlined. Note the short N-terminal region composed of only 10 amino acids. **B,** Picture of the hypothetical transmembrane domains generated by the SOSUI program (Hirokawa et al. 1998).

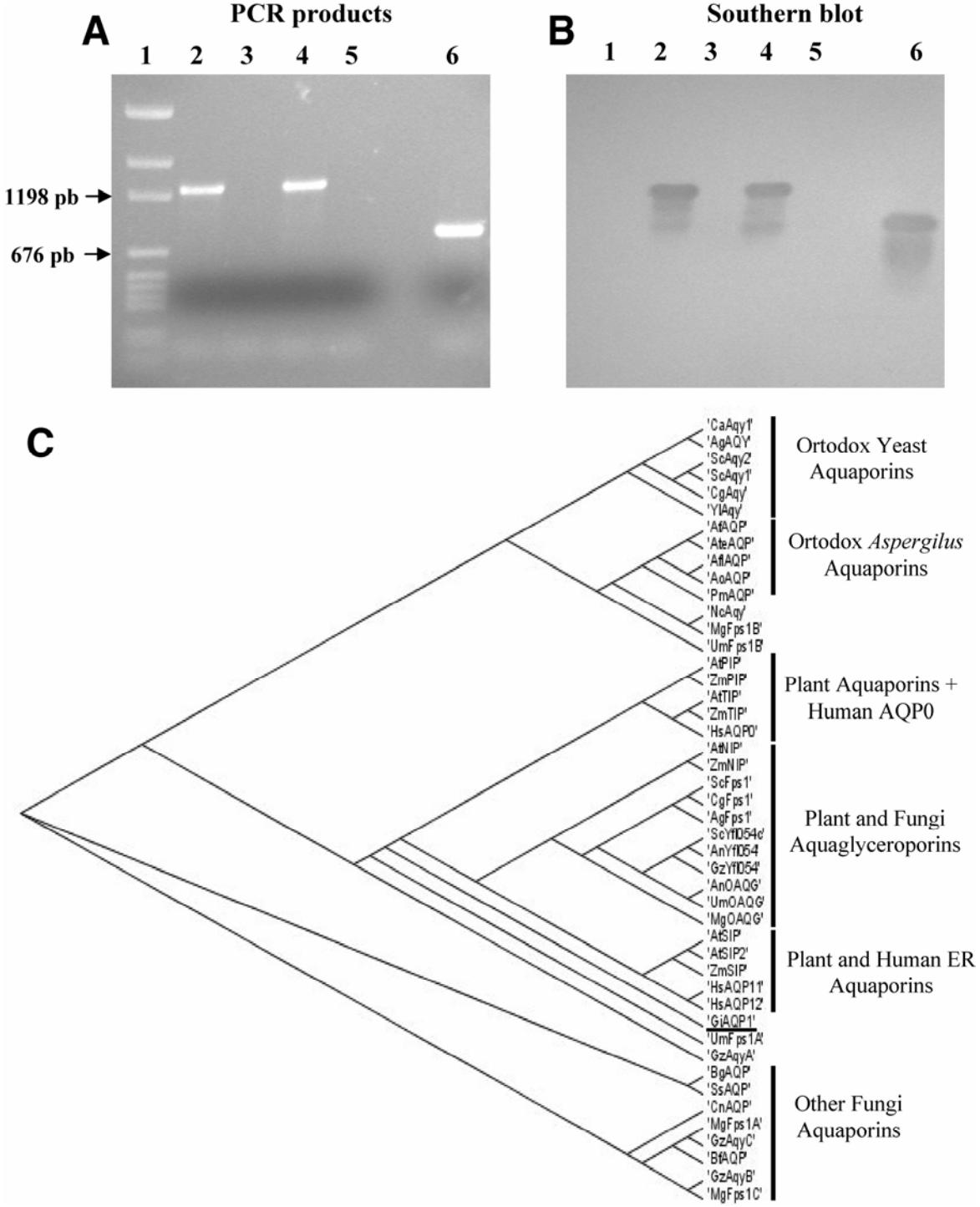

Fig. 2. A, Electrophoresis on 1.2% agarose gel for polymerase chain reaction (PCR) products obtained with specific primers designed on the *GintAQP1* gene. **B,** Southern blot hybridization using the full open reading frame (ORF) of the *GintAQP1* as a probe. Lane 1, Molecular weight marker; 2, genomic DNA from *Glomus intraradices* spore; 3, genomic DNA from *Daucus carota* root; 4, genomic DNA from *G. intraradices* mycelium; 5, negative control (DNA-free water); 6, positive control (pGEM plasmid carrying the ORF cDNA of *GintAQP1*). **C,** Phylogenetic tree of various human, plant, and fungi aquaporins, including *GintAQP1* (underlined), using neighbor-joining method. The abbreviations for species names are Ca (*Candida albicans*), Ag (*Ashbya gossypii*), Sc (*Saccharomyces cerevisiae*), Cg (*Candida glabrata*), Yl (*Yarrowia lipolytica*), Af (*Aspergillus fumigatus*), Ate (*Aspergillus terreus*), Afl (*Aspergillus flavus*), Ao (*Aspergillus oryzae*), Pm (*Penicillium marnefei*), Nc (*Neurospora crassa*), Mg (*Magnaporthe grisea*), Um (*Ustilago maydis*), At (*Arabidopsis thaliana*), Zm (*Zea mays*), Hs (*Homo sapiens*), An (*Aspergillus nidulans*), Gi (*Glomus intraradices*), Gz (*Gibberella zeae*), Bg (*Blumeria graminis*), Ss (*Sclerotinia sclerotiorum*), Cn (*Cryptococcus neoformans*), and Bf (*Botryotinia fuckeliana*). The accession numbers of the amino acid sequences used are CaAqy1 (EAK96766), AgAQY (AAS54225), ScAqy2 (AAD10058), ScAqy1 (BAG50026), CgAqy1 (CAG57715), YlAqy (CAG77656), AfAQP (EDP47366), AteAQP (EAU37108), AflAQP (EED52460), AoAQP (BAE64845), PmAQP (EEA19904), NcAqy (EAA35947), MgFps1B (EDJ99463), UmFps1B (EAK84000), AtPIP (CAB71073), ZmPIP (X82633), AtTIP (AAD31569), ZmTIP (AF037061), HsAQP0 (AAH74913), AtNIP (CAA16748), ZmNIP (AF326483), ScFps1 (CAA38096), CgFps1 (CAG58224), AgFps1 (AAS51160), ScYfl054c (AAT92622), AnYfl054 (EAA63393), GzYfl054 (EAA71472), AnOAQG (EAA59224), UmOAQG (EAK82563), MgOAQG (EDJ96530), AtSIP (AAF26804), AtSIP2 (CAB72165), ZmSIP (AF326497), HsAQP11 (EAW75031), HsAQP12 (BAC45006), GiAQP1 (FJ861239), UmFps1A (EAK81040), GzAqyA (EAA70757), BgAQP (CAD66431), SsAQP (EDN98188), CnAQP (AAW42227), MgFps1A (EDJ99173), GzAqyC (EAA75170), BfAQP (EDN26017), GzAqyB (EAA71493), and MgFps1C (EDJ94979).

Therefore, we analyzed how *GintAQP1* gene expression was regulated in the root compartment (RC), mainly composed of colonized carrot roots together with small amount of extraradical mycelium, when NaCl was added to the HC. Curiously, the expression of *GintAQP1* was higher in RC than in HC under control conditions but, more interestingly, the expression increased in the RC compartment upon exposing HC to 100 mM NaCl (Fig. 4). In a second experiment, we added the same concentration of NaCl to the RC and checked the *GintAQP1* expression in both compartments. Unexpectedly, *GintAQP1* expression profusely rose in the HC and only marginally and nonsignificantly in the RC (Fig. 4).

Carrot aquaporins regulation under salt stress.

From the previous data, we grew interested in knowing how carrot aquaporins could be regulated by the AM symbiosis as well as by salt stress applied in both plate compartments. Thus, using the same methods and degenerate primers described for

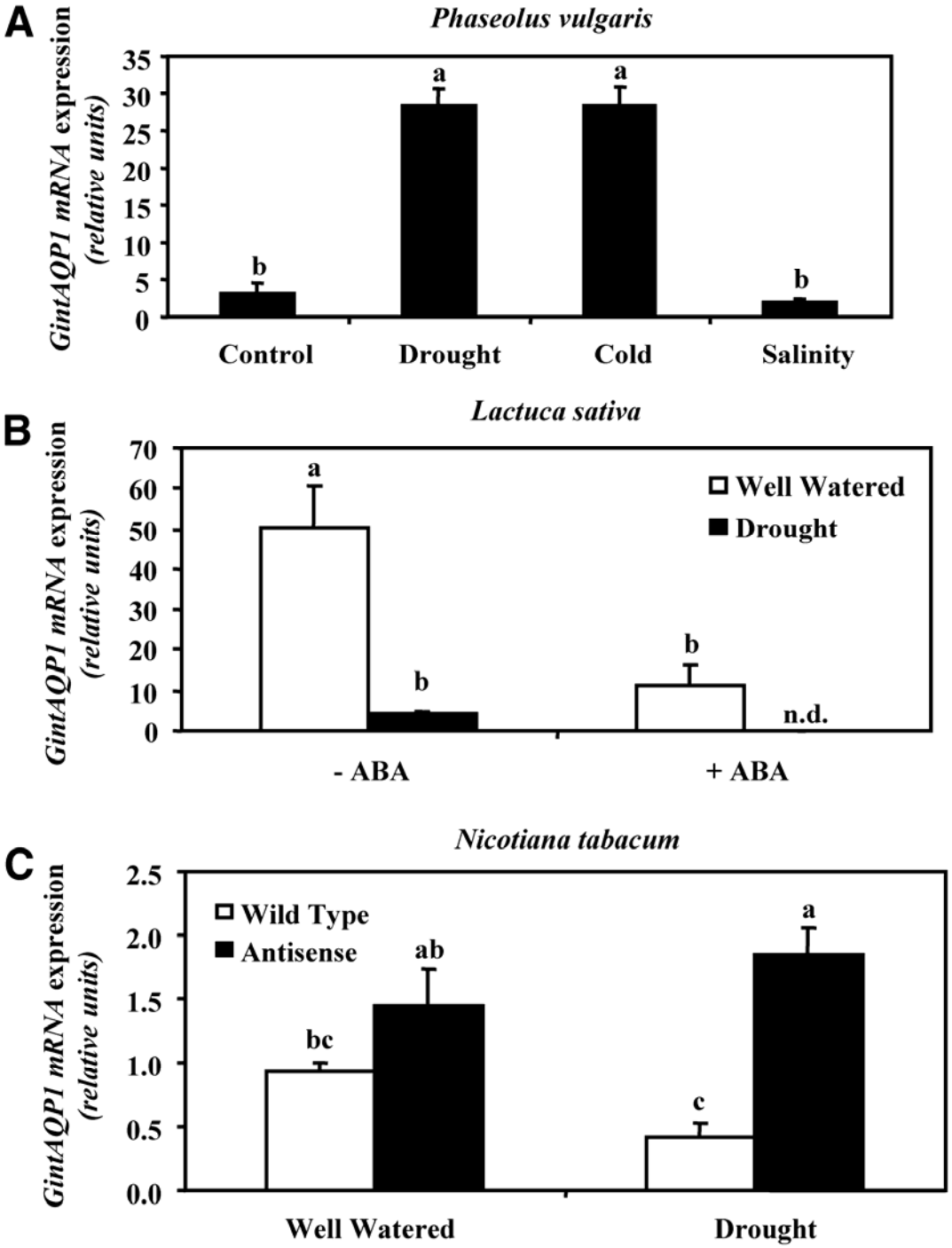

Fig. 3. *GintAQP1* gene expression analyses in several host roots and environmental conditions. **A,** *GintAQP1* gene expression in *Phaseolus vulgaris* roots colonized by *Glomus intraradices* untreated (Control) without watering for 4 days (Drought), subjected to 4°C for 2 days (Cold), and subjected to 60 mM NaCl (Salinity). **B,** *GintAQP1* gene expression in *Lactuca sativa* roots colonized by *G. intraradices* and cultivated under well-watered conditions (white bars) or grown under 70% soil-water-holding capacity for 10 days (Drought, black bars), treated or not with 100 μM abscisic acid (ABA); n.d. = not detected. **C,** *GintAQP1* gene expression in *Nicotiana tabacum* wild-type (white bars) or *NtAQP1* antisense (black bars) roots colonized by *G. intraradices* and cultivated under well-watered conditions or under 70% soil-water-holding capacity for 10 days (Drought). Bars represent mean ± standard error ($n = 6$). Bars with the same letter indicate nonsignificant differences ($P > 0.05$) after analysis of variance and least significant difference tests.

the cloning of the *GintAQP1* gene and RNA extracted from carrot roots growing in vitro without any AM fungus, we obtained two different clones of putative carrot aquaporins. After RACE experiments, we obtained the full-length cDNAs of the two clones and called them *DcTIP1;1* (accession number FJ861240) and *DcTIP2;1* (accession number FJ861241), based on similarity to *Arabidopsis* and maize aquaporins (Fig. 5A). *DcTIP1;1* gene expression was unaltered by mycorrhization or by salt stress when the root was uncolonized (Fig. 5B). However, when the roots were colonized by *G. intraradices*, its expression increased wherever the salt was added (RC or HC), although the increase was higher when the salt was added to RC (Fig. 5B). In contrast, *DcTIP2;1* gene expression was downregulated by AM colonization and did not change when salt stress was added to HC (Fig. 5B). Finally, *DcTIP2;1* expression rose under salt stress in noncolonized roots but was undetected when the roots were colonized (Fig. 5B).

DISCUSSION

Although the transport of water from the AM hyphae to the host roots has been described for a long time (Hardie 1985; Khalvati et al. 2005; Marualanda et al. 2003; Ruiz-Lozano and Azcón 1995), here, an aquaporin from the AM fungus *G. intraradices*, which could be involved in such transport, is reported for the first time. Based on phylogenetic analyses and on transmembrane domain analyses, the *GintAQP1* gene seems to be related to ER aquaporins, such as plant SIP and human *AQP11* and *AQP12* (Maeshima and Ishikawa 2008). *GintAQP1* has a very short N-terminal tail and loop C similar to ER aquaporins, especially to plant SIP (Maeshima and Ishikawa 2008). However, contrary to ER aquaporins, *GintAQP1* has conserved the two NPA motifs and has a lower predicted isoelectric point. On the other hand, *GintAQP1* does not possess any diacidic motif in its N-terminal tail, similar to plant SIP (Maeshima and Ishikawa 2008), likely to favor their retention in the ER (Zelazny et al. 2009). We failed to detect any water, glycerol, or urea transport of *GintAQP1* protein when it was expressed in *Xenopus* oocytes (data not shown). These negative results could be caused by the retention of *GintAQP1* in the ER of *Xenopus* oocytes and, therefore, the lack of transport to the plasma membrane. This problem in detecting aquaporin activity using oocytes experiments was also reported for PIP1 plant aquaporins (Fetter et al. 2004), which are retained in ER membranes (Zelazny et al. 2009). In fact, *Arabidopsis SIP1;1* and *SIP1;2*, and human *AQP11* were described to transport water by using stopped-flow light-scattering assays of membrane vesicles from yeast cells expressing SIP (Ishikawa et al. 2005), or via proteoliposomes reconstituted with purified recombinant *AQP11* (Yakata et al. 2007), avoiding the inconvenience of ER retention. Therefore, more accurate studies are needed to localize *GintAQP1* and to characterize its putative transport substrates.

Based on the results of *GintAQP1* gene expression analyses in roots of soil-grown plants and on the expression of aquaporins from these plants, analyzed in previous studies, it seems that the expression of *GintAQP1* is coordinated with that of plant aquaporins. Therefore, in the *P. vulgaris* experiment, *GintAQP1* did not change its expression under salt stress, whereas the four *P. vulgaris PIP* genes analyzed increased their expression (Aroca et al. 2007). *GintAQP1* was upregulated under drought or cold conditions, whereas two of four *P. vulgaris PIP* genes decreased their expression and the expression of the other two remained constant (Aroca et al. 2007). These data could be interpreted as a compensatory mechanism between host plant aquaporins and the AM fungal aquaporin. In fact, in lettuce plants treated with ABA, that caused an increase of one *PIP2* aquaporin gene (Aroca et al. 2008); also, a decrease on *GintAQP1* gene expression was observed. However, contrary to what happened in *P. vulgaris*, lettuce plants were unable to exude any water from the roots under drought conditions, indicating a severe drought stress (Aroca et al. 2008). Therefore, this reduction in *GintAQP1* gene expression could be interpreted as a water-saving mechanism (Porcel et al. 2006a). In contrast, the possible compensatory relationship between plant host aquaporins and the AM fungal one could be observed in tobacco plants. Under drought conditions, a major expression of *GintAQP1* was found in the roots of antisense plants with downregulated PIP1 aquaporins. Although this compensatory mechanism could be inferred from the data of soil-grown plants, more comprehensive experiments are needed in order to confirm such a possible mechanism.

It is known that several *G. intraradices* genes are developmentally and spatially regulated, with some of them being highly expressed in the intraradical structures (Seddas et al. 2008). In the present research, *GintAQP1* was not regulated in the extraradical mycelium by any of the treatments imposed (ABA, PEG [polyethylene glycol], NaCl, or cold). However, its expression was higher in the intraradical mycelium (considering that RNA from the RC was mainly from colonized roots and not from associated extraradical mycelium) (Recobert et al. 2009), and it was even higher after addition of NaCl to the

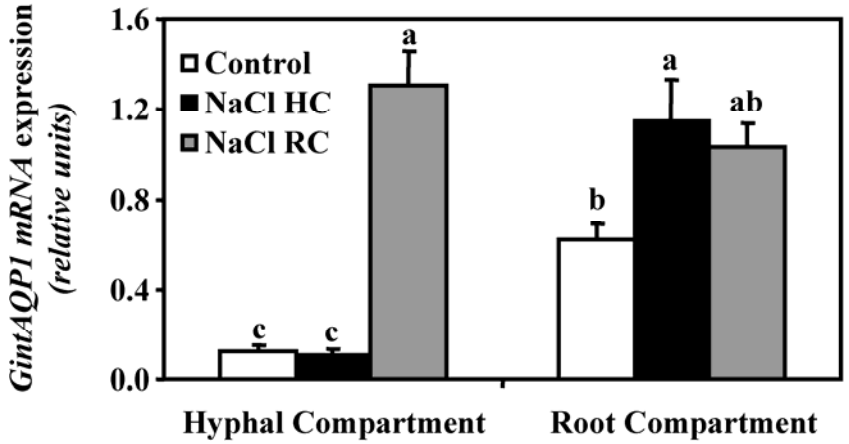

Fig. 4. *GintAQP1* gene expression in arbuscular mycorrhizal fungal mycelium from the hyphal compartment (HC) or from the root compartment (RC, predominantly intraradical hyphae). Treatments are untreated (Control, white bars), 100 mM NaCl added to HC (black bars), or 100 mM NaCl added to RC (gray bars). Bars represent mean ± standard error ($n = 6$). Bars with the same letter indicate non significant differences ($P > 0.05$) after analysis of variance and least significant difference tests.

HC. These results show that *GintAQP1* was upregulated by exposure of the extraradical mycelium to an osmotic stress, although the intraradical mycelium was not subjected to NaCl stress. This upregulation of *GintAQP1* in the RC could be related to the transport of water from the isotonic medium (RC) to the hypertonic one (HC), contributing to alleviate the osmotic stress of the HC mycelium. This hypothesis was supported by the fact that *GintAQP1* expression also increased in the HC (now the isotonic medium) mycelium when NaCl was applied to the RC (now the hypertonic medium), while nonsignificant changes in its expression were observed in the RC. Therefore, it is likely to presume a cross-talk or communication between the fungal mycelium growing in the two plate compartments when an osmotic stress was applied to one of the compartments. These results showed regulation of an AM fungal gene expression in one fungal structure not subjected to any stress, caused by environmental changes affecting another fungal structure. The different signaling mechanisms carrying out this communication from the stressed fungal structure to the nonstressed one may be of a hormonal nature, like ABA (Herrera-Medina et al. 2007); hydraulic nature (Christmann et al. 2007); or electrical nature (Zimmermann et al. 2009). Besides the possible production of the hormone ABA by the AM fungal partner (Esch et al. 1994), no evidences of hydraulic or electrical signaling mechanisms have been described thus far in AM fungi. However, it is known that phytopathogenic fungi alter the membrane electrical potential gradient in the host tissues (Felle et al. 2008). How electric potential gradients could be transmitted along the AM hyphae remains unknown.

It is known that AM symbiosis alters the aquaporin expression pattern of the host roots (Aroca et al. 2007; Ouziad et al. 2006; Porcel et al. 2006a; Ruiz-Lozano and Aroca 2008). So, we were interested in knowing whether the communication observed between the AM mycelia from both plate compartments also was valid for the host carrot aquaporins. The two TIP aquaporins cloned from *D. carota* cDNA roots were regulated differently by the AM symbiosis. *DcTIP2;1* was downregulated by the symbiosis, and even more when NaCl was applied to the RC, where no gene expression was detected, in contrast to noncolonized roots, where a significant increase was observed. An opposite trend was observed for *DcTIP1;1*, which only increased its expression under salt conditions when roots were colonized. These results agree with those in the literature because each aquaporin gene may respond specifically to a given stress and also depending on the presence or absence of the AM symbiont (Aroca et al. 2007). However, the most interesting result was that *DcTIP1;1* gene expression also was upregulated when NaCl was applied to HC. This result supports the possible communication between the two parts of the AM mycelium subjected to different environmental conditions, regulating also the gene expression of the host roots not exposed directly to the stressful conditions. As far as we know, this is the first report where this kind of communication has been described at the gene level. The upregulation of *GintAQP1* and *DcTIP1;1* upon exposure of HC to NaCl or the upregulation of *GintAQP1* in the HC when NaCl was applied to RC could be interpreted as a mechanism of transporting water from an iso-osmotic medium to a hyperosmotic one through the AM mycelium. In fact, Egerton-Warburton and associates (2007) found water transport from well-watered plants to

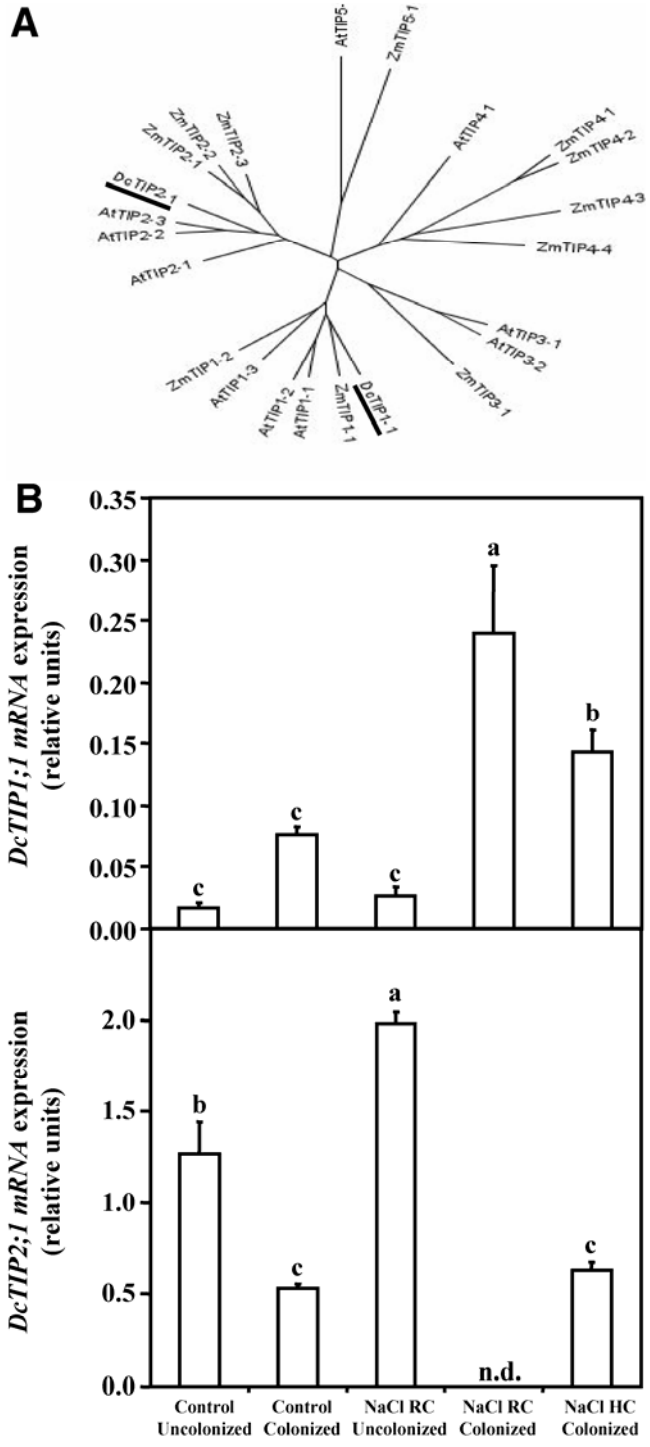

Fig. 5. A, Phylogenetic tree of *Arabidopsis*, maize and, carrot tonoplast intrinsic proteins (TIPs) based on neighbor-joining method. *DcTIP1;1* and *DcTIP2;1* are underlined. The abbreviations for species names are At (*Arabidopsis thaliana*), Zm (*Zea mays*), and Dc (*Daucus carota*). The accession numbers of the amino acid sequences used are AtTIP1-1 (AAD31569), AtTIP1-2 (BAB01832), AtTIP1-3 (AAC62778), AtTIP2-1 (BAB01264), AtTIP2-2 (CAB10515), AtTIP2-3 (BAB09071), AtTIP3-1 (AAG52132), AtTIP3-2 (AAF97261), AtTIP4-1 (AAC42249), AtTIP5-1 (CAB51216), ZmTIP1-1 (AF037061), ZmTIP1-2 (AF326500), ZmTIP2-1 (AF326501), ZmTIP2-2 (AF326502), ZmTIP2-3 (AF326503), ZmTIP3-1 (AF326504), ZmTIP4-1 (AF326505), ZmTIP4-2 (AF326506), ZmTIP4-3 (AF326507), ZmTIP4-4 (AF326508), ZmTIP5-1 (AF326509), DcTIP1-1 (FJ861240), and DcTIP2-1 (FJ861241). **B,** *DcTIP1;1* (upper panel) and *DcTIP2;1* (lower panel) gene expression in carrot roots grown in vitro (from left to right) untreated and uncolonized by *Glomus intraradices*, untreated and colonized by *G. intraradices*, uncolonized and treated with 100 mM NaCl in the root compartment (RC) for 1 week, colonized and treated with 100 mM NaCl in the RC for 1 week, and colonized and harvested after 1 week of adding 100 mM NaCl to the hyphal compartment. Bars with the same letter indicate nonsignificant differences ($P > 0.05$) after analysis of variance and least significant difference tests; n.d. = not detected.

plants subjected to drought through ectomycorrhizal and AM hyphae, increasing the transport rate as the drought was more severe.

In addition to the first description of an aquaporin gene from an AM fungus, the main findings of the present research could be summarized as follows. i) *GintAQP1* expression responded differently to each environmental stress and host plant, although a certain compensatory mechanism between the host aquaporins and *GintAQP1* expressions could be inferred. ii) *GintAQP1* increased its expression in the unstressed part of the mycelium when the other part was subjected to an osmotic stress by NaCl, implicating a communication between the two parts of the mycelium that allows a spatial regulation of gene expression. iii) Similarly, the host aquaporin *DcTIP1;1* also increased its expression when only the extraradical mycelium from HC was treated with NaCl, also supporting a communication mechanism involving the extra and intraradical mycelium and the host root. Further studies are needed to determine precisely where the *GintAQP1* is localized inside the mycelium and what its putative transport substrates could be.

MATERIALS AND METHODS

Cloning of *G. intraradices* aquaporin gene.

G. intraradices was established in a monoxenic culture (St.-Arnaud et al. 1996), as described by Porcel and associates (2006b) and Jahromi and associates (2008). Briefly, clone DC2 of carrot (*D. carota* L.) Ri-T DNA-transformed roots were cultured with the AM fungus *G. intraradices* Smith and Shenk (DAOM 197198, Biosystematic Research Center, Ottawa, Canada) in two-compartment petri dishes. Culture was initiated in one compartment (RC) of each plate, which contained minimal medium. Fungal hyphae but not roots were allowed to grow over to the second compartment (HC), which contained liquid minimal medium without sucrose (M-C medium). The plates were incubated in the dark at 24°C for 3 months. Fungal mycelium was carefully removed from the HC, and RNA was extracted by using the RNesay plant mini kit (Qiagen, Valencia, CA, U.S.A.). cDNA was synthesized from such RNA as previously described by Porcel and associates (2006a). Synthesized DNA was amplified by PCR using degenerate primers designed from alignment of different plant and fungal aquaporins: DEGAQPFor, 5′-GG(A/T)GG(C/T)(A/T)(A/T) TTIAA (C/T)CC(A/T) GC(A/C/T)GT-3′ and DEGAQPRev, 5′-CC(A/C/T)A(A/G)(A/G)GA(C/T)C(G/T)(A/T)GC(A/T)GG(A/G)TT-3′. The PCR program was as described by Aroca and associates (2006). Amplified products were separated in 2% agarose gel, and visible bands of the expected size (approximately 400 kb) were eluted with QIAquick gel extraction kit (Qiagen) and cloned into pGEM plasmid (Promega Corp., Madison, WI, U.S.A.) and used to transform *Escherichia coli* DH-5α-competent cells. The plasmid DNA was purified and sequenced, which allowed identification of a clone with a putative aquaporin sequence.

For the determination of the full-length sequence of the obtained cDNA, 5′- and 3′-RACE technique was carried out on total RNA using the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, U.S.A.). After 5′- and 3′-RACE, new primers were designed covering the full gene and PCR was performed with high-fidelity *Pfu* DNA polymerase (Stratagene, La Jolla, CA, U.S.A.).

The alignment of *GintAQP1* with other plant, human, and fungal aquaporins was carried out by ClustalW XXL method. The phylogenetic tree was obtained by using TreeIllustrator VO.52 Beta using the neighbor-joining method. The hypothetical plasma membrane domains of *GintAQP1* were predicted by the SOSUI program (Hirokawa et al. 1998).

Later, and based on *GintAQP1* expression analyses, two different carrot aquaporins were cloned and identified following the same procedure as described above. The original RNA was isolated from the same carrot roots used above but grown without any AM fungus.

Southern blot.

Southern blot was carried out as described by Ruiz-Lozano and Bonfante (2000). Briefly, specific primers flanking the full open reading frame (ORF) of the *GintAQP1* were designed (forward [For], ATGGGATTAAAGGATGACTTTGTG and reverse [Rev], TTAATCCTTACCAAGATTTTCCCTA), and PCR at an annealing temperature of 56°C was carried out with genomic DNA extracted from spores and mycelium of *G. intraradices* and from *D. carota* roots. PCR products were transferred to Hybond-N^+ nylon membrane by capillarity; DNA was denatured and fixed by exposition to UV light. The DNA corresponding to the full ORF was labeled by a chemiluminiscent detection kit (ECL direct DNA labeling and detection system, Amersham, U.K.) and hybridized with the membrane overnight at 42°C with the buffer supplied by the kit. After washing, an X-ray film was exposed to the membrane and developed. Genomic DNAs were isolated as described previously by Ruiz-Lozano and Bonfante (2000).

GintAQP1 transport activity assays.

Analysis of *GintAQP1* water transport activity was carried out by expressing the ORF of the gene in *Xenopus laevis* oocytes as previously described by Mut and associates (2008). Briefly, the full-length ORF of *GintAQP1* and *AtPIP2;3* used as a positive control (Daniels et al. 1994) were cloned into pSP64T-derived vector carrying 5′ and 3′ untranslated sequences of a *β-globin* gene from *X. laevis* (Agre et al. 1999) and complementary RNAs were synthesized (Mut et al. 2008). After 3 days of cRNA or distilled water injection, mature *X. laevis* oocytes were transferred to a hypotonic medium and their osmotic water permeability coefficient was calculated as described by Agre and associates (1999). Measurements of glycerol and urea transport activities were carried out in *X. laevis* oocytes as previously described by Soto and associates (2008).

Biological material.

Two different kinds of experiments were carried out. First, the expression of *GintAQP1* was analyzed in colonized roots of *P. vulgaris*, *L. sativa*, or *N. tabacum* plants. Plants growing in pots filled with a mixture of sterilized soil and sand (1:1) were inoculated with the AM fungus *G. intraradices* isolate BEG 123 (Aroca et al. 2007, 2008; Porcel et al. 2005). *P. vulgaris* plants were subjected to cold, drought, or salt stress as previously described by Aroca and associates (2007). *L. sativa* plants were subjected to drought and supplied with exogenous ABA as described by Aroca and associates (2008). Two *N. tabacum* plants lines, wild-type or NtAQP1 antisense line (Siefritz et al. 2002), were subjected to drought as described by Porcel and associates (2005). At harvest, roots were carefully cleaned with tap water before being stored at –80°C in order to avoid contaminations with extraradical mycelium. Total root RNA from different plants was extracted by a phenol/chloroform method followed by precipitation with LiCl (Kay et al. 1987), and cDNAs were synthesized as described by Aroca and associates (2007).

The second set of experiments were carried out in vitro, with the AM fungus *G. intraradices* growing in monoxenic cultures (Jahromi et al. 2008; Porcel et al. 2006b), or with uninoculated carrot roots growing under the same conditions but in absence of the fungus (see above). Three different treatments were applied to the HC and one to the whole plate. Water stress

was imposed to HC by adding 25% of PEG 6000 (Sigma-Aldrich Co., Madrid, Spain) to the liquid minimal medium, corresponding to a water potential of –1.5 MPa. The mycelium grew under this condition for 5 days (Porcel et al. 2006b). Salt stress was imposed to HC by applying a final concentration of 100 mM NaCl; the mycelium was grown under this condition for 1 week. The NaCl concentration was selected based on the study by Jahromi and associates (2008), because it was the lowest concentration with a significant effect on hyphal growth and spore production. Exogenous ABA was applied to the HC at a final concentration of 100 μM and the mycelium was grown there for 1 day. Finally, the whole plates were kept at 5°C in the dark for 3 days. At the end of all treatments, untreated and treated mycelia from HC were isolated, immersed in liquid N_2, and stored at –80°C until RNA was extracted by the RNesay plant mini kit (Qiagen). In a separate set of experiments, carrot roots colonized or not with the AM fungus were subjected to a final concentration of 100 mM NaCl for 1 week. Roots were removed from the medium and hyphal debris were carefully removed with forceps before they were immersed in liquid N_2 and stored at –80°C. Total root RNA was extracted as described for the mycelium protocol.

Gene expression analyses.

GintAQP1 and carrot aquaporin *DcTIP1;1* and *DcTIP2;1* gene expression analyses were carried out by quantitative reverse-transcription (qRT)-PCR using iCycler (BioRad, Hercules, CA, U.S.A.), as described by Porcel and associates (2006b), with an annealing temperature of 57°C. cDNA was synthesized as described above from the RNA samples. Each reaction mixture of 25 μl contained 1 μl of a 1:10 dilution of the corresponding cDNA, 200 nM dNTPs, 400 nM each primer, 3 mM $MgCl_2$, 2.5 μl of 1× SYBR Green (Molecular Probes, Eugene, OR, U.S.A.), and 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.) in 1× PCR buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl). The primers used in the qRT-PCR experiments were as follows: for *GintAQP1*, For, 5′-AGGACTCGGAGGTAGTGATGC-3′ and Rev, 5′-GCCGGATATATCACTCCAAAGC-3′; for *Gint18S rRNA*, For, 5′-TGTTAATAAAAATCGGTGCGTTGC-3′ and Rev, 5′-AAAACGCAAATGATCAACCGGAC-3′ (Gónzalez-Guerrero et al. 2005; Porcel et al. 2006b); for *DcTIP1;1*, For, 5′-GTGGCTGTCGGAGCTAATATC-3′ and Rev, 5′-CAGTGGAGCCAAGACACTGAG-3′; for *DcTIP2;1*, For, 5′-GGATCCTGCTGGATTAGTAGC-3′ and Rev, 5′-TGTTGGCTGCTATGGACACTC-3′; and for carrot actin gene, For, 5′-GTGACGTTGATATCAGAAAGG-3′ and Rev, 5′-CGTCATATTCACCCTTCGAG-3′ (Kihara et al. 2003). Experiments were repeated three times using cDNAs originated from two RNAs extracted from two different biological samples.

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