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Impairment of *NtAQP1* gene expression in tobacco plants does not affect root colonisation pattern by arbuscular mycorrhizal fungi but decreases their symbiotic efficiency under drought

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Abstract We investigated in two tobacco (*Nicotiana tabacum*) plant lines (wildtype or antisense mutant) whether impairment in expression of the plasma membrane aquaporin gene (*NtAQP1*) affects the arbuscular mycorrhizal (AM) fungal colonisation pattern or the symbiotic efficiency of AM fungi. These two objectives were investigated under well-watered and drought stress conditions. Both plant lines had a similar pattern of root colonisation under well-watered and drought stress conditions. In contrast, under drought stress, AM wildtype plants grew faster than mycorrhizal antisense plants. Plant gas exchange also appeared to depend on the expression of *NtAQP1* and parallelled the determined growth increments. The implications of enhanced symplastic water transport via NtAQP1 for the efficiency of the AM symbiosis under drought stress conditions are further discussed.

Keywords Aquaporin · Antisense mutant · Arbuscular mycorrhizae · Drought · Symbiotic efficiency

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Introduction

In arbuscular mycorrhiza (AM), the AM fungus occupies a protected ecological niche and receives plant photosynthates, while the plant's ability to take up nutrients and its tolerance to biotic and abiotic stresses is improved (Smith and Read 1997). Among abiotic stresses, water deficit is considered one of the most important factors limiting plant growth and yield (Kramer and Boyer 1997). Several eco-physiological studies investigating the role of AM symbiosis in protection against drought stress have demonstrated that the symbiosis often results in altered rates of water movement into, through, and out of the host plants, with consequences on tissue hydration and plant physiology (for reviews, see Augé 2001; Ruiz-Lozano 2003).

Some studies have demonstrated that AM symbiosis induces the expression of a gene encoding a tonoplast-located aquaporin in parsley and in alfalfa (Roussel et al. 1997; Krajinski et al. 2000). Aquaporins facilitate membrane water transport along a water potential gradient. These proteins belong to the large major intrinsic protein (MIP) family of transmembrane proteins and are represented in all kingdoms (Chrispeels and Agre 1994). Two major classes of plant aquaporins, located in the plasma membrane or tonoplast, respectively, have been identified so far (Johnson et al. 1990; Kammerloher et al. 1994). It has been suggested that vacuolar and plasma membrane aquaporins, acting in concert, are responsible for the cytosolic osmoregulation that is necessary for maintaining normal metabolic processes. However, inhibition studies of aquaporins in vivo and antisense mutant studies have also suggested that, in addition to cytosolic osmoregulation, aquaporins are important for the bulk flow of water in plants (Grote et al. 1998; Kjellbom et al. 1999; Martre et al. 2002; Javot et al. 2003). The high expression of genes encoding aquaporins in tissues involved in water transport suggests a role in transcellular water flow through living cells (Barrieu et al. 1998; Aharon et al. 2003).

The NtAQP1 aquaporin of tobacco has been isolated and characterised as a plasma membrane intrinsic aquaporin

(Biela et al. 1999). Using RNA gel blot and whole mount hybridisation, Otto and Kaldenhoff (2000) found *NtAQP1* gene expression in almost all organs of tobacco, with the highest levels in the root. In situ immunological studies indicated NtAQP1 protein accumulation in the root exodermis and endodermis, in the cortex, close to the vascular bundles, in the xylem parenchyma, and in cells of the stomatal cavities. The aquaporin was found at sites of anticipated high water fluxes from and to the apoplast or symplast. In a subsequent report, Siefritz et al. (2002) could attribute NtAQP1 function to cellular and whole-plant water relations.

Since AM fungi have been shown to enhance gene expression of tonoplast-located aquaporins during root colonisation, the goal of the present work was to investigate whether impairment of *NtAQP1* gene expression affects the AM fungal colonisation pattern and to find out if such an impairment has any effect on symbiotic efficiency. These two objectives were investigated under well-watered and drought stress conditions in wildtype and antisense tobacco plants previously shown to have an 80% reduction in *NtAQP1* gene expression (Siefritz et al. 2002). Two AM fungi with diverse colonisation patterns, *Glomus mosseae* and *Glomus intraradices*, were examined.

Materials and methods

Experimental design and statistical analysis

The experiment consisted of a three factor randomised complete block design of: (1) two tobacco plant lines, (2) mycorrhizal treatment consisting of two *Glomus* species or uninoculated control plants, and (3) two water treatments (well-watered or drought stressed), with five replications per treatment totalling 60 pots (one plant per pot).

Within each water treatment, data were subjected to analysis of variance (ANOVA) with plant line, AM fungus and plant line-AM fungus interaction as sources of variation, and followed by Duncan's multiple range test (Duncan 1955). As percentage values do not follow a normal distribution (they cannot be less than 0 or more than 100), they were arcsin transformed for normalisation before statistical analysis.

Soil and biological materials

Loamy soil was collected from the Zaidin Experimental Station (Granada, Spain), sieved (2 mm), diluted with quartz-sand (<1 mm; 1:1, soil/sand, v/v) and sterilised by steaming (100°C for 1 h on 3 consecutive days). The soil had a pH of 8.1 (water); 1.81% organic matter, and nutrient concentrations of (mg kg^{-1}): N, 2.5; P, 6.2 ($NaHCO_3$-extractable P); K, 132.0. The soil texture was made up of 35.8% sand, 43.6% silt and 20.5% clay.

Seeds of *Nicotiana tabacum* L. (wildtype and *NtAQP1* antisense mutant) were surface-sterilised with 70% ethanol for 2 min and with 2.5% sodium hypochloride for 10 min, then washed several times with sterile water to remove any trace of chemical that could interfere with seed germination. After sterilisation, wildtype seeds were sown on plates containing 1/2 MS medium (Murashige and Skoog 1962) and antisense seeds on plates containing the same medium supplemented with kanamycin (100 μg/ml) for selection of antisense plants (Siefritz et al. 2002). After 10 days of incubation at 25°C, seedlings were transferred to pots containing 500 g sterilized soil/sand mixture.

Mycorrhizal inoculum was bulked in an open-pot culture of *Zea mays* L. and consisted of soil, spores, mycelia and infected root fragments. The AM species were *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe, isolate BEG 122 and *Glomus intraradices* Schenck and Smith, isolate BEG 121. Aliquots (10 g) of each inoculum, with similar infection characteristics (an average of 50 propagules per gram according to the most probable number test), were placed below tobacco seedlings. This amount of inoculum was selected in preliminary tests as optimal to produce a good infection level according to the total amount of soil in the pot. Non-mycorrhizal treatments received the same quantity of autoclaved inoculum together with a 2-ml aliquot of a filtrate (<20 μm) of the AM inoculum in an attempt to provide a general microbial population free of AM propagules.

Growth conditions

Plants were grown in a controlled environmental chamber with 70–80% relative humidity, day/night temperatures of 25/15°C, and a photoperiod of 16 h at a photosynthetic photon flux density (PPFD) of 350 μmol m^{-2} s^{-1} (Licor, Lincoln, Neb., model LI-188B).

Soil moisture was measured with a ML2 ThetaProbe (AT Delta-T Devices, Cambridge, UK), which measures volumetric soil moisture content by responding to changes in the apparent dielectric constant of moist soil (Roth et al. 1992; White et al. 1994). Volumetric soil water content is the ratio between the volume of water present and the total volume of the soil sample. It is a dimensionless parameter, expressed either as a percentage (% vol) or as a ratio (m^3 m^{-3}). For one-half of the plants, water was supplied daily to maintain constant soil water content close to field capacity (17% volumetric soil moisture). For the rest of the plants, water was supplied daily to maintain constant soil water content close to 70% field capacity (10% volumetric soil moisture) and maintained under such conditions during the entire experiment. In order to control the level of water stress, the pot water content was daily measured with the ThetaProbe ML2 (at the end of the afternoon) and the amount of water lost was added to each pot in order to maintain soil water content at the desired 17% (well-watered plants) or 10% (droughted plants) of volumetric soil moisture.

Parameters measured

Biomass production

At harvest (8 weeks after planting) the root system was separated from the shoot and fresh weights (FW) were recorded. Shoot dry weight was determined after drying in a forced draught oven at 70°C for 2 days.

Symbiotic development

The roots were carefully washed and stained by the normal non-vital Trypan Blue (TB) staining of all fungal tissues (Phillips and Hayman 1970). Mycorrhizal development was evaluated by the method of Trouvelot et al. (1986). The colonisation frequency (F%) is the ratio between colonised root fragments and total number of root fragments observed. It gives an estimation of the root length colonised by the fungus. The colonisation intensity (M%) is an estimation of the amount of root cortex occupied by fungal structures. Finally, the arbuscule abundance (A%) gives an estimation of the arbuscule richness in the root system. Four replicates per treatment were used.

Solute accumulation

At harvest, free proline and total soluble sugars (TSS) were extracted from 1 g FW leaves as described by Bligh and Dyer (1959). The methanolic phase was used for quantification of both substances. Proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrin reaction, according to Bates et al. (1973). TSS were analysed by reacting 0.1 ml alcoholic extract with 3 ml freshly prepared anthrone [200 mg anthrone +100 ml 72% (w/w) H_2SO_4] and placing in a boiling water bath for 10 min according to Irigoyen et al. (1992). After cooling, the absorbance at 620 nm was determined in a Shimadzu UV-1603 spectrophotometer (Shimadzu, Kyoto, Japan). A calibration curve was prepared using glucose in the range of 20–400 μg/ml.

Gas exchange

The CO_2 exchange rate (CER), transpiration rate, instantaneous water use efficiency (WUE), and stomatal conductance were measured before harvest on the third leaf from each plant. Atmospheric CO_2 was measured 5 m above ground level. The PPFD was 1,180 μmol m^{-2} s^{-1} in order to ensure that no limitation in photon irradiance occurred. Light was provided by a halogen lamp (General Electric 300 PAR 56/WFL). A model LCA-3 portable, integrated infrared CO_2 analyzer (Analytical Development, Hoddesdon, UK) was used for these determinations. Measurements were made 2 h after the light was turned on. Precautions were taken according to Long and Hällgren (1987) to prevent diurnal, intra-plant and inter-plant variations in plant gas exchange.

Results

Shoot and root biomass production

Under well-watered conditions, uninoculated wildtype and antisense plants did not show any significant difference in shoot or root growth (Fig. 1). Inoculation with *G. mosseae* increased shoot biomass production more than 8-fold in wildtype plants and more than 7-fold in the antisense plants. Inoculation with *G. intraradices* also enhanced

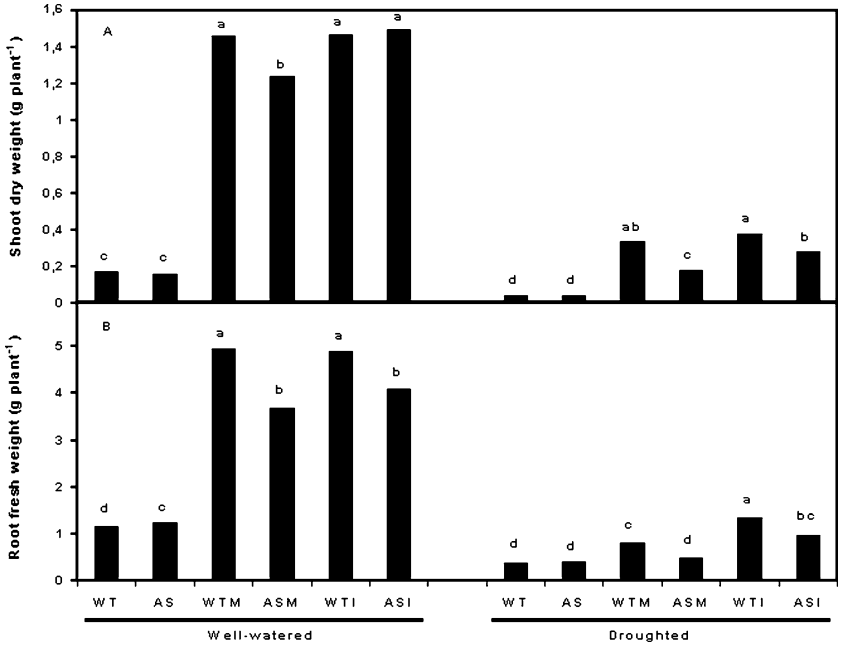

Fig. 1 Shoot dry weight (**A**) and root fresh weight (**B**) in wildtype (*WT*) or antisense (*AS*) tobacco plants inoculated or not with *Glomus mosseae* (*M*) or *Glomus intraradices* (*I*) and cultivated under well-watered conditions or subjected to drought stress. Means followed by the same letter are not significantly different ($P<0.05$) as determined by Duncan's multiple-range test ($n=5$)

shoot biomass production in both wildtype and antisense plants to a comparable level. Root FW was enhanced by mycorrhization slightly more in wildtype plants than in antisense plants (Fig. 1).

Under drought stress conditions, uninoculated control plants of both phenotypes again showed similar shoot and root biomass production, and inoculation with both AM fungi increased shoot dry weight in both plant lines. In all cases, the increase in shoot dry weight was more effective in wildtype plants (8-fold) than the antisense plants (4-fold). In addition, for antisense plants under drought stress, *G. intraradices* enhanced (55%) shoot biomass production more than *G. mosseae*. Similarly, root FW was not enhanced by *G. mosseae* in antisense plants.

AM colonisation

Following AM infection, a similar colonisation frequency (F) was obtained under all treatments investigated (Fig. 2).

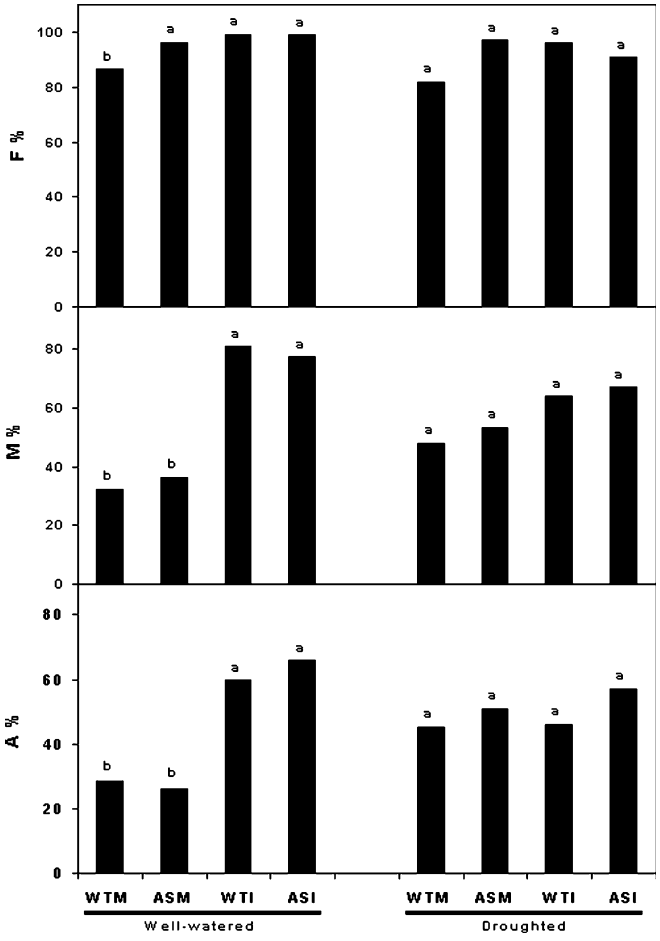

Fig. 2 Frequency (F%) and intensity (M%) of root AM colonisation, and arbuscule richness (A%) in wildtype (*WT*) or antisense (*AS*) tobacco plants inoculated or not with *G. mosseae* (*M*) or *G. intraradices* (*I*) and cultivated under well-watered conditions or subjected to drought stress. Means followed by the same letter are not significantly different ($P<0.05$) as determined by Duncan's multiple-range test ($n=5$)

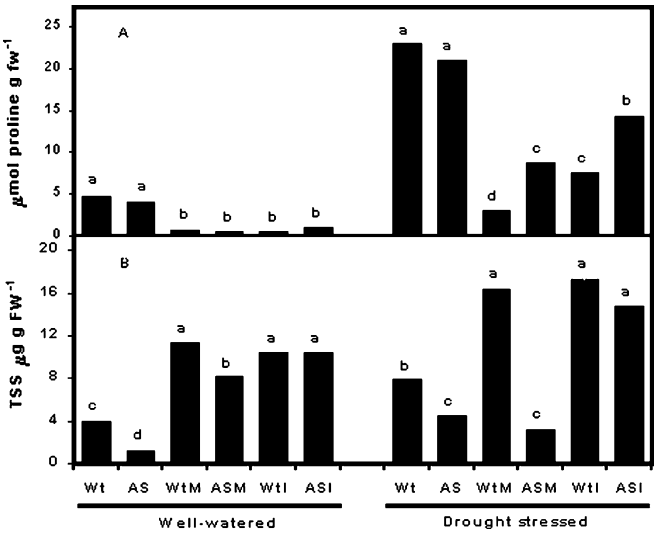

Fig. 3 Proline (**A**) and total soluble sugar (**B**) accumulation in wildtype (*WT*) or antisense (*AS*) tobacco plants inoculated or not with *G. mosseae* (*M*) or *G. intraradices* (*I*) and cultivated under well-watered conditions or subjected to drought stress. Means followed by the same letter are not significantly different ($P<0.05$) as determined by Duncan's multiple-range test ($n=5$)

In contrast to drought conditions, where similar values were observed, well-watered wild type plants inoculated with *G. mosseae* showed a slight decrease in F values. M% and A% were lower in *G. mosseae*-colonised plants.

Solute accumulation

Nonmycorrhizal wildtype and antisense plants accumulated more proline than mycorrhizal plants (Fig. 3A). Under well-watered conditions, no differences in proline accumulation were observed among mycorrhizal treatments. In contrast, under drought stress, antisense plants inoculated with either AM fungus accumulated more proline than the corresponding wildtype plants inoculated with the same fungus. The increase was 200% for *G. mosseae*-colonised plants and 93% for *G. intraradices*-colonised plants.

TSS increased in both plant lines as a consequence of mycorrhization under both well-watered and drought stress conditions (Fig. 3B). The only exception was found in antisense plants colonised by *G. mosseae*, which, under drought stress conditions, showed TSS accumulation equal to that of nonmycorrhizal plants.

Gas exchange measurement

Well-watered plant lines exhibited a similar transpiration rate after AM colonisation (Table 1). Antisense lines colonised by *G. mosseae* displayed slightly lower transpiration. Under drought, both mycorrhizal wildtype plants showed increased transpiration rates in comparison to mycorrhizal antisense plants.

Table 1 CO_2-exchange rate (CER, μmol CO_2 m^{-2} s^{-1}), transpiration (mmol H_2O m^{-2} s^{-1}), water use efficiency (WUE, mmol CO_2/mol H_2O) and stomatal conductance (mmol H_2O m^{-2} s^{-1}) in wildtype (*WT*) or antisense (*AS*) tobacco plants inoculated or not with *Glomus mosseae* (*M*) or *Glomus intraradices* (*I*) and cultivated under well-watered conditions or subjected to drought stress. Means followed by the same letter are not significantly different ($P<0.05$) as determined by Duncan's multiple-range test ($n=5$). Significance of the sources of variation is also displayed

Treatment	CER	Transpiration	WUE	Conductance
Well-watered				
WT	8.3 d	1.6 c	5.3 a	82 d
AS	7.2 d	1.9 c	3.9 bc	95 d
WTM	61.0 a	14.1 a	4.4 abc	580 b
ASM	48.6 b	10.7 b	4.6 ab	446 c
WTI	38.6 c	14.4 a	2.7 d	540 b
ASI	48.1 b	13.7 ab	3.5 cd	690 a
Significance of sources of variation				
Plant line (P)	***	*	*	***
Mycorrhizal fungus (F)	**	*	**	**
P×F	***	**	*	**
Droughted				
WT	2.0 d	1.0 d	2.0 c	40 d
AS	1.5 d	0.9 d	1.8 cd	30 d
WTM	9.3 b	3.9 a	2.4 b	180 a
ASM	3.2 c	2.0 c	1.6 d	85 c
WTI	11.2 a	3.9 a	2.9 a	150 b
ASI	8.7 b	2.8 b	3.1 a	140 b
Significance of sources of variation				
Plant line (P)	***	***	*	*
Mycorrhizal fungus (F)	**	*	**	**
P×F	***	**	*	**

*$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$

Like the transpiration rates, CER was enhanced by mycorrhization (Table 1). Under well-watered conditions, wildtype plants exhibited higher photosynthetic activity when colonised by *G. mosseae* than when colonised by *G. intraradices*. Antisense plants showed similar photosynthetic rates with both AM fungi. Under drought stress conditions, mycorrhizal wildtype plants had higher photosynthetic activity than the corresponding mycorrhizal antisense plants.

Under well-watered conditions, WUE was lower in wildtype plants colonised by *G. intraradices* than in nonmycorrhizal plants and no significant differences were observed among the other treatments (Table 1). In contrast, under drought stress, both plant lines colonised by *G. intraradices* showed the highest WUE. No significant differences between mycorrhizal and nonmycorrhizal plants were found for each *G. mosseae*-inoculated plant line.

Stomatal conductance was also enhanced by mycorrhization in both plant lines (Table 1). Under well-watered conditions, no differences were found in wildtype plants as a consequence of the colonising fungus. In contrast, in antisense plants *G. mosseae* produced the lowest stimulation of stomatal conductance, while *G. intraradices* produced the highest. Under drought stress conditions, colonisation by *G. mosseae* enhanced more stomatal conductance in wildtype than in antisense plants, while *G. intraradices* behaved similarly with both plant lines.

Discussion

The AM system is an excellent model for the extensive morphological alterations undergone by plant root cells in order to accommodate the presence of symbionts. Cytoskeletal elements are rearranged, the nucleus increases in size, amyloplasts lose their starch content, and changes occur in the membrane systems of arbuscule-containing cells. 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 surface of the plant cell (Bonfante and Perotto 1995; Gianinazzi-Pearson 1996).

Since most of the mycorrhiza-induced changes in plant root cells concern cytoplasmic or vacuolar membrane systems, variations in the expression patterns of genes encoding membrane-associated proteins can be expected (Krajinski et al. 2000). Accordingly, gene activity of plant-encoded aquaporins has been shown to be upregulated in mycorrhizal roots, and expression is localised in the highly compartmentalised vacuole of arbuscule-containing cells (Roussel et al. 1997; Krajinski et al. 2000). It has been proposed that these aquaporins could optimise nutrient and water exchange between both symbiotic partners. They may also permit efficient osmoregulation of the highly compartmentalised root cells (Maurel et al. 2003). Considering that, and the fact that the periarbuscular membrane is derived from the plasma membrane rather than the tonoplast, we have studied the effect of the plasma-membrane intrinsic aquaporin NtAQP1 on the pattern of mycorrhizal colonisation and/or on fungal symbiotic efficiency. Data from this study show that plant lines with either a reduced or a natural level of NtAQP1 had a similar pattern of root colonisation under well-watered and drought stress conditions. The differences found under well-watered conditions concern the AM fungal species involved in the symbiosis. *G. mosseae* showed lower colonisation intensity (M%) and arbuscule abundance (A%) than *G. intraradices*, as has been observed previously (Graham et al. 1996). With regard to colonisation efficiency, *G. intraradices* is a more aggressive AM fungus than *G. mosseae*. The lack of effect of impairment in *NtAQP1* gene expression on AM fungal colonisation ability would suggest either that NtAQP1 function is irrelevant for the process of root colonisation, or that the impairment in *NtAQP1* gene expression has been compensated by other mechanisms, such as alterations in the abundance or activity of other aquaporins (Eckert et al. 1999; Johansson et al. 2000). However, this latter possibility is unlikely; Siefritz et al. (2002) analysed the consequences of antisense *NtAQP1* on expression of other aquaporins and found that only the closely related *NtPIP1a*

gene showed some reduced expression, which was less severe than that of *NtAQP1* gene. The RNA levels of other aquaporin genes belonging to different subfamilies were unaffected by antisense *NtAQP1* expression. Thus they suggested that results obtained by subsequent analysis of the antisense plants using plant physiology techniques can be ascribed solely to the function of *NtAQP1* and closely related *PIP1* genes.

Plant growth and development depend strongly on water absorption from the soil and its movement from the roots to other plant parts. Moreover, plant water status is important not only for growth under favourable environmental conditions; the ability of plants to tolerate water deficits and high salt levels also depends heavily on their water status, which is altered in response to environmental conditions (Aharon et al. 2003). Long-distance water transport is carried out in the vascular tissues, where water is transported by bulk flow, and membrane barriers are in most cases non-existent. In contrast, short-distance transport and transport in non-vascular tissues frequently involve transport across membranes, which includes transport through proteinaceous water channels (aquaporins). It has been proposed that rapid transmembrane water flow is possible due to the presence of aquaporins, and that the rate of water flux may be controlled by changing aquaporin abundance or activity (Eckert et al. 1999; Johansson et al. 2000).

In this study, both plant lines showed severely stunted growth when nonmycorrhizal, whether cultivated under well-watered conditions or under drought stress. Under well-watered conditions, shoot biomass production improved drastically in both antisense and wildtype plants after mycorrhizal colonisation, and the accumulation of TSS in mycorrhizal plants could have contributed to such an effect (Kameli and Lösel 1993). Under drought stress, however, mycorrhizal wildtype plants grew more than mycorrhizal NtAQP1 antisense plants, indicating that the symbiotic efficiency of both AM fungi (in terms of plant biomass production) was greater with wildtype than with NtAQP1 antisense plants. A low symbiotic efficiency of *G. mosseae* with antisense plants was evidenced not only under drought stress, but also under well-watered conditions. When the colonising fungus was the more aggressive *G. intraradices*, the ability of the wildtype plants to increase membrane water permeability by NtAQP1 seemed to be more beneficial in terms of biomass production. The ability of the mycorrhizal plants to grow better as an adaptation to altered environmental conditions appears to be due to the function of NtAQP1, as illustrated by Siefritz et al. (2004) in their study on the developmental regulation and function in growth processes of NtAQP1. The plant gas exchange also appears to depend on the expression of *NtAQP1*, because increases and decreases in transpiration, photosynthetic activity and stomatal conductance, parallel growth increments in the mycorrhizal plants. Aharon et al. (2003) and Uehlein et al. (2003) recently showed that overexpression of an *Arabidopsis* plasma membrane aquaporin, or of *NtAQP1* in tobacco, significantly increased plant growth rate, transpiration rate, stomatal density and photosynthetic efficiency when plants were cultivated under favourable growth conditions. In contrast to our results, such overexpression had no beneficial effect under salt stress and was deleterious during drought (Aharon et al. 2003). However, the mycorrhization status was not determined in these experiments and an artificial increase in membrane water permeability by overexpression of an arbitrarily chosen aquaporin gene in all tissues of the plant could have unpredictable effects on plant water relations and also on cell stability.

The lower proline content in wildtype plants could be an indication that these plants had a better water status and needed lower osmotic adjustment. In contrast, antisense plants had to accumulate more proline in order to decrease osmotic potential in their tissues (more active osmoregulation, also suggesting a possibly lower water status). This is in agreement with the fact that nonmycorrhizal plants always accumulated more proline (mainly under drought stress), indicating that they were more drought stressed than the mycorrhizal plants, as has been reported previously (Augé 2001; Ruiz-Lozano 2003).

Taken together, the present results indicate that enhanced symplastic water transport via the plasma membrane aquaporin NtAQP1 is important for the efficiency of AM symbiosis, at least under drought stress conditions. This confirms the results obtained by Siefritz et al. (2002), which show a measurable and visible effect of *NtAQP1* expression under drought stress, and the report by Ruiz-Lozano (2003) indicating that AM symbiosis contributes to the modulation of the total amount of water, thereby modifying plant water status and plant growth.

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