The Role of Tobacco Aquaporin1 in Improving Water Use Efficiency, Hydraulic Conductivity, and Yield Production Under Salt Stress[1][C][W][OA]

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Tobacco (*Nicotiana tabacum*; C3) plants increase their water use efficiency (WUE) under abiotic stress and are suggested to show characteristics of C4 photosynthesis in stems, petioles, and transmitting tract cells. The tobacco stress-induced Aquaporin1 (NtAQP1) functions as both water and CO_2 channel. In tobacco plants, overexpression of NtAQP1 increases leaf net photosynthesis (A_N), mesophyll CO_2 conductance, and stomatal conductance, whereas its silencing reduces root hydraulic conductivity (L_p). Nevertheless, interaction between NtAQP1 leaf and root activities and its impact on plant WUE and productivity under normal and stress conditions have never been suggested. Thus, the aim of this study was to suggest a role for NtAQP1 in plant WUE, stress resistance, and productivity. Expressing NtAQP1 in tomato (*Solanum lycopersicum*) plants (TOM-NtAQP1) resulted in higher stomatal conductance, whole-plant transpiration, and A_N under all conditions tested. In contrast to controls, where, under salt stress, L_p decreased more than 3-fold, TOM-NtAQP1 plants, similar to maize (*Zea mays*; C4) plants, did not reduce L_p dramatically (only by approximately 40%). Reciprocal grafting provided novel evidence for NtAQP1's role in preventing hydraulic failure and maintaining the whole-plant transpiration rate. Our results revealed independent, albeit closely related, NtAQP1 activities in roots and leaves. This dual activity, which increases the plant's water use and A_N under optimal and stress conditions, resulted in improved WUE. Consequently, it contributed to the plant's stress resistance in terms of yield production under all tested conditions, as demonstrated in both tomato and Arabidopsis (*Arabidopsis thaliana*) plants constitutively expressing NtAQP1. The putative involvement of NtAQP1 in tobacco's C4-like photosynthesis characteristics is discussed.

Aquaporins (AQPs) are integral membrane proteins that increase the permeability of membranes to water as well as to other small molecules such as CO_2, glycerol, and boron (Uehlein et al., 2003; Kaldenhoff and Fischer, 2006). Of all the kingdoms, plants contain the largest AQP family, consisting of over 30 members (e.g. 35 in Arabidopsis [*Arabidopsis thaliana*; Johanson et al., 2001]; 36 in maize [*Zea mays*; Chaumont et al., 2005]; and 37 in tomato [*Solanum lycopersicum*; Sade et al., 2009]). AQPs were suggested to play a key role in plant water balance and water use efficiency (WUE; Knepper, 1994; Tyerman et al., 2002; Aharon et al., 2003; Kaldenhoff and Fischer, 2006; Maurel, 2007).

Raising ambient CO_2 levels can potentially improve the growth rates of some C3 plants under optimal growth conditions (Kimball and Idso, 1983; Besford et al., 1990; Li et al., 2007), suggesting that membrane permeability to CO_2 is a growth-limiting factor in these plants (Loreto et al., 1992; Evans and von Caemmerer, 1996; Evans et al., 2000; Flexas et al., 2008). Impaired CO_2 conductance in the mesophyll following treatment with the nonspecific AQP inhibitor $HgCl_2$ was one of the first pieces of evidence suggesting AQPs's ability to conduct CO_2 (Terashima and Ono, 2002). The first direct evidence of this ability was reported for tobacco (*Nicotiana tabacum*) Aquaporin1 (NtAQP1) expressed in *Xenopus* oocytes (Uehlein et al., 2003). Those authors also induced NtAQP1 expression in tobacco plants and reported significant increases in photosynthetic rate, stomatal opening, and leaf growth rate. A role for NtAQP1 in the photosynthetic mechanism has recently been reported in tobacco plants overexpressing NtAQP1, which showed a 20% increase in photosynthetic rate relative to controls, while *NtAQP1* antisense plants showed a 13% decrease (Flexas et al., 2006). That study provided firm evidence (obtained by three independent research groups) for

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the role of NtAQP1 in increasing mesophyll CO_2 conductance (g_m), net photosynthesis (A_N), and stomatal conductance (g_s). However, no morphological, growth rate, or yield parameters were reported for any plants overexpressing the NtAQP1.

NtAQP1's cellular localization has been reported in both the mesophyll plasma membrane (PM) and the chloroplast inner membrane (CIM) in mesophyll and guard cells (Uehlein et al., 2008). However, these two membranes showed opposite permeability coefficients: while the water permeability of the CIM was 3-fold that of the PM, its CO_2 permeability was about five times lower than that of the PM. Moreover, while silenced plants showed an approximately 10-fold decrease in CIM CO_2 permeability, they did not show significantly decreased PM CO_2 permeability, and vice versa for water permeability (Uehlein et al., 2008). These results indicate a localization-function relationship in NtAQP1 cellular activity.

In addition to its role in controlling CO_2 and water permeability of mesophyll tissues and g_s (Uehlein et al., 2003; Flexas et al., 2006), NtAQP1 was originally suggested to control root hydraulic conductivity (L_P). Based on its high abundance in the roots, especially around the xylem vessels (Otto and Kaldenhoff, 2000; Siefritz et al., 2001), its impact on increasing the water permeability of *Xenopus* oocytes (Biela et al., 1999), and the decrease in root L_P of *NtAQP1*-silenced plants (Siefritz et al., 2002), NtAQP1 was suggested to play a role in controlling root L_p. This activity might be related to abiotic stress conditions, since *NtAQP1* is a stress-induced gene, it is regulated by an abscisic acid (ABA)-sensitive promoter (Siefritz et al., 2001), and it shows a significant increase in root transcript level during drought stress (Mahdieh et al., 2008). Moreover, *NtAQP1*-silenced plants reveal higher drought sensitivity than control plants (Siefritz et al., 2002).

Considerable progress has been made in the study of NtAQP1. Research on its water and CO_2 transport and stress resistance activities has progressed in parallel, but without much interaction. Furthermore, tobacco plants are known to significantly improve their WUE under drought conditions (Cakir and Cebi, 2009) and are suggested to have C4 photosynthesis characteristics in stems, petioles, and transmitting tract cells (Jansen et al., 1992; Hibberd and Quick, 2002). Nevertheless, to the best of our knowledge, a specific role for AQPs in these mechanisms has never been suggested. Thus, the aim of this study was to address NtAQP1's role in the plant stress resistance mechanisms by testing the hypothesis that NtAQP1 plays a key role in improving WUE and yield under abiotic stress.

We constructed transgenic tomato (*Solanum lycopersicum*) plants (a well-characterized model crop plant) constitutively expressing NtAQP1 (TOM-NtAQP1) and compared their A_N, g_s, CO_2 concentration in the substomatal cavity (C_i), stomatal aperture, stomatal density, whole-plant transpiration, WUE, root L_p, and yield production with control and reciprocally grafted plants under normal and salinity stress conditions. Our results revealed independent, albeit closely related, water and CO_2 activities induced by NtAQP1 in the roots and leaves, respectively. This dual activity, which increases the plant's water use and photosynthetic rate under optimal conditions and consequently improves its WUE, contributed to the plant (tomato and Arabidopsis) yield production under all tested conditions.

RESULTS

NtAQP1 Increases Osmotic Water Permeability of Tobacco Mesophyll Cells

In addition to its role as a CO_2 channel, NtAQP1 expression in *Xenopus* oocytes proved its activity as a water channel (Biela et al., 1999). It was also suggested to be involved in regulating the mesophyll osmotic water permeability coefficient (P_f) and root L_p, in particular under stress. However, NtAQP1's specific contribution to the water permeability of these tissues was never characterized in planta. Thus, our first objectives were to measure and characterize NtAQP1 water channel activity in tobacco mesophyll cells and to estimate its role in regulating whole-plant L_p.

The impact of NtAQP1 on the P_f value of tobacco mesophyll protoplasts was measured by cell-swelling assay. Mesophyll protoplasts were isolated from tobacco (Uehlein et al., 2003) expressing NtAQP1 under a 35S tetracycline-inducible promoter. The induced cells had three times higher P_f values than the control noninduced cells (Fig. 1), indicating NtAQP1's activity as a functional water channel in mesophyll cells.

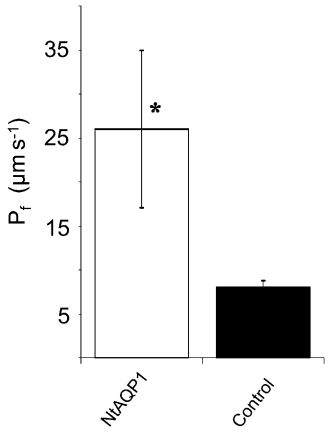


Figure 1. NtAQP1 expression increases P_f of tobacco mesophyll membrane. Columns represent mean P_f (±SE) of tobacco mesophyll protoplasts expressing NtAQP1 (white bar; $n = 15$) and control protoplasts (black bar; $n = 9$). * Significant difference between the means (*t* test, $P < 0.05$).

The Impact of NtAQP1 Water Conductivity and Photosynthesis in Tomato Plants under Normal and Salt Stress Conditions

To determine NtAQP1's impact on whole-plant water conductivity, A_N, and abiotic stress resistance (which we defined as fruit yield under stress relative to controls), we introduced the *NtAQP1* gene into tomato, producing TOM-NtAQP1.

Three different independent transgenic tomato lines expressing NtAQP1 under the constitutive promoter 35S were identified at the genomic DNA, mRNA, and membrane protein levels (Supplemental Fig. S1).

The impact of NtAQP1 on leaf photosynthesis and stomatal conductivity was tested using a gas-exchange measuring system that enables monitoring CO_2 assimilation rate. As expected and similar to Uehlein et al. (2003) and Flexas et al. (2006), our TOM-NtAQP1 plants showed significantly higher A_N and g_s, while the C_i concentration remained the same relative to control plants under normal conditions (Table I; Supplemental Fig. S2). A higher g_s can result from changes in stomatal density and/or stomatal pore area. An anatomical characterization of the leaf epidermis of both TOM-NtAQP1 and control plants was performed to explore this issue. Imprints of the abaxial leaf surface revealed no significant change in stomatal density among plants, whereas a significantly larger stomatal pore area was measured in TOM-NtAQP1 plants under both normal and salt stress conditions (Table I). The higher A_N was maintained under 100 mM NaCl, suggesting that NtAQP1's ability to transport CO_2 provides a substantial benefit in improving carbon's diffusive permeability under stress conditions. Calculating the instantaneous WUE (IWUE) revealed improvement in TOM-NtAQP1 plants under normal and salt-stress growth conditions compared with the control plants, with the highest improvement seen in TOM-NtAQP1 plants under salt stress (Table I).

The rate of tension-induced root exudation from the detopped root system of TOM-NtAQP1 plants differed from that of controls under normal irrigation. Moreover, salt application caused a considerable decrease in root exudation in control plants, while TOM-NtAQP1 plants retained their original rate of sap exudation (Supplemental Fig. S3). Root L_p, accounting for the total cross-sectional area of the xylem, in TOM-NtAQP1 plants did not differ from that in control plants under normal irrigation. However, when irrigated with water containing 50 mM NaCl, the TOM-NtAQP1 plants decreased their L_p only by about 40%, while L_P of control plants decreased more than 3-fold (Table I).

Both plant types yielded a low and stable sodium concentration in their xylem sap of around 2 mM Na^+ during the 3 h prior to irrigation with 50 mM NaCl-containing water. Within a short time after irrigation with the salt-containing water, the sodium concentration rose dramatically in the xylem sap of both plants, with control plants reaching over 26 mM Na^+, almost twice the Na^+ concentration in the xylem sap of TOM-NtAQP1 plants. However, when the lower exudate volume collected from the control plants following the salt treatment relative to the TOM-NtAQP1 plants was taken into account, the amount of Na^+ that was taken up by the detopped root systems (Na^+ discharge) did not differ between the two treatments (Table I).

The higher g_s in TOM-NtAQP1 compared with control plants was supported by the wider stomatal aperture measured; however, under salinity stress, the g_s values in both plants were similar, while TOM-NtAQP1 plants continued to show significantly wider stomatal aperture (Table I). This phenomenon suggested that the timing of the stomatal conductivity measurements might be important. Therefore, we conducted continuous daily monitoring of plant transpiration.

Using a multiple lysimeter system normalized for vapor-pressure deficit (VPD) and plant leaf area, we measured the daily transpiration rates in all TOM-NtAQP1 and control plants simultaneously. Daily transpiration rate in the former was higher than in control plants under normal growth conditions (Fig. 2A), mainly during the hours of high water demand

Table I. *Photosynthetic characteristics including transpiration (T_r; as determined at 1,200 μmol m^{-2} s^{-1}, approximately 25°C, and 370 μmol mol^{-1} CO_2) and root hydraulic characteristics of TOM-NtAQP1 and control plants treated with normal irrigation and 100 mM NaCl in a controlled greenhouse*

Data are given as means ± SE (*n*, number of replicates, as indicated). Different letters in a row indicate significant differences (*t* test, $P < 0.05$). * For the root hydraulic measurements (L_p and Na discharge), plants were treated with 50 mM NaCl.

Variable	Normal Irrigation		Salt Irrigation	
	Tom-NtAQP1 (*n*)	Control Plants (*n*)	Tom-NtAQP1 (*n*)	Control Plants(*n*)
A_N (μmol CO_2 m^{-2} s^{-1})	24 ± 1.3 (20) a	17 ± 1.5 (10) b,c	17 ± 0.8 (13) b	15 ± 0.8 (9) c
g_s (mol water m^{-2} s^{-1})	0.43 ± 0.04 (20) a	0.31 ± 0.03 (10) b	0.16 ± 0.02 (13) c	0.16 ± 0.01 (9) c
C_i (μmol CO_2 mol^{-1})	260 ± 8 (20) a	248 ± 6 (10) a	151 ± 15 (13) b	158 ± 10 (9) b
T_r (mmol water m^{-2} s^{-1})	5 ± 0.4 (20) a	4 ± 0.3 (10) b	2.4 ± 0.3 (13) c	2.5 ± 0.3 (9) c
Stomatal density (0.01 mm^2)	22 ± 1 (18) a	24 ± 3 (12) a	19 ± 1 (18) a	21 ± 2 (12) a
Stomatal pore area (μm^2)	52.9 ± 1.1 (445) a	29.2 ± 2.0 (110) b	25 ± 1.1 (200) b	16.1 ± 1.5 (74) c
IWUE (μmol CO_2 $mmol^{-1}$ water)	4.9 ± 0.3 (20) a	4.3 ± 0.2 (10) b	8.2 ± 1 (13) c	6.3 ± 0.4 (9) d
L_p (mm s^{-1} MPa^{-1})*	229 ± 68 (6) a	225± 65 (6) a	141 ± 53 (6) b	66 ± 15 (6) c
Na discharge (pmol s^{-1})*	6 ± 2 (4) a	5 ± 2 (4) a	38 ± 2 (4) b	35 ± 2 (4) b

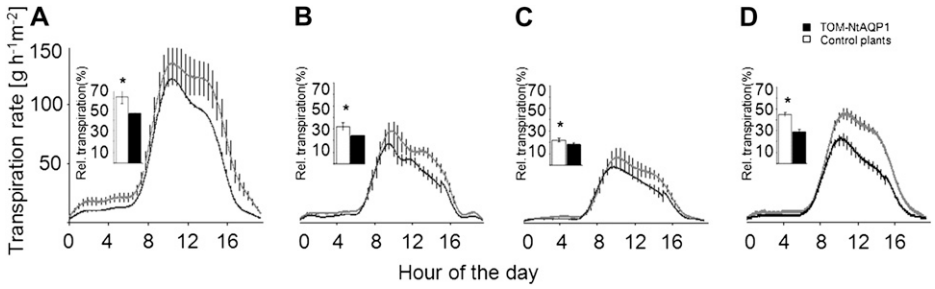


Figure 2. Whole-plant daily transpiration rate and relative transpiration of TOM-NtAQP1 and control plants grown under normal irrigation and salt stress in a controlled greenhouse. A, Mean daily transpiration rate (±SE; normalized to leaf area) of TOM-NtAQP1 (gray line; $n = 3$) and control plants (black line; $n = 3$) under normal irrigation regime. B to D, Mean daily transpiration rate (±SE; normalized to leaf area) in plants after 1 d (B) and 3 d (C) of 100 mM NaCl salinity stress and on day 1 of recovery (D; receiving normal irrigation again; $n_{NtAQP1} = 3$, $n_{control} = 3$). All insets represent mean daily relative (rel.) transpiration (%), normalized to VPD and leaf area, of the plants presented in the graphs. Data are given as means ± SE for each 10th sampling point. When not seen, SE is smaller than the symbol. * Significant difference between means (*t* test, $P < 0.05$).

(11:00 AM to 3:00 PM), resulting in significantly higher relative transpiration levels for the entire day (Fig. 2A, inset). Total daily transpiration was defined by the area under the daily transpiration rate curve. The differences in transpiration between TOM-NtAQP1 and control plants were significant during the salinity stress treatment (Fig. 2, B and C) and during subsequent recovery from salinity stress (Fig. 2D).

NtAQP1's Role in Preventing Root-Shoot Hydraulic Failure and Improving Whole-Plant Stress Resistance

Increasing g_s and transpiration, on the one hand, while maintaining normal root L_p under osmotic stress conditions, on the other hand, suggests dual independent roles for the NtAQP1 channel in whole-plant hydraulic control. To estimate the relations between these activities of NtAQP1 and the relative importance of each in the whole plant's response to stress, a reciprocal grafting experiment was conducted. Whole-plant transpiration rates and relative daily transpiration of all grafted plants were simultaneously measured (using the multiple lysimeter system and normalization of each plant to VPD and leaf area; see "Materials and Methods"). The grafting process did not affect the plants' behavior, as reflected by the fact that the control grafted plants, TOM-NtAQP1 over TOM-NtAQP1 (T/T) and control over control (C/C), maintained similar transpiration rate patterns as their nongrafted counterparts (Fig. 2), that is, higher transpiration rate and relative daily transpiration of the transgenic plants under both normal and salt treatments (Fig. 3, A and B). Interestingly, the T/C grafted plants exhibited a considerable reduction, starting at midday, in the rate of the whole-plant daily course of transpiration. This midday "break" in transpiration rate in T/C plants (clearly seen under both normal and salt stress conditions) might be explained by stomatal closure. Another explanation for the break might be a failure in L_p resulting from their higher g_s and lower L_p (as was demonstrated previously in the nongrafted plants).

To estimate the relative impact of root hydraulic signals on shoot g_s and A_N under salt stress in the reciprocal grafted plants, both were measured in the grafted plants. These measurements were taken during two time intervals: morning (8:00–11:00 AM) and noontime (11:00 AM–2:00 PM). During the morning period, no change in whole-plant transpiration rate (Fig. 3B) or in either g_s or A_N could be detected in the grafted plants (Fig. 3, C and E). During the noon period, however, a break in transpiration rate was clearly seen in all but the T/T plants (Fig. 3B). While the reduction in whole-plant transpiration rate could be explained by stomatal closure for the C/C and C/T plants, this could not explain the high g_s and A_N values of T/C plants, which remained higher than controls and similar to T/T plants (Fig. 3, D and F): both T/T and T/C plants maintained similar and significantly higher g_s and A_N values than C/C or C/T plants.

TOM-NtAQP1 Plants Show Higher Yields Than Controls under Normal and Salt Treatments

The fact that TOM-NtAQP1 plants showed significant increases in whole-plant transpiration and A_N, resulting in increased WUE, suggests that NtAQP1 may contribute to plant vigor, biomass, and yield parameters under both optimal and stress conditions. Thus, we conducted a greenhouse experiment to determine the impact of NtAQP1 on plant productivity under normal and salinity conditions.

Three independent transgenic TOM-NtAQP1 plants were grown in a controlled greenhouse under optimal or 100 mM NaCl conditions for an entire growing season. In each trial, the transgenic genotypes were compared with nontransformed plants as controls. In the salt stress trial, all of the plants were continuously irrigated with water containing 100 mM NaCl. TOM-

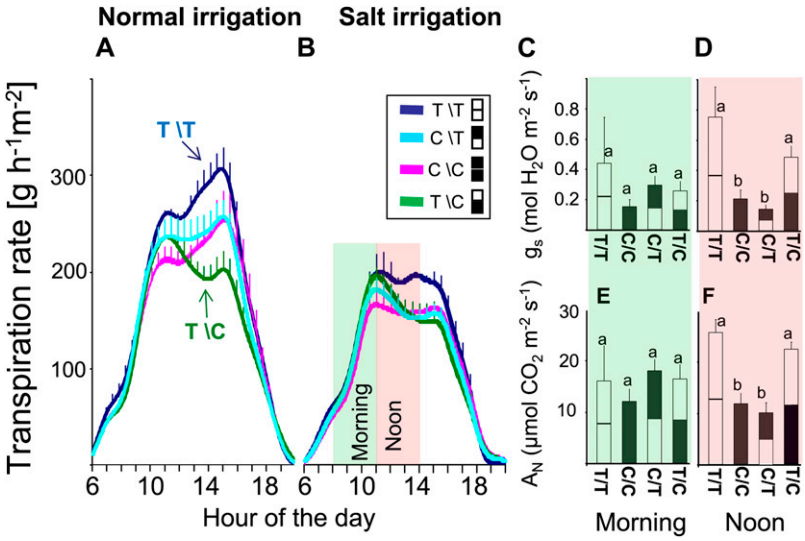


Figure 3. Daily transpiration rate, g_s, and A_N for grafted plants under normal and 100 mM NaCl irrigation. A and B, Whole-plant mean daily transpiration rate normalized to leaf area of all reciprocal grafted TOM-NtAQP1 and control plants measured simultaneously under normal irrigation (A) and after 3 d of 100 mM NaCl irrigation (B; T/T $n = 3$, C/C $n = 3$, C/T $n = 8$, T/C $n = 6$). SE is given for each 10th sampling point; when not seen, SE is smaller than the line. The green area represents morning hours, 8:00 to 11:00 AM, and the light-red area represents noon hours, 11:00 AM to 2:00 PM. At these hours and under the same salt conditions, parallel measurements of reciprocal grafted scions were conducted for g_s and leaf A_N, during the morning hours (C and E) and the noon hours (D and F). Bars represent means ± SE of grafted plants (white for TOM-NtAQP1 and black for control; T\T $n = 3$ for morning and noon, C/C $n = 4$ for morning and $n = 11$ for noon, C/T $n = 6$ for morning and $n = 5$ for noon, T/C $n = 5$ for morning and $n = 6$ for noon). Different letters indicate significant differences (*t* test, $P < 0.05$). All measurements were performed in a semicontrolled greenhouse (see "Materials and Methods"); gas-exchange measurements were conducted with a portable apparatus (Li-6400) on young, fully expanded leaves.

NtAQP1 plants did not appear to be more vigorous than control plants under either control or stress irrigation; nevertheless, TOM-NtAQP1 plants showed improved yield parameters, relative to controls, under both favorable and stressed growth conditions (Fig. 4). To rule out the possibility that NtAQP1's impact is unique to tomato or to the Solanaceae, we conducted a complementary experiment with transgenic Arabidopsis plants expressing NtAQP1 (Supplemental Fig. S4). These plants showed better tolerance to a salt stress of 100 mM NaCl than control plants, as reflected by their 125% higher dry biomass under salt stress and 81% higher dry biomass under control irrigation (Fig. 5).

DISCUSSION

NtAQP1 is one of the few plant AQPs that has been thoroughly studied in the last decade. Early reports suggested that NtAQP1 controls root L_p, in particular under stress (Siefritz et al., 2002). Later studies suggested novel CO_2 transport activity for this channel, launching fresh studies into its role in the mechanism governing plant photosynthesis (Uehlein et al., 2003, 2008; Flexas et al., 2006). However, despite the extensive research on this AQP, there have been no studies on the integrated impact of NtAQP1 on whole-plant WUE in general and under stress. Moreover, the fact that NtAQP1 overexpression triggers an increase in leaf g_s, which leads to a higher rate of whole-plant transpiration, conflicts with the fact that this gene is naturally induced under stress. To expand our understanding of NtAQP1's role in the whole-plant response to stress, we investigated its role in the water and CO_2 balance of tomato plants grown under optimal and abiotic stress conditions.

NtAQP1's Impact on Transpiration Use Efficiency

Although NtAQP1 was shown to be an active water channel in *Xenopus* oocytes (Biela et al., 1999), its contribution to the water permeability of mesophyll cells was not obvious. Furthermore, the dual localization of NtAQP1 in the CIM and PM (Uehlein et al., 2008) led us to measure NtAQP1 water transport activity in planta. Here, we show that NtAQP1 is indeed an active water channel in tobacco protoplasts, significantly increasing their P_f level relative to controls (Fig. 1). Thus, we provide, to our knowledge, the first proof of its water transport activity, in addition to its previously reported function as a CO_2 channel, in tobacco mesophyll cells. Interestingly, NtAQP1 has a dual impact not only at the cellular level but also at the whole-plant level, where its expression increases both transpiration and net photosynthetic fluxes. In addition, the fact that this channel is stress induced (Siefritz et al., 2001; Mahdieh et al., 2008; Mahdieh and

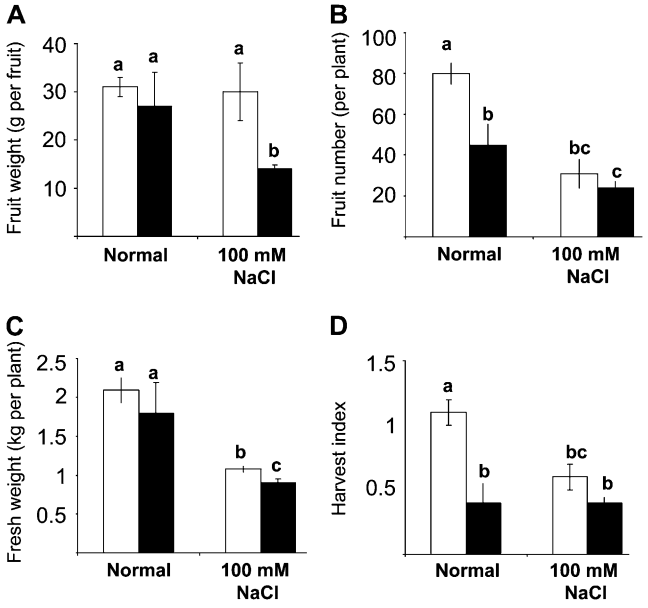


Figure 4. Yield parameters of TOM-NtAQP1 (white bars) and control plants (black bars). Plants grown for 3 months in a controlled greenhouse under normal ($n_{NtAQP1} = 8$, $n_{control} = 3$) and 100 mM NaCl ($n_{NtAQP1} = 5$, $n_{control} = 5$) irrigation conditions. Columns represent means ± SE of individual fruit weight (A), fruit number (B), shoot fresh weight (C), and harvest index (D). Different letters indicate significant differences (*t* test, $P < 0.05$).

Mostajeran, 2009) suggests its unique involvement in the plant's mechanism of transpiration use efficiency (biomass produced per unit of water transpired), particularly under stress. Reduced plant production under stress conditions can be explained and evaluated by the crop's transpiration use efficiency ratio, which is linear and depends on crop characteristics (deWit, 1958; Sinclair et al., 1984; Kemanian et al., 2005).

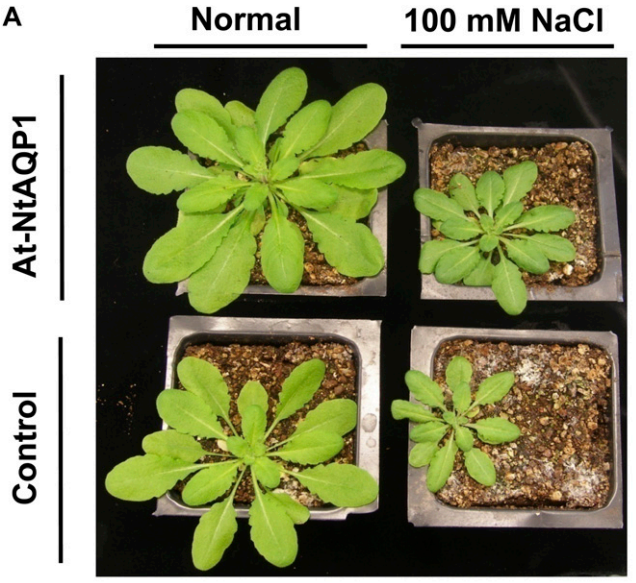
As expected from previous reports (Uehlein et al., 2003; Flexas et al., 2006), TOM-NtAQP1 plants showed an increased response of A_N to C_i under normal growth conditions. These plants retained this photosynthetic advantage under stress conditions (Supplemental Fig. S2). In addition, their leaf g_s and whole-plant transpiration rate were significantly increased due to wider stomatal aperture (Table I; Fig. 2), resulting in improved yield parameters in all treatments relative to controls (Fig. 4).

Under normal growth conditions, the above changes yielded a major improvement in productivity due to the high photosynthetic rate; however, this process was accompanied by a proportionally higher transpiration rate, thereby revealing only slightly higher IWUE (Table I). On the other hand, under stress conditions, the ratio between CO_2 assimilation and transpiration increased, as reflected by the improved IWUE of TOM-NtAQP1 plants (Table I), which eventually led to higher biomass and/or yield (in comparison with controls) under stress in both tomato and Arabidopsis plants (Figs. 4 and 5).

The involvement of NtAQP1 in the mechanism controlling g_s, A_N, C_i, and g_m has been reported previously (Uehlein et al., 2003; Flexas et al., 2006). Those studies showed increased g_s in NtAQP1-overexpressing tobacco plants and decreased g_s in *NtAQP1* antisense plants. This impact on the guard cells might be related to a direct effect of NtAQP1 in transporting CO_2 (Uehlein et al., 2003; Flexas et al., 2006) or water, as demonstrated in this study, although we cannot rule out other indirect effects; nevertheless, based on our grafting experiments, we suggest that the impact of NtAQP1 on g_s and A_N is independent of the conventional root-to-shoot signal.

Root-to-Shoot Signaling and Its Impact on g_s and A_N

Signals initiated under water stress (salinity or drought) are sensed by the roots, transported to the



B

Treatment / Plant	Normal dry weight (mg)	100 mM NaCl dry weight (mg)
At-NtAQP1	123 ± 9a	54 ± 5b
Control	71 ± 5c	24 ± 3d

Figure 5. NtAQP1 impact on Arabidopsis plant dry weight under normal and 100 mM NaCl irrigation. A, Arabidopsis plants (45 d old) constitutively expressing NtAQP1 (top row) and control plants (bottom row) grown in a controlled growth room under normal irrigation regime (left) and 100 mM NaCl irrigation regime (right) for 33 d. B, Mean ± SE shoot dry weight of Arabidopsis plants overexpressing NtAQP1 and control plants under normal ($n_{NtAQP1} = 11$, $n_{control} = 8$) and 100 mM NaCl ($n_{NtAQP1} = 12$; $n_{control} = 6$) irrigation conditions. Different letters indicate significant differences (*t* test, $P < 0.05$). [See online article for color version of this figure.]

leaf, and ultimately reduce g_s. These root-to-shoot signals may be either chemical or hydraulic (for review, see Schachtman and Goodger, 2008). A hydraulic signal may form due to the sharp decrease in root L_p in response to abiotic stress. Such a decrease has been reported as a general reaction in plants to many abiotic stresses (Steudle, 2000) and was recorded also in our control plants (more than 3-fold reduction in L_p) in response to 50 mM NaCl. In contrast, TOM-NtAQP1 plants reduced their L_p by less than 40% under the same salt stress (Table I). A reduced root hydraulic signal might explain TOM-NtAQP1's higher transpiration, g_s, and A_N under stress conditions compared with stressed control plants. Yet, a TOM-NtAQP1 scion grafted on a control rootstock (T/C) and exposed to salt stress still exhibited higher g_s and A_N, similar to T/T plants (Fig. 3, C–F). This suggests that NtAQP1's activity in controlling g_s and A_N is dominant and nearly independent of root signals.

However, T/C plants showed a midday drop in transpiration rate under both normal and stressed conditions (Fig. 3, A and B). This "drop" came just after these plants had reached their daily peak transpiration rate (assumed to be coupled with peak xylem tension). Replacing the control rootstock with TOM-NtAQP1 (T/T) revealed a mirror image of these results (i.e. a peak instead of a drop in midday transpiration rate), thereby indicating the roots' involvement in this process. Moreover, since g_s of T/C plants remained high during the midday drop in transpiration (Fig. 3, C–F), we can conclude that this drop is not due to stomatal closure and that it might be due to hydraulic failure resulting from the combination of high g_s and low L_p, leading to a decrease in plant water content.

Interestingly, maize (a C4 plant) exhibits a similar strategy of increasing root ZmPIP expression level and L_p in response to stress or ABA (Hose et al., 2000; Parent et al., 2009). Those authors suggested that ABA has long-lasting effects on plant hydraulic properties via ZmPIP activity, which contribute to maintenance of favorable plant water status. Moreover, a recent study revealed that inhibiting maize root L_P does not reduce plant transpiration under low VPD (Ehlert et al., 2009). A similar impact of ABA on tomato root L_p (Thompson et al., 2007) and on tobacco root AQP (*NtAQP1*, *NtPIP1;1*, and *NtPIP2;1*) transcript levels and an increase in L_p (Mahdieh and Mostajeran, 2009) have been recently reported.

We hypothesize that TOM-NtAQP1's stress resistance behavior is tightly related to NtAQP1 water transport activity in the roots. Accordingly, NtAQP1 might act as the root's "emergency" hydraulic valve (i.e. release hydraulic tension by increasing root L_p under higher transpiration rate or other stress, which in turn decreases xylem tension), thereby preventing hydraulic failure in the xylem system. Nevertheless, the fact that our model plants overexpressed NtAQP1 constitutively puts the physiological relevance of our findings on the in vivo role of NtAQP1 in tobacco into question. In other words, have we really learned something about the role of NtAQP1, or are we just seeing what happens when we artificially increase membrane permeability?

The Endogenous Role of NtAQP1 in Improving Tobacco Plant WUE

To address the above question, we looked to related studies on the impact of other AQPs on the whole-plant water balance. Interestingly, constitutive expression of several AQPs has been reported to increase plant L_p, suggesting that increasing membrane permeability might be the dominant role of AQP in its impact on the whole plant. However, while overexpression of stress-induced AQPs resulted in improving the plant's stress resistance, expressing AQPs that are down-regulated under abiotic stress had the opposite effect. For example, the rice (*Oryza sativa*) PIP1 AQP *OsPIP1;3* is a stress-induced gene that increases its transcript level in both leaves and roots of the stress-resistant upland rice (subspecies *indica* cv Zhonghan 3) in response to stress (Lian et al., 2004, 2006). Overexpressing OsPIP1;3 in the stress-sensitive lowland rice (subspecies *japonica* cv Zhonghua 11) improved its water status during conditions of water deficit and revealed an increase in whole-plant transpiration and L_p (Lian et al., 2004). In another study, constitutive expression of the tomato stress-induced AQP SlTIP2;2 (Sade et al., 2009) led to an increase in the transgenic plants' whole-plant transpiration and dramatically improved their stress resistance. In contrast, tobacco plants overexpressing AtPIP1b (which is down-regulated under drought stress; Alexandersson et al., 2005) showed a significant increase in transpiration rate yet became more sensitive to water stress (Aharon et al., 2003). Unlike this latter study but similar to the previous ones, in our study, plants overexpressing stress-induced AQPs presented superior stress resistance relative to their controls. In addition, the differential impacts of the different AQPs on the plant stress response (regardless of their constitutive expression profile) suggest that studying their involvement in the stress resistance mechanism by overexpressing them in heterologous plants is a justifiable approach.

On the basis of our results, we suggest an explanation for the tobacco "C4-like" stress resistance mechanism. It is possible that during evolution, tobacco plants developed C4-like characteristics of higher maximal CO_2 assimilation rates and L_p as stress resistance mechanisms. Interestingly, tobacco plants have two other C4-analogous behaviors: (1) photosynthetic cells of stems and petioles that surround the xylem and phloem possess high activities of enzymes characteristic of C4 photosynthesis, which allow the decarboxylation of four-carbon organic acids from the vascular system and not from the stomata, similar to the bundle sheath cells of C4 plants (Hibberd and Quick, 2002); and (2) tobacco transmitting tract cells lack Rubisco. Photosynthesis in these cells is function-

ally coupled to phospho*enol*pyruvate carboxylase-mediated "dark reactions," which result in the formation of malate (Jansen et al., 1992). The fact that NtAQP1 is highly expressed in xylem-surrounding cells and in flowers (Biela et al., 1999; Otto and Kaldenhoff, 2000; Siefritz et al., 2001) gives additional support to this hypothesis.

CONCLUSION

We suggest that the stress-induced AQP *NtAQP1* is involved in improving the stressed plant's transpiration use efficiency by a combination of three independent but complementary means: the first involves increasing mesophyll CO_2 permeability, resulting in increased photosynthetic rate; the second involves increasing the stomatal aperture; and the third involves maintaining a nearly constant root L_p, thus preventing hydraulic failure under higher xylem tensions. Obviously, this mechanism cannot function under severe stress. Nevertheless, as we show in this work, moderating the decrease of root L_p allows a higher transpiration rate, and together with a higher CO_2 assimilation rate, the plant reveals significant improvement in productivity under normal and stress treatments.

MATERIALS AND METHODS

Construction of Transgenic Plants

The full-length cDNA of *NtAQP1* from tobacco (*Nicotiana tabacum*; a gift from Prof. Ralf Kaldenhoff) was digested from pCRII TOPO (Invitrogen) using *Bam*HI and *Xho*I restriction enzymes. *NtAQP1* was then cloned, using the same restriction enzymes, into binary plasmid pBIN203 (courtesy of Dr. Orit Edelbaum) under the regulation of the 35S constitutive promoter. M82 tomato (*Solanum lycopersicum*) lines were genetically transformed using disarmed *Agrobacterium tumefaciens* transformation methods (Barg et al., 1997). Arabidopsis (*Arabidopsis thaliana*) plants were genetically transformed using the floral dip transformation method (Clough and Bent, 1998). Plants were assayed for the presence of *NtAQP1* by PCR (4-min initial denaturation at 94°C, followed by 33 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, and a final step at 72°C for 15 min) using the following primers, sense primer 35Sprom-FWD (5′-TATCCTTCGCAAGACCCTCC-3′) and the reverse complementary primer NtAQP1-REV (5′-TGCCTGGTCTGTGTTGTAGAT-3′), amplifying a 930-bp *NtAQP1* DNA fragment.

mRNA Extraction and cDNA Construction

Leaf tissue (100 mg) was taken from transgenic and control plants, and total RNA was extracted using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's protocol. To rule out the effect of any residual genomic DNA in the preparation, RNA was treated with TURBO DNA-*free* (Ambion) according to the manufacturer's instructions. Total RNA (1 μg) was taken for reverse transcription-PCR using ReverseTranscriptase Moloney murine leukemia virus RevertAid according to the manufacturer's protocol (Fermentas). cDNA amplification was performed using the sense primer NtAQP1-RNA SENSE (5′-CCGGGCAGGTGTACTATCC-3′) and the reverse complementary primer NtAQP1-REV (5′-TGCCTGGTCTGTGTTGTAGAT-3′), amplifying an 830-bp *NtAQP1* cDNA fragment.

Protein Extraction and Detection

Leaf tissue (100 mg) was taken from transgenic and control plants and homogenized in three volumes of homogenization buffer (330 mM Suc, 100 mM KCl, 1 mM EDTA, 50 mM Tris/0.05% MES, pH 7.5, 5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). The sample was then centrifuged twice for 15 min, once at 1,000*g* (supernatant collected) and then at 10,000*g* (supernatant collected). Finally, the sample was centrifuged at 48,000*g* for 75 min to extract the microsomal phase. The pellet (microsomal fraction) was resuspended in membrane buffer (330 mM Suc, 20 mM dithiothreitol, and 50 mM Tris/0.05% MES, pH 8.5).

Protein extracts were diluted in sample buffer (10% [v/v] glycerol, 5% [v/v] mercaptoethanol, 0.125 M Tris-HCl, pH 6.8, 3% [w/v] SDS, and 0.05% [w/v] bromphenol blue) and subjected to 10% SDS-PAGE. After electrophoresis, proteins were electroblotted onto a Hybond-C Extra membrane (Amersham Life Science) at 4°C for 2 h at 110 V, using transfer buffer (25 mM Tris-HCl, pH 8.3, and 192 mM Gly) supplemented with 10% (v/v) methanol. The membranes were blocked for 1 h at 22°C to 25°C with 2% (w/v) bovine serum albumin in 10 mM Tris-HCl, pH 7.5, and 150 mM NaCl containing 0.1% (w/v) Tween 20 (TBS-T). Briefly, membranes were incubated for 18 h at 4°C with primary antibody (1:5,000 dilution; a kind gift from Prof. Ralf Kaldenhoff). All subsequent steps were performed at 22°C to 25°C. Following five washes of 10 min each in TBS-T, membranes were incubated for 1 h with horseradish peroxidase-linked secondary antibody. After intensive washes with TBS-T, immobilized conjugates were visualized by enhanced chemiluminescence (Amersham Life Science), followed by exposure to x-ray film.

Plant Material and Growth Conditions

The experiments were conducted on T2 generation plants from three independent transgenic M82 tomato lines overexpressing the tobacco PM AQP gene *NtAQP1*: TOM-NtAQP1 and nontransgenic plants as controls. The plants were transplanted to 3.9-L pots with ready mixed growing substrate and were grown for approximately 3 months (May to August) in a controlled greenhouse at 25°C and 50% to 60% relative humidity. The experimental design was completely randomized. Fertilization was added to the irrigation system automatically. Normal fertigation consisted of approximately 500 mL, three times a day. Salt treatment was applied by treating the plants with 1.5 L of 100 mM NaCl solution in the fertigation solution, given once a day.

The Arabidopsis experiment consisted of two independent T2 transgenic Arabidopsis lines overexpressing NtAQP1 and nontransgenic plants as controls. All plants were grown in a controlled growth chamber at 22°C under short-day conditions (10 h of light) in 200-mL pots with commercial growing medium containing slow-release fertilizers. Plants were irrigated with tap water or 100 mM NaCl solution until shoot harvesting (45 d from transplanting).

Yield Parameters

Total number and weight of fruits from the transgenic TOM-NtAQP1 and control plants were measured for each plant under normal and salt stress (100 mM NaCl) conditions. Average fruit weight was calculated by dividing the total weight of the fruits by their number. The fresh weight of the above-ground shoots was measured. Harvest index was calculated by dividing total weight of fruits per plant (fruit number × individual fruit weight) by fresh weight per plant.

Gas-Exchange Measurements

The response of A_N to C_i was recorded in three independent T2 transgenic TOM-NtAQP1 and control plants inside a controlled greenhouse on fully expanded leaves, under all tested irrigation conditions, using an Li-6400 portable gas-exchange system (LI-COR). All measurements were conducted between 8:00 AM and 2:00 PM.

Photosynthesis was induced in saturating light (1,200 μmol m^{-2} s^{-1}) with 370 μmol mol^{-1} CO_2 surrounding the leaf (C_a). The amount of blue light was set to 15% photosynthetically active photon flux density to optimize stomatal aperture. The leaf-to-air VPD was kept at around 1 to 2.5 kPa during all measurements. Leaf temperature for all measurements was approximately 26°C (ambient temperature). Once a steady state was reached, a CO_2 response curve was measured and, finally, the A_N-C_i curve was plotted.

P_f Measurements

Protoplasts were isolated from tobacco leaf mesophyll (Uehlein et al., 2003) and subjected to 10 mg L^{-1} tetracycline for approximately 1 h to induce

NtAQP1 expression. P_f was measured from the initial (videotaped) rate of volume increase in a single protoplast in response to hypotonic solution. The P_f was determined by a numerical approach (offline curve-fitting procedure using several algorithms), which has been proven to yield accurate P_f values over a large range of water permeability values. The analyses were performed with the P_fFit program incorporating these equations, as described in detail by Moshelion et al. (2002) and Volkov et al. (2007).

Measurements of Whole-Plant Transpiration Rate

The calculation of whole-plant transpiration rate was based on the plant's rate of weight loss. The examined plants were planted in 3.9-L pots. Each pot was placed on a temperature-compensated load cell with digital output. To monitor the temporary variation in water demand in the greenhouse, we used a vertical wet wick, made of 0.14-m^2 cotton fibers, that was partially submerged in a 1-L water tank. The wick system was located on a load cell. Evaporation from the growth medium surface was prevented by covering the pot surface with aluminum foil. Each pot was immersed in a nontransparent plastic container (13 × 21.5 × 31.5 cm [height, width, length]) through a hole in its upper cover. The container was sealed to prevent evaporation. The load cell output was monitored every 10 s, and the average readings over 3 min were logged in a data logger for further analysis. The whole-plant transpiration rate was calculated by a numerical derivative of the load cell output after a data-smoothing process. The plant daily transpiration rate was normalized to the total leaf area (measure by LI-COR area meter model Li-3100) and the neighboring submerged wick (i.e. mean daily evaporation was averaged for a given treatment over all plants; wick daily amount = 100%).

The plants were fertigated once a day by adding a commercial fertilizer solution to the container. The salinity stress consisted of a solution of 100 mM NaCl to which the normal dosage of nutrients was added. The salinity treatment was applied for 3 consecutive d. Normal irrigation was resumed at the end of the stress treatment to examine the plant recovery patterns.

Stomatal Aperture and Density

Abaxial leaf stomata were imprinted on glass as detailed by Geisler and Sack (2002). All samples were collected at around 11:00 AM (at peak transpiration). Counting and photographing were performed with a bright-field inverted microscope (1M7100; Zeiss) mounted with a Hitachi HV-D30 CCD camera. Stomatal images were later analyzed to determine aperture using the ImageJ software (http://rsb.info.nih.gov/ij/) area measurement tool. A microscopic ruler (Olympus) was used for the size calibration.

Grafting Experiment

Seedlings from three independent T2 transgenic TOM-NtAQP1 plants (2 weeks after germination) were used as scions on control rootstocks and vice versa. As controls, we used both TOM-NtAQP1 plants and control plants grafted on themselves. Two weeks after grafting, the plants were transplanted to 3.9-L pots and grown in a semicontrolled greenhouse, cooled by a compact evaporative air-cooling unit that allows lowering the temperature of the air passing through the evaporative pad (approximately 30°C). Plants (8 weeks old) were exposed to a series of measurements including whole-plant transpiration rate and gas exchange (leaf temperature was approximately 30°C, ambient temperature) as described above.

L_p

Two independent T2 transgenic TOM-NtAQP1 plants and control plants were used. On the night before the experiment, the main stem was cut with a razor 5 cm aboveground and the stump was sleeved with a silicone tube with an air-tight seal. The plants were then irrigated with fresh nutrient solution until drainage. The next morning (8:00 AM), the plants were irrigated again and a vacuum pump (RK 400; Today's Instruments) was connected to the sleeve via a custom-made liquid trap (for details, contact R. Seligmann) and vacuum was adjusted to a suction of 80 kPa. The first 15 min of exuded sap was discarded, and thereafter the sap was collected every 30 min. After 3 h, excess salt solution was added (50 mM NaCl), and sap collection was continued for another 3 h. Sap fractions were weighed, and plant discharge was calculated. Samples were then stored in the cold (5°C) for later mineral element analysis. Stem cross-sections (250 μm) were taken using a vibratom (VT1000S; Leica; http://www.leica.com) and photographed with a bright-field inverted microscope (Olympus-IX8 Cell-R) mounted with an Orca-AG CCD camera (Hamamatsu). Image analysis of the sections was used to determine the area of the xylem elements using the ImageJ software area measurement tool. A microscopic ruler (Olympus) was used for the size calibration. Sap discharge was normalized to flux (average velocity) based on conductive area.

L_p was calculated using the general flow equation and accounted for both hydrostatic and osmotic pressure gradients (Joly, 1989). The osmotic component included only sodium concentrations, as the osmotic component calculated for the other cations in the sap was relatively small; therefore, it was neglected. The reflection coefficient of the entire root system was assumed to be 0.5 (based on Steudle, 2001).

Sap Ion Concentration

Inductively coupled plasma mass spectrometry analysis (ARCOS-SOP; Spectro Analytical Instruments) was used to determine Na^+ sap concentration, and Na^+ uptake rate was quantified by multiplying Na^+ concentration by sap discharge.

Statistical Analysis

Statistically significant differences between plants with altered NtAQP1 expression and their respective controls were analyzed using Student's *t* test for comparison of the means. In all cases, the level of significance was set at $P < 0.05$. *P* value was computed using Fisher's exact test statistic. All of the results, unless otherwise specified, are presented as means ± SE. Root L_p and sap ion concentration results were grouped into pre and post salt treatment and analyzed for significance using the EMS procedure followed by Student's *t* test with $\alpha = 0.05$ (JMP; SAS Institute).

Sequence data from this article can be found in GenBank data libraries under accession number AJ001416 (*NtAQP1*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Screening of tomato plants that regenerated following cocultivation of explants with *Agrobacterium*.

Supplemental Figure S2. The response of A_N to substomatal C_i in control and TOM-NtAQP1 transgenic plants.

Supplemental Figure S3. Root system sap exudate discharge, measured from detopped plants under vacuum, before and after application of 50 mM NaCl.

Supplemental Figure S4. Screening of Arabidopsis plants following flower cocultivation with *Agrobacterium*.

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