Improving plant stress tolerance and yield production: is the tonoplast aquaporin SlTIP2;2 a key to isohydric to anisohydric conversion?

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Summary

• Anisohydric plants are thought to be more drought tolerant than isohydric plants. However, the molecular mechanism determining whether the plant water potential during the day remains constant or not regardless of the evaporative demand (isohydric vs anisohydric plant) is not known.

• Here, it was hypothesized that aquaporins take part in this molecular mechanism determining the plant isohydric threshold. Using computational mining a key tonoplast aquaporin, tonoplast intrinsic protein 2;2 (SlTIP2;2), was selected within the large multifunctional gene family of tomato (*Solanum lycopersicum*) aquaporins based on its induction in response to abiotic stresses. *SlTIP2;2*-transformed plants (TOM-SlTIP2;2) were compared with controls in physiological assays at cellular and whole-plant levels.

• Constitutive expression of *SlTIP2;2* increased the osmotic water permeability of the cell and whole-plant transpiration. Under drought, these plants transpired more and for longer periods than control plants, reaching a lower relative water content, a behavior characterizing anisohydric plants. In 3-yr consecutive commercial glasshouse trials, TOM-SlTIP2;2 showed significant increases in fruit yield, harvest index and plant mass relative to the control under both normal and water-stress conditions.

• In conclusion, it is proposed that the regulation mechanism controlling tonoplast water permeability might have a role in determining the whole-plant ishohydric threshold, and thus its abiotic stress tolerance.

Introduction

The global shortage of fresh water is one of the most severe agricultural problems affecting plant growth and crop yield (Toenniessen *et al.*, 2003). Studies in recent years have identified a large number of genetic and molecular networks underlying plant adaptation to adverse environmental growth conditions (Sreenivasulu *et al.*, 2007). All of these studies emphasize the complexity of the different traits and their polygenic nature. The current notion is that the defense mechanisms of plants against stress conditions are tightly associated with their growth habits, and hence every claim of tolerance enhancement needs to be tested on a crop-yield basis, coupled with its economic significance from an agricultural point of view. However, despite the large number of attempts to improve the abiotic stress tolerance of commercial crop plants through genetic engineering, no major progress has been made (Flowers, 2004; Passioura, 2007).

A number of previous studies have reported the regulatory role of aquaporins (AQPs) in cellular water transport

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(Chrispeels & Maurel, 1994; Knepper, 1994; Heymann & Engel, 1999; Aharon *et al.*, 2003). AQPs are considered to be the main channels for the transport of water, as well as small neutral solutes and CO_2, through the plant cell membrane (Tyerman *et al.*, 2002; Uehlein *et al.*, 2003). The total number of AQPs in plants, as compared with animals, appears to be surprisingly high, with 35 *AQP* genes identified in the Arabidopsis genome (Johanson *et al.*, 2001; Quigley *et al.*, 2002; Boursiac *et al.*, 2005), 36 in maize (*Zea mays*) (Chaumont *et al.*, 2001) and 33 in rice (*Oryza sativa*) (Sakurai *et al.*, 2005). The expression of *AQP* genes in plants is differentially regulated not only in various tissues, but also under different physiological states and different environmental conditions (Alexandersson *et al.*, 2005; Boursiac *et al.*, 2005).

In spite of their role in controlling cellular water permeability, AQPs have never been mentioned, to the best of our knowledge, in the context of partitioning plants between isohydric and anisohydric groups. Isohydric plants differ from anisohydric plants: Isohydric plants reduce leaf water potential as soil water potential decreases and the air vapour pressure deficit increases at the start of a day, reaching a constant leaf water potential during midday, which is similar in both well irrigated and droughted plants, at least for a certain eriod. Anisohydric plants, on the other hand, markedly decrease their water potential following the evaporative demand during the day, permitting a lower leaf water potential in droughted relative to well-watered plants (Tardieu & Simonneau, 1998). Anisohydric plants are considered more tolerant to drought than isohydric plants (McDowell *et al.*, 2008).

Although AQPs are considered to play a key regulatory role in water transport, attempts to utilize them to improve crop tolerance to abiotic stresses have yielded contradictory results. Lian *et al.* (2004) showed that overexpression of the *AQP RWC3*, a member of the rice plasma-membrane intrinsic protein 1 (*PIP1*) subfamily, in transgenic rice plants improves the general water status of the plants under water deficit, whereas overexpression of an Arabidopsis *PIP1b* in transgenic tobacco (*Nicotiana tabacum*) plants had no beneficial effects under salt stress and even had a negative effect during water stress (Aharon *et al.*, 2003). Recently, overexpression of *Ginseng* (*Panax ginseng*) tonoplast intrinsic protein 1 (*PgTIP1*) in transgenic Arabidopsis plants has been shown to significantly enhance seed size and mass, as well as increase growth rate and salinity tolerance (Lin *et al.*, 2007; Peng *et al.*, 2007). Thus, the overexpression of different individual *AQP* genes might have opposite outcomes with respect to the response of the whole plant to abiotic stresses, emphasizing the importance of accurately selecting the right candidate *AQP* genes from this large and functionally variable family to improve plant response to various stresses. In this study, by using a biologically driven computational approach, we identified a candidate *AQP* gene member, out of the family of 37 *AQP* genes found in tomato (*Solanum lycopersicum*), which improved plant tolerance to water stress in terms of growth and other yield-related parameters. In contrast to previous studies in which PIPs were mainly selected as candidates for the improvement of plant abiotic stress tolerance, apparently on the basis of educated guesses, we selected a tonoplast-localized *TIP* gene isoform named *SlTIP2;2* using an effective computational program. We demonstrated that plants overexpressing *SlTIP2;2* adjusted their whole-plant transpiration regulation and relative water content under different conditions. These findings, in addition to the fact that *SlTIP2;2*-transformed plants (TOM-SlTIP2;2) showed significant increases in fruit yield, support the hypothesis that the constitutive expression of *SlTIP2;2* might convert tomato plants from isohydric growth behavior to drought-tolerant anisohydric growth behavior.

Materials and Methods

Database assembly and mining

The bioinformatics filtering approach used for gene discovery and data-mining consisted of three phases. (1) Clustering and assembly: expressed sequence tags (ESTs) and mRNA sequences of the different plant species were extracted from GenBank, clustered and assembled using Compugen's LEADS clustering and assembly platform (Compugen, Rockville, MA, USA) (Yelin *et al.*, 2003). Automatically extracted EST library annotations were manually curated and classified by anatomy, developmental stage, abiotic stress treatments and cultivars. The results were loaded into the Oracle database. The predicted proteins were then annotated using INTERPRO(2) (http://www.ebi.ac.uk/interpro/). (2) All clusters from tomato that contained the major intrinsic protein domain (IPR000425) were selected for further analysis ($n = 37$). (3) Via a digital-expression approach, we obtained the expression profile of all clusters in terms of plant anatomy (the tissues/organs in which the gene was expressed), developmental stage (the developmental stage at which the gene could be found) and treatment profile (the physiological conditions under which a gene was expressed, such as drought, cold and pathogen infection). Digital-expression computations were performed as follows: fold overexpression was computed as $m/(n[M/N])$, where N is the total number of ESTs of a specific organism, M is the number of ESTs in a given library/tissue/category, n is the total number of ESTs in a given contig, and m is the number of ESTs from the library/tissue/category in the contig; the P-value was computed using Fisher's exact test statistic.

Phylogenetic analysis and AQP naming

The open reading frames (ORFs) of all 37 tomato *AQP* clusters were translated into amino acid sequences (using the EXPASy translation tool; http://www.expasy.ch) which were aligned together with the 35 AQPs of Arabidopsis (TIPs, PIPs, nodulin26-like intrinsic proteins [NIPs] and small and basic intrinsic proteins [SIPs] from SwissProt [http://www.expasy.ch/

sprot/], using ClustalX 1.8 [ftp://ftp.ebi.ac.uk/pub/software/clustalw2]). The tomato AQP names were given based on similarity to known Arabidopsis AQPs. Drawing and analysis of the phylogenetic tree were accomplished using TreeIllustrator software (http://nexus.ugent.be/geert/).

Generation of transgenic Arabidopsis and tomato plants

To construct the plasmid for plant transformation, *SlTIP2;2* cDNA was cloned under the regulation of the EVO205 promoter. The EVO205 promoter is a proprietary promoter (IP No. WO2004/081173) that is constitutively expressed in all organs, and strongly expressed in seeds, cork and xylem, as can be inferred from the expression analysis of the downstream-encoded gene (AT3G14230) described in the Arabidopsis eFP Browser (http://bbc.botany.utoronto.ca/efp/cgi-bin/). *SlTIP2;2* cDNA was isolated from tomato (*Solanum lycopersicum* L.) seedlings and subcloned into the pBluescript II KS vector (Fermentas, Glen Burnie, MA, USA) between *Bam*HI and *Sac*I restriction sites. Subcloning was followed by transfer of the *SlTIP2;2* ORF from the pBluescript II KS vector using the same restriction enzymes into the pPI vector. The pPI vector is a modified pBI101.3 plasmid, with a polyA signal inserted after the Nopaline synthase (NOS) terminator of the Neomycin phosphotransferase (NPTII) cassette. The EVO205 promoter was inserted into the pPI*SlTIP2;2* vector before the *SlTIP2;2* ORF using *Hin*dIII and *Sal*I restriction sites. Arabidopsis plants were genetically transformed using the floral-dip transformation method (Clough & Bent, 1998). Miniature tomato lines (MicroTom) were genetically transformed using disarmed *Agrobacterium tumefaciens* transformation methods. Transgenic MicroTom tomato plants overexpressing the *SlTIP2;2* gene were cross-pollinated with plants of the commercial variety M82. Transgenic MicroTom lines representing four independent transgenic insertion events (randomly selected from approximately six events) were used as female parents. The resulting F_1 hybrids were utilized for further field trial evaluation. For this purpose, the segregating F_1 populations were divided into two isogenic populations: one consisting of plants overexpressing the *SlTIP2;2* gene and the other of nontransgenic plants (null segregants). Null segregants were further used as negative controls. PCR screening was used to distinguish between transgenic and nontransgenic plants.

Arabidopsis seedling salt-stress assay

Seeds of transgenic Arabidopsis plants harboring the *EVO205::SlTIP2;2* gene or *35S::GUS* gene (used as a negative control) were sown in half-strength Murashige and Skoog (MS) medium containing 40 mg l^{-1} kanamycin for selection. Selected seedlings were subcultured in half-strength MS agar sterile medium containing 0 or 150 mM NaCl. Plants were grown for a period of 3 wk. Results are the average for four independent transgenic lines that were analyzed in four repeated experiments. For the determination of shoot dry weight, the shoots of plants were collected and dried for 24 h at 60°C and then weighed.

Salt-stress field trial

All field trials were performed in a light soil, in an open field (net-house) near Rehovot, Israel. The F_1 hybrids of four independent transgenic lines of the cross between *SlTIP2;2*-transgenic MicroTom plants and M82 plants were grown for the first 3 wk in a nursery under normal irrigation conditions. The seedlings were then transplanted into rows and grown in a commercial net-house. The salt-stress trial was divided into four blocks. In each block, two different irrigation systems were established: a normal water regime for tomato cultivation and a regime of continuous irrigation with saline water (addition of 180–200 mM NaCl). Each block consisted of a total of 60 plants divided as follows: six plants per insertion event and six seedling null segregants were planted in the control row and a similar number of plants were planted in the salt-stressed row. At the stage of *c*. 80% red fruits *in planta*, fruit yield, plant fresh weight, and harvest index were calculated. Harvest index was calculated as yield/plant biomass.

Water deficiency stress field trial

All field trials were performed in a light soil, in an open field (net-house) near Rehovot, Israel. The F_1 hybrids of the four independent transgenic lines were initially grown as described above. Three-week-old seedlings were transplanted into a net-glasshouse. The experiment was structured in four blocks containing three rows irrigated with different amounts of water and at different intervals (WLI-0, WLI-1, WLI-2; see below). In each block, six transgenic plants per event analyzed and six nontransgenic plants were transplanted into each row. Seedlings were transplanted after 4 wk. The amount of water used to uniformly irrigate before transplanting reached maximum water capacity (20% w/w) at a depth of 60 cm, but without the creation of water overload. Each plant was transplanted near a dripper, with a 30-cm distance between plants according to a commercial growth protocol. Soil water capacity was measured using the standard procedures by sampling soil from the following three depths: 0–20, 20–40, and 40–60 cm. The water content in these soil layers was routinely measured every week. The soil contained 5% hygroscopic water while the maximum water capacity of the soil was 20%. All fertilizers were applied to the soil before plant transplantation. The amount of both phosphorus and potassium was calculated to be sufficient for the entire seasons. Nitrogen was applied as recommended, equally to all treatments, through the irrigation system. Each row contained three dripping irrigation lines creating coverage of nine drippers per 1 m^2. The different water regimes were begun only 4 wk after transplanting when plants initiated the flowering stage. The

amount of water supplied every week during the assay was calculated at the beginning of every week following the recommendations of standard growth protocols. The WLI-0 treatment (control) received the recommended total weekly irrigation volume divided into three irrigations. In the WLI-1 treatment, irrigation was performed three times a week, but the amount of water supplied was half that supplied in WLI-0. At the end of every week, WLI-1 plants received the amount of water required to reach maximum soil water capacity. WLI-2 plants were irrigated only once a week until soil saturation was achieved. The water-stress experiment lasted throughout the flowering period (23 d), corresponding to four cycles of the above-described stresses. Afterwards, all treatments received the recommended amount of water. The calculated water amount was equal to the difference between the water contents in dry soil and in soil with maximum water capacity. At the end of each stress cycle, the water amounts were compared between treatments according to the actual water content in the soil (S3).

Transient expression and subcellular localization of SlTIP2;2 in Arabidopsis protoplasts

Protoplasts were isolated from Arabidopsis leaf mesophyll and the chimer *GFP::SlTIP2;2* was transiently expressed using the polyethylene glycol (PEG) transformation method (Locatelli *et al.*, 2003). Protoplasts transiently expressing cytosolic GFP were used as a control. Imaging of GFP was performed using a fully motorized epifluorescence inverted microscope (Olympus-IX8 Cell-R; Olympus, Tokyo, Japan) with the following features: objective lens, plan apochromat, ×60, oil immersion, and a numerical aperture of 1.42. The CCD camera used was a 12-bit Orca-AG (Hamamatsu, Hamamatsu, Japan). The filter sets were GFP-3035B-000 and TXRED-4040B, with zero pixel shift (Semrock, Rochester, NY, USA). All images were processed using Olympus imaging software Cell-R for Windows.

Osmotic water permeability coefficient (P_f) measurements

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 using a numerical approach (an off-line curve-fitting procedure using several algorithms), which has been shown 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 previously (Moshelion *et al.*, 2002, 2004; Volkov *et al.*, 2007).

Whole-plant transpiration measurements

To measure whole-plant transpiration and transpiration rate, plants from two independent transgenic lines of the F_1 hybrid cross between transgenic TOM-SlTIP2;2 plants were grown in 3.9-l pots. Pots were placed on electronic temperature-compensated load cells, located in a controlled-environment glasshouse. A vertical wet wick consisting of 0.7-m^2 cotton fibers (partly submerged in a 1-l water tank) was placed on a similar load cell. Its weight variation was used as a reference for the temporal variations in potential transpiration rate. The load cell readings, taken every 10 s and averaged over 3-min periods, were recorded by data logger. The daily transpiration rates were normalized to the total leaf area of the plant (Li-Cor area meter, model Li 3100; Li-Cor, Lincoln, NE, USA) and the mean daily evaporation of the neighboring wet wick and were averaged for a given treatment over all plants (wick daily amount = 100%). Evaporation from the growth medium was prevented by covering the pots with aluminum foil. Each pot was immersed in an opaque plastic container (height × width × length $13 \times 21.5 \times 31.5$ cm) through a hole in its upper cover. The plants were fertigated once a day by adding a commercial fertilizer solution to the container. Two stress treatments were applied to the transgenic and control plants: salinity and water deficiency. The salinity stress included a solution of 100 mM NaCl to which the nutrients were added at their normal doses. The higher salinity was maintained for 3 d. The water deficiency treatment consisted of eliminating irrigations until the plant showed significant turgor loss. Normal irrigation was resumed at the end of the stress treatments to examine the patterns of plant recovery. For the salinity and water deficiency stress assays, plants were irrigated again and the recovery rate was also recorded. Plant transpiration and wick evaporation rates were calculated from the first derivative of the measured weight–time series.

Measurement of stomatal pore area

Stomatal pore area was measured in the peeled tomato epidermis from two independent transgenic lines of TOM-SlTIP2;2 plants harboring the *SlTIP2;2* gene. The peeled epidermis was floated on a solution of 50 mM KCl, 0.01 mM $CaCl_2$ and 10 mM MES (2-N-Morpholino ethanesulfonic acid), pH 6.5, at 25°C and a light intensity of 235 μmol m^{-2} s^{-1} for 2 h before the measurements. Counting was performed, and photographs taken, using a bright-field inverted microscope (Zeiss 1M7100; Zeiss, Jena, Germany) mounted with a Hitachi (Hitachi, Japan) 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 microscope ruler (Olympus) was used for the size calibration.

Relative water content

Leaf relative water content was measured in control and TOM-SlTIP2;2 plants. Fresh weight (FW) was immediately recorded; leaves were then soaked for 8 h in distilled water at room temperature in the dark, and the turgid weight (TW) was recorded.

Total dry weight (DW) was recorded after drying these leaves at 70°C to a constant weight. Relative water content (RWC) was calculated as (FW − DW)/(TW − DW) × 100.

Ion content analysis

TOM-SlTIP2;2 tomato plants and nontransgenic tomato plants were grown in hydroponics tanks (0.5 mM Hoagland medium completed to 10 mM $CaCl_2$) for 10 d before treatment with NaCl, which was gradually added (on days 1 to 3 of the experiment) to a final concentration of 175 mM. After day 5 of the experiment all plants were washed once with 20 mM $CaCl_2$ and twice with double-distilled water. Leaves and roots were separately collected and dried at 65°C for 3 d. Samples were then ground to a fine powder and 0.1 g was digested with 14.6 N nitric acid for 4 h at 120°C. Na^+ and K^+ contents were determined using a Flame Photometer (410 Corning; Corning Science Products, Essex, UK) according to the manufacturer's instructions.

Root osmolarity measurement

TOM-SlTIP2;2 and control tomato roots treated as described in the previous sections were collected and frozen (−80°C for 5 h). The roots were thawed and centrifuged in a 1.5-ml tube to extract the sap. The osmolarity of the samples was measured using an osmometer (OsMomat 030; Gonotec, Berlin, Germany) according to the manufacturer's instructions.

Statistical analysis

Field trials statistical analysis, performed in four repeats per each independent transgenic line and six repeats per control line. Student's *t*-test was used for comparison of means, which were deemed significantly different at $P < 0.05$.

Results

Transcriptome identification of tomato AQPs

The contradictory reports regarding the contributions of different plant AQP isoforms to the resistance of plants to abiotic stresses, together with the large size of this family and its diverse functions in regulating plant water homeostasis, suggest that the AQP family may be a good model for selection of suitable candidate genes to improve stress-associated traits in crop plants. We have chosen to focus on AQPs from tomato plants for several reasons: (1) the relatively high tolerance of this crop species to salt stress; (2) the availability of well-defined yield parameters for this species; and (3) the availability of relevant genomic databases. In this study, we analyzed the transcript expression levels of different tomato *AQP* genes by digital gene expression analysis (also known as electronic northern blot).

By mining the database of tomato *AQP* gene sequences, we identified 37 different *AQP* genes (see Supporting Information Table S1). To the best of our knowledge, this is the largest *AQP* gene family detected in any single species tested to date. A comprehensive phylogenetic analysis was conducted to establish groups of homology within the tomato *AQP* gene family. As shown in Fig. 1, the 37 AQPs were classified into 18 PIP, nine TIP, six NIP, and three SIP isoforms. We also identified a new AQP with high similarity to the novel plant AQP subfamily XPIP recently reported in poplar (*Populus*) (Danielson & Johanson, 2008), and named it *Solanum lycopersicum* XIP (SlXIP; Fig. 1).

Next, we used our computational approach to select *AQP* genes that are overrepresented under various abiotic stresses, such as nutrient deficiency, heat, salinity and heavy metal stresses, as well as biotic stresses, such as application of elicitors and pathogens, taking into consideration the possibility of a large overlap in the expression patterns of *AQP* genes under many abiotic and biotic stresses. As shown in Table 1, of the *AQP* genes analyzed, *SlTIP2;2* was the only one that was highly overexpressed in roots (6-fold; $P \leq 1.01$ E-24) as well as under both biotic (2-fold, $P \leq 4.6$ E-02) and abiotic (3.6-fold, $P \leq 4.49$E-04) stresses. Thus, we chose to test in field trials the effect of overexpression of this tonoplast *AQP* gene isoform on stress tolerance and yield parameters under favorable growth conditions and under salt and drought stresses.

Transgenic tomato plants expressing *SlTIP2;2* show improved tolerance to salt and water stresses under field conditions

The coding DNA sequence of *SlTIP2;2* was fused to a constitutive proprietary promoter (EVO205) and to 3′ terminator (see the Materials and Methods) and the *EVO205::SlTIP2;2* construct was transformed into tomato plants. Four independent F_1 transgenic tomato genotypes overexpressing *SlTIP2;2* in the heterozygous form (TOM-SlTIP2;2 plants) were evaluated for their tolerance to water deficiency (a single field trial including two different water-limiting regimes) and salt stress (two different field trials) with respect to plant vigor and yield parameters. In each field trial, the transgenic genotypes were compared with their segregated counterparts exhibiting no antibiotic resistance as controls. In both salt-stress field trials, performed in the autumn of 2004 (see Table S2) and the summer of 2005 (Fig. 2), the transgenic and control tomato plants were continuously irrigated with water containing 180–200 mM NaCl. TOM-SlTIP2;2 plants appeared on average to be more vigorous than the control plants in both field trials, as indicated by their big size and less severe symptoms of leaf and shoot necrosis (Fig. 2j), and also accumulated higher fruit yield than the controls (Fig. 2a). Under water deficiency stresses, TOM-SlTIP2;2 plants developed significantly higher (26%) plant biomass than the control plants (Fig. 2) and this was also associated with a

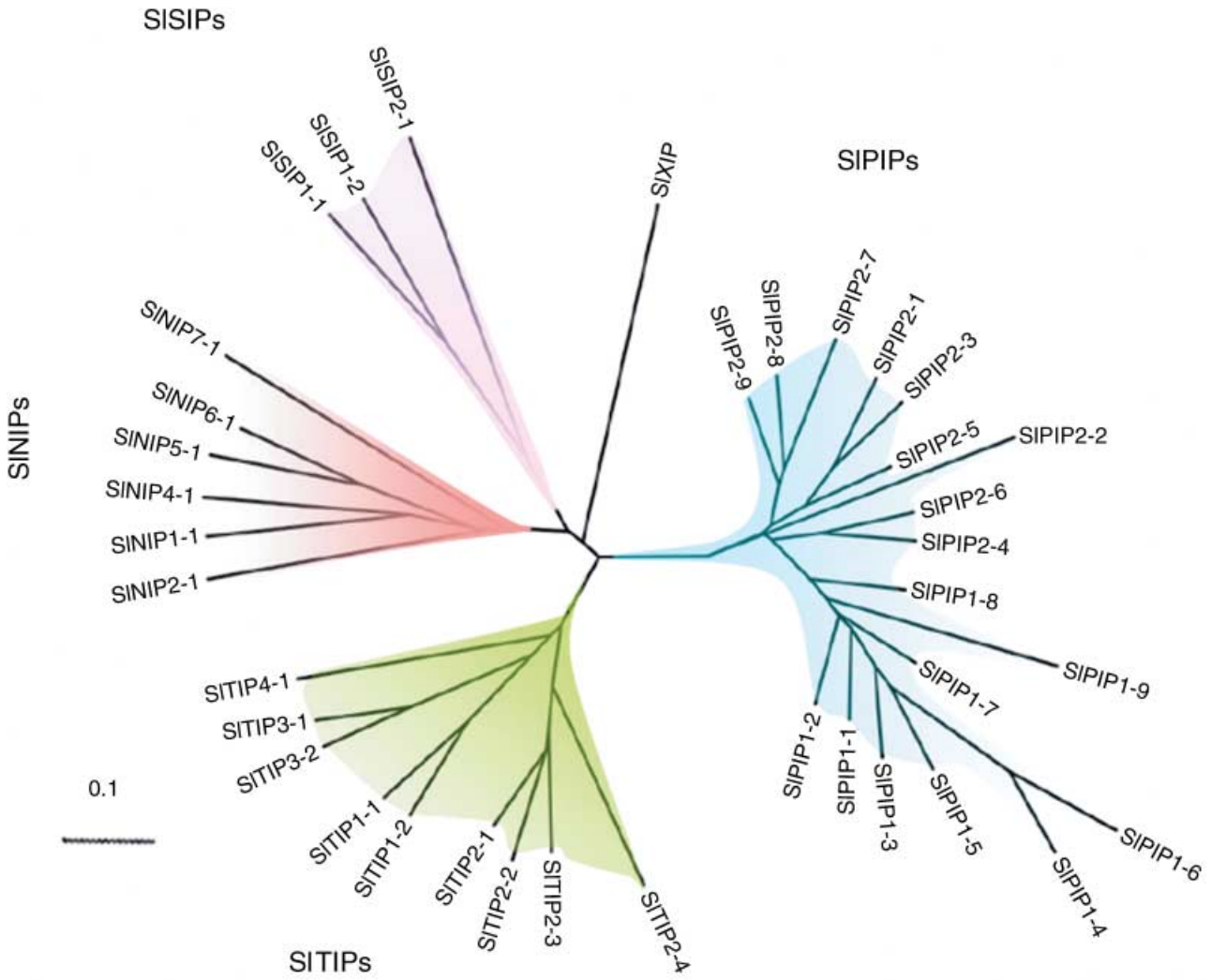

Fig. 1 Phylogenetic analysis of 37 tomato (*Solanum lycopersicum*) aquaporin proteins. The bar length is proportional to the number of amino acid substitutions (accession numbers are shown in Supporting Information Table S1).

significant (up to 21%) increment of fruit yield under relatively mild (WLI-1) and more severe (WLI-2) regimens of water deficiency stress (see details in the Materials and Methods). In addition, the harvest index of the TOM-SlTIP2;2 plants was significantly higher (20%) than that of the control under mild water stress (WLI-1) and remained similar to that of control plants under more severe drought stress (WLI-2; Fig. 2f).

All in all, the results from the three field trials suggest that overexpression of *SlTIP2;2* improves yield parameters when compared with the control, under both favorable growth conditions and exposure to salt and drought stresses.

A complementary experiment with transgenic Arabidopsis plants expressing the *SlTIP2;2* construct showed increased tolerance to a salt stress of 150 mM NaCl compared with control plants, as reflected in 42% higher fresh biomass and 60% higher dry biomass (Table S3).

Subcellular localization of SlTIP2;2

Based on its amino acid sequence, SlTIP2;2 is predicted to be a tonoplast AQP. We thus wished to confirm this prediction by transient expression of a chimeric GFP::SlTIP2;2 fusion protein in Arabidopsis mesophyll protoplasts. Pinpointing the location of any tonoplast protein is a tricky task as the vacuole takes up > 90% of the cell volume, is surrounded by many organelles and is often touching the plasma membrane (PM). Our approach here was to use the chloroplasts as a cytoplasmic 'data-point' separating the PM from the tonoplast. The relatively large chloroplasts which were very densely packed in the cytoplasm (Fig. 3a) and emitted red autofluorescence (Fig. 3b) were surrounded by cytosolic GFP (Fig. 3c), as expected, and located above the green fluorescent signal coming from the GFP::SlTIP2;2 (Fig. 3d), thus strongly indicating its vacuolar localization. In addition, we extracted

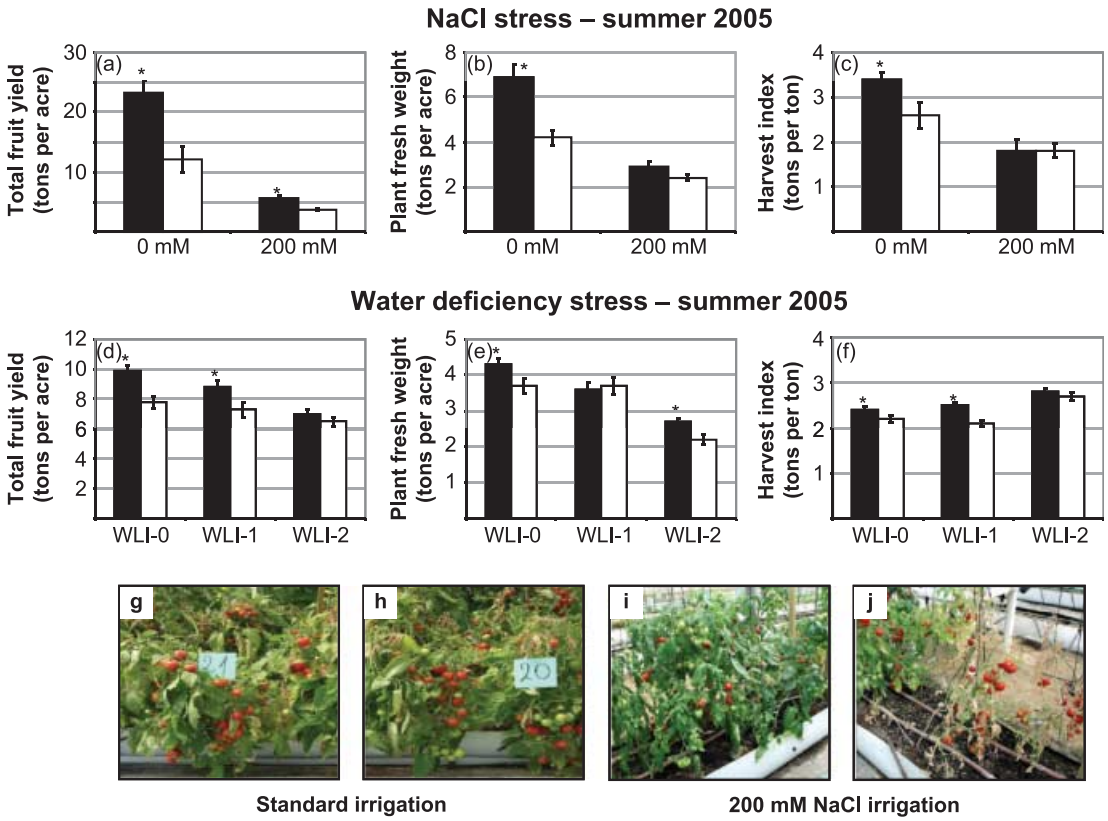

Fig. 2 Total economic fruit yield, plant biomass and harvest index for *Solanum lycopersicum* tonoplast intrinsic protein 2;2 (*SlTIP2;2*)-transformed (TOM-SlTIP2;2) (black bars) vs control (white bars) plants growing in a commercial glasshouse under a 200 mM NaCl irrigation regime (a–c, respectively), or under two different water stress regimes (WLI-1 and WLI-2; compared with the standard irrigation regime (WLI-0); d–f, respectively). (g) TOM-SlTIP2;2 plants and (h) control plants growing under standard irrigation conditions. (i) TOM-SlTIP2;2 plants and (j) control plants after growth under a 200 mM NaCl irrigation regime during the entire growth season. Results represent the average of the four independent transgenic lines (analyzed in four repeats) ± SE. *Significantly different at $P \leq 0.05$.

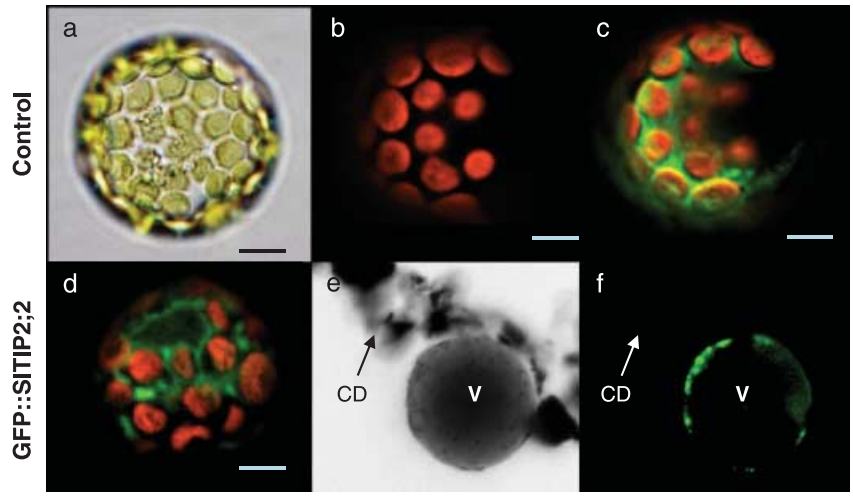

Fig. 3 Epifluorescent images of a transient expression GFP::SlTIP2;2 fusion protein (GFP, green fluorescent protein; SlTIP2;2, *Solanum lycopersicum* tonoplast intrinsic protein 2;2) and control GFP in Arabidopsis mesophyll protoplasts. (a) Bright field image of the protoplast. (b) Chloroplast autofluorescence in a nonexpressing protoplast under 488 nm excitation and 650 nm emission. (c) Image of GFP overexpression (excitation 488 nm; emission 520 nm) superimposed on its autofluorescence image; note the expected signal from the diffuse cytosolic GFP. (d) Image of GFP::SlTIP2;2 overexpression (excitation 488 nm; emission 520 nm) superimposed on its autofluorescence image. (e) An intact vacuole (V), loaded with neutral red dye, surrounded by cytoplasmic debris (CD) after short mechanical pressure, and (f) GFP fluorescence localized to the tonoplast; note that no GFP was detected at the plasma membrane. Bar, 10 mm.

Table 1 Aquaporin clusters overrepresented (≥ 2-fold increment and *P*-value ≥ 0.05) in roots and under different abiotic stresses (nutrient deficiency, heat, salinity and heavy metal stresses) and biotic stresses (elicitors and pathogens)

Clusters overrepresented in	Aquaporin	Fold*	*P*-value
Roots	**SlTIP2.2**	**6.01**	**1.01E-24**
	SlPIP2.6	4.63	8.14E-24
	SlTIP2.3	10.69	2.31E-12
	SlPIP2.3	5.76	3.21E-08
	SlPIP1.6	2.92	1.61E-07
Abiotic stresses	SlPIP2.3	6.00	2.06E-04
	SlTIP2.2	**3.64**	**4.49E-04**
	SlPIP2.2	5.08	1.10E-03
	SlPIP1.3	5.00	2.44E-03
	SlPIP2.8	4.00	6.15E-03
Biotic stresses	SlTIP1.1	3.51	1.40E-05
	SlPIP1.7	3.28	9.70E-03
	SlPIP1.6	2.05	3.30E-02
	SlTIP2.2	**2.01**	**4.60E-02**
	SlPIP1.3	2.69	6.00E-02

*Fold, increase in expression compared with the average of all organs or treatments.
Genes were ranked based on the calculated *P*-value.
Sl, *Solanum lycopersicum*; PIP, plasma-membrane intrinsic protein; TIP, tonoplast intrinsic protein.

the vacuoles of cells expressing GFP::SlTIP2;2 and detected the GFP-labeled AQP only in the tonoplast, while no traces of GFP were detectable in the cell debris (Fig. 3e,f).

SlTIP2;2 increases cell water permeability

Taking into account the major effect of overexpression of *SlTIP2;2* with respect to tomato yield parameters under normal growth conditions as well as drought and salt stresses, we were further interested to unravel the mechanism through which the SlTIP2;2 AQP exerts its cellular physiological effects. To address this issue, we measured the impact of SlTIP2;2 on the osmotic water permeability coefficient (P_f) of isolated plant cells, using Arabidopsis as a model system. Using a cell-swelling assay, we measured the P_f value of Arabidopsis mesophyll cells transiently expressing *GFP::SlTIP2;2*. Notably, the *SlTIP2;2*-expressing cells revealed significantly higher P_f values than control cells, indicating that SlTIP2;2 indeed functions as a water channel (Fig. 4). These results suggested a regulatory role of SlTIP2;2 in cellular water homeostasis regulation.

TOM-SlTIP2;2 plants revealed the anisohydric behavior of increasing their transpiration performance up until turgor loss

To further elucidate the impact of this regulatory function on the water balance of the whole plant, we measured the whole-plant transpiration rate under favorable growth conditions as

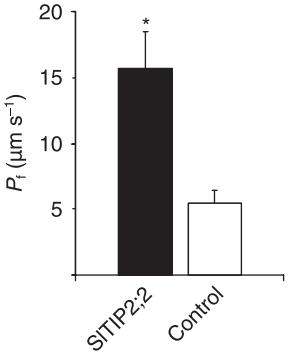

Fig. 4 The protoplast osmotic water permeability coefficient (P_f) (mean (± SE)) determined in Arabidopsis protoplasts transiently expressing GFP::SlTIP2;2 (GFP, green fluorescent protein; SlTIP2;2, *Solanum lycopersicum* tonoplast intrinsic protein 2;2) (n = 12) and in control protoplasts transiently expressing GFP (n = 11). *Statistically significant values; $P < 0.05$.

well as under salt and water stresses. Transpiration rates as well as relative transpiration of TOM-SlTIP2;2-transgenic plants were significantly higher than those of control plants under normal growth conditions (Fig. 5a,b), on the 1st and 3rd days after the application of 100 mM NaCl (Fig. 5c,d), and on the 1st day of recovery from this salt stress (Fig. 5e). Note that the plants returned to their pre-stress transpiration as soon as the salinity stress was removed (Fig. 5e). Despite the fact that transpiration levels of all plants were dramatically decreased by the water stress treatment, the relative transpiration level of TOM-SlTIP2;2 plants remained significantly higher than that of the control plants up to the point of leaf turgor loss (Fig. 5f), where it became similar to that of the control plants (Fig. 5g) and on recovery from the water stress was comparable to that of the control plants (Fig. 5h). Moreover, the significantly larger stomatal aperture in TOM-SlTIP2;2 plants vs control plants (Fig. S1) probably accounted for the higher transpiration of TOM-SlTIP2;2 plants. This higher and more prolonged water loss resulted in a lower relative water content of TOM-SlTIP2;2 plants compared with control plants subjected to drought stress (Fig. 6).

Discussion

The growing world-wide limitations on fresh water and high-quality soils are becoming two major humanitarian and economic factors that limit crop yield (Toenniessen *et al.*, 2003), motivating both conventional breeders and seed companies to maximize plant yield potentials under a wide range of suboptimal environmental conditions. Many abiotic stresses, such as soil salinity and drought, cause rapid decreases in plant hydraulic conductance (Steudle, 2000; Boursiac *et al.*, 2005), resulting in stomatal closure and thereby reducing transpiration. Unfortunately, this process also causes a reduction in photosynthesis, plant growth and yield. It has also been strongly

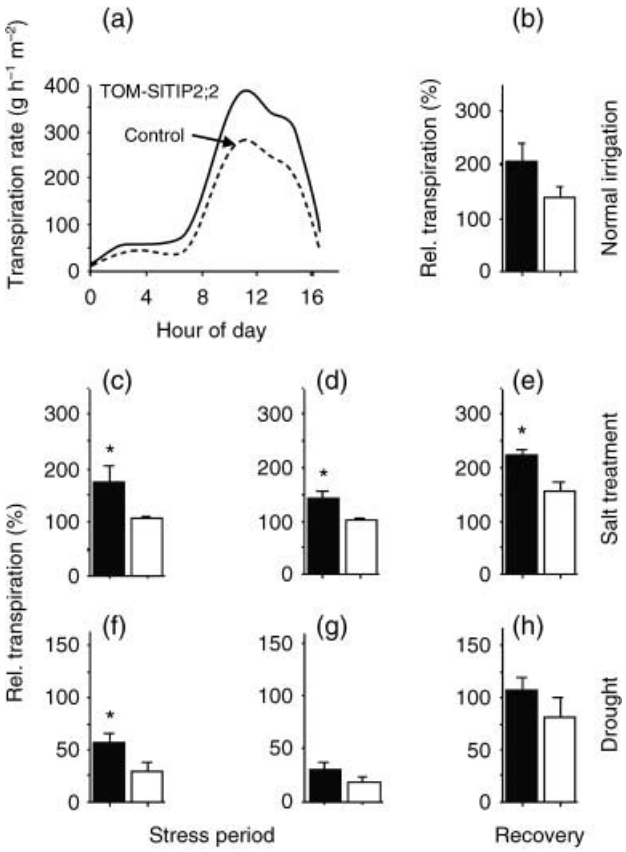

Fig. 5 Relative transpiration rate of *Solanum lycopersicum* tonoplast intrinsic protein 2;2 (*SlTIP2;2*)-transformed (TOM-SlTIP2;2) (black bars) vs control (white bars) plants grown in pots in a commercial glasshouse. (a) Daily transpiration rate of TOM-SlTIP2;2 and control plants under normal irrigation. (b) Normalized, mean daily transpiration under normal irrigation ($n = 12$ for TOM-SlTIP2;2; $n = 7$ for the control). (c, d) Normalized, mean daily transpiration on the 1st and 3rd days of salinity stress (100 mM NaCl, respectively) and (e) normalized, mean daily transpiration on the subsequent, recovery, day ($n = 6$ for TOM-SlTIP2;2; $n = 3$ for the control). (f, g) Normalized, mean daily transpiration on the day before and the day during which the plant lost turgor as a result of water deficiency stress. (h) Normalized, mean daily transpiration on the subsequent, recovery, day ($n = 6$ for TOM-SlTIP2;2; $n = 4$ for the control). Data represent the mean ± SE of two independent transgenic lines (the number of plants is indicated for each condition). *Statistically significant values; $P < 0.05$.

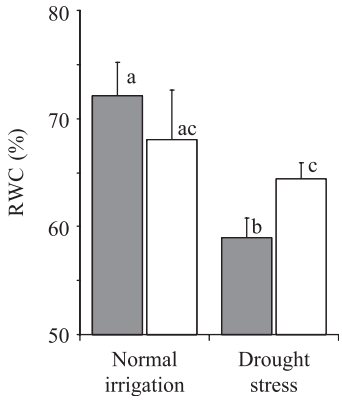

Fig. 6 Average relative water content (% RWC) of 8-wk-old tomato (*Solanum lycopersicum*) plants before and after 1 d of drought stress: open bars, control plants ($n = 4$); closed bars, plants of two independent *S. lycopersicum* tonoplast intrinsic protein 2;2 (*SlTIP2;2*)-transformed (TOM-SlTIP2;2) transgenic lines ($n = 4$). Data are given as mean ± SE. (a, b) Bars with different letters are significantly different at $P < 0.05$.

suggested that salt and drought stresses predominantly affect CO_2 diffusion in the leaves, via a decrease in stomatal and mesophyll conductance, rather than the biochemical CO_2-assimilation capacity (Flexas *et al.*, 2004). Anisohydric plants, which regulate this mechanism differently from isohydric plants, are considered to be more tolerant to drought than isohydric plants (McDowell *et al.*, 2008). In this study, we hypothesized that specific AQPs, which take part in plant water balance regulation, might also have a regulatory role in determining the isohydric behavior of plants, and hence could be used to convert an isohydric plant to a more tolerant anisohydric plant. For the selection of suitable AQP candidates to improve stress tolerance, because of the large size of this gene family, we used a computational mining process focusing on the expression patterns of different isoforms of the *AQP* gene family. In contrast to many previous trials to select suitable AQP candidates to improve plant drought and salt tolerance, our ability to select an AQP isoform that improved the tolerance of tomato plants to drought and salt stress under field conditions illustrates the strength of the computational system applied. In addition, our results show that manipulation of AQP function on the vacuolar tonoplast membrane is of central importance to improve water homeostasis as well as salt and drought tolerance under field conditions.

Our results suggest that SlTIP2;2 regulates cell water permeability, and probably does not participate in other putative stress resistance mechanisms, such as absorption of Na^+ ions into the vacuole or regulation of the plant osmoticum (Yamaguchi & Blumwald, 2005; see also Fig. S2 and Table S4).

Our results also show that *SlTIP2;2* expression has multiple functions, increasing transpiration under normal growth conditions, limiting the reduction in transpiration under drought and salt stresses, and also speeding up the revival of transpiration upon recovery from these stresses. Nevertheless, under prolonged dehydration stress, TOM-SlTIP2;2 plants did not lose their ability to significantly reduce transpiration. The characteristics attributable to overexpression of the *TIP* isoform *SlTIP2;2* do not appear to exist in many *PIP*-overexpressing plants. In fact, *PIP* overexpression seems to induce deleterious effects in response to water deficiency stress (Aharon *et al.*, 2003). The mechanism underlying the differential effects of *TIP* and *PIP* overexpression on salt and drought tolerance is

still unknown. However, it is probably related to the fact that the vacuole is by far the largest organelle in the adult plant cell, comprising up to 95% of its volume. The water permeability of the tonoplast is known to be much higher than that of the plasma membrane and is considered a means of improving the vacuole osmotic buffering capacity of the cytoplasm (Tyerman *et al.*, 1997; Morillon & Lassalles, 1999). Under stress conditions, tonoplast water permeability appears to be reduced (Vera-Estrella *et al.*, 2004).

In conclusion, we propose that overexpression of the tonoplast *AQP SlTIP2;2* can bypass the stress-induced down-regulation of the endogenous *AQP* genes of the tonoplast, and thus prevent the slowdown of tonoplast osmotic water permeability coefficient values j. The high water permeability of the tonoplast extends the capacity of the vacuole for osmotic buffering of the cytoplasm under stress conditions, allowing the leaf to attain a lower water potential (Fig. 6; see also Peng *et al.*, 2007) and preserving the water potential of the cytoplasm at a constant value. This constitutes, in fact, a conversion of the tomato plants from isohydric to anisohydric behavior. In other words, overexpression of the tonoplast *AQP* extends the ability of the plant to maintain relatively normal physiological functions and also growth and yield production – even under relatively severe stress conditions. Thus, our results support the hypothesis that 'isohydric and anisohydric water potential regulation may partition species between survival and mortality' (McDowell *et al.*, 2008). The benefit of maintaining a certain amount of transpiration during stress, as opposed to a complete shutdown of transpiration, ensures not only continuous CO_2 uptake, but also a continued supply of nutrients and a reduction in leaf temperature, promoting plant growth (Idso *et al.*, 1987). This offers a new approach for the application of biotechnology in various agricultural crops exposed to different environmental stresses.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Mean stomatal aperture of two *Solanum lycopersicum* tonoplast intrinsic protein 2;2 (*SlTIP2;2*)-transformed (TOM-SlTIP2;2) independent transgenic lines ($n = 44$) vs control plants ($n = 30$).

Fig. S2 K^+ and Na^+ contents measured in two *Solanum lycopersicum* tonoplast intrinsic protein 2;2 (*SlTIP2;2*)-transformed (TOM-SlTIP2;2) independent transgenic lines ($n = 4$) (upper and middle panels, respectively) showed no significant change from control plants ($n = 3$) both in roots and leaves when treated with NaCl (2 mM or 175 mM).

Table S1 List of the 37 aquaporins (AQPs) found in tomato (*Solanum lycopersicum*)

Table S2 The salt-stress field trial carried out in autumn 2004

Table S3 The Arabidopsis salt-stress assay

Table S4 The root osmolarity of two *Solanum lycopersicum* tonoplast intrinsic protein 2;2 (*SlTIP2;2*)-transformed (TOM-SlTIP2;2) independent transgenic lines and control tomato plants grown in normal hydroponic solution and in this growth medium after the addition of 175 mM NaCl

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