Relationship between Hexokinase and the Aquaporin PIP1 in the Regulation of Photosynthesis and Plant Growth

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Abstract
Increased expression of the aquaporin NtAQP1, which is known to function as a plasmalemma channel for CO₂ and water, increases the rate of both photosynthesis and transpiration. In contrast, increased expression of Arabidopsis hexokinase1 (AtHXK1), a dual-function enzyme that mediates sugar sensing, decreases the expression of photosynthetic genes and the rate of transpiration and inhibits growth. Here, we show that AtHXK1 also decreases root and stem hydraulic conductivity and leaf mesophyll CO₂ conductance (ĝm). Due to their opposite effects on plant development and physiology, we examined the relationship between NtAQP1 and AtHXK1 at the whole-plant level using transgenic tomato plants expressing both genes simultaneously. NtAQP1 significantly improved growth and increased the transpiration rates of AtHXK1-expressing plants. Reciprocal grafting experiments indicated that this complementation occurs when both genes are expressed simultaneously in the shoot. Yet, NtAQP1 had only a marginal effect on the hydraulic conductivity of the double-transgenic plants, suggesting that the complementary effect of NtAQP1 is unrelated to shoot water transport. Rather, NtAQP1 significantly increased leaf mesophyll CO₂ conductance and enhanced the rate of photosynthesis, suggesting that NtAQP1 facilitated the growth of the double-transgenic plants by enhancing mesophyll conductance of CO₂.

Introduction
Aquaporins (AQPs), also known as MIPs (major intrinsic proteins), are integral membrane proteins that increase the permeability of membranes to water, as well as small uncharged molecules [1]. Of all kingdoms, the plant kingdom contains the largest known AQP family consisting over 30 members [2,3]. There are 35 AQPs in Arabidopsis (Arabidopsis thaliana [4]), 36 in maize (Zea mays [1]) and 37 in tomato (Solanum lycopersicum [5]). Based on sequence similarities, AQPs have been divided into five subgroups: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs) and X intrinsic proteins (XIP) [4,6]. Plant PIPs can be divided into two major groups, PIP1 and PIP2, on the basis of their sequences and water-channel activity. PIP2 proteins exhibit high levels of water-channel activity in Xenopus oocytes and yeast vesicles; whereas PIP1 proteins often have relatively low permeability to water [7–12].

Evidence for the role of PIP1 aquaporin in planta has come from mutant analyses and the manipulation of PIP1 expression in plants. Analysis of Arabidopsis mutants has shown that AtPIP1,2 can account for a significant portion of aquaporin-mediated leaf water transport [13]. The antisense expression of AtPIP1,2 in Arabidopsis has been associated with reductions in the membrane hydraulic conductivity of isolated protoplasts and decreased total root hydraulic conductivity [14,15]. Antisense suppression of NtAQP1 (a member of the PIP1 subgroup) in tobacco (Nicotiana tabacum) lowered the level of expression of several PIP1 homologues and resulted in a significant decrease in protoplast membrane water permeability, reduced root hydraulic conductivity and decreased transpiration [16,17].

The results of heterologous expression in Xenopus oocytes suggest that, in addition to functioning as a water channel, NtAQP1 is also a membrane CO₂ pore that facilitates the transport of CO₂ across membranes [7,18]. The movement of CO₂ between the substomatal cavities and the sites of carboxylation within chloroplasts, through plasma and chloroplast membranes, is generally termed leaf mesophyll conductance (ĝm) [19]. The ability of NtAQP1 and its Arabidopsis homolog AtPIP1,2 to function as CO₂ membrane transport facilitators has been demonstrated in vivo experiments. Increased expression of NtAQP1 in tobacco plants enhanced CO₂ incorporation and stomatal conductance; whereas antisense suppression of NtAQP1 had the opposite effect [18]. In other
studies, overexpression of \textit{AtPIP1,2} or \textit{NtAQP1} in tobacco plants significantly enhanced the rates of growth, transpiration and photosynthesis [20–22]; whereas antisense suppression of \textit{NtAQP1} in tobacco plants and T-DNA insertion Arabidopsis mutants in \textit{AtPIP1,2} reduced \( \mu \)m and led to lower rates of photosynthesis [21,23,24].

Unlike \textit{NtAQP1}, overexpression of Arabidopsis hexokinase (\textit{AtHXK1}) in Arabidopsis and tomato plants decreased photosynthesis, transpiration, and growth [25,26]. \textit{AtHXK1} is a sugar-sensing enzyme that monitors glucose levels, most likely in mesophyll cells of photosynthetic tissues. When glucose levels are sufficiently high, this enzyme inhibits the expression of photosynthetic genes, decreases chlorophyll levels and reduces the rate of photosynthesis [25–29]. In addition, \textit{AtHXK1} also stimulates stomatal closure and decreases transpiration in response to photosynthesis [25,26]. AtHXK1 also stimulates the expression of photosynthetic genes, decreases chlorophyll levels and reduces the rate of photosynthesis [25,26].

\textbf{Materials and Methods}

\textbf{Construction of transgenic AQP1 plants}

Cloning of the full-length cDNA of the tobacco (\textit{Nicotiana tabacum}) \textit{NtAQP1} under the control of the 3SS constitutive promoter was performed as described in [22]. MP-1 lines (\textit{Solanae lycopersicon cv. MP-1}) were transformed using the \textit{Agrobacterium tumefaciens} transformation method [31]. Plants were assayed for the presence of \textit{NtAQP1} by PCR using the following primers: 3SSprom-Fwd: TATCCTTCGCAAGACCCTCC, and NtAQP1-Rev: TGCC-TGTTCTGTGTTGTAGAT.

\textbf{Plant material}

All experiments were conducted using wild-type (WT) tomato (\textit{Solanum lycopersicon cv. MP-1}), isogenic independent transgenic homozygous tomato line expressing different levels of the Arabidopsis \textit{AtHXK1} (HK37, HK4 and HK38 lines), as previously described in Dai [25], and an isogenic \textit{NtAQP1}-expressing transgenic homozygous line \textit{AQP1}. Double-transgenic homozygous plants \textit{NtAQP1xAtHXK1} (\textit{AQP1xHK4}) were generated by crossing the \textit{AQP1} and HK4 parental lines. After self-pollination of the F1 hybrid plants, screening for F2 plants homozygous for both genes was performed using the highly sensitive Taq-Man DNA quantitative PCR method with specific probes, as described by German et al. [32]. Further validation of homozygosity was carried out by PCR analysis of tens of F3 plants using specific primers for \textit{NtAQP1} (3SSprom-Fwd-TATCCTTCGCAAGACCCTCC, \textit{NtAQP1}-Rev-TGCCCTGGTTCTGGTTGTAGAT) and \textit{AtHXK1} (Fwd-CGGGAAGCAAGAGCGTGTT, Rev-CTCCTCGGGTTGCTATGAT).

\textbf{Measurements of root hydraulic conductance}

The hydraulic conductance of the tomato root system (\( L_r \)) was assessed using plants grown hydroponically and was determined by measuring the flow induced in response to 1 bar of applied pressure. De-topped root systems were fitted with a plastic tube filled with deionized water and connected to a beaker located on a balance (Sartorius \( \pm 0.01 \) mg). The root system was sealed in a chamber containing the hydroponic solution in which the plants had been grown. The pressure in the chamber was regulated using a needle valve, which was adjusted to allow a small leak into the chamber, so that the air used to pressurize the chamber also served to aerate the medium. Water flow through the root system was automatically recorded by a computer at 30 s intervals. At the end of each experiment, the roots were dried in an oven for 72 h at 90°C and the dry weight of the root system was then measured.

\textbf{Measurements of stem hydraulic conductivity}

Stem hydraulic conductivity was assessed on five to seven stems of each genotype. Short sections of stems (\( \approx2–3\) cm long) were cut under water directly from the intact plants to prevent embolisms caused by air entering into the cut vessels. Stems were connected to a balance (Sartorius \( \pm 0.1 \) mg) by a plastic tube and a filtered 10 mM KCl solution, used as a perfusion solution, was located on the balance in a beaker. Stem segments were first perfused under elevated pressure (0.2 MPa) to remove any embolisms and hydraulic conductivity (\( K_s \)) was then calculated as the flow rate multiplied by the length of the stem segment and divided by the pressure gradient.

Xylem cross-sectional area was microscopically determined for each stem to allow the calculation of the xylem-specific stem conductivity (\( K_{sx} \), which equals \( K_s \) divided by total xylem area). Free-hand cross-sections were excised and stained for a few seconds in a diluted Safranin solution. The sections were then rinsed in deionized water for few minutes and photographed under a compound microscope. Xylem area was later determined using the ImageJ software (http://rsweb.nih.gov/ij/).

\textbf{Measurements of whole-plant transpiration}

Whole-plant transpiration rates and relative daily transpiration (RDT) were determined using lysimeters, as described in detail by Sade et al. [22]. WT, \textit{AQP1}, \textit{HK4}, \textit{AQP1xHK4} and grafted plants were planted in 3.9-L pots and grown under controlled conditions. Each pot was placed on a temperature-compensated load cell with digital output and was sealed to prevent evaporation from the surface of the growth medium. A wet, vertical wick made of 0.14 m\(^2\) cotton fibers partially submerged in a 1-L water tank was placed on a similar load cell and used as a reference for the temporal variations in the potential transpiration rate. The output of the load cells was monitored every 10 s and the average readings over 5-min intervals were logged in a data logger for further analysis. The whole-plant transpiration rate was calculated as a numerical derivative of the load cell output following a data-smoothing process [22]. The plant’s daily transpiration rate was normalized to the total leaf area [measured using a LL-COR area meter, model LI-3100; (Lincoln, Nebraska, USA)] or to total plant weight, and to the data for neighboring submerged wick. These figures were averaged for each line and graft type (amount taken up by the wick daily \( \approx 100\% \)).

\textbf{Protein extraction and analysis of hexokinase activity}

Protein extraction and hexokinase activity measurements were performed as described by Dai et al. [25].

\textbf{RNA extraction, cDNA preparation and quantitative real-time PCR}

Leaf tissue was harvested from WT, \textit{AQP1}, \textit{HK4} and \textit{AQP1xHK4} plants and total RNA was extracted from that tissue using EZ-RNA kit (Biological Industries Co., Beit Haemek, Israel) according to the manufacturer’s protocol. The RNA was treated with DNase (Ambion, Austin, TX, USA), according to the manufacturer’s instructions, to degrade any residual DNA. The presence of RNA was confirmed by gel electrophoresis and DNA
degradation was confirmed by PCR. For cDNA preparation, total RNA (1 μg) was taken for reverse transcription-PCR using MMLV RT (Promega, Madison, WI, USA) in a 25-μl reaction with 2 μl of random primers (Promega, Madison, WI, USA) and 1 μl of mixed poly-dT primers. cDNA samples were diluted 1:7 in RNase-free DEPC (Diethylpyrocarbonate) water. Quantitative real-time PCR reactions were performed using SYBR Green mix (Thermo-Scientific, Waltham, Massachusetts, USA). Reactions were run in a RotorGene 6000 cycler (Corbett, Mortlake, New South Wales, Australia). Following an initial pre-heating step at 95°C for 15 min, there were 40 cycles of amplification consisting of 10 s at 95°C, 15 s at 55°C, 10 s at 60°C and 20 s at 72°C. Results were analyzed using RotorGene software. Data were normalized using SlAtG (cyclphinin – accession; M55019) as a reference gene. The following primers were used for amplification: SlCAB1 (Fwd-CGTCGTGTTTGGAACCAG, Rev-AAGGCCATTCTGCCGAAAG), SlCyP (Fwd-TTGTGTTGATGGGAGCCGT, Rev-AAGGCCATCTGGTAGG), TRAMP (Fwd-GTGAAGGGCTTCATGGTAGG, Rev-CTGTTGCTTCATGGTAGG). For each line tested, five to six independent samples were examined, with two replicates per sample.

Gas-exchange measurements and estimation of \( g_m \) based on gas exchange and chlorophyll fluorescence

Gas exchange was measured using a Li-6400 portable gas-exchange system (LI-COR, Lincoln, Nebraska, USA). Analysis was performed on fully expanded leaves (3rd–6th leaf from top) of plants growing under favorable conditions. All measurements were conducted between 10:00 AM and 1:00 PM. Photosynthesis was conducted between 10:00 AM and 1:00 PM. Photosynthesis was induced in saturating light (1200 μmol m\(^{-2}\) s\(^{-1}\)) with 370 μmol mol\(^{-1}\) CO\(_2\) surrounding the leaf (\( C_m \)) and 15% photosynthetically active photon flux density. The flow rate was set to 500 μmol air \(^{-1}\). The leaf-to-air vapor pressure deficit was kept around 1-2.5 kPa during all measurements. Leaf temperature was ~28°C (ambient temperature).

Chlorophyll fluorescence was measured using the LI-6400 open gas-exchange system with an integrated fluorescence chamber head (LI-6400-40; LI-COR). The actual photochemical efficiency of photosystem II (\( \Phi_{PSII} \)) was calculated using Equation 1. Steady-state fluorescence (\( F'_m \)) and maximum fluorescence were measured during a light-saturating pulse of ca. 8000 μmol m\(^{-2}\) s\(^{-1}\) (\( F'_m' \)), following the protocol used by Genty [33]. This procedure was repeated four times with similar results.

\[
\Phi_{PSII} = (F'_m - F_m) / F'_m
\]

The electron transport rate (\( J \)) was then calculated using Equation 2, in which PPFD is the photosynthetically active photon flux density, \( \alpha \) is leaf absorbance and \( \beta \) reflects the partitioning of absorbed quanta between photosystem II and photosystem I (PSII and PSI). Leaf absorbance (\( \alpha \)) was measured between wavelengths of 400–700 nm, using an integrated sphere device (LI-COR, 1800–12s), as described by [34–36]. There were six to eight independent biological repeats for each line. A \( \beta \) value of 0.5 was used as described in [37–40].

\[
J = \Phi_{PSII} \times \text{PPFD} \times \alpha \times \beta
\]

From combined gas-exchange and chlorophyll-fluorescence measurements, the mesophyll conductance for CO\(_2\) (\( g_m \)) was estimated as:

\[
g_m = \Delta S / (C_t - \Delta F_s) / (\Delta F_s + \Delta F_t)
\]

where \( \Delta S \) and \( \Delta F_s \) were obtained from gas-exchange measurements, as described by [41]. A value of 49.2 μmol mol\(^{-1}\) for the CO\(_2\) compensation point under non-respiratory conditions (\( \Delta F \)) was used, after [42]. Respiration in the light (\( R_t \)) was determined from dark respiration values (\( R_d \)) obtained with the Li-6400 instrument at 25°C (~1.4±0.2 μmol CO\(_2\) m\(^{-2}\) s\(^{-1}\)). A value equal to half of the dark respiration was used as a surrogate for \( R_t \) [43].

**Results**

**AtHXK1 decreases root and stem hydraulic conductivity**

To examine the effects of *AtHXK1* on hydraulic proprieties, we measured the root conductance and stem hydraulic conductivity of tomato lines expressing elevated levels of *AtHXK1* (Fig. 1). HK37, HK4 and HK38 are very well characterized independent isogenic transgenic tomato lines that express *AtHXK1* at different levels [25]. These lines exhibit HKX activity that is about 2, 5 and 6 times higher than that of WT plants, respectively [25]. The root hydraulic conductance (\( L_r \)) and xylem-specific stem hydraulic conductivity (\( K_{sx} \)) of HK4 and HK38 lines with high levels of *AtHXK1* expression were significantly lower than those of WT plants (Fig. 1A and 1B).

**NtAQP1 complements AtHXK1-mediated growth inhibition**

While *AtHXK1* decreases hydraulic conductivity, photosynthesis and growth [25,26], *NtAQP1* increases hydraulic conductivity and enhances photosynthesis and growth [20,22]. In light of these opposite effects of *AtHXK1* and *NtAQP1*, we were interested in...
exploring the relationship between \textit{NtAQP1} and \textit{AtHXK1} at the whole-plant level. To that end, we developed tomato line expressing \textit{NtAQP1} against the same genetic background (MP1 [31]) as that of the HK lines and assigned it \textit{AQP1}. Expression of the \textit{NtAQP1} gene and the level of \textit{NtAQP1} protein were determined by quantitative PCR and Western blot analysis, respectively (Fig. S1). We then created double-transgenic plants expressing both \textit{AtHXK1} and \textit{NtAQP1} simultaneously by crossing \textit{AQP1} lines with the HK4 line. Plants homozygous for both genes were identified and are referred to as \textit{AQP1xHK4}.

\textit{AQP1xHK4} plants were taller and had more leaf area than the HK4 parent line (Fig. 2), suggesting that \textit{NtAQP1} complemented the growth-inhibition effects of \textit{AtHXK1}. To verify that this complementation effect was not the result of lowered expression of \textit{AtHXK1}, HXK activity and the sugar-sensing effects of HXK were checked. HXK activity in the double-transgenic plants was similar to that of the HK4 parent plants, about 7-fold higher than that of the control WT and the \textit{AQP1} (homozygote) parent plants (Fig. 2D). We also examined the effect of HXK on the expression of the well-established sugar-sensing photosynthesis marker gene \textit{CAB1}, which is known to be repressed by \textit{AtHXK1} [26,27,29]. \textit{CAB1} expression in \textit{AQP1xHK4} was repressed to levels similar to those observed in the HK4 plants (Fig. 2E), indicating that \textit{AtHXK1} mediated sugar-sensing effects in the double-transgenic plants. These results suggest that the growth complementation effects of \textit{NtAQP1} do not stem from suppression of HXK activity, but rather are probably due to epistatic physiological effects of \textit{NtAQP1}.

\subsection*{Growth complementation of AQP1xHXK is related to \textit{NtAQP1} copy number}

The epistatic effects of \textit{NtAQP1} on plant growth were observed primarily in plants homozygous for both genes, \textit{NtAQP1} and \textit{AtHXK1} (Fig. 3). Crossing \textit{AQP1xHK4} with WT, HK4 or \textit{AQP1} lines yielded plants that were heterozygous or homozygous for \textit{NtAQP1}, \textit{AtHXK1} or both genes. Only plants that were homozygous for \textit{AtHXK1} exhibited significant growth inhibition, and \textit{NtAQP1} enhanced the growth of \textit{AtHXK1} homozygous plants only when present in the homozygous state (Fig. 3). Plants that were heterozygous for \textit{NtAQP1} and lacked \textit{AtHXK1} displayed slightly improved growth, but that effect was abolished in the presence of one or two copies of \textit{AtHXK1}, suggesting a dosage effect in the relationship between \textit{NtAQP1} and \textit{AtHXK1}.

\subsection*{\textit{NtAQP1} enhances the stomatal conductance and transpiration of \textit{AtHXK1} plants}

In previous studies, overexpression of \textit{AtHXK1} decreased stomatal conductance and transpiration; whereas overexpression of \textit{NtAQP1} increased stomatal conductance and transpiration [18,20–22,26,30]. Therefore, we tested the combined effects of \textit{AtHXK1} and \textit{NtAQP1} on stomatal conductance and transpiration. Stomatal conductance (\textit{gs}) of HK4 plants was significantly lower than that of WT plants (Table 1; [30]). Meanwhile, the \textit{gs} of the double-transgenic plants was similar to that of the WT plants (Table 1). Continuous measurements of whole-plant transpiration per unit leaf area over the course of the day revealed significantly lower transpiration rates in HK4 plants, as compared to WT and \textit{AQP1} plants (Fig. 4). Yet, the double-transgenic plants had intermediate-level transpiration rates that were higher than those of HK4 plants (Fig. 4). These results indicate that \textit{NtAQP1} enhanced stomatal conductance and compensated for the limitations imposed on transpiration rates by \textit{AtHXK1}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{\textit{NtAQP1} complements growth inhibition of \textit{AtHXK1}. (A) Representative images of 5-week-old tomato plants homozygous for \textit{NtAQP1} (\textit{AQP1}), \textit{AtHXK1} (\textit{HK4}) or both genes (\textit{AQP1xHK4}). (B) Height (\textit{n}=8) and (C) leaf area (\textit{n}=6) of 9-week-old plants. (D) Hexokinase activity was determined using protein extracted from mature leaves of WT, \textit{AQP1}, \textit{HK4} and \textit{AQP1xHK4} plants. Data are means of five independent biological repeats ± SE. (E) Relative expression of \textit{SICAB1} (\textit{Solanum lycopersicum} a/b binding protein) in WT, \textit{AQP1}, \textit{HK4} and \textit{AQP1xHK4} plants. Data are means of five-six independent biological repeats ± SE. (B–E) Different letters indicate a significant difference (\textit{t}\ test, \textit{P}<0.05). doi:10.1371/journal.pone.0087888.g002}
\end{figure}

\subsection*{Growth and transpiration complementation occurs when \textit{NtAQP1} and \textit{AtHXK1} are simultaneously expressed in the shoot}

To examine whether the decreased transpiration and growth imposed by \textit{AtHXK1} and its complementation by \textit{NtAQP1} emanate from distinct effects on roots or shoots, we created reciprocal grafts, in which WT, \textit{AQP1} and HK4 shoots were grafted onto WT, \textit{AQP1} and HK4 roots, covering all nine possible combinations. (Five combinations involving \textit{AQP1} are shown in Figure 5A and the other four combinations are shown in Figure 2A of [30].) \textit{AtHXK1} inhibited growth only when expressed in shoots, independent of the root genotype (Fig. 5A and [30]). Similarly, measurements of cumulative whole-plant relative daily transpiration of the grafted plants indicated that \textit{AtHXK1} decreased transpiration by about 50% only when expressed in shoots,
independent of the root genotype (Fig. 5B), in line with our recent discovery that AtHXK1 stimulates stomatal closure and reduces transpiration when expressed in shoots (i.e., NtAQP1 in roots had no complementation effect on HK4 shoots) (Fig. 5A). These results show that separate expression of NtAQP1 and AtHXK1 in roots or shoots is insufficient to achieve complementation of AtHXK1 phenotypes by NtAQP1 and that the complementation of AtHXK1 effects by NtAQP1 occurs only when both genes are expressed simultaneously in the shoots.

NtAQP1 does not improve hydraulic conductance of AtHXK1 plants, but does increase the conductance of CO₂ in the leaf mesophyll and the rate of photosynthesis.

The enhanced transpiration of the double-transgenic plants relative to HK4 plants might suggest that NtAQP1 could potentially improve the low hydraulic properties of HK4 plants. We, therefore, measured the root and stem hydraulic conductivity of the WT, AQP1, HK4 and double-transgenic plants. NtAQP1 did not improve the root conductance or xylem-specific stem hydraulic conductivity (Lᵣ and Kₓₓ, respectively) of the double-transgenic plants, which remained low, as in the HK4 plants (Table 1). However, gas-exchange analysis of the double-transgenic plants revealed that NtAQP1 increased photosynthesis rates (Aₛ), CO₂ conductance (gₘₐₜ) and stomatal conductance (gₛ), with no effect on intracellular CO₂ concentration (Cᵢ), as compared to the low Aₛ, gₘₐₜ and gₛ values observed in the HK4 plants (Table 1). In addition, NtAQP1 increased both the concentration of CO₂ in the chloroplasts (Cᵥ) and the electron transport rate (J), as compared to the HK4 plants (Table 1). We,
therefore, suggest that the complementation of AtHXK1 effects by NtAQP1 is primarily due to the role of NtAQP1 as a CO2 facilitator, which enhances the conductance of CO2 in the mesophyll thereby elevating the rate of photosynthesis despite the low expression of CAB1 in AQP1xHK4 plants.

### Discussion

PIP1-AQPs were shown to enhance cell permeability to both CO2 and water [7,13,18]. Overexpression of NtAQP1 in tobacco plants enhanced leaf mesophyll CO2 conductance (gms), hydraulic conductance of water (Lr), xylem-specific stem hydraulic conductivity (Ksx), net photosynthesis (AN), stomatal conductance (gs), mesophyll CO2 conductance (gms), substomatal CO2 concentration (Ci), Chloroplast CO2 concentration (Cc), and the rate of electron transport (J).

#### Table 1. Photosynthetic and hydraulic characteristics of WT, AQP1, AQP1xHK4 and HK4 plants.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>AQP1</th>
<th>AQP1xHK4</th>
<th>HK4</th>
</tr>
</thead>
<tbody>
<tr>
<td>L_r (gH2O s^-1 MPa^-1)</td>
<td>0.00319±0.0003 (7) a</td>
<td>0.00269±0.0005 (7) ab</td>
<td>0.00201±0.0004 (10) b</td>
<td>0.00178±0.00008 (6) b</td>
</tr>
<tr>
<td>K_sx (gH2O s^-1 m^-1 MPa^-1)</td>
<td>1501.48±167.7 (7) a</td>
<td>1081.73±196.2 (7) a</td>
<td>381.61±36.6 (5) b</td>
<td>274.25±16.8 (6) b</td>
</tr>
<tr>
<td>AN (μmol CO2 m^-2 s^-1)</td>
<td>28.189±0.68 (18) a</td>
<td>27.580±0.60 (20) a</td>
<td>28.073±0.49 (15) a</td>
<td>20.831±1.34 (13) b</td>
</tr>
<tr>
<td>g_s (mol H2O m^-2 s^-1)</td>
<td>0.702±0.04 (18) a</td>
<td>0.637±0.04 (20) ab</td>
<td>0.697±0.03 (15) a</td>
<td>0.525±0.06 (13) b</td>
</tr>
<tr>
<td>g_m (mol CO2 m^-2 s^-1)</td>
<td>0.248±0.019 (18) a</td>
<td>0.232±0.014 (20) ab</td>
<td>0.2004±0.007 (15) b</td>
<td>0.148±0.015 (13) c</td>
</tr>
<tr>
<td>C_i (μmol CO2 mol^-1)</td>
<td>0.325±2.67 (18) a</td>
<td>305.1±4.39 (20) a</td>
<td>313.27±2.39 (15) a</td>
<td>311.29±5.33 (13) a</td>
</tr>
<tr>
<td>C_c (μmol CO2 mol^-1)</td>
<td>185.6±6.27 (18) a</td>
<td>183.8±6.34 (20) a</td>
<td>173.9±3.09 (15) a</td>
<td>154.9±6.26 (13) b</td>
</tr>
<tr>
<td>J (μmol m^-2 s^-1)</td>
<td>233.3±3.19 (18) b</td>
<td>238.8±3.5 (20) ab</td>
<td>247.7±2.16 (15) a</td>
<td>204.05±6.22 (13) c</td>
</tr>
</tbody>
</table>

L_r, root hydraulic conductance; K_sx, xylem-specific stem hydraulic conductivity; AN, net photosynthesis; g_s, stomatal conductance; g_m, mesophyll CO2 conductance; C_i, substomatal CO2 concentration; C_c, Chloroplast CO2 concentration; J, the rate of electron transport. Presented data are means ± SE (n, number of replicates, as indicated in parentheses). Different letters in a row indicate significant differences (t test, P<0.05).

**Figure 5. Reciprocal grafting and whole-plant relative daily transpiration.** Reciprocal grafting of WT, AQP1 and HK4 plants was performed at the seedling stage. The plants were photographed (A and Fig. 2A in [30]) and their transpiration was measured about 4 weeks after grafting. (B) Whole-plant relative daily transpiration of reciprocal-grafted plants. Data were normalized to the total plant weight and the amount of water taken up by the neighboring submerged fixed-size wick each day, which was set to 100%. Presented data are means of four independent repeats ± SE. Different letters indicate a significant difference (t test, P<0.05).

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conductivity, stomatal conductance ($g_s$), transpiration and photosynthesis ($A$) [18,20,21]. Expression of NtAQP1 in tomato plants also enhanced photosynthesis, stomatal conductance and transpiration [22]. However, in our study, NtAQP1 did not enhance photosynthesis, stomatal conductance or hydraulic conductivity relative to WT plants (Fig. 5, Table 1) and enhanced transpiration only slightly (Fig. 4). These differences may be due to the different tomato genotype used in our study (MP1 [31], an indeterminate variety) or to different expression levels of NtAQP1. Nevertheless, photosynthesis, stomatal conductance and transpiration were elevated by NtAQP1 in the double-transgenic plants (AQP1xHK4), as compared to the HK4 parental (isogenic) line. Yet, the hydraulic conductivity of AQP1xHK4 remained low as in the HK4 plants, implying that the increased transpiration that was observed is not directly related to hydraulic characteristics. Rather, the increased transpiration is most likely due to high $g_s$ values in the mesophyll, which opens stomata and increases the influx of CO2 to help maintain constant levels of $C_i$ in the substomatal cavity [44,45]. High levels of $A$, $g_s$ and $g_m$, accompanied by constant $C_i$, were also reported in previous studies of tobacco plants overexpressing NtAQP1 [18,21,42].

$AtHXK1$ is a sugar-sensing enzyme that inhibits the expression of photosynthetic genes, decreases chlorophyll levels and reduces the rate of photosynthesis in response to increasing sugar levels [25–29]. As a result, tomato and Arabidopsis plants with high levels of $AtHXK1$ expression display severe growth inhibition directly correlated to $AtHXK1$ expression and activity levels [25,26]. It is likely that part of the growth inhibition imposed by $AtHXK1$ is the result of insufficient photosynthesis, since the increased photosynthesis rate observed in AQP1xHK4 plants partially eliminated this growth inhibition.

The increased rate of photosynthesis observed in AQP1xHK4 plants, despite the low level of expression of the photosynthetic gene CAB1 in those plants, can probably be attributed to NtAQP1, which accelerates CO2 mesophyll conductance ($g_m$) [21,22]. The CO2 mesophyll conductance of HK4 plants is significantly lower than that of WT plants and is enhanced by simultaneous expression of NtAQP1, indicating that CO2 mesophyll conductance significantly affects growth. It appears that, in addition to its known sugar-sensing effect (reducing expression of photosynthetic genes and reducing the rate of photosynthesis in response to increasing sugar levels [25–29]), $AtHXK1$ expression in tobacco plants with high levels of $AtHXK1$ expression also enhances photosynthesis, stomatal conductance and transpiration. The effect of $AtHXK1$ on $g_m$ suggests that HXK might coordinate photosynthesis with sugar levels by several mechanisms in different cell types. It inhibits expression of photosynthetic genes [25,46] and reduces $g_m$ most likely in mesophyll photosynthetic cells. In guard cells HXK mediates stomatal closure in response to sugars and reduces stomatal conductance ($g_s$) [26,30]. These findings support the existence of a multilevel feedback-inhibition mechanism that is mediated by HXK in response to sugars. When sugar levels are high, likely when the rate of photosynthesis exceeds the rate at which the sugar is loaded and carried by the phloem, the surplus of sugar is sensed by HXK in mesophyll and guard cells, which respond in concert to reduce both unnecessary investments in photosynthetic capacity and water loss. This response includes reducing the expression of photosynthetic genes, slowing chlorophyll production, diminishing mesophyll CO2 conductance and closing the stomata.

In addition to these effects in shoots, HXK reduces the hydraulic conductivity of stem and roots via an as yet unknown mechanism. This reduction in hydraulic conductivity occurs independently of stomatal conductance, as it also happens in the double-transgenic plants that have WT levels of stomatal conductance (Table 1). Nevertheless, grafting experiments indicate that neither overexpression of $AtHXK1$ in roots nor expression of $AtHXK1$ in the stem has any visible physiological effects. Rather, overexpression of $AtHXK1$ in shoots is necessary and sufficient to obtain a photosynthesis effect and growth inhibition [25,30]. The dominant effect of $AtHXK1$, lowering hydraulic conductance in AQP1xHK4, might be the reason for the intermediate transpiration rate of AQP1xHK4 plants, which is lower than that of WT plants (Fig. 4), despite the increase in stomatal conductance to levels similar to that of WT plants (Table 1). It has been suggested that NtAQP1 might play independent roles in leaves and roots, a hydraulic role in roots and a membrane CO2 permeability role in shoots [22]. The improved $g_m$ observed in the double-transgenic plants supports the notion that, in leaves, NtAQP1 functions as a CO2 transmembrane facilitator and that the complementation effect of NtAQP1 may be primarily attributed to its effect on CO2 conductance in leaf mesophyll. The roles of HXK and PIP1 in the regulation of photosynthesis, stomatal conductance and transpiration are well established [18,20–22,25–29]. This study suggests that HXK and PIP1 together may influence these central properties of plant physiology and, eventually, plant growth.

Supporting Information

Figure S1 Expression analysis of NtAQP1 in AQP1 transgenic line: Presence of NtAQP1 DNA, RNA and protein. (A) The presence of NtAQP1 was assayed by PCR using NtAQP1-specific primers; transgenic AQP1 plants yielded the expected 930-bp product. WT is a negative non-transformed wild-type plant. + stands for a positive PCR control with a plasmid containing NtAQP1. Ladder: 100-bp ladder. (B) cDNA of AQP1 was subjected to semi-quantitative PCR using NtAQP1-specific primers; Fwd-CCGGGCCAGGTGACTATCC, Rev-TGCTCCTGGTGTCGGTTAGAT. Amplification was performed using 35 PCR cycles. $SlQpP$ (cyclophilin – accession: M55019) was used as a control. (C) Western blot analysis of protein extracts from AQP1 plants probed with NtAQP1-specific antibody (upper panel); Ponceau red staining of the Western blot indicating equal protein loading (lower panel). Western blot analysis and Ponceau staining were performed exactly as described in Sade et al. [22]. (TIF)
**Figure S2 Expression of the TRAMP is suppressed by AtHXK1.** Expression level of TRAMP (tomato ripening associated membrane protein, accession no. NM_001247210), the tomato NaAQP1 homolog, was determined by quantitative real-time PCR using cDNA extracted from leaves of WT and HK4 plants. Data are means of five independent biological repeats ± SE. Different letters indicate a significant difference (t-test, P≤0.05). SigCYP (cyclophilin) was used for normalization. (TIF)

**References**


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Author Contributions

Conceived and designed the experiments: GK NMH MM DG. Performed the experiments: GK NS FS MZ AL. Analyzed the data: GK NS ZA FS MZ AL DG. Contributed reagents/materials/analysis tools: FS MZ NMH VA MM DG. Wrote the paper: GK NS FS MZ MM DG.

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