Running head: Water balance and virus resistance

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Research area:
Signaling and Response
Water balance, hormone homeostasis and sugar signaling are all involved in tomato resistance to Tomato yellow leaf curl virus (TYLCV)

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Summary
Tonoplast water channels (TIPS) are involved in mediating Tomato yellow leaf curl virus (TYLCV) resistance through hormone homeostasis and sugar signaling.
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Abstract

Vacuolar water movement is largely controlled by membrane channels called tonoplast-intrinsic aquaporins (TIP-AQPs). Some TIP-AQP genes, such as *TIP2;2* and *TIP1;1*, are up-regulated upon exposure to biotic stress. Moreover, *TIP1;1* transcript levels are higher in leaves of a tomato line resistant (R) to *Tomato yellow leaf curl virus* (TYLCV) than in those of a susceptible line (S) with a similar genetic background. Virus-induced silencing of *TIP1;1* in the tomato R line and the use of an *Arabidopsis thaliana* tip1;1 null mutant showed that resistance to TYLCV is severely compromised in the absence of *TIP1;1*. Constitutive expression of tomato *TIP2;2* in transgenic TYLCV-susceptible tomato and *Arabidopsis* plants was correlated with increased TYLCV resistance, increased transpiration, decreased ABA levels and increased SA levels at the early stages of infection. We propose that TIP-AQPs affect the induction of leaf ABA, which leads to increased levels of transpiration and gas exchange, as well as better SA signaling.
**Introduction**

Plants manage their water balance in different ways. Some plants maintain a strict transpiration rate, ensuring nearly constant leaf water potential and relative water content; this conservative strategy is referred to as isohydric. Others allow leaf water potential to decrease while transpiration increases during the day; this less conservative strategy is referred to as anisohydric (Sade et al., 2012b; Tardieu and Simonneau, 1998). Compared to isohydric plants, anisohydric plants exhibit differential stomatal regulation and higher rates of transpiration and photosynthesis. Anisohydric plants generally exhibit mild to moderate resistance to abiotic stress (McDowell et al., 2008; Sade et al., 2012b).

The movement of water in a plant is largely controlled by membrane channels called aquaporins (AQPs; Kaldenhoff et al. 2007; Maurel et al., 2008; Tyerman et al., 2002). AQPs regulate cellular water transport (Chrispeels and Maurel, 1994; Heymann and Engel, 1999; Knepper, 1994) and transpiration (Aharoni et al., 2003; Parent et al., 2009). Tonoplast intrinsic proteins (TIPs), a family of proteins belonging to the AQP super-family associated with large vacuoles, play a major role in cell water balance (Maurel et al., 2008, Reuscher et al., 2013). The unique roles of the cell vacuole and tonoplast intrinsic aquaporins (TIP-AQPs) in the regulation of water homeostasis have been demonstrated by the over-expression of these proteins in plants. For example, *Arabidopsis* plants expressing the ginseng (*Panax ginseng*) TIP-AQP gene *PgTIP1* showed altered drought and salt tolerance (Peng et al., 2007).

Recently, we have shown that over-expression of the TIP-AQP gene *SlTIP2;2* in an isohydrictic tomato genotype modified the plant's water-management, so that it became anisohydric. This switch was accompanied by increased transpiration and fruit set under
mild to moderate (but not severe) drought conditions (Sade et al., 2009). Moreover, in an analysis of the expression levels of different aquaporin clusters in the presence of different types of biotic stress (elicitors and pathogens), the homologous genes \textit{SlTIP2;2} and \textit{SlTIP1;1} exhibited 2 and 3.5 fold increases in expression, respectively (Sade et al., 2009). According to comparisons of their amino acid sequences, all TIPs belong to a single phylogenetic group of tonoplast major intrinsic proteins, which includes four TIP sub-families (TIP1-4). Interestingly, although the expression of both \textit{SlTIP1;1} and \textit{SlTIP2;2} is affected by biotic stress in tomato (Eybishtz et al., 2009; Sade et al., 2009), these two genes are the most distantly related members of their sub-family (Reuscher et al., 2013).

TYLCV is a begomovirus transmitted by the whitefly \textit{Bemisia tabaci}, which threatens tomato crops around the world (Navot et al., 1991). Breeders have been working to develop lines that are resistant to TYLCV using the wild tomato species \textit{Solanum habrochaites} as a source of resistance. Two lines that differ only in their susceptibility to TYLCV [susceptible (S) and resistant (R)] were developed by Vidavsky and Czosnek (1998). It has been postulated that the genes that are expressed at higher levels in R plants (as compared to S plants) are related to resistance and that silencing these genes will lead to the collapse of resistance (Eybishtz et al., 2009). This concept has been verified for a number of genes, including the tomato hexose transporter \textit{LeHT1} (Sade et al., 2012a; Eybishtz et al., 2010). Among the genes preferentially expressed in R plants upon TYLCV infection is the tomato TIP-AQP gene \textit{SlTIP1;1} (Eybishtz et al., 2009).

Here, we used molecular and physiological tools to investigate whether TIP-AQPs contribute to TYLCV resistance by cross-talk regulation of water, hormone
balance and hexose signaling upon virus infection. We compared R tomato plants in which the transcript level of the aquaporin gene *SlTIP1;1* was up-regulated, R plants in which *SlTIP1;1* had been silenced using TRV-VIGS and a TYLCV-susceptible tomato genotype rendered TYLCV-resistant by constitutive over-expression of the aquaporin gene *SlTIP2;2*. The results obtained with tomato were confirmed using an Arabidopsis mutant in which the endogenous *AtTIP1;1* (The tomato ortholog; Sade et al., 2009; Reuscher et al., 2013) gene has been knocked-out and transgenic *Arabidopsis* plants in which the tomato *SlTIP2;2* gene was over-expressed. The results presented here indicate that TIP-AQPs are involved in TYLCV resistance, which is controlled by cellular water balance and transpiration, with subsequent effects on hormonal balance and sugar signaling.

**Results**

TYLCV-resistant (R) and TYLCV-susceptible (S) tomato plants present different water regulation patterns; higher transpiration in R tomato plants correlates with higher levels of *TIP1;1* transcription

R plants exhibited higher levels of leaf transpiration (E) than S plants (Fig. 1A). Leaf relative water content (RWC) and midday water potential (*ψ*<sub>leaf</sub>) were lower in the R plants than in the S plants (Fig. 1B, C). In parallel, the amount of *TIP1;1* transcripts was higher in R plants than in S plants (Fig. 1D). Analysis of the water status of the plants as the soil water content decreased showed that R plants exhibited anisohydric behavior
These results indicate that high TIP1;1 expression is correlated with enhanced transpiration and attenuated conservative water regulation. Similar behavior was observed among transgenic tomato plants over-expressing the TIP2;2 gene (Tom-TIP2;2 plants), underscoring the role of TIP-AQPs in water management (Sade et al., 2012b).

**TIP-AQPs are involved in resistance to TYLCV in tomato and Arabidopsis**

The putative role of TIP-AQPs was investigated by 1) silencing the TIP1;1 gene in tomato (by TRV-VIGS) and Arabidopsis (using a T-DNA insertion mutant) and 2) constitutively over-expressing the TIP2;2 gene in transgenic tomato and transgenic Arabidopsis. The behavior of these plants was assessed following TYLCV infection.

The TIP-AQP gene TIP1;1 is expressed at higher levels in R plants than in S plants (Eybishtz et al., 2009). The putative involvement of TIP1;1 in TYLCV resistance was assessed by gene silencing. In R plants in which TIP1;1 was VIGS-silenced [R(0):TRV-TIP1;1], the expression of TIP1;1, but not TIP1;2, was significantly down-regulated, confirming the specificity of TRV-based silencing (Supplemental Fig. S2). Thirty days after inoculation (30 dpi), infected R plants [R(i)] remained free of symptoms, while infected silenced R plants [(R(i):TRV-TIP1;1] exhibited the leaf yellowing and curling associated with infected S [S(i)] plants (Fig. 2A). At 7 dpi, the amount of virus in the R(i):TRV-TIP1;1 plants was already significantly higher than the amount of virus in the R(i) plants, but lower than in the S(i) plants (Fig. 2B). At 21 dpi, the amount of virus in the R(i):TRV-TIP1;1 plants was similar to that in the S(i) plants, about 100 times higher
than in the R(i) tomato plants (Supplemental Fig. S3A). These results indicate that once 
TIP1;1 is silenced, the resistance of R plants is broken.

The role played by TIP-AQPs in the resistance phenomenon was confirmed using the 
Arabidopsis mutant tip1;1. This T-DNA insertion mutant does not express detectable levels of TIP1;1 (Supplemental Fig. S4A; Schüessler et al., 2008). Upon TYLCV inoculation, the tip1;1 mutant was more susceptible to whitefly-mediated virus infection than the wild-type plants. The infected mutant plants [tip1;1(i)] remained stunted and, at 7 dpi, the amount of virus in their leaves was at least five times higher than that observed in the infected wild-type plants [Col-wt(i)] (Fig. 2C).

Another TIP-AQP gene, TIP2;2, has been found to be involved in tomato resistance to a variety of biotic agents (Sade et al., 2009, 2012b). The TYLCV-susceptible tomato genotype Tom-TIP2;2 (F1 hybrid cv. M82 x MicroTom; Sade et al., 2009), in which the endogenous SlTIP2;2 gene is constitutively over-expressed (Supplemental Fig. S4D), was tested for TYLCV resistance. Following TYLCV inoculation, the transgenic tomato plants over-expressing TIP2;2 were more vigorous than the non-transgenic control plants (Tom-wt) and presented attenuated symptoms (Fig. 3A). At 7 dpi, the infected Tom-TIP2;2(i) plants contained about 50 times less virus than the non-transgenic Tom-wt(i) plants. At 21 dpi, the infected Tom-TIP2;2(i) plants contained about 200 times less virus than the non-transgenic Tom-wt(i) plants (Fig. 3B, Supplemental Fig. S3B). The specific role of TIP-AQPs was demonstrated by over-expressing another Solanaceae AQP that localizes to the plasma membrane and not the tonoplast. Over-expression of this AQP, Nicotiana tabacum AQP1 (NtAQP1), was previously reported to improve tomato plant abiotic stress tolerance (these mutant tomato plants were referred to as Tom-NtAQP;
Sade et al., 2010). Here, these Tom-NtAQP1 plants did not show any resistance to TYLCV upon inoculation (Supplemental Fig. S5).

The results obtained with tomato plants were confirmed using transgenic Arabidopsis constitutively expressing the tomato gene SliTIP2;2 (these mutants are referred to as Col-SliTIP2;2, Supplemental Fig. S4C). Upon infection, the Arabidopsis Col-SliTIP2;2(i) plants behaved like the Tom-SliTIP2;2 tomato plants. At 7 dpi, the Col-SliTIP2;2(i) plants contained less viral DNA than the wild-type Arabidopsis plants, Col-wt(i) (Fig. 3C). In addition, at 7 dpi, the Arabidopsis mutants tip2;2, which does not express TIP2;2 (Supplemental Fig. 4B), contained significantly more viral DNA than the infected wild-type plants (Fig. 3D).

ABA/SA homeostasis is involved in resistance to TYLCV in tomato and in Arabidopsis

The transcription level of TIPs is known to be regulated by external stresses, as well as phytohormones (Maurel and Chrispeels, 2001). We first examined the transcript levels of hormone-related marker genes in the tomato and Arabidopsis genotypes that present different patterns of TIP expression. At 7 dpi, a difference in the transcript levels of abscisic acid (ABA)- and salicylic acid (SA)-responsive sentinel genes including ABA8 and AtNCED1 (Neill et al., 1998; Saito et al., 2004) and PR1 and AtICS1 (Chen et al., 2009; Durrant and Dong 2004)] was observed in the resistant plants as compared to the susceptible plants. In contrast, jasmonic acid (JA) transcript [OPR3 and AtOPR3 (Stintzi et al., 2001)] levels were the same in the different genotypes (Supplemental Fig. S6). The transcript levels of these hormone-related marker or hormone biosynthesis genes
in tomato and *Arabidopsis* are known to be correlated with actual hormone levels. These associations were confirmed in this study (Fig. 4 and Supplemental Fig. S6).

Determination of abscisic acid (ABA) and salicylic acid (SA) levels by GC-MS, revealed no significant differences between non-infected tomato and *Arabidopsis* leaves (Fig. 4). At 7 dpi, the level of ABA in the R(i) leaves was significantly lower than in the S(i) leaves (Fig. 4A). In contrast, the level of SA at 7 dpi was significantly higher in R(i) than in S(i) leaves (Fig. 4A). In addition, while S plants did not exhibit any significant change in hormone levels upon infection [S(0) vs. S(i)], R plants showed a significant increase in SA levels and a significant decrease in ABA levels following infection (Fig. 4). Infected over-expressing *TIP2;2* tomato plants and non-transgenic plants (Tom-TIP2;2 and Tom-wt) exhibited trends similar to those observed among the R and S plants (Fig. 4B). Although ABA levels increased upon infection in both Tom-TIP2;2 and Tom-wt, the Tom-TIP2;2 plants exhibited a milder increase than the Tom-wt plants, which was accompanied by a significant increase of SA in Tom-TIP2;2 plants (Fig. 4B).

Hormone levels were also compared in *Arabidopsis* plants upon TYLCV infection (Fig. 4C). The TIP1;1 mutant (*tip1;1*), the over-expressing Tom-TIP2;2 (Col-SITIP2;2) and the wild-type (Col-wt) plants exhibited behavior similar to that of their tomato counterparts. Following inoculation, *tip1;1* plants displayed the highest levels of ABA. There was little difference between the ABA levels of the Col-SITIP2;2 plants and the Col-wt plants. At 7 dpi, the Col-SITIP2;2 plants had higher levels of SA than the other plants.

The changes in hormone homeostasis genes in tomato plants were already noticeable at 1 dpi (Fig. 5). Interestingly, in contrast to what was observed at 7 dpi, at 1 dpi, the JA biosynthesis gene *OPR3* transcript level was higher in the R plants than in the S plants.
To further confirm the involvement of ABA and SA in the accumulation of viral amounts in the plants, we conducted an experiment using Arabidopsis mutants that were either ABA-deficient (aba1; Koornneef et al., 1982) or SA-activated (cim10; Maleck et al., 2002). These plants were infected with viruliferous whiteflies. Compared with wt plants, the aba1 and cim10 plants (with low ABA and high SA levels, respectively) exhibited stronger resistance at 7 dpi (Supplemental Fig. S7).

Enhancing the SA pathway by treating TYLCV-susceptible plants with acibenzolar-S-methyl enhances their resistance to the virus

To further establish the link between TYLCV resistance and ABA/SA homeostasis, the SA pathway was up-regulated by treating TYLCV-susceptible plants (S tomato plants and wild-type Arabidopsis plants) with acibenzolar-S-methyl (Actigard), a compound that mimics SA in the plants’ natural systemic activated resistance (SAR) response (Walters et al., 2013). The effects of this treatment on the transcript levels of the tomato hormone synthesis genes PR1 (a marker of SA) and ABA8 (a marker of ABA biosynthesis) and on the Arabidopsis genes ICS1 (SA) and NCED1 (ABA) indicated that, as expected, the SA genes were up-regulated while the ABA genes were down-regulated (Fig. 6A, B, respectively). In this regard, the treated S plants behaved like R plants. Seven days after the acibenzolar-S-methyl treatment, the plants were inoculated with TYLCV. At 7 dpi, S tomato and wild-type Arabidopsis plants contained significantly less virus than the untreated control plants and the R tomato plants contained about the same amounts of virus as the untreated plants (Fig. 6C). These results confirm that SA is part of the TYLCV resistance network.
Hierarchy of genes involved in TYLCV resistance in R tomato plants: \textit{TIP1;1} is upstream of the extracellular invertase \textit{Lin6} gene and the hexose transporter \textit{LeHT1}; whereas \textit{Lin6} is upstream of \textit{LeHT1}

It has been postulated that the genes up-regulated upon TYLCV infection of R plants are part of an interconnected hierarchical network that confers resistance (Eybishtz et al., 2009). We have recently shown that \textit{Lin6} and \textit{LeHT1} are involved in the resistance to TYLCV in R plants, likely leading to enhanced carbon utilization and photosynthesis (Sade et al., 2013). In this work, we hypothesized that the silencing of one of these genes will cause a decrease in the transcript levels of genes downstream in the network. 

\textit{TIP1;1} transcript levels were examined in infected R plants in which \textit{LeHT1} had been silenced [R(i):\textit{TRV-LeHT1}]. We found that in the \textit{LeHT1}-silenced R plants, the transcript level of \textit{TIP1;1} was not affected (Fig. 7A). In contrast, the \textit{LeHT1} transcript levels were decreased in R plants in which \textit{TIP1;1} had been silenced [R(i):\textit{TRV-TIP1;1}] (Fig. 7B). Moreover, the transcript level of \textit{Lin6} was strongly down-regulated in R(i):\textit{TRV-TIP1;1} plants, but was not altered in \textit{LeHT1} silenced R plants [R(i):\textit{TRV-LeHT1}] (Fig. 7C). These results indicate that in the hierarchy of the gene network conferring TYLCV resistance, these three genes are interconnected: \textit{TIP1;1} is upstream of \textit{Lin6} and \textit{LeHT1}, and \textit{Lin6} is upstream of \textit{LeHT1}.

\textit{Pearson analyses in Arabidopsis} revealed a strong correlation between the expression of TIP-AQPs, sugar metabolism and hormone homeostasis during early stages of biotic stress
We have previously shown (Sade et al., 2013) and confirmed here (Figs. 5 through 7) that TIP-AQPs, LeHT1, Lin6 and hormone homeostasis genes are all involved in the resistance of R tomatoes to TYLCV. In order to ascertain whether the transcript levels of these genes are affected by pathogens, we data-mined Arabidopsis microarray analyses (there is not enough available data for tomato) upon infestation with fungi and bacteria (not enough data for viruses; NASCArray, http://affy.arabidopsis.info/narrays/experimentbrowse.pl). This analysis included gene members of the Arabidopsis aquaporin TIP family, of the hexose transporter STP and of the cell wall invertase AtcwINVs. We also included hormone-signaling genes of the SA (ICS1, SID2, BGL2, WRK28), JA (OPR3, AOS, PDF1.2A) and ABA (NCED1, CYP707A2, ZEP, NSY, XD, ABA ox8) pathways (Fig. 8 and Supplemental Table SII). The hierarchical clustering of the correlation intensities (Fig. 8) showed that, in most instances, the correlation patterns of members of a specific gene family were not similar. However, there were correlations between the behavior of Arabidopsis genes and that of their tomato homologues. TIP1;1 expression was positively correlated with STP1 expression (the tomato LeHT1 homolog) and with specific cwINV1 expression at an early stage of infection (24 hours after inoculation). Moreover, TIP1;1, STP1 and cwINV1 expression levels were positively correlated with the SA and JA pathways, and negatively with the ABA pathway. These analyses strongly suggest that TIP1;1 AQPs, sugar metabolism and hormone homeostasis are linked.
Discussion

Plants’ water balance management is carefully regulated via stomatal conductance and changes in response to changing environmental conditions. Different plants manage their water budgets differently and the molecular and physiological mechanisms of this regulation are not yet fully understood. In addition to abiotic stresses, biotic stresses are also known to affect plant water regulation (Gudesblat et al., 2009), raising the possibility that these two types of stress activate the same signal transduction pathways (Fujita et al., 2006). Plant AQPs are involved in regulating both transpiration and hydraulic conductivity (Aharon et al., 2003; Boursiac et al., 2005; Postaire et al., 2010; Pou et al., 2012). Differential levels of AQP expression correlate with differences in the rate of leaf transpiration under normal and abiotic/biotic stress conditions (Parent et al., 2009; Sade et al., 2010, 2012b; Siefritz et al., 2002).

Plants’ responses to stress involve changes in phytohormones and protein interactions (Fujita et al., 2006), which lead to reduced stomatal conductance ($g_s$), decreased CO$_2$ assimilation ($A_N$) and changes in water utilization (Gudesblat et al., 2009; Kyseláková et al., 2011). Resistance to biotic stress is strongly associated with homeostasis of hormones such as SA and JA (Glazebrook, 2005; Thaler et al., 2010). Tomato plants (*Solanum lycopersicum*) grown in an CO$_2$-enriched atmosphere exhibited increased resistance to the begomovirus *Tomato yellow leaf curl virus* (TYLCV), as well as increased SA levels (Huang et al., 2012). Infection of *Arabidopsis* plants with the begomovirus *Cabbage leaf curl virus* (CaLCuV) led to an early pathogen response involving the SA pathway (Ascencio-Ibáñez et al., 2008). Moreover, antagonistic cross-talk between ABA
(associated with abiotic stress) and SA and JA (both associated with biotic stress) has been observed upon bacterial, fungal and viral infection (Audenaert et al., 2002; de Torres Zabala et al., 2009; Yasuda et al., 2008). Hence, it has been suggested that carbon surpluses (McDowell et al., 2008, 2011) and hormone homeostasis are involved in resistance to stress (Audenaert et al., 2002; Huang et al., 2012; Ascencio-Ibáñez et al., 2008).

These finding led us to hypothesize that vacuole water channels may be involved in plant resistance to TYLCV (Fig. 1). We suggest that greater permeability of the vacuole membrane to water during the onset of viral infection will continue to buffer the osmotic and mineral concentration in the cytoplasm and thus delay the initiation of a stress signal in the cytoplasm. Hence a slower stomatal response is expected to be controlled by a mechanism similar to that suggested for isohydric/anisohydric behavior (Sade et al., 2009). The prolonged stress signal might be a result of lower levels of ABA (a phytohormone extensively involved in responses to abiotic stress) acting as a negative regulator of disease resistance (Mauch-Mani and Mauch, 2005). In contrast, SA plays a central role in signaling upon pathogen infection. SA and ABA have antagonistic effects. Thus, the ABA/SA cross-talk will result in higher SA levels, leading to resistance to TYLCV (Huang et al., 2012).

By screening tomato ESTs from TYLCV-resistant (R) and -susceptible (S) genotypes, we found that TIP1;1 was up-regulated upon TYLCV infection (Fig. 1 and Eybishtz et al., 2009). Indeed, R tomato plants displayed less conservative water management than S plants, maintaining relatively high transpiration and lower midday water potentials and RWC than S plants (Fig. 1 and Supplemental Fig. S1; Sade et al., 2013). This resembles
the anisohydric behavior observed in tomato lines transformed with \textit{SI}TIP2;2 (Sade et al., 2009, 2012b). The unique role of TIP-AQPs in regulating whole-plant water balance and abiotic-stress resistance has been demonstrated in other studies as well (Lin et al., 2007; Peng et al., 2007; Pou et al., 2012).

The role of TIP-AQPs was demonstrated in both tomato and \textit{Arabidopsis}. When a reverse-genetic approach (TRV-VIGS in tomato and transposon-mediated knock-out in \textit{Arabidopsis}) targeting \textit{TIP1;1} was used, tomato resistance was impaired and \textit{Arabidopsis} became more susceptible to infection, as compared to untreated control plants (Fig. 2). In addition, anisohydric transgenic Tom-TIP2;2 tomato plants and transgenic \textit{Arabidopsis} plants over-expressing \textit{SI}TIP2;2 (Col-SI)TIP2;2 displayed enhanced TYLCV resistance (Fig. 3). These results can be compared with those of a previous study in which anisohydric \textit{TIP2;2}-overexpressing tomato plants exhibited a higher level of resistance to \textit{Botrytis cinerea} than the isohydric controls (Sade et al., 2012b). In contrast, transgenic tomato plants over-expressing a \textit{Nicotiana tabacum} plasma membrane AQP1 (NtAQP1) did not show any resistance to TYLCV (Supplemental Fig. S5), emphasizing the specific role of the tonoplast AQPs in resistance. Moreover, tomato plants that have mutations in the ABA biosynthesis pathway that prevent them from closing their stomata (and as such could referred as extremely anisohydric) displayed greater resistance to \textit{B. cinerea} infection, lower ABA levels and higher levels of SA, as compared to the isohydric controls (Audenaert et al., 2002).

Generally, anisohydric plants display lower amounts of, or sensitivity to the stress hormone ABA (Loewenstein and Pallardy, 1998; Loveys and During, 1984; Soar et al., 2006; Tardieu and Simonneau, 1998). Low levels of ABA can lead to high levels of SA
due to antagonistic interactions between these two phytohormones (Audenaert et al., 2002; de Torres Zabala et al., 2009; Jiang et al., 2010; Mohr and Cahill, 2007). SA is well known for its role in resistance to numerous types of biotic stress (Hammond Kosack et al., 1996; Thomma et al., 1998), including geminiviruses (Ascencio-Ibáñez et al., 2008, Huang et al., 2012). This suggests that anisohydric resistance to biotic stress may be related to a plant defense mechanism regulated by ABA and SA. Indeed, analysis of ABA and SA levels at 7 dpi revealed that the anisohydric R and the Tom-2;2 transgenic tomato plants contained larger amounts of SA and smaller amounts of ABA than the isohydric plants (S line and Tom-wt; Fig. 4A, B).

The same trend was observed when the two TYLCV-infected Arabidopsis plants Col-SITIP2;2 and tip1;1 were compared. The transgenic plants contained larger amounts of SA and smaller amounts of ABA than the mutant plants (Fig. 4C). A close correlation was also observed between hormone levels and the transcript levels of hormone marker and biosynthesis genes in these plants (with the exception of the amounts of ABA in the Col-SITIP2;2 plants; Supplemental Fig. S6). It is possible that the heterologous expression of SITIP2;2 in Arabidopsis caused a bias between ABA hormone level and gene expression at 7 dpi. Additionally, no change was observed in the amounts of JA or the transcript levels of the JA biosynthesis genes in the different lines at 7 dpi (data not shown and Supplemental Fig. S6).

The changes in hormone homeostasis in tomato plants were noticeable as early as at 1 dpi (Fig. 5). Interestingly, at 1 dpi, the JA biosynthesis gene OPR3 was more highly expressed in R plants than in S plants (Fig. 5A), emphasizing the involvement of this hormone in the early stages of infection and the activation of resistance. JA has been
shown to be a crucial component of the plant defense response to sucking insects such as whiteflies (Kempema et al., 2007; Zarate et al. 2007). Hence, over-expression of the JA-associated gene may be related to insect feeding rather than to virus infection per se. Moreover, combined applications of exogenous SA and JA induced stronger resistance to TYLCV than the application of either SA or JA alone (Huang et al., 2012). Therefore, it appears that the modulated interaction between SA and JA in the R line may significantly contribute to the observed TYLCV resistance.

As expected, the lower levels of ABA were accompanied by higher transpiration rates in the anisohydric tomato plants Tom-TIP2;2 and R (Fig. 1; Sade et al., 2009). This phenomenon has also been associated with increased photosynthetic activity (Sade et al., 2013). Interestingly, the TYLCV resistant Arabidopsis Col-SITIP2;2 plants also exhibited improved photosynthesis compare to wt (Supplemental Fig. S8).

It was recently shown (Huang et al., 2012) that tomatoes grown under elevated CO2 show enhanced resistance to TYLCV and to Phytophthora parasitica, a phenomenon likely related to changes in SA/ABA levels (Jwa and Walling, 2001; Zavala et al., 2008). In addition, it has been previously demonstrated that in R plants in which the hexose transporter gene LeHT1 has been silenced, hexoses are not taken up by the cells and, therefore, do not act as defense-signaling molecules, provoking the collapse of TYLCV resistance (Sade et al., 2013). Moreover, both cell wall invertases, Lin6 and LeHT1, were differentially expressed in R plants soon after infection (1dpi; Sade et al., 2013).

We further hypothesized that this interconnecting network is not specific to tomato and can be identified in Arabidopsis as well. The over-expression of TIPs was correlated with TYLCV resistance; whereas down-regulation of TIP expression was correlated with
susceptibility (Figs. 2C and 3C). This was further connected to ABA/SA homeostasis (Figs. 4 and 6 and Supplemental Fig. S6). Co-expression analysis of Arabidopsis under several types of biotic stress at early stages of infection (<1 dpi) revealed a positive correlation between the TIP-AQP, hexose transport and hormone pathways (Fig. 8). Interestingly, *TIP1;1* was positively correlated with *STP1* (homolog of the tomato *LeHT1*; McCurdy et al., 2010), with *cwINV1* and with the SA and JA pathways. In contrast, a negative correlation was observed between *TIP1;1* expression and activation of the ABA pathway. This unique pattern was not observed for other TIPs, other STPs or *cwINV*, emphasizing the specific interconnection of these genes at early stages of infection. Therefore, it is likely that cross-talk between sugar and hormone signaling pathways in plants leads to an effective immune response (Bolouri Moghaddam and Vanden Ende, 2012; Herbers et al., 1996).

Since *TIP1;1*, *LeHT1* and *Lin6* are preferentially expressed in R plants and are over-expressed upon TYLCV infection (Sade et al., 2013 and Fig. 7), we postulated that they belong to an interconnected hierarchical network that confers resistance to this virus. We tested the hypothesis that silencing one of these genes would influence the transcript levels of genes downstream in the gene hierarchy, but not those upstream. The step-wise silencing and gene transcript-level analyses indicated that *TIP1;1* is upstream of both *Lin6* and *LeHT1* and that *Lin6* is upstream of *LeHT1* (Fig. 7). Therefore, it is likely that endogenous *TIP1;1* in R plants leads to more efficient gas exchange (i.e., transpiration and photosynthesis), which induces a subsequent effect on sugar (i.e., hexose transport) and hormone signaling (i.e., SA).
SA plays a major role in resistance to TYLCV at the early stages of infection (7 dpi) and can also act as a resistance signal, activating pathogen-resistance genes (PR) through the SAR signaling pathway (Vlot et al., 2009). Analysis of gene expression in Arabidopsis plants infected with the geminivirus CaLCuV revealed that that virus triggers an early pathogen response via the SA pathway (Ascencio-Ibáñez et al., 2008). Moreover, Arabidopsis cpr1 plants that constitutively expressed PR1 (Bowling et al., 1994) were less susceptible to CaLCuV infection than Col-0 wild-type plants; symptoms in those plants were attenuated and developed much later than in the wt plants. In our study, Arabidopsis plants with an up-regulated SA pathway were more resistant to TYLCV (Supplemental Fig. S7). These findings and our results indicate that up-regulation of the SA pathway and SAR triggering (and subsequent down-regulation of the ABA pathway) impair geminivirus infection (Fig. 6B, C and Supplemental Fig. S7).

Conclusion

We have shown that TIP-AQPs play a significant role in mediating TYLCV resistance and that this involves changes in water balance regulation, hexose signaling and hormone homeostasis. A flow chart of events triggered by TIP1;1 in R and S plants is presented in Figure 9. The question of how these pathways are interconnected should be explored in further research.
Materials and Methods

Plants

Tomato. Two inbred tomato lines were used, line 902 and line 906-4. These lines were generated in a breeding program aimed at introgressing resistance to TYLCV from *S. habrochaites* (Vidavsky and Czosnek, 1998) into the domesticated tomato. Line 902 is resistant to the virus (R); whereas line 906-4 is susceptible (S). Upon infection, R plants develop normally, produce fruit and contain low amounts of virus. In contrast, S plants exhibit stunted growth, produce small quantities of fruit and contain large amounts of virus. In addition, two independent lines of the tomato M82 x MicroTom hybrid expressing the tomato aquaporin gene *SlTIP2;2* (called TOM-TIP2;2 plants; Sade et al., 2009) and non-transgenic controls were used. The plants were transplanted into 2-L pots and grown for about 3 weeks in a climate-controlled greenhouse that was kept at 18 to 25°C and 50 to 60% relative humidity before being infected with the virus via viruliferous whiteflies. Fertilizer was applied through the automatic irrigation system.

Arabidopsis thaliana. Plants were transformed using the floral-dip method (Clough and Bent, 1998). Two independent T2 transgenic lines (ecotype Columbia) over-expressing the tomato gene *TIP2;2* and a line with a transposon insertion mutation in the endogenous *TIP1;1* gene (line *tip1;1*, SM_3_32402 plants, Schüssler et al., 2008) were used, together with their non-transgenic control counterparts. Homozygous mutants *tip2;2* (Salk 151945) and *aba1-1* were obtained from arabidopsis biological resource center (ABRC). All plants were grown in 200-mL pots in a climate-controlled growth chamber (22°C,
8h/16h dark/light regimen). After 28 days of growth, these plants were infected using viruliferous whiteflies (about 30 insects per plant).

Whitefly-mediated inoculation of tomato and *Arabidopsis* plants with TYLCV. Whiteflies, *Bemisia tabaci* B biotype, were reared on cotton plants, as described previously (Zeidan and Czosnek, 1991). TYLCV (Navot et al., 1991) was maintained in tomato plants. We inoculated the tomato plants (3 weeks after sowing) and Arabidopsis plants (4 weeks after sowing; Polston et al., 2001) by placing them together with viruliferous whiteflies (approximately 30 insects per plant) in insect-proof, wooden boxes in a growth chamber kept at 24 to 27°C. After 3 days, the insects were killed with imidacloprid (Bayer Crop Science, Manheim am Rhein, Germany) and the plants were returned to the greenhouse.

Gas-exchange measurements.

Gas exchange was recorded using a Li-6400 portable gas-exchange system (Li-Cor, Inc., Lincoln, NE). All measurements were taken between 11 am and 1 pm, under saturating light (1200 μmol m⁻² s⁻¹) and with 400 μmol mol⁻¹ CO₂ surrounding the leaf (C_a). The amount of blue light (signal for stomata opening) was set to 10% photosynthetically active photon flux density (PFD) to optimize stomatal aperture. The leaf-to-air vapor pressure deficit (VPD) was kept at 1 to 2.5 kPa while the data were being collected. All measurements were taken at 25°C.
Water potential.

Leaf water potential ($\Psi_{\text{leaf}}$) was measured between 11 am and 1 pm using a pressure chamber (ARIMAD-3000; MRC, http://www.mrclab.com/htmls/home.aspx). A leaflet (third to fourth apical) was harvested, placed in the chamber and observed under a binocular microscope (SZ; Olympus, Intralux 5000; Volpi AG). The $\text{N}_2$ pressure in the tightly closed chamber was slowly increased until water oozed out of the petiole cut.

Relative water content (RWC).

After $\Psi_{\text{leaf}}$ was determined, the very same leaves were kept in previously weighed, zipper-locked plastic bags to avoid weight loss by evaporation-transpiration and taken to the laboratory for measurement of fresh weight and relative water content, between 11 am and 1 pm. The sealed plastic bag with the leaf was weighed and the weight of the bag was subtracted from the total weight, in order to get the fresh weight (FW). Two mL of 5 mM CaCl$_2$ were added to each plastic bag and the leaf petioles were left to soak in the dark at room temperature for 7 to 9 h. Then, the leaves were gently removed from the bags and placed between two paper towels to absorb excess water and the turgid weight (TW) was recorded. The leaves’ total dry weight (DW) was measured after they had been dried in an oven at 60°C for 72-84 h. Leaf RWC was calculated as ($\%$) = ($\text{FW} - \text{DW/TW} - \text{DW}$) × 100.

Silencing of the tomato aquaporin gene $TIP1;1$

A fragment of the tomato aquaporin gene $TIP1;1$ (nt 420 to 722, TC170408) was cloned into a pDrive vector (Qiagen, USA). The gene fragments were excised from the vector
using XbaI and KpnI and ligated to the TRV RNA2 vector (Liu et al., 2002) using the same restriction sites, resulting in the silencing vector TRV- TIP1;1. The plasmid was introduced into Agrobacterium LB4404 cells by electroporation. The agrobacteria containing TRV- TIP1;1 and TRV RNA1 were cultured in YEB medium for 48 h at 28°C. A mixture of TRV RNA1 and TRV- TIP1;1 was introduced into 30 R and S tomato seedlings at the 6- to 8-leaf stage by agro-inoculation. Five days later, 20 silenced plants were inoculated with TYLCV. We did this by placing the plants in cages with viruliferous whiteflies for a 3-day period. In a previous study, we found that inoculation with TRV does not affect TYLCV replication or resistance in R tomato plants (Eybishitz et al., 2009). Samples from at least two independent experiments were analyzed in triplicate.

Analysis of gene expression and TYLCV amounts by quantitative PCR (qPCR)

**Gene transcript level.** At the times indicated, the two youngest leaves of each plant were harvested. Total RNA was extracted using Tri-Reagent (Molecular Research Center, USA) and treated with RNase-free DNase (Fermentas; Vilnius, Lithuania). cDNA was synthesized using the EZ-First Strand cDNA Synthesis kit (Biological Industries, Israel) according to the manufacturer's instructions. qPCR was performed in the presence of SYBR Green I (Takara, Japan) in a Corbett Research Rotor-Gene 6000 cycler. The tomato β-actin gene (TC198350; Sade et al., 2012a) and the Arabidopsis ACTIN2 (Alexandersson et al., 2005) were used as references for the standardization of cDNA quantities. The reaction was carried out as follows: 30 s at 94°C, followed by 40 cycles consisting of 10 s at 94°C, 30 s at 58°C and 20 s at 72°C.
**Virus amounts.** To estimate the relative amount of TYLCV (GenBank X15656), DNA was extracted from the youngest two leaves (Bernatzky and Tanksley, 1986) and subjected to qPCR with virus-specific primers. Tomato β-actin and Arabidopsis UBQ10 were used as calibrating genes. The primers used are listed in Supplemental Table I.

Determination of hormone levels by GC-MS.

Frozen young leaves were treated with 1.5 mL of 90% methanol. The extract was sonicated for 15 min and cell debris was removed by centrifugation at 13,000 × g for 10 min. The supernatant was dried in glass tubes at 40°C. The dried residues were dissolved in 1 mL of 4M HCl and hydrolyzed at 80°C for 1 h. The hydrolyzed mixtures were treated with cyclopentane/ethyl acetate (1:1 v/v) and the organic phase of each mixture was collected and dried at 40°C under nitrogen. ABA and JA were extracted overnight from 1 g of frozen tissue in 20 mL of 80% methanol. After extraction, each sample was reduced *in vacuo* and diluted with 20 mL of water. The pH of the aqueous phase was adjusted to pH 2.8 with 1M HCl and partitioned four times with equal volumes of ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness. The residues were then dissolved in 1 mL of 10% methanol and applied to a pre-equilibrated C18 cartridge (www.phenomenex.com). The column was washed with aqueous acetic acid (pH 3.0) and JA and ABA were then eluted with 80% methanol. After evaporation to dryness, the samples from the SA, ABA and JA analyses were dissolved in 40 µL of 20 mg/mL methoxyamine hydrochloride in pyridine for 2 h at 37°C to protect the carbonyl moieties. Acidic protons were derivatized by treatment with 70 µL of *N*-methyl-*N*-(trimethylsilyl)-trifluoracetamide for 30 min at 37°C. A volume of 1 µL of each sample
was injected into a GC-TOF-MS system (Pegasus III, Leco; St. Joseph, MO, USA) using an autosampler system (PAL Agilent; Santa Clara, CA, USA). Gas chromatography was performed on a 30-m MDN-35 column. The injection temperature was 230ºC and the transfer line and ion source temperatures were set to 250ºC. The initial oven temperature (85ºC) was gradually increased (15ºC per minute) to a final temperature of 360ºC. The samples were analyzed by GC-MS and quantified using external SA, ABA and JA standards.

Acibenzolar-S-methyl treatment.
Acibenzolar-S-methyl (Actigard 50WG, Syngenta, USA) was applied to tomato plants (3 weeks after sowing) and Arabidopsis plants (4 weeks after sowing) by twice spraying a mixture of 98 μL/L on the plants. The two spray applications were made 3 days apart.

Pearson correlation coefficient.
Pearson correlation coefficient values were calculated for all of the gene pairs in the matrix using the web-based tool Expression Angler (Toufighi et al., 2005). A subset of expression values derived from the AtGenExpress Pathogen Compendium were used for the calculation. This subset included all the expression values of experiments in response to infection by four different pathogens (Phytophthora infestans, Botrytis cinerea, Pseudomonas and Erysiphe orontii).

Accession numbers
A list of all the accession numbers is provided in Supplemental Tables I and II (available online).

**Supplemental Data**

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

**Table S I.** List of primers used for qRT-PCR analyses of gene expression.

**Table S II.** Analysis of the co-expression of several *Arabidopsis* genes (sorted by family) in the presence of biotic stress (*Phytophthora infestans, Botrytis cinerea, Pseudomonas* and *Erysipheorontii*).

**Figure S1.** Relative water content (RWC) of leaves and leaf water potential at decreasing relative soil volumetric water content (VWC) levels in S and R plants.

**Figure S2.** *SlTIP1;1* and *SlTIP1;2* expression in R(0) plants and R(0):TRV-*TIP1;1* plants as measured by qPCR.

**Figure S3.** Effects of the expression of the tomato genes *TIP1;1* and *TIP2;2* on TYLCV infection of tomato plants at 21 dpi.

**Figure S4.** Relative expression levels of the *Arabidopsis TIP1;1, Arabidopsis TIP2;2* and tomato *TIP2;2* genes in plants with different genotypes.

**Figure S5.** Effect of the expression of the *Nicotiana tabacum* plasma membrane AQP gene *NtAQPI* on TYLCV infection of tomato plants transformed with *NtAQPI* (Tom-NtAQPI plants), as compared with non-transgenic tomato plants (wt).

**Figure S6.** Expression of hormone-related marker and biosynthesis genes (ABA: *ABA8, AtNCED1; SA: PRI, AtICS1* and JA: *OPR3, AtOPR3*) in tomato and *Arabidopsis* plants at 7 dpi.
Figure S7. Effects of hormone levels on TYLCV infection of *Arabidopsis* mutants at 7dpi.

Figure S8. Photosynthesis and transpiration of wild-type *Arabidopsis* plants (Col-wt) and *Arabidopsis* plants expressing *SlTIP2;2*.

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Reference


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Figure legends

Figure 1. Comparison of water management in R (white bars) and S (black bars) tomato plants. (A) Leaf transpiration, E. (B) Leaf relative water content, RWC. (C) SlTIP1;1 expression as measured by qPCR. (D) Midday leaf water potential, $\psi_{\text{leaf}}$. * indicates a significant difference ($t$ test, $P < 0.05$). Data points are means ± SE ($N = 7$ to $30$).

Figure 2. Effect of SlTIP1;1 silencing on the responses of tomato and Arabidopsis plants to TYLCV infection. (A) In tomato at 30 dpi, the S [S(i)] plants and the R plants in which the TIP1;1 gene had been silenced [R(i):TRV-TIP1;1] showed symptoms of infection, but the R [R(i)] plants did not. (B) qPCR estimation of relative amounts of TYLCV in tomato plants at 7 dpi; S(i): black bar, R(i):TRV-TIP1;1: gray bar and R(i): white bar. Tomato $\beta$-actin was used as the calibrating gene. Different letters above the columns indicate significant differences ($t$ test, $P < 0.05$). (C) qPCR estimation of relative amounts of TYLCV in Arabidopsis at 7 dpi; wild type Col-wt(i): white bar; mutant tip1;1(i): black bar. Arabidopsis UBQ10 was used as the calibrating gene. * indicates a significant difference ($t$ test, $P < 0.05$). Data points are means ± SE ($N = 7$ to $9$).

Figure 3. Effect of SlTIP2;2 over-expression on TYLCV infection of tomato and Arabidopsis. (A) Appearance of symptoms in transgenic tomato plants over-expressing the tomato gene TIP2;2 [Tom-TIP2;2(i)] and in non-transgenic control plants [Tom-wt(i)] at 30 dpi. Note that the transgenic tomato grew better than the wild type and exhibited less severe symptoms. (B-D) qPCR estimations of relative amounts of TYLCV in tomato and Arabidopsis plants at 7 dpi. Tomato $\beta$-actin and Arabidopsis UBQ10 were
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**Figure 4.** Amounts of abscisic acid (ABA) and salicylic acid (SA) in TYLCV-infected tomato and *Arabidopsis* plants at 7 dpi. (A) R (white bars) and S tomatoes (black bars) before [R(0), S(0)] and after [R(i), S(i)] infection. (B) Tom-TIP2;2 tomato plants (white bar) and Tom-wt (black bar) before [Tom-Tip2;2(0), Tom-wt(0)] and after infection [Tom-Tip2;2(i), Tom-wt (i)]. (C) *Arabidopsis* Col-SITIP2;2 (white bar), Col-wt (gray bar) and tip1;1 mutant plants (black bar) before [Col-SITIP2;2(0), Col-wt(0), tip1;1(0)] and after infection [Col-SITIP2;2(i), Col-wt(i), tip1;1(i)]. Hormone levels are expressed as μg per g leaf fresh weight. Data points are means ± SE ($N = 3$ to $6$). Different letters above the columns indicate significant differences (t test, $P < 0.05$).

**Figure 5.** Expression of hormone-related marker or biosynthesis genes in tomato (ABA: *ABA8*; SA: *PR1* and JA: *OPR3*) at 1 dpi. (A) Tomato: susceptible line (black bar, i = infected, 0 = uninfected) and resistant line (white bar, i = infected, 0 = uninfected). (B) Tom-wt tomato plants (black bar, i = infected, 0 = uninfected) and Tom-TIP2;2 tomato plants (white bar, i = infected, 0 = uninfected). Data points are means ± SE ($N = 4$ to $7$). Different letters above the columns indicate significant differences (t test, $P < 0.05$).
**Figure 6.** Effects of acibenzolar-S-methyl (Actigard) on the expression of hormone-marker genes (SA: *PR1* and *AtICS1*, and ABA: *ABA8* and *AtNCED1*) and on resistance to TYLCV in R and S tomato and wt *Arabidopsis* plants. (A, B) qPCR analysis of hormone-related marker or biosynthesis genes associated with SA (A) and ABA (B) in uninfected tomato and *Arabidopsis* plants (0 dpi) that had or had been treated with Actigard, as compared with untreated control plants. The tomato *β*-actin gene and the *Arabidopsis* *Actin2* or *UBQ10* genes were used as calibrators. (C) qPCR estimation of relative amounts of TYLCV in infected, Actigard-treated tomato and *Arabidopsis* plants at 7 dpi as compared with untreated control plants. The numbers above the columns represent the values. Data points are means ± SE (*N* = 4 to 10). * indicates a significant difference (*t* test, *P* < 0.05).

**Figure 7.** Hierarchy in the expression of *TIP1;1, LeHT1* and *Lin6*. Gene expression in infected R [R(i)] and S [S(i)] tomato plants (blue and orange lines) and in infected R plants in which *TIP1;1* and *LeHT1* had been silenced [R(i):TRV-*TIP1;1* (black line) and R(i):TRV-*LeTH1* (black broken line), respectively]. (A) Expression of *TIP1;1*, (B) *LeHT1* (C) and *Lin6*. qPCR analysis was performed at 0, 1, 3 and 7 dpi. Tomato *β*-actin was used as the calibrator gene. Note that the expression of *LeHT1* and *Lin6* was inhibited in *TIP1;1*-silenced R plants, but the expression of *TIP1;1* and *Lin6* was not inhibited in *LeHT1*-silenced R plants. Data points are means ± SE (*N* = 4 to 9).

**Figure 8.** Hierarchical clustering of co-expression microarray data derived from *Arabidopsis* in the presence of several sources of biotic stress (the different pathogens
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Figure 9. Flow chart describing the possible role of TIP1;1 in hormone- and sugar-signaling leading to resistance to TYLCV in R and S plants. [1] Sade et al., 2012. [2] Fig. 1 and Supplemental Fig. S1. [3] Figs. 4, 5, 6 and S6 and Huang et al., 2012. [4] Proles et al., 2009. [5] Fig. 7. [6] Figs. 4, 5, 6 and S6. [7] Fig. 2.
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