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The Tomato DELLA Protein PROCERA Promotes Abscisic Acid Responses in Guard Cells by Upregulating an Abscisic Acid Transporter[1]

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Plants reduce transpiration through stomatal closure to avoid drought stress. While abscisic acid (ABA) has a central role in the regulation of stomatal closure under water-deficit conditions, we demonstrated in tomato (*Solanum lycopersicum*) that a gibberellin response inhibitor, the DELLA protein PROCERA (PRO), promotes ABA-induced stomatal closure and gene transcription in guard cells. To study how PRO affects stomatal closure, we performed RNA-sequencing analysis of isolated guard cells and identified the ABA transporters *ABA-IMPORTING TRANSPORTER1.1* (*AIT1.1*) and *AIT1.2*, also called *NITRATE TRANSPORTER1/PTR TRANSPORTER FAMILY4.6* in Arabidopsis (*Arabidopsis thaliana*), as being upregulated by PRO. Tomato has four *AIT1* genes, but only *AIT1.1* and *AIT1.2* were upregulated by PRO, and only *AIT1.1* exhibited high expression in guard cells. Functional analysis of *AIT1.1* in yeast (*Saccharomyces cerevisiae*) confirmed its activity as an ABA transporter, possibly an importer. A clustered regularly interspaced short palindromic repeats-Cas9–derived *ait1.1* mutant exhibited an increased transpiration, a larger stomatal aperture, and a reduced stomatal response to ABA. Moreover, *ait1.1* suppressed the promoting effects of PRO on ABA-induced stomatal closure and gene expression in guard cells, suggesting that the effects of PRO on stomatal aperture and transpiration are AIT1.1-dependent. Previous studies suggest a negative crosstalk between gibberellin and ABA that is mediated by changes in hormone biosynthesis and signaling. The results of this study suggest this crosstalk is also mediated by changes in hormone transport.

The growth-promoting hormone GA regulates central developmental processes throughout the plant life cycle, from germination to stem elongation, leaf expansion, flowering, and fruit development (Yamaguchi, 2008). GA also affects plant response to abiotic stresses, such as salinity and drought (Achard et al., 2006; Colebrook et al., 2014, Nir et al., 2017). The output of GA activity on plant development and response to the environment depends on complex interactions with other hormones (Weiss and Ori, 2007). The negative interaction between GA and the stress hormone abscisic acid (ABA) has been studied for many years in numerous plant species. These studies suggest that GA and ABA negatively affect each other's biosynthesis and signaling (Shu et al., 2018).

The nuclear DELLA proteins suppress almost all GA responses by interacting with various transcription factors (Hauvermale et al., 2012; Locascio et al., 2013). When GA binds to its receptor GIBBERELLIN-INSENSITIVE DWARF1 (GID1), it increases the affinity of the latter to DELLA. The generation of GID1-GA-DELLA complex leads to DELLA degradation via the ubiquitin-proteasome pathway, which is mediated by the F-box protein SLEEPY1 (Sasaki et al., 2003; Dill et al., 2004; Griffiths et al., 2006; Harberd et al., 2009; Hauvermale et al., 2012). DELLA destruction in the proteasome leads to transcriptional reprogramming and activation of GA responses. The ability of DELLA to interact with numerous transcriptional regulators is a key factor in the crosstalk between GA and other hormones. For example, DELLA interaction with JASMONATE ZIM DOMAIN proteins mediates the effect of GA on jasmonic acid (JA) activity (Hou et al., 2010), and its interaction with BRASSINAZOLE-RESISTANT1 mediates the crosstalk with brassinosteroids (Li et al., 2012).

The N-terminal region of DELLA (the DELLA domain) is important for the interaction with GID1 and therefore, mutations in this region interfere with the interaction (Harberd et al., 2009). These dominant, gain-of-function

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H.S. performed most of the experiments; Y.K. performed transport assays in yeast; N.I.-E. provided some of the materials used in this study; H.S., M.S., and D.W. designed the experiment and analyzed the experimental data; and H.S., M.S., and D.W. wrote the article.

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mutations stabilize DELLA, leading to constitutive inhibition of GA responses. The C-terminal region of DELLA (the GRAS domain) plays a major role in repressing GA responses by interacting with numerous transcription factors (Yoshida et al., 2014). Mutations in the C-terminal region are recessive, and exhibit constitutive GA responses (Sun and Gubler, 2004; Harberd et al., 2009). Tomato (*Solanum lycopersicum*) has one DELLA protein, called PROCERA (PRO; Jasinski et al., 2008; Livne et al., 2015). The tomato loss-of-function mutant *pro* is tall and exhibits increased GA responses (Van Tuinen et al., 1999; Bassel et al., 2008; Fleishon et al., 2011), whereas the gain-of-function *proGF* mutant is dwarf due to constitutive inhibition of GA responses (Zhu et al., 2019).

The crosstalk between GA and ABA has been studied for many years, mainly in seeds (Piskurewicz et al., 2008; Liu and Hu, 2018; Shu et al., 2018). The balance between the two hormones regulates dormancy versus germination; high ABA to GA ratio promotes dormancy, whereas the opposite promotes germination (Razem et al., 2006). The transcription factor ABSCISIC ACID-INSENSITIVE4 (ABI4) promotes seed dormancy in Arabidopsis (*Arabidopsis thaliana*) by the suppression of GA accumulation and the promotion of ABA biosynthesis (Shu et al., 2013). In *Sorghum bicolor*, *SbABI4* promotes the transcription of the GA deactivating gene *SbGA2ox3* (Cantoro et al., 2013). Moreover, the transcription factor GERMINATION INSENSITIVE TO ABA MUTANT2 promotes GA biosynthesis while

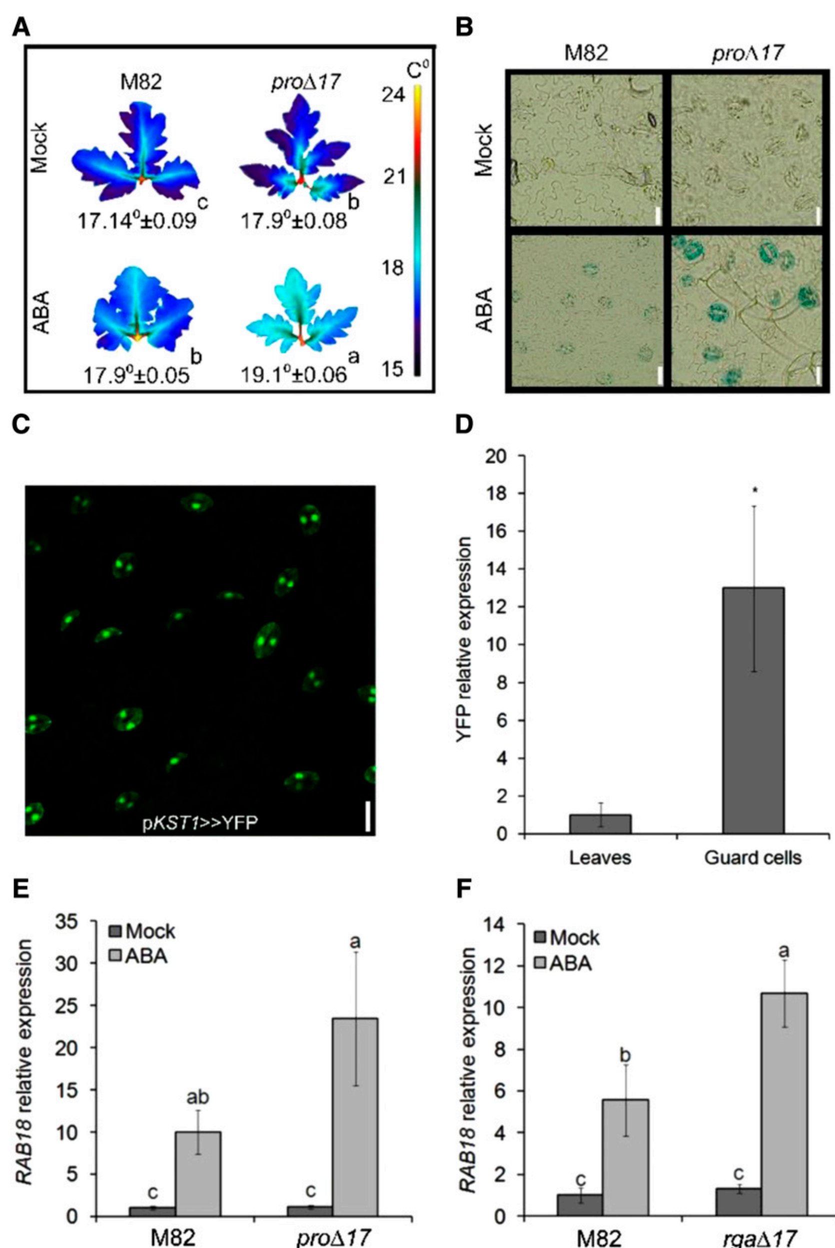

Figure 1. PRO promotes ABA responses in guard cells. A, Thermal imaging of leaves (leaf no. 4 below the apex) taken from M82 and *35S:pro*Δ*17* treated or not (Mock) with 10 μM of ABA. Leaves were digitally extracted for comparison. Number below leaves are the calculated leaf-surface temperature and the values are means of three plants, measured 20 times ± SE. Small letters above the numbers represent significant differences between respective treatments (Tukey–Kramer HSD test, $P < 0.05$). B, Representative images of GUS staining of epidermal peels treated or not (Mock) with 10 μM of ABA. Peels were taken from leaf no. 4 below the apex of M82 and *35S:pro*Δ*17* expressing the reporter GUS under the regulation of the *MAPKKK18* promoter. C, YFP signal in guard cells of *pKST1*>>*YFP* transactivated epidermal peel. Scale bars = 20 μm. D, YFP expression in whole leaf tissue and guard-cell–enriched samples. Values are means of four biological replicates ± SE. Stars above the columns represents significant differences between respective treatments by Student's *t* test ($P < 0.05$). E and F, RT-qPCR analysis of *RAB18* expression in guard-cell–enriched samples isolated from leaves no. 3 and 4 below the apex of M82 and *35S:pro*Δ*17* (E) or *35S:rga*Δ*17* (F). Values in E and F are means of four biological replicates ± SE. Small letters above the columns represent significant differences between respective treatments by Tukey–Kramer HSD ($P < 0.05$). The value for leaves in D was set to 1 and the value for M82 Mock in E and F was set to 1.

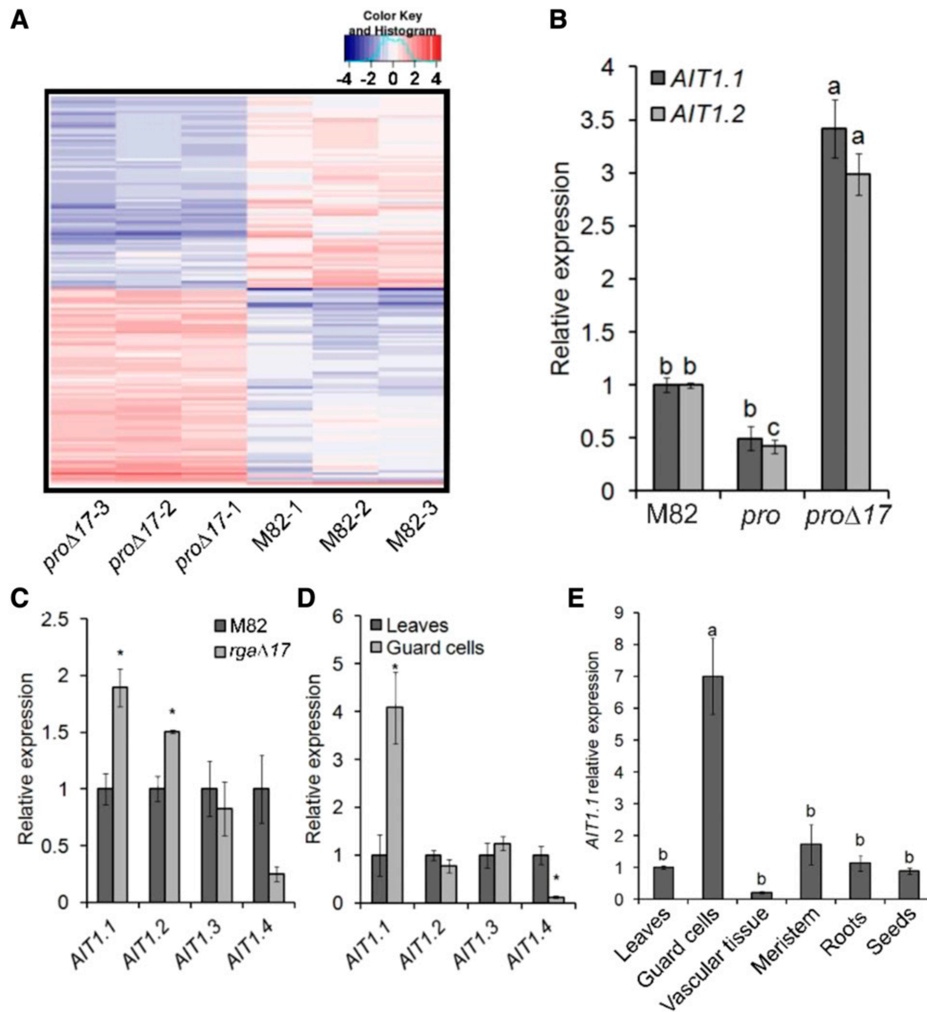

Figure 2. RNA-seq analysis identified the ABA transporter AIT1.1 as upregulated by PRO in guard cells. A, Clustered heatmap of PRO-regulated genes (*proΔ17* versus M82, three samples each) generated from RNA-seq analysis shows 81 PRO upregulated and 81 downregulated genes. Genes were grouped based on their pattern of expression. Coloring of the genes is according to the color bar on the upper-right side (Log2 fold change). The complete list of PRO-regulated genes is provided in Supplemental Dataset 1. B, RT-qPCR analysis of *AIT1.1* and *AIT1.2* expression in M82, *pro*, and *35S:proΔ17* (*proΔ17*) guard cells isolated from leaves no. 3 and 4 below the apex. Values are means of three biological replicates ± SE. Lowercase letters represent significant differences between lines by Tukey–Kramer HSD ($P < 0.05$). C, Expression of all tomato *AIT1* genes in M82 and *35S:rgaΔ17* (*rgaΔ17*) isolated guard cells. D, Expression of all tomato *AIT1* genes in leaves and isolated guard cells. E, Expression of AIT1.1 in different tissues: leaves (leaf no. 4 below the apex), guard cells, vascular tissue (isolated from leaf no. 4 below the apex), meristems (apices including leaf primordia), young roots, and imbibed seeds. Values in C, D and E are means of four replicates ± SE. Stars (C and D) and lowercase letters (E) above the columns represent significant differences between respective treatments by Student's *t* test ($P < 0.05$). The values for M82 in B and C were set to 1 and the values for leaves in D and E were set to 1.

reducing ABA production (Xiong et al., 2018). In Arabidopsis seeds, DELLA promotes the expression of the RING ubiquitin E3 ligase XERICO that is involved in ABA accumulation. It also increases the expression of the transcription factor *ABI5* that inhibits seed germination, and interacts with the ABA signaling components ABI3 (Lim et al., 2013). ABA, in turn, stabilizes the Arabidopsis DELLA protein REPRESSOR OF GA1-3 LIKE-2 and inhibits GA signaling (Piskurewicz et al., 2008). In tomato, the lack of DELLA activity in seeds suppresses desiccation tolerance due to inhibition of ABA-induced gene expression (Livne et al., 2015). Taken together, these studies suggest that GA and ABA negatively interact at the hormone biosynthesis and signaling levels (Shu et al., 2018).

Previously we suggested a crosstalk between GA/DELLA and ABA in the regulation of stomatal movement in tomato. Overexpressing the Arabidopsis *GA METHYLTRANSFERASE1* gene in tomato reduces GA levels and whole-plant transpiration (Nir et al., 2014). Transgenic tomato plants overexpressing the Arabidopsis or the tomato stable DELLA mutant proteins

rgaΔ17 or *proΔ17*, respectively, exhibit lower GA activity and reduced stomatal aperture and transpiration compared with wild-type controls. Overexpressing *proΔ17* specifically in guard cells was sufficient to reduce stomatal aperture. On the other hand, *pro* loss-of-function mutant plants exhibit increased transpiration rate, faster water loss under water-deficit conditions, and larger stomatal pore area (Nir et al., 2017). The effects of *proΔ17* on stomatal closure and water loss were suppressed in the ABA-deficient *sitiens* mutant, indicating that these effects of DELLA are ABA-dependent (Nir et al., 2017). We found that DELLA promotes ABA responses, including ABA-induced stomatal closure and reactive oxygen species accumulation in guard cells after ABA application. Because DELLA is a transcription regulator, it is yet unclear how PRO affects ABA-induced stomatal closure. PRO did not affect ABA accumulation in leaves, thus we speculated that it affects ABA signaling or uptake into guard cells via transcriptional regulation of ABA signaling component or transporter genes (Nir et al., 2017).

Several ABA transporters have been identified and characterized in Arabidopsis, including the ATP-BINDING CASSETTE (ABC) transporters ABCG25 and ABCG40, and ABA-IMPORTING TRANSPORTER1 (AIT1), also called NITRATE TRANSPORTER1.2 (NRT1.2), or NRT1/PTR TRANSPORTER FAMILY4.6 (NPF4.6). ABCG25 is expressed in vascular tissues and functions as an ABA exporter (Kuromori et al., 2010). ABCG40 is an ABA importer that was localized to the guard-cell plasma membrane (Kang et al., 2010). *AIT1* is expressed in the vascular tissues of inflorescence stems and the *ait1* mutant exhibited increased water loss due to open stomata (Kanno et al., 2012). Loss of *ABCG25* increases the sensitivity to ABA whereas the loss of *ABCG40* and *AIT1* reduce the sensitivity to the hormone (Kuromori et al., 2018).

Here we studied the mechanism by which the tomato DELLA protein PRO increases ABA responses in guard cells. RNA-sequencing (RNA-seq) analysis of isolated guard cells identified the ABA transporter AIT1.1 as upregulated by PRO. The loss of AIT1.1 suppressed the effect of PRO on guard-cell ABA responses.

RESULTS

PRO Promoted ABA Responses in Guard Cells

To support our previous suggestion that DELLA promotes ABA responses in guard cells (Nir et al., 2017), we tested the effect of PRO on ABA-inhibition of transpiration and ABA-induced gene expression in guard cells. Thermal imaging of M82 and transgenic plants overexpressing the stable DELLA protein *proΔ17* (*35S:proΔ17*; Nir et al., 2017) showed higher leaf-surface temperature in the transgenic line, after the application of ABA, indicating lower transpiration rate (Fig. 1A). To examine the effect of DELLA on ABA-induced transcription, we have generated transgenic M82 plants expressing the *GUS* reporter gene under the regulation of the Arabidopsis ABA-induced promoter *MAPKKK18* (Okamoto et al., 2013). The transgene was then introgressed into *35S:proΔ17* plants by crosses. The GUS signal in ABA-treated leaves was observed in guard cells and was stronger in *35S:proΔ17* compared to M82 (Fig. 1B). These results suggest that PRO promotes ABA physiological and transcriptional responses in guard cells.

Because DELLA is a transcription regulator, we hypothesized that PRO affects transpiration and stomatal movement by regulating the expression of ABA/stomatal-related genes in guard cells. To study the interaction between DELLA and ABA in the regulation of gene expression, we first developed a rapid and efficient guard-cell isolation protocol to minimize the

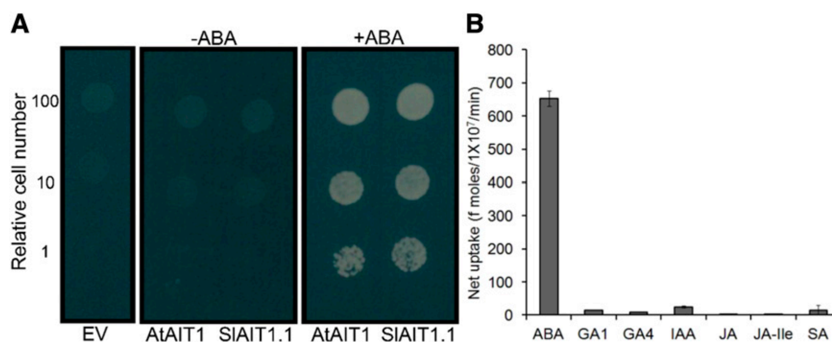

Figure 3. AIT1.1 mediates ABA uptake into yeast cells. A, Effects of AIT1.1 on the interactions between the ABA receptor and protein phosphatase 2C. Tomato AIT1.1 (*SlAIT1.1*) or Arabidopsis AIT1 (*AtAIT1*) was expressed in yeast containing a yeast two-hybrid system with the Arabidopsis PYR1 ABA receptor fused to the GAL4 DNA binding domain and the ABI1 protein phosphatase fused to the GAL4 activation domain, and the cells were inoculated on selection media (SD, -Leu, -Trip, -Ura, and -His) containing 0.5 μM of ABA (+ABA) or without ABA (−ABA). An empty vector (EV) was transformed as a negative control. Photos were taken 3 d after inoculation. B, Hormone transport activities of AIT1.1. Yeast cells expressing tomato AIT1.1 were incubated with solutions containing 10 μM of ABA, GA_1, GA_4, IAA, JA, JA-Ile, or salicylic acid (SA), and the amounts of compounds taken into the cells were quantified with LC-MS/MS.

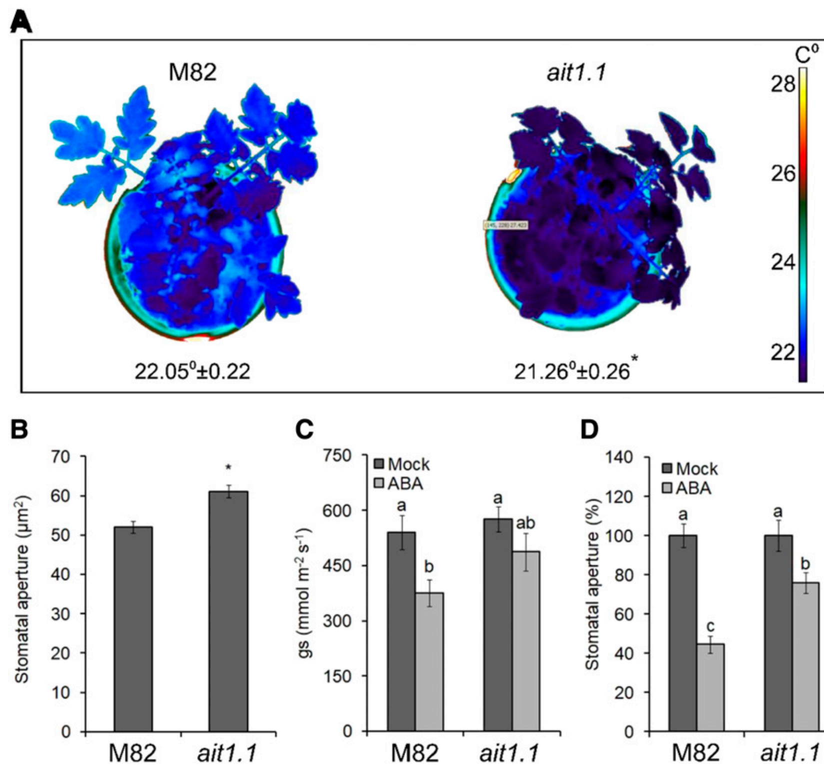

Figure 4. Loss of the ABA transporter *AIT1.1* increased transpiration and inhibited ABA responses in guard cells. A, Thermal imaging of M82 and CRISPR-Cas9–derived *ait1.1* mutant. Images were digitally extracted for comparison. Numbers below plants are the leaf-surface temperature, and the values are means of three replicates, each measured 20 times ± SE. Star above the number represents significant differences between lines by Student's *t* test ($P < 0.05$). B, Stomatal aperture measured on imprints of abaxial epidermis taken at 11:00 AM from leaves no. 3 and 4 below the apex. Values are means of four replicates, each with ~100 measurements (stomata) ± SE. Star above the column represents significant difference between respective treatments (Student's *t* test, $P < 0.05$). C, Stomatal conductance (g_s) in the fourth leaf below the apex in M82 and *ait1.1* plants, 1 h after treatment with 10 μM of ABA (or Mock). Values are means of six measurements taken from three different plants ± SE. D, Stomatal aperture in M82 and *ait1.1* epidermal peels (taken from leaves no. 3 and 4 below the apex) treated or not treated (Mock) with 10 μM of ABA. One hour after the ABA treatment, stomatal aperture was measured. Values are mean percentage of mock of four replicates, each with ~100 measurements (stomata) ± SE. Different letters above the columns in C and D represent significant differences between lines and treatments by Tukey–Kramer HSD test ($P < 0.05$).

effect of the isolation on gene expression (see "Materials and Methods"). To validate the procedure, we have used plants expressing the YELLOW FLUORESCENT PROTEIN (YFP) under the guard-cell–specific promoter *KST1* (Fig. 1C; Nir et al., 2017). Quantitative reverse-transcription PCR (RT-qPCR) analysis of RNA extracted from whole leaf tissue or isolated guard cells showed ~13-fold higher YFP expression in the guard-cells enriched samples (Fig. 1D). We then used this procedure to test the response of the ABA-induced gene *RAB18* (Nir et al., 2017) to ABA in guard-cell enriched samples taken from M82, *35S:pro*Δ*17*, and *35S:rga*Δ*17*. The expression of *RAB18* after ABA treatment was higher in the transgenic lines (Fig. 1, E and F).

Global Expression Response to PRO in Guard Cells

We next explored the mechanism by which DELLA promotes ABA-induced stomatal closure. To this end, we examined the global effect of PRO on guard-cell–transcriptional activity by performing RNA-seq analysis to guard-cell–enriched samples taken from M82, *35S:pro*Δ*17*, and *pro*. Using a twofold increase or decrease cutoff (adjusted *P* value for multiple comparisons ≤ 0.05), we identified 162 PRO-regulated genes (81 upregulated and 81 downregulated; Fig. 2A; Supplemental Dataset S1; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE143999). We then searched for differentially expressed genes related to stomatal closure and/or ABA. Among these genes (Supplemental Table S1), we identified three putative ABA transporters: the tomato homologs of the Arabidopsis *ABCG40* and two *AIT1* genes, also called in Arabidopsis *NPF4.6* or *NRT1.2* (Supplemental Fig. S1; Kanno et al., 2012). We named the tomato proteins AIT1.1 and AIT1.2. The expression of *ABCG40* and *AIT1.2* in the RNA-seq analysis was extremely low (Supplemental Table S1). It is worth noting that although we reported previously that two ABA receptors

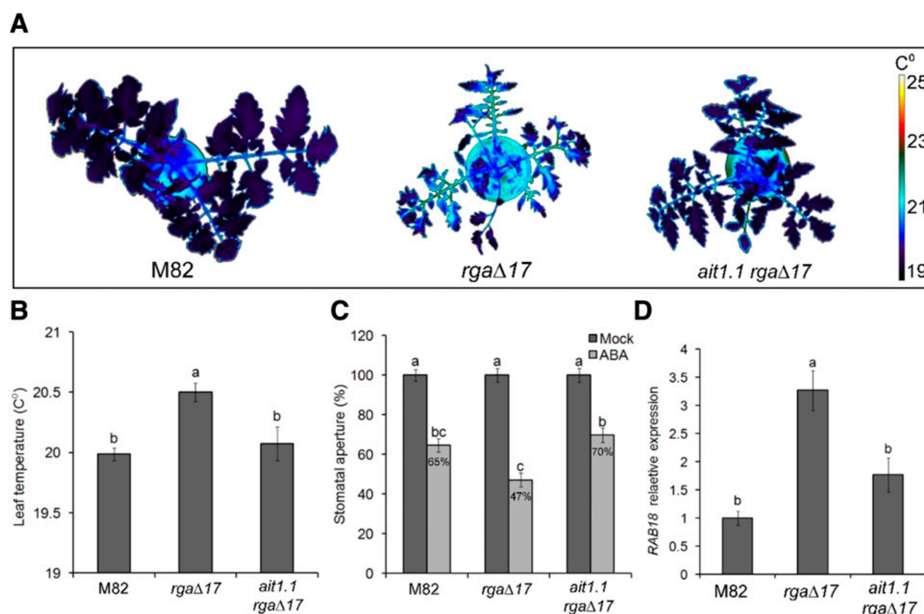

Figure 5. *ait1.1* suppressed the effect of PRO on ABA responses in guard cells. A, Thermal imaging of M82, *35S:rgaΔ17* (*rgaΔ17*), and *rgaΔ17 ait1.1*. Images were digitally extracted for comparison. B, Leaf-surface (leaves no. 3 and 4 below the apex) temperature of M82, *rgaΔ17*, and *rgaΔ17 ait1.1*. plants. Values are means of three replicates measured 20 times ± SE. C, Stomatal aperture in M82, *rgaΔ17*, and *rgaΔ17 ait1.1* epidermal peels (from leaves no. 3 and 4 below the apex) treated or not treated (Mock) with 10 μM of ABA. One hour after the ABA treatment, stomatal aperture was measured. Values are mean percentage of Mock of four replicates, each with ~100 measurements (stomata) ± SE. D, RT-qPCR analysis of *RAB18* expression in M82, *rgaΔ17*, and *rgaΔ17 ait1.1* guard cells, isolated from leaves no. 3 and 4 below the apex. Values are means of four biological replicates ± SE. Different letters above the columns in B and D represent significant differences between lines by Student's *t* test ($P < 0.05$). Different letters above the columns in C represent significant differences between lines and treatments by Tukey–Kramer HSD test ($P < 0.05$). The values for M82 were set to 1.

PYRABACTIN RESISTANCE1(*PYR1*) and *PYR1-like8-1* are upregulated in *35S:proΔ17* (Nir et al., 2017), in the RNA-seq analysis we did not find them among the differentially expressed genes.

We first validated the results of the RNA-seq for the effect of PRO on the expression of *ABCG40*, *AIT1.1*, and *AIT1.2* in guard-cell–enriched samples by RT-qPCR. This analysis did not confirm the effect of PRO on *ABCG40* (Supplemental Fig. S2), but it did confirm PRO's effect on *AIT1.1* and *AIT1.2*. These genes were up- and downregulated in *35S:proΔ17* and *pro*, respectively (Fig. 2B). Tomato has four *AIT1* homologs that we named *AIT1.1* to *AIT1.4* (Supplemental Fig. S1). We analyzed the expression of all four *AIT1* homologs in M82 and *35S:rgaΔ17* guard cells and only *AIT1.1* and *AIT1.2* were upregulated by DELLA in guard cells (Fig. 2C). We then analyzed the expression of all *AIT1s* in M82 guard-cell–enriched samples compared to whole leaf tissue, and only *AIT1.1* exhibited significantly higher expression in guard cells (Fig. 2D). Kanno et al. (2012) found that the Arabidopsis *AIT1* is expressed in the vascular tissue, using the reporter line. We used the same approach and generated transgenic M82 plants expressing the GUS reporter under the regulation of the *AIT1.1* promoter (~1,400 bp upstream of the start codon). The tomato *AIT1.1* also showed high GUS activity in vascular tissues (Supplemental Fig. S3), but not in guard cells. We therefore analyzed *AIT1.1* expression in various tissues of M82 plants by RT-qPCR (mature leaves, shoot apices, vascular tissue, guard cells, roots, and imbibed seeds), and found the highest expression in guard cells (Fig. 2E). These results suggest that the promoter used to express GUS did not provide the authentic spatial expression pattern.

PRO Promoted ABA Responses via the ABA Transporter *AIT1.1*

Functional analysis of the Arabidopsis AIT1 protein in yeast cells suggests that it operates as an ABA importer (Kanno et al., 2012). When expressed in yeasts, (*Saccharmyces cerevisiae*) the tomato AIT1.1 induced the interactions between the Arabidopsis ABA receptor PYR1 and the protein phosphatase 2C ABI1 under a relatively low ABA concentration (0.5 μM) in the growth media, as the Arabidopsis AIT1 protein did, while the interactions were not observed in the absence of ABA (Fig. 3A). We further confirmed that AIT1.1 mediated cellular ABA uptake by directly quantifying the molecules taken into the yeast cells by liquid chromatography tandem mass-spectrometry (LC-MS/MS; Fig. 3B). We also examined the substrate selectivity of AIT1.1 against several other hormones. It appeared that AIT1.1

transported GA1, GA4, and indole-3-acetic acid (IAA) to some extent; however, the selectivity was much lower compared to ABA (Fig. 3B).

Although *AIT1.2* was upregulated by PRO, it showed very low expression levels (~20-fold lower than *AIT1.1*; Supplemental Table S1) and its expression in guard cells was similar to whole leaf tissue. We therefore focused further on *AIT1.1*. We generated clustered regularly interspaced short palindromic repeats-Cas9 (CRISPR-Cas9)–derived *ait1.1* mutant and obtained two independent alleles. Both mutations caused a frame shift and premature stop codon (Supplemental Fig. S4). Homozygous plants of the two lines exhibited mild growth suppression (Supplemental Fig. S5, A and B). Thermal imaging showed lower leaf-surface temperature in *ait1.1*, suggesting that transpiration in *ait1.1* is higher than in M82 (Fig. 4A; Supplemental Fig. S5C). The *ait1.1* plants exhibited larger stomatal aperture (Fig. 4B; Supplemental Fig. S5D), higher stomatal conductance (measured as g_s) after ABA treatment (Fig. 4C), and partial inhibition of stomatal closure in response to application of ABA to epidermal peels (Fig. 4D).

We then introgressed the *35S:rgaΔ17* transgene into the *ait1.1* background by crosses and generated homozygous *ait1.1* plants overexpressing *rgaΔ17*. We confirmed the presence of the transgene (*35S:rgaΔ17*) by the phenotype (shorter stem and smaller, darker, and more serrated leaves; Supplemental Fig. S6), and the *ait1.1* mutation by sequencing. Stable DELLA (*35S:rgaΔ17*) inhibited germination, but the loss of *AIT1.1* suppressed this effect and promoted germination (Supplemental Fig. S7), suggesting that the inhibition effect of DELLA on germination in tomato (Zhu et al., 2019) is AIT1.1-dependent. We next tested the effect of the mutation on DELLA activity in guard cells. The loss of *AIT1.1* suppressed the effect of stable DELLA overexpression on transpiration as indicated by the lower leaf-surface temperature in *35S:rgaΔ17 ait1.1* plants compared to *35S:rgaΔ17* (Fig. 5, A and B; Supplemental Fig. S8). In addition, stomatal response to ABA in epidermal peels, and the expression of the ABA-induced gene *RAB18* in isolated guard cells, were suppressed in *35S:rgaΔ17 ait1.1* compared to *35S:rgaΔ17* (Fig. 5, C and D). These results suggest that AIT1.1 is required for DELLA to promote ABA responses in guard cells.

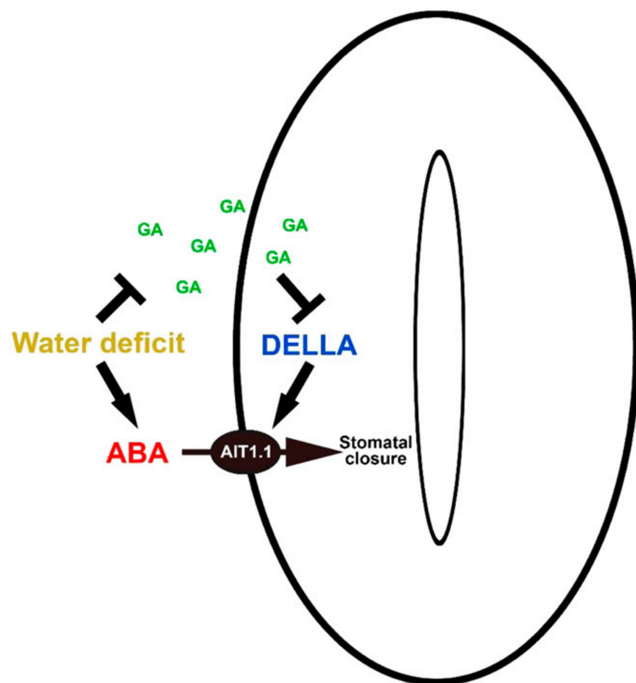

Figure 6. Suggested model of the crosstalk between GA and ABA in guard cells. Water-deficit conditions suppress GA accumulation (Colebrook et al., 2014), leading to DELLA (PRO) stabilization. In guard cells, PRO promotes the expression of the ABA importer *AIT1.1*, facilitating ABA uptake into guard cells, and stomatal closure.

DISCUSSION

Drought avoidance is a major plant-adaptation strategy to survive transient water-deficit conditions (Kooyers, 2015). To avoid dehydration, plants close their stomata to reduce transpiration and can use the available water in the soil more slowly and for a longer time before the next rain comes (Martin-St Paul et al., 2017; Gupta et al., 2020). While ABA has a major role in the regulation of transpiration under water-deficit conditions, accumulating evidence suggests that GA antagonizes these ABA responses. Increased GA levels or activity promote stomatal opening in *Commelina benghalensis*, *Vicia faba*, Arabidopsis, and tomato (Santakumari and Fletcher, 1987; Göring et al., 1990; Nir et al., 2017; Sukiran et al., 2020). Reduced GA activity and DELLA accumulation suppress canopy expansion and xylem hydraulic conductivity and promotes stomatal closure, all leading to lower transpiration (Nir et al., 2014, 2017; Illouz-Eliaz et al., 2020). It was suggested that water deficiency inhibits GA accumulation to promote adaptation to drought (Colebrook et al., 2014).

Because ABA-induced stomatal closure is mediated by the phosphorylation of ion channels and not by activation of gene transcription (Cutler et al., 2010; Kim et al., 2010; Munemasa et al., 2015), it was unclear how the transcriptional regulator DELLA (PRO) affects ABA-induced stomatal closure. We hypothesized that PRO affects the transcription of either the ABA signaling component or the ABA transporter (Nir et al., 2017). RNA-seq analysis of isolated guard cells (M82, *35S:proΔ17*, and *pro*) identified the ABA transporter *AIT1.1* as upregulated by PRO. The Arabidopsis *AIT1*, also called *NRT1.2* or *NPF4.6*, is an ABA importer (Kanno et al., 2012). This gene is also upregulated by DELLA in the Arabidopsis shoot apical meristems (Serrano-Mislata et al., 2017). The Arabidopsis *ait1* mutant exhibited increased water loss due to open stomata (Kanno et al., 2012); however, expression

analysis (based on reporter line) suggests that AIT1 is active in the vascular tissue of inflorescence stems, but not in stomata. Although the promoter:*GUS* line suggested that the tomato *AIT1.1* gene is also active in the vascular tissue, RT-qPCR analysis showed that the highest expression levels of this gene is in guard cells. This, together with the open-stomatal phenotype of the *ait1.1* mutant, suggest that protein function in tomato to be an ABA transporter, possibly as an importer, in guard cells. The source for ABA that stimulates stomatal closure under water-deficit conditions is still not clear. Several studies suggest that ABA is produced in the phloem companion cells and/or in guard cells (Bauer et al., 2013; Kuromori et al., 2014; Merilo et al., 2018). Other studies suggest that the leaf mesophyll cells are the major site of ABA production under water-deficit conditions (McAdam and Brodribb, 2018). The localization of AIT1.1 in guard cells and its possible activity as an importer, regulating ABA influx into guard cells, supports the hypothesis that at least part of the ABA that stimulates stomatal closure in response to drought comes from other cells—either leaf mesophyll or companion cells.

Because *ait1.1* mutant exhibited only partial inhibition of stomatal closure in response to exogenous ABA, AIT1.1 is probably not the only ABA transporter in tomato guard cells. Still, among the AIT1 group of transporters, AIT1.1 is the most dominant one, based on its expression level. In Arabidopsis, an importer from another group, the ABC transporter ABCG40, is active in guard cells (Kang et al., 2010; Kuromori et al., 2014). We identified the *ABCG40* homolog in the RNA-seq analysis of isolated guard cells as PRO-upregulated. However, this was not confirmed by RT-qPCR analysis. Thus, ABCG40 may contribute to ABA uptake into tomato guard cells, but probably does not mediate the effect of PRO on guard-cell ABA responses.

The up- and downregulation of *AIT1.1* by stable PRO and *pro* loss of function, respectively, and the suppression of PRO-promoted ABA responses in *ait1.1* guard cells, suggests that *AIT1.1* mediates the effect of PRO on ABA-induced stomatal closure. It is possible that water deficiency reduces the levels of active GAs (Colebrook et al., 2014), leading to PRO accumulation in guard cells (Nir et al., 2017). PRO promotes the expression of the ABA importer *AIT1.1*, facilitating ABA uptake into guard cells, leading to faster stomatal closure (Fig. 6).

The crosstalk between GA and ABA has been investigated for many years (Weiss and Ori, 2007; Shu et al., 2018). This crosstalk is largely dependent on DELLA; DELLA promotes ABA synthesis and signaling, and ABA promotes DELLA stability—and therefore inhibits GA signaling (Achard et al., 2006; Piskurewicz et al., 2008; Lim et al., 2013; Liu and Hou, 2018). Here we bring evidence that PRO (DELLA) promotes ABA responses in guard cells via the upregulation of the ABA transporter *AIT1.1*, suggesting that the crosstalk between GA and ABA is mediated at multiple levels through hormone biosynthesis, signaling, and transport.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Hormone Treatments

Tomato (*Solanum lycopersicum*) plants in M82 background (*sp/sp*) were used throughout this study. The *pro* mutant was in the M82 background (Fleishon et al., 2011). The CRISPR-derived *ait1.1* mutant and the transgenic lines 35S:*rga*Δ*17* (Livne et al., 2015), 35S:*pro*Δ*17*, *pKST1*:LhG4, OP:YFP (Nir et al., 2017), and *pMAPKKK18*:GUS were generated in the M82 background. Plants were grown in a growth room set to a photoperiod of 12/12-h night/day, light intensity of 200 μmol m^{-2} s^{-1} and 25°C, and irrigated to saturation. In other experiments, plants were grown in a greenhouse under natural day-length conditions, light intensity of 700 to 1,000 μmol m^{-2} s^{-1}, and temperature of 18°C to 30°C. The seeds were harvested from ripe fruits and treated with 1% (v/v) sodium hypochlorite followed by 1% (w/v) Na_3PO_4 and 12 water, and incubated with 10% (w/v) Suc overnight in 37°C. Seeds were stored dry at room temperature. (±)-ABA dissolved in DMSO (Sigma-Aldrich) was applied to plants by spraying.

CRISPR/Cas9 Mutagenesis, Tomato Transformation, and Selection of Mutant Alleles

Four single-guide RNAs (sgRNAs; Supplemental Table S2) were designed to target the *AIT1.1* gene, using the CRISPR-P tool (http://cbi.hzau.edu.cn/crispr). Vectors were assembled using the Golden Gate cloning system, as described by Weber et al. (2011). Final binary vectors, pAGM4723, were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. The constructs were transferred into M82 cotyledons using transformation and regeneration methods described by McCormick (1991). Kanamycin-resistant T0 plants were grown and independent transgenic lines were selected and self-pollinated to generate homozygous transgenic lines. The genomic DNA of each plant was extracted, and genotyped by PCR for the presence of the Cas9 construct. The CRISPR-Cas9–positive lines were further genotyped for mutations using a forward primer to the upstream sequence of the sgRNA1 target and a reverse primer downstream of the sgRNA2 target sequence. The target genes in all mutant lines were sequenced. Several homozygous and heterozygous lines were identified, and independent mutant lines for each gene were selected for further analysis. The Cas9 construct was segregated out by crosses to M82.

Molecular Cloning/Construct and Transactivation

To generate the ABA-reporter transgenic plants, the plasmid containing the *AtMAPKKK18* promoter were kindly supplied by Assaf Mosquna (Okamoto et al., 2013). To generate *pMAPKKK18:GUS* the *MAPKKK18* promoter was inserted into the pRITA vector downstream to the GUS start codon into the *Kpn*I and *Bam*HI sites (Steiner et al., 2012). Primers sequences for cloning are presented in Supplemental Table S3. This construct was then cloned into the pART27 binary vector and introduced to the *A. tumefaciens* strain GV3101 by electroporation to generate transgenic M82 tomato plants (as described above). To specifically express YFP in guard cells, the LhG4 transactivation system (Moore et al., 1998) with the *KST1* promoter was used, with *pKST1*:LhG4 as the driver line and OP:YFP as the responder line. The cross between these lines generated the transactivated line *pKST1*>>YFP.

pAIT1.1:*GUS* Molecular Cloning

The 1,380 bp upstream to the *AIT1.1* start codon were amplified by PCR (−49 to −1,429). *pAIT1.1* was inserted into the *Kpn*I and *Bam*HI sites in the pRITA vector downstream to the GUS start. Primers sequences for cloning are presented in Supplemental Table S3. This construct was then cloned into the pART27 binary vector and introduced to *A. tumefaciens* strain GV3101 by electroporation to generate transgenic M82 tomato plants (as described above).

Library Constructions and Sequencing

Total RNA was extracted from isolated guard cells using an RNeasy Plant Mini Kit (Qiagen). Libraries were prepared at the Crown Institute for Genomics (The Nancy & Stephen Grand Israel National Center for Personalized Medicine [G-INCPM], Weizmann Institute of Science). Five-hundred nanograms of total RNA for each sample were processed using the in-house poly-A–based

RNA-seq protocol. Libraries were evaluated with the tools Qubit and TapeStation (the INCPM Unit, Weizmann Institute of Science). Sequencing libraries were constructed with barcodes to allow multiplexing of nine samples on one lane of a HiSeq 2500 machine (Illumina), using the Single-Read 60 protocol (v4), yielding a median of 29.4 million reads per sample.

Sequence Data Analysis

Stretches of Poly-A/T and Illumina adapters were trimmed from the reads using the tool Cutadapt (Martin, 2011); resulting reads shorter than 30 bp were discarded. Reads were mapped to the *S. lycopersicum* reference genome (release SL3.00) using the program STAR (with "End-To-End" option and "out Filter Mismatch Nover Lmax = 0.04"; Dobin et al., 2013). The annotation file was downloaded from the International Tomato Genome Sequencing Project (3.2; Sol Genomics). Expression levels for each gene were quantified with the program htseq-count (Anders et al., 2015), using the gtf file given above (in "Union" mode). Differential expression gene analysis was performed using the program DESeq2 (Love et al., 2014) with the "betaPrior," "cooksCutoff," and independent filtering parameters set to "False." Raw *P* values were adjusted for multiple testing using the procedure of Benjamini and Hochberg (1995). The RNA-seq data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through Gene Expression Omnibus Series accession number GSE:143999 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE143999).

RNA Extraction and cDNA Synthesis

Total RNA was extracted with an RNeasy Plant Mini Kit (Qiagen). For synthesis of cDNA, SuperScript II reverse transcriptase (18064014; Invitrogen) and 3 mg of total RNA were used according to the manufacturer's instructions.

RT-qPCR Analysis

RT-qPCR analysis was performed using an Absolute Blue qPCR SYBR Green ROX Mix kit (AB-4162/B; Thermo Fisher Scientific). Reactions were performed using a Rotor-Gene 6000 Cycler (Corbett Research). A standard curve was obtained using dilutions of the cDNA sample. The expression was quantified using the Rotor-Gene's software (Corbett Research). Three independent technical repeats were performed for each sample. Relative expression was calculated by dividing the expression level of the examined gene by that of *SlACTIN*. The target-gene-to-*ACTIN* ratio was then averaged. All primer sequences are presented in Supplemental Table S3.

Isolation of Guard Cells

Fully expanded leaves (leaf no. 3 or 4 below the apex) without the central veins were ground twice, for 60 s each time, in a blender containing 100 mL of cold distilled water. The blended mixture was poured onto a 100-μm nylon mesh (Sefar) and the remaining epidermal peels were rinsed thoroughly with 0.5 L of cold deionized water. The peels were then transferred into 2-mL tubes and frozen in liquid nitrogen.

Microscopy and Confocal Imaging Analysis

Imaging was done using a confocal laser scanning microscope model SP8 (Leica Microsystems) with an HCX PL APO CS 20×/0.70 dry objective (Leica Microsystems) for YFP in epidermal strips. YFP was excited with the 514-nm laser line, and the 520- to 560-nm filter was used for emission. Images were later analyzed using the "Fit-Ellipse" tool from the software ImageJ (http://rsb.info.nih.gov/ij/).

Thermal Imaging

Thermal images were obtained using a model no. A655sc FOV 15 camera (FLIR Systems). The camera was mounted vertically about 50 cm above the plants. Mean temperature of leaflets from leaves no. 3 and 4 below the apex were calculated using the customized region-of-interest tool, according to the manufacturer's instructions.

GUS Staining

Histochemical detection of GUS activity was performed using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide as described in Donnelly et al. (1999). Samples were put on glass coverslips and photographed under a model no. ICC50 W bright-field inverted microscope (Leica Microsystems). Images were later analyzed using the software ImageJ. A microscopic ruler (Olympus) was used for size calibration.

Transport Assays

Yeast two-hybrid assays were performed using the ProQuest Two-Hybrid System with Gateway Technology (Thermo Fisher Scientific). Arabidopsis (*Arabidopsis thaliana*) *ABI1* and *PYR1* cDNAs cloned in pENTR-D-TOPO were introduced into pDEST22 and pDEST32, respectively, by LR recombination reactions. Tomato *AIT1.1* cloned in pENTR-D-TOPO was introduced into pYES-DEST52 in which the GAL1 promoter had been replaced with the *AHD* promoter (Kanno et al., 2012). Arabidopsis *AIT1* was cloned in pYES-DEST52 as in a previous study (Kanno et al., 2012). The pDEST22, pDES32, and pYES-DEST52 constructs were transformed into a yeast (*Saccharmyces cerevisiae*) strain BY20249. Several (~10) independent colonies were mixed and precultured in media (synthetic defined [SD], -Leu, -Trp, and -Ura) overnight at 30°C and then inoculated on selection media (SD, -Leu, -Trp, -Ura, -His, and 1 mM of 3AT) with (+) or without (−) ABA.

For direct measurements of transport activities by LC-MS/MS, AIT1.1 was cloned into the standard pYES-DEST52 vector and transformed into a yeast strain INVSc1. Assays were performed as described in Kanno et al. (2016). Hormones were extracted from yeast cells with 1 mL of acetone containing 1% (v/v) acetic acid, and the supernatants after centrifugation were dried with N_2 gas. The extracts were dissolved in 1 mL of water containing 1% (v/v) acetic acid and loaded onto a cartridge column (1-cc Oasis Wax; Waters) that had been pretreated with 0.5 mL of acetonitrile and then with water containing 1% (v/v) acetic acid. After washing with 1 mL of water containing 1% (v/v) and then with 80% (v/v) acetonitrile, ABA, GA_1, GA_4, IAA, JA, and JA-Ile were eluted with 80% (v/v) acetonitrile containing 1% (v/v) acetic acid. Salicylic acid was then eluted with 2 mL of acetonitrile containing 5% (v/v) formic acid. The eluents containing hormones were dried with N_2 gas and then dissolved in 50 μL of water containing 1% (v/v) acetic acid to be analyzed by LC-MS/MS. LC-MS/MS analysis was performed as described in Kanno et al. (2016).

Stomatal Aperture Measurements

Stomatal aperture was determined using the rapid imprinting technique described by Geisler et al. (2000). Light-bodied vinylpolysiloxane dental resin (eliteHD+; Zhermack Clinical) was attached to the abaxial side of the terminal leaflet (leaf no. 4 below the apex) and then removed as soon as it dried (minutes). The resin epidermal imprints were covered with transparent nail polish, which was removed after it dried, and served as a mirror image of the resin imprint. The nail-polish imprints were put on glass coverslips and photographed under a light microscope, as described above.

Stomatal Response to ABA

Abaxial epidermal peels taken from leaf no. 4 below the apex were incubated in stomatal opening buffer, containing 20 mM of potassium chloride and 5 mM of MES at pH 6.15 (Wigoda et al., 2006) for 2 h under light conditions (400 μmol m^{-2} s^{-1}). The peels were then transferred to a fresh stomatal opening buffer with or without 10 μM of ABA, under the same light conditions. After 60 min, the peels were put on glass coverslips and photographed under the bright-field inverted microscope as described above. Stomatal images were later analyzed using the software ImageJ for stomatal aperture measurements as described above.

Germination Assay

Fresh seeds were germinated in petri dishes on Murashige & Skoog medium in a growth room set to a photoperiod of 12/12-h night/day, with a light intensity of 200 μmol m^{-2} s^{-1}, and at a temperature of 25°C. Germination was scored when the radicle pierced the seed coat.

Statistical Analyses

All assays were conducted with three or more biological replicates and analyzed using the software JMP (SAS Institute). Means comparison was conducted using ANOVA with post hoc Tukey–Kramer Honestly Significant Difference (HSD; for two comparisons) and Student's *t* tests (for one comparison; $P < 0.05$). Summary of the statistical parameters, and significant differences, are presented in Supplemental Table S4.

Accession Numbers

Sequence data from this article can be found in the Sol Genomics Network (https://solgenomics.net/) under the following accession numbers: *ACTIN*, Solyc11g005330; *RAB18*, Solyc05g053600 *ABCG40*, Solyc02g084850; *AIT1.1*, Solyc05g006990; *AIT1.2*, Solyc05g007000; *AIT1.3*, Solyc04g005790; and *AIT1.4*, Solyc03g113250.

Supplemental Data

The following supplemental data are available.

Supplemental Figure S1. Molecular phylogenetic analysis of AIT1 protein sequences in tomato and Arabidopsis.

Supplemental Figure S2. PRO activity has no effect on the expression of the tomato *ABCG40* homolog.

Supplemental Figure S3. *AIT1.1* promoter drives expression in the vascular tissue.

Supplemental Figure S4. Sequence analyses of *ait1.1* no. *1* and *ait1.1* no. *7* CRISPR mutant alleles.

Supplemental Figure S5. Loss of *AIT1.1* suppresses growth, and increases transpiration and stomatal aperture.

Supplemental Figure S6. Plant and leaf phenotypes of 35S:*rgaΔ17 ait1.1*.

Supplemental Figure S7. Loss of *AIT1.1* promotes germination of 35S:*rgaΔ17* seeds.

Supplemental Figure S8. Thermal imaging of M82, 35S:*rgaΔ17*, and 35S:*rgaΔ17 ait1.1* leaves.

Supplemental Table S1. ABA- and guard-cell–related PRO-regulated genes (fold change > ±2).

Supplemental Table S2. Guide RNAs used in this study.

Supplemental Table S3. Primers used in this study.

Supplemental Table S4. Summary of statistical parameters and significant differences.

Supplemental Dataset S1. Results of the RNA-seq analysis.

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