The Tomato DELLA Protein PROCERA Acts in Guard Cells to Promote Stomatal Closure

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\textbf{Short title:} DELLA promotes stomatal closure

\textbf{One-sentence summary:} The tomato DELLA protein PROCERA, a negative regulator of gibberellin signaling, increases the sensitivity of guard cells to abscisic acid, leading to stomatal closure and increased drought tolerance.

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\textbf{ABSTRACT}

Plants employ stomatal closure and reduced growth to avoid water deficiency damage. Reduced levels of the growth-promoting hormone gibberellin (GA) lead to increased tolerance to water deficit, but the underlying mechanism is unknown. Here, we show that the tomato (\textit{Solanum lycopersicum}) DELLA protein PROCERA (PRO), a negative regulator of GA signaling, acts in guard cells to promote stomatal closure and reduce water loss in response to water deficiency by increasing abscisic acid (ABA) sensitivity. The loss-of-function \textit{pro} mutant exhibited increased stomatal conductance and rapid wilting under water-deficit stress. Transgenic tomato overexpressing constitutively active stable DELLA proteins (S-\textit{della}) displayed the opposite phenotype. The effects of S-\textit{della} on stomatal aperture and water loss were strongly suppressed in the ABA-deficient mutant \textit{sitiens} (\textit{sit}), indicating that these effects of S-\textit{della} are ABA-dependent. While DELLA had no effect on ABA levels, guard cell ABA responsiveness was increased in S-\textit{della} and reduced in \textit{pro} plants compared to wild type. Expressing S-\textit{della} under the control of a
guard-cell-specific promoter was sufficient to increase stomatal sensitivity to ABA and to reduce water loss under water-deficit stress but had no effect on leaf size. This result indicates that DELLAs promote stomatal closure independently of its effect on growth.

INTRODUCTION

Water deficit has a marked impact on plant development and productivity, as expressed by the suppression of growth, flowering and fruit development (Zhu, 2002; Chaves et al., 2003; Munns and Tester, 2008). Plants have adopted various strategies to cope with drought, including maintaining their water status by rapid stomatal closure and altered growth and development. Both rapid stomatal movement and integrated growth plasticity involve long-distance communication between different organs, which is primarily mediated by the stress-related hormone abscisic acid (ABA; Munns, 2002; Sachs, 2005). Accumulating evidence also suggests that reduced gibberellin (GA) activity promotes plant tolerance to water-deficit stress (Magome et al., 2004; Magome et al., 2008; Li et al., 2012; Nir et al., 2014; Colebrook et al., 2014). GA-dependent responses are inhibited by nuclear proteins known as DELLAs, which interact with and regulate numerous transcription factors (Locascio et al., 2013). DELLAs, which are a subgroup of the GRAS transcription factor family, can transactivate transcription, but they lack a DNA binding domain (Hirano et al., 2012). When GA levels increase, this hormone binds to its receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1), which in turn interacts with DELLAs. This complex binds to SCF E3 ubiquitin ligase via a specific F-box protein, and DELLAs are polyubiquitinated and then degraded by the 26S proteosome to relieve GA responses (Harberd et al., 2009, Hauvermale et al., 2012).

The DELLAs N-terminal region, which contains the conserved DELLA and VHYNP motifs (Locascio et al., 2013), interacts with GID1 to form the GID1-GA-DELLA complex (Murase et al., 2008). The C-terminal region includes the GRAS domain and plays a major role in repressing GA responses (Sun et al., 2012). Various gain-of-function, dominant mutations affecting the N-terminal region of DELLAs interfere with GA-induced DELLAs.
degradation by blocking the integration of DELLA into the GID1-GA complex (Harberd et al., 2009). These mutant proteins are stable (S-della), and therefore, constitutively active. By contrast, null and loss-of-function, recessive mutations in the DELLA genes lead to a constitutive GA-response phenotype (Sun and Gubler, 2004; Weston et al., 2008; Harberd et al., 2009). Tomato (Solanum lycopersicum) has only one DELLA gene, called PROCERA (PRO) (Jasinski et al., 2008; Livne et al., 2015). Tomato plants homozygous for the weak loss-of-function pro mutation exhibit increased GA responses but also retain some responsiveness to GA (Jones 1987; Jupe et al., 1988; Van Tuinen et al., 1999; Bassel et al., 2008; Fleishon et al., 2011). Recently, we identified a GA-insensitive, null mutant (proGRAS), with a mutation that likely truncates the protein prior to the GRAS domain (Livne et al., 2015). We found that seeds of this mutant are highly sensitive to desiccation and proposed that the loss of DELLA activity in seeds reduces ABA-induced desiccation tolerance.

Osmotic stress suppresses GA levels and signaling activity (Achard et al., 2006; Wang et al., 2008; Colebrook et al., 2014), which, in turn, promotes tolerance to the abiotic stresses, including drought (Magome et al., 2004; Achard et al., 2006; Shan et al., 2007; Li et al., 2012; Colebrook et al., 2014). While the mechanism of this stress tolerance is not fully understood, indirect effects on transpiration due to decreased plant size (Magome et al., 2008; Achard et al., 2006), activation of various stress-related genes (Wang et al., 2008; Tuna et al., 2008) and suppression of reactive oxygen species (ROS) accumulation (Achard et al., 2008) have been suggested. We recently showed that reducing GA levels in tomato by overexpressing GA METHYLTRANSFERASE1 (GAMT1) increased tolerance to water-deficit stress (Nir et al., 2014). The transgenic plants maintained higher leaf water content for a longer period under water-deficit conditions than wild type due to reduced whole-plant transpiration. While the reduced transpiration was associated with smaller leaves, we noted that the stomatal aperture of the transgenic plants was reduced, raising the possibility that GA may also regulate transpiration directly in guard cells. This hypothesis is also in line with the previously reported role for GA as a positive regulator of stomatal opening (Santakumari and Fletcher, 1987; Göring et al., 1990).
In this study, we investigated the role of guard cell GA signaling in regulating transpiration by examining the responses of guard cell to water deficiency in DELLAla mutants and transgenic tomato plants expressing S-della proteins. DELLAla affected the guard cell’s response to ABA and promoted stomatal closure in response to soil water deficit. Moreover, while increasing DELLAla activity in guard cells alone did not affect leaf size, it was sufficient to reduce stomatal aperture and delay the negative effects of water limitation. These results suggest that DELLAla increases the sensitivity of guard cells to ABA, leading to earlier stomatal closure.

RESULTS

The tomato pro mutant exhibits increased transpiration

To examine DELLAla's effect on plant water status, we tested the response of the DELLA loss-of-function tomato mutant pro (Bassel et al., 2008) to water deficit stress. Control (M82) and pro seedlings were grown until they produced six expanded leaves, after which irrigation was stopped to induce dehydration. Before the onset of the water-deficit treatment, all plants were irrigated to saturation. After 4 days, non-irrigated pro plants began to wilt, while control plants remained turgid (Figure 1A). Four days after the initiation of the drought treatment, the relative water content (RWC) of leaves was reduced in pro by approximately 30% and in M82 by approximately 10% (Figure 1B).

We then examined whether the rapid water loss in pro plants was caused by higher stomatal conductance and increased transpiration. Stomatal conductance measured at 10:00 AM in irrigated pro plants was approximately 50% higher than that measured in M82 (Figure 1C). We monitored whole-plant transpiration in irrigated plants growing in a greenhouse using an array of load cells (lysimeters, see Methods) that simultaneously followed the daily weight loss of each plant. The daily transpiration rate (normalized to plant weight to eliminate the effect of plant size) of pro plants was significantly higher than that measured for M82 plants (Figure 1D). Microscopic analysis of imprints taken from the abaxial leaf epidermis of irrigated pro and M82 plants revealed a much larger stomatal pore area in pro (72 µm²) versus M82 (37 µm², Figure 1E) plants. pro stomata were slightly larger than those of M82 and their density was similar (Supplemental Figure 1). These results suggest
that the rapid water loss observed in pro resulted from increased stomatal pore area, which in turn led to increased stomatal conductance and transpiration.

**Increased DELLA activity reduces stomatal conductance**

We next aimed to determine how increased DELLA activity affects plant water status. To this end, transgenic tomato plants overexpressing the *Arabidopsis* S-*della* protein RGAΔ17 (Livne et al., 2015), which lacks the DELLA domain and is stable in the presence of GA (Dill et al., 2001), were subjected to water-deficit conditions. Relatively weak, semi-dwarf 35S<sub>pro</sub>:rgaΔ17 lines were used to minimize the effect of plant size. After 5 days without irrigation, the control plants began to wilt, while the transgenic plants remained turgid (Figure 2A). At this same time point, leaf RWC was reduced by 25% in M82 plants but remained unchanged in 35S<sub>pro</sub>:rgaΔ17 (Figure 2B). After 10 days without irrigation, 35S<sub>pro</sub>:rgaΔ17 plants wilted (Figure 2A). After 14 days without irrigation, the plants were rehydrated and the recovery process was monitored. M82 plants failed to recover (Figure 2A), but 35S<sub>pro</sub>:rgaΔ17 plants fully recovered, and only small necrotic lesions were noted on several leaves. Irrigated 35S<sub>pro</sub>:rgaΔ17 plants displayed significantly lower whole-plant transpiration rates compared to M82 plants (Figure 2C). Since 35S<sub>pro</sub>:rgaΔ17 had smaller leaflets (Supplemental Figure 2A), the reduced transpiration may have been the result of lower stomata counts per leaf. However, stomatal density was higher in 35S<sub>pro</sub>:rgaΔ17 leaflets (Supplemental Figure 2B) and the total number of stomata per leaf and per plant was similar between the two genotypes (Supplemental Figure 2C). 35S<sub>pro</sub>:rgaΔ17 stomata were only slightly smaller than those of M82 (Supplemental Figure 2D), but their pore area (25 µm<sup>2</sup>) was much smaller as compared to M82 (42 µm<sup>2</sup>, Figure 2D).

We measured stomatal conductance in M82 and 35S<sub>pro</sub>:rgaΔ17 during the drought treatment. Control and 35S<sub>pro</sub>:rgaΔ17 plants with 7 expanded leaves were exposed to water-deficit conditions (see above). The stomatal conductance of the 4<sup>th</sup> leaf below the apex was measured each day at noon using a porometer. At the same time, the soil relative volumetric water content (VWC) was determined using a soil moisture sensor. Stomatal conductance was lower in 35S<sub>pro</sub>:rgaΔ17 plants throughout the experiment (Figure 2E).
35S<sub>pro</sub>:rgaΔ17 stomata closed when VWC reached approximately 40% and M82 stomata only closed when VWC reached approximately 20%. These results suggest that high DELLA activity increases the sensitivity of stomata to reduced soil water content.

We also generated transgenic tomato plants overexpressing proΔ17, a tomato S-della mutant gene. All transgenic lines presented a dwarf phenotype and resistance to GA<sub>3</sub> treatment (Supplemental Figure 3A). The transgenic plants displayed significantly lower daily whole-plant transpiration that M82 plants (normalized to plant weight, Supplemental Figure 3C). In addition, the transgenic plants maintained higher leaf water content for a longer period under water-deficit conditions (see below Figure 4). Under normal irrigation, stomatal pore area at noon was much smaller in 35S<sub>pro</sub>:proΔ17 versus M82 plants (Supplemental Figure 3D), but stomatal size was similar (Supplemental Figure 3B).

The findings that 35S<sub>pro</sub>:rgaΔ17 and 35S<sub>pro</sub>:proΔ17 plants have reduced transpiration, increased leaf water content under water-deficit stress and smaller stomatal pore area than wild type, raised the possibility that DELLA proteins act in guard cells to reduce water loss under both irrigation and water-deficit conditions by promoting stomatal closure. To confirm the expression of PRO and proΔ17 in guard cells, we extracted RNA from isolated guard cells (Supplemental Figure 4A). qRT-PCR analysis revealed the expression of PRO in M82 guard cells and showed approximately 60-fold higher expression level of proΔ17 in transgenic guard cells vs. M82 (Supplemental Figure 4B).

To determine the contribution of leaf size to water loss, we generated trans-activated tomato plants expressing a **GREEN FLUORESCENT PROTEIN (GFP)proΔ17** fusion under the control of the **FILAMENTOUS FLOWER (FIL)** promoter (**FIL<sub>pro</sub>>GFP-proΔ17**). We used the transgenic line **FIL<sub>pro</sub>-LhG4** as a driver (Shani et al., 2010) and **OP-GFP-proΔ17** as a responder line. The **FIL** promoter is active during a limited developmental window in leaf primordia and initiating leaflets, but not later during leaf expansion or in mature leaves (Lifschitz et al., 2006). The expression of GFP-proΔ17 in young leaf primordia and initiating leaflets of **FIL<sub>pro</sub>>GFP-proΔ17** plants was confirmed by confocal microscopy (Supplemental Figure 5A).
However, in epidermal peels from mature $FIL_{pro} \gg >GFP-pro\Delta 17$ leaves, no GFP signal was detected in pavement or guard cells (Supplemental Figure 5B). As expected, the trans-activated plants featured normal stem length but smaller leaflets than wild type (Figure 3A and B; Supplemental Figure 5C). When subjected to water-deficit conditions, M82 and $FIL_{pro} \gg >GFP-pro\Delta 17$ plants wilted at the same time (after 5 days) and exhibited similar RWC at this time (Figure 3A and C). In addition, stomatal pore area was similar in irrigated $FIL_{pro} \gg >GFP-pro\Delta 17$ and M82 plants (Figure 3D). Stomatal density in $FIL_{pro} \gg >GFP-pro\Delta 17$ was higher than in M82, but the total number of stomata per plant was similar (Supplemental Figure 5D and E). These results indicate that the reduced leaf size in S-della-overexpressing plants is not the main determinant of reduced transpiration.

**DELLA activity promotes the stomatal response to ABA**

The shortage of water and loss of turgor induce ABA biosynthesis (McAdam and Brodribb, 2016), which, in turn promotes stomatal closure. The stomatal aperture of the ABA-deficient tomato mutant sitiens (sit) is larger than in wild-type plants (Tal, 1966). Pores in pro stomata were larger than those of wild-type plants, resembling those of sit (Figure 4B and C, Supplemental Figure 6). Therefore, we investigated the possible interaction between DELLA and ABA in the regulation of stomatal movement. To this end, $35S_{pro} : pro\Delta 17$ was introgressed from one of the transgenic M82 lines into sit. qRT-PCR analysis confirmed that the transgene was expressed in both M82 and sit (Supplemental Figure 7). sit leaves were smaller and stems were shorter than those of M82 (Figure 4A). Overexpression of pro\Delta 17 reduced the leaf size and stem length of both M82 and sit, but the effects were greater in the sit background. After one day without irrigation, sit and $35S_{pro} : pro\Delta 17$ sit began to wilt, whereas M82 only began to wilt after 4 days. Even after 7 days without irrigation, $35S_{pro} : pro\Delta 17$ M82 plants were turgid (Figure 4A). After 7 days under water-deficit conditions, we resumed irrigation and scored recovery 10 days later. $35S_{pro} : pro\Delta 17$ did not show any sign of damage, and M82 exhibited minor damage in 1 or 2 leaves. In contrast, sit and $35S_{pro} : pro\Delta 17$ sit leaves failed to recover (Figure 4A). Microscopic analysis of imprints of the
abaxial leaf epidermis of irrigated M82, 35S\textsubscript{pro::pro\textgreek{A}17}, sit and 35S\textsubscript{pro::pro\textgreek{A}17} sit plants revealed widely opened stomata in sit and 35S\textsubscript{pro::pro\textgreek{A}17} sit and small pores in 35S\textsubscript{pro::pro\textgreek{A}17} (Figure 4B and C; Supplemental Figure 8). These results indicate that the effect of S\textit{-della} on stomatal aperture under irrigation and under water-deficit conditions is ABA-dependent. In addition, the results show that the effect of DELLA on plant size is not linked to its effect on stomatal closure.

The suppression of the effect of S\textit{-della} on stomatal closure by sit suggested that DELLA either promotes ABA production or affects downstream ABA-associated responses. To test and distinguish between these possibilities, we first analyzed the levels of ABA in irrigated versus water-deprived M82, pro and 35S\textsubscript{pro::pro\textgreek{A}17} leaves. In the water-deficit treatment, a similar soil VWC was maintained for all plants by adding small amounts of water to the pots that dried faster. All plants started to wilt 8 days after the beginning of the treatment, and leaves were collected for ABA analysis one day later. At this time, the soil VWC of all plants reached 40%. Under irrigation, no significant differences in ABA content were recorded between the different lines (Figure 5A). While the water-deficit treatment increased ABA levels in all lines, these levels were lower in 35S\textsubscript{pro::pro\textgreek{A}17} leaves than in M82 or pro leaves. This may have been the result of feedback inhibition induced by higher ABA activity (Liu et al., 2016). These results suggest that DELLA does not promote ABA accumulation in tomato leaves.

To evaluate whether DELLA affects stomatal response to ABA, we treated peeled abaxial epidermal strips taken from M82, pro and 35S\textsubscript{pro::rga\textgreek{A}17} leaves with different ABA concentrations and monitored stomatal closure. Incubation with 10\textmu M ABA reduced stomatal aperture in M82 by 30%, in pro by 24% and in 35S\textsubscript{pro::rga\textgreek{A}17} by 50% (Figure 5B). Application of 100\textmu M reduced stomatal aperture in M82 by 55%, in pro by 38% and in 35S\textsubscript{pro::rga\textgreek{A}17} by 75%. In addition, a stronger stomatal response to ABA was observed in 35S\textsubscript{pro::pro\textgreek{A}17} (60% stomatal closure in 35S\textsubscript{pro::pro\textgreek{A}17} and 30% in M82, Figure 5C), but not in \textit{FIL}\textsubscript{pro}>>GFP-pro\textgreek{A}17 epidermal peels (Supplemental Figure 9). These results indicate that DELLA activity affects the response of the guard cells to ABA. In these experiments, no significant difference in the pore area between untreated M82 and S\textit{-della}
stomata was detected. It is possible that the buffer used in this experiment to force stomatal opening in the epidermal peels masked the effect of DELLA in the mock treatment.

In addition to its effect on stomatal closure, ABA also promotes gene expression (Hubbard et al., 2010). We analyzed the expression of two tomato ABA-responsive genes (González-Guzmán et al., 2014), DELTA 1-PYRROLINE-5-CARBOXYLATE SYNTHASE (SlP5CS1- Solyc06g019170) and RESPONSIVE TO ABA18 (SlRAB18- Solyc02g084850), in isolated M82 and 35S<sub>pro</sub>:proΔ17 guard cells. The expression of both genes was significantly higher in 35S<sub>pro</sub>:proΔ17 guard cells than in M82 (Figure 5D and 5E), suggesting that S-della constitutively promotes ABA responses in guard cells.

We then analyzed H<sub>2</sub>O<sub>2</sub> accumulation, one of the most immediate responses to ABA in guard cells (Pei et al., 2000), following exposure of peeled abaxial epidermal strips to ABA. H<sub>2</sub>O<sub>2</sub> was detected with the fluorescent dye 2′,7′-dichlorofluorescin (H2DCF, Zhang et al., 2001). 35S<sub>pro</sub>:proΔ17 guard cells displayed a significantly stronger response to ABA than those of M82. Conversely, pro guard cells exhibited less of a response than the wild type (Figure 5E and Supplemental Figure 10).

To determine whether DELLA activity affects stomatal movement typically induced by reduced apoplastic osmotic potential, we incubated abaxial leaf epidermal strips of M82, pro and 35S<sub>pro</sub>:proΔ17 plants in 50mM sorbitol to induce stomatal closure (Yuan et al., 2014). Sorbitol induced similar levels of stomatal closure (~45%) in all examined lines (Figure 5F), suggesting that DELLA activity is not involved in ABA-independent stomatal closure.

To determine if DELLA affects guard cells in a cell-autonomous manner, we examined the effects of exclusive expression of proΔ17 in guard cells on responses to water deficiency. In tomato plants, the promoter of KST1 (a K<sup>+</sup> channel gene) is active exclusively in guard cells (Kelly et al., 2013). Transgenic KST<sub>1</sub><sub>pro</sub>:LhG4 plants were crossed with OP:GFP-proΔ17 plants to transactivate proΔ17 expression in guard cells. The phenotype of the transactivated plants was indistinguishable from that of M82 (Figure 6A and C). Confocal microscopy analysis detected the expression of GFP only in
guard cells (Figure 6B). After 7 days under water-deficit conditions, M82 plants wilted but KST1pro>>GFP-proΔ17 plants remained turgid (Figure 6C). At this same time point, leaf RWC was reduced by 30% in M82 plants but remain unchanged in the trans-activated KST1pro>>GFP-proΔ17 leaves (Figure 6D). Stomatal pore area in the third leaf below the apex of irrigated KST1pro>>GFP-proΔ17 plants was smaller than that of M82 leaves (Figure 6E) and KST1pro>>GFP-proΔ17 guard cells exhibited increased response to ABA (Figure 6F). These results suggest that exclusive expression of DELLA in guard cell is sufficient to promote stomatal closure. The results also demonstrate that DELLA can affect stomatal closure and transpiration without affecting leaf size.

Since DELLA proteins are transcriptional regulators, S-della may affect ABA sensitivity in guard cells by transcriptionally regulating ABA signaling component(s). To start addressing this possibility, we analyzed the expression of several ABA signaling genes in isolated M82 and 35Spro:proΔ17 guard cells. These included all putative ABA receptor genes Pyrabactin Resistance1 (PYR1)/Regulatory Component of ABA Receptor (RCAR) (SIPYR1), SIPYR1-Like 2-1 (SIPYL2-1), SIPYL2-2, SIPYL4-1, SIPYL4-2, SIPYL4-3, SIPYL6-1, SIPYL6-2, SIPYL6-3, SIPYL8-1, SIPYL8-2, SIPYL9-1 and SIPYL9-2, the ABA signaling inhibitor phosphatase type 2C, subclass III SnRK2 kinase Open Stomata 1 and NADPH oxidase (homolog of the Arabidopsis RBOH1, (SIRBOH1). It should be noted that the names of these tomato genes do not necessarily reflect their relatedness to the Arabidopsis genes. qRT-PCR analysis revealed approximately 2-fold higher expression of the ABA-receptor genes SIPYR1 and SIPYL8-1 in 35Spro:proΔ17 compared to M82 (Figure 7A). We then analyzed the expression of these two genes in pro. The expression of both genes was downregulated in pro compared to M82, although these changes were significant only for SIPYR1 (Student’s t test, P < 0.05, Figure 7B). DELLA activity had no effect on the expression of all other receptor genes and not on SIPP2C, SIOST1-like gene and SIRBOH1 (Figure 7C).

DISCUSSION
Various abiotic stresses, including water deficit, promote DELLA stability and activity via the suppression of GA biosynthesis (Colebrook et al., 2014). The accumulated DELLA induces transcriptional reprogramming, which leads to growth suppression and improves plant adaptation to the changing environment (Achard et al., 2008; Colebrook et al., 2014). Here, we show that the DELLA protein PRO acts in guard cells to promote tolerance to water deprivation via a mechanism independent of its effect on plant growth. Reduced DELLA activity in the tomato pro mutant increased stomatal conductance and transpiration and suppressed its response to water deficiency and ABA treatment. On the other hand, overexpression of S-della reduced stomatal conductance and transpiration and promoted stomatal closure under mild water-deficit stress.

Studies in Arabidopsis have found that DELLA proteins promote ABA biosynthesis in seeds (Zentella et al., 2007; Piskurewicz et al., 2008). Furthermore, Lim et al. (2013) showed that the Arabidopsis DELLA protein GAI interacts in seeds with the ABA signaling components ABA-INSENSITIVE3 (ABI3) and ABI5, and Ariizumi et al. (2013) showed that seeds of the Arabidopsis della gain-of-function mutant gai-1 are more sensitive to ABA than wild type. Taken together, these studies suggest that DELLAs affect both ABA biosynthesis and signaling in Arabidopsis seeds.

The expression of S-della in the ABA-deficient mutant sit failed to promote stomatal closure and tolerance to water deprivation, indicating that this effect of S-della is ABA-dependent. While ABA levels were unaffected in pro and S-della plants, high DELLA activity increased the response of guard cells to ABA and low DELLA activity reduced this response, suggesting that DELLA affects stomatal movement by increasing sensitivity to ABA. This increased response to ABA could explain the earlier stomatal closure during drought found in plants overexpressing S-della. The gradual exposure of plants to increasing intensities of water deficiency leads to gradual increases in ABA levels (Du et al., 2013); under these conditions, enhanced sensitivity to ABA should lead to earlier stomatal closure.

We also found that expression of the ABA-responsive genes SIP5CS1 and SIRAB18 (González-Guzmán et al., 2014) was higher in S-della guard cells than in wild type. This suggests that the DELLA-mediated increase in
ABA sensitivity in guard cells is not limited to ABA-induced stomatal movement and that DELLA also promotes ABA-induced transcriptional activity (Hubbard et al., 2010). The results also indicate that overexpression of S-della constitutively promotes ABA activity in guard cells regardless of water availability, leading to smaller stomatal aperture and increased expression of ABA-responsive genes under both irrigation and water-deficit stress. It is unlikely that DELLA also affects stomatal movement in an ABA-independent manner, for example, by affecting leaf structure or cell wall elasticity (Marshall and Dumbroff 1999). DELLA activity did not influence sorbitol-induced stomatal closure, which involves an ABA-independent pathway. The site of DELLA-driven regulation of stomatal movement is likely guard cells, as shown by the exclusive expression of S-della in guard cells, which was sufficient to increase their sensitivity to ABA and promote tolerance to water-deficit stress.

Since DELLAs are transcription regulators, it is unclear yet how they affect ABA sensitivity to promote rapid cytosolic responses, such as stomatal movement. Perhaps DELLAs affect the transcription of component(s) in the ABA signaling machinery. ABA binds to its receptors, PYR1/PYL/RCAR, which triggers a conformational change within the receptor, thereby increasing its affinity to the signaling suppressor phosphatases PP2C. Inhibition of PP2C activity by the activated receptor releases downstream SnRK2 kinases to phosphorylate downstream proteins, such as transcription factors, ion channels and NADPH oxidase, which in turn regulate gene transcription and stomatal movement (Hubbard et al., 2010; Kim et al., 2010; Cai et al., 2017). The effect of DELLA activity on ABA-induced H$_2$O$_2$ accumulation (reduced and increased response in pro and S-della, respectively) suggests that DELLAs affects early event(s) in the ABA response pathway, upstream or at the level of NADPH oxidase. This impact may be mediated by enhanced (e.g., PYR/PYL receptors, SnRK2 kinases or NADPH oxidase) or attenuated (e.g. PP2C) transcription of components in the ABA signaling pathway. The expression of two genes encoding ABA receptors, SIPYR1 and SIPYL8-1, was upregulated in S-della and reduced in pro guard cells compared to wild type. Other receptor and ABA signaling components were not affected by DELLAs. While these results support the hypothesis that the effect of DELLAs on ABA sensitivity is mediated by a transcriptional effect on these two ABA
receptor genes, further research is required to address this possibility. Although increased sensitivity to ABA is our preferred hypothesis, we cannot exclude the possibility that DELLA affects ABA uptake into guard cells via transcriptional regulation of ABA transporter genes (Kang et al., 2010).

The present findings, as well as earlier studies, suggest that DELLA play a role in plant adaptation to water-deficit stress. It has been previously suggested that water deficiency reduces GA concentrations (Colebrook et al., 2014) and thereby increases DELLA accumulation. Water deficit also induces ABA accumulation, which in turn stabilizes DELLA (Achard et al., 2006; Jiang and Zhang, 2002; Endo et al., 2008; Guo et al., 2014). We propose that DELLA plays a role in both early and long-term responses to water-deficit stress. Our model (Figure 8) suggests that DELLA increases the sensitivity of guard cells to ABA by promoting the transcription of specific ABA-receptor genes. The increased sensitivity to ABA promotes ABA-induced stomatal closure. These actions protect plants from transient and/or mild water-deficit stress. In the case of persistent drought, DELLA accumulates to high levels and suppresses growth. The inhibition of plant growth further reduces transpiration and water loss and redirects the available energy to protection and adaptation processes (Achard et al., 2008).

METHODS

Plant materials and growth conditions
Tomato (Solanum lycopersicum) M82 (sp/sp) plants were used throughout this study. The pro allele was in the M82 background (Fleishon et al., 2011; Livne et al., 2015) and the sit allele, originally in 'Alisa Craig' (Harrison et al., 2011), was backcrossed three times to M82. All plants were grown in a growth room set to a photoperiod of 12/12 h night/days, light intensity (cool-white bulbs) of approximately 250 µmol m^{-2} s^{-1}, and 25 °C. In other experiments, plants were grown in a greenhouse under natural day length conditions, at 700–1200 µmol m^{-2} s^{-1} and 18-29 °C.

DNA constructs, the transactivation system and plant transformation
Truncated PRO (proΔ17) was generated using PCR-based "overlap extention" mutagenesis (Hoa et al., 1989). proΔ17 was inserted into the
pART7 vector downstream to the 35S promoter into the KpnI and BamHI sites. The pro\(\Delta\)17 coding sequence was also inserted into the pART7-GFP vector into the KpnI and BamHI sites to create GFP-pro\(\Delta\)17. This construct was then cloned downstream of Operator array (OP) sequences from E. coli into the KpnI and BamHI sites to create OP:GFP-pro\(\Delta\)17. To specifically express GFP-pro\(\Delta\)17 in guard cells or in leaf primordia, the LhG4 transactivation system (Moore et al., 1998) with the KST1 and FIL promoters, respectively, was used. The KST1 promoter was inserted into the pBJ36 vector, upstream of LhG4, in the Sall and PstI sites to create KST1\_pro:LhG4. KST1\_pro:LhG4 and FIL\_pro:LhG4 (Plesch et al., 2001; Shani et al., 2010) were used as driver lines and OP:GFP-pro\(\Delta\)17 as the responder line. The cross between these lines generated the trans-activated lines KST1\_pro:>>GFP-pro\(\Delta\)17 and FIL\_pro:>>GFP-pro\(\Delta\)17. The constructs were sub-cloned into the pART27 binary vector and introduced into Agrobacterium tumefaciens strain GV3101 by electroporation. The constructs were transferred into M82 cotyledons using transformation and regeneration methods described by McCormick (McCormick, 1991). Kanamycin-resistant T0 plants were grown and at least four independent transgenic lines were selected and self-pollinated to generate homozygous transgenic lines.

**RNA extraction and cDNA synthesis**

Total RNA was isolated from tomato seedlings or leaves from mature plants. RNA extraction and cDNA synthesis were performed as previously described (Nir et al., 2014). Frozen tissues were ground, re-suspended in guanidine HCl and combined with phenol/chloroform. The samples were mixed by vortexing for 30 s and after 30 min, they were centrifuged at 4°C for 45 min. Ethanol (100%) and 1 M acetic acid were added, and the samples were mixed and stored overnight at -80°C. NaAc (3 M) was added and the samples were washed with cold 70% ethanol. SuperScript II reverse transcriptase (18064014, Invitrogen, Waltham, MA, USA) and 3 µg of total RNA were used to synthesize cDNA according to the manufacturer’s instructions.

**qRT-PCR analysis**

qRT-PCR analysis was performed using an Absolute Blue qPCR SYBR Green ROX Mix (AB-4162/B) kit (Thermo Fisher Scientific, Waltham, MA
USA). Reactions were performed using a Rotor-Gene 6000 cycler (Corbett Research, Sydney, Australia). A standard curve was obtained using dilutions of the cDNA sample. The expression was quantified using Corbett Research Rotor-Gene software. 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 TUBULIN. Gene-to-TUBULIN ratios were then averaged and are presented as the proportion of the control treatment, which was set to a value of 1. All primer sequences are presented in Supplemental Table 1.

**Measurements of stomatal pore area, length and density**

Stomatal pore area and density were determined using the rapid imprinting technique (Geisler et al., 2000). This approach allowed us to reliably and simultaneously score hundreds of stomata from each experiment. Briefly, light-body vinylpolysiloxane dental resin (eliteHD+, Zhermack Clinical, Badia Polesine, Italy) was attached to the abaxial side of the leaf, dried for ~1 min and then removed. The resin epidermal imprints were covered with transparent nail polish, which was removed once it dried and served as a mirror image of the resin imprint. The nail-polish imprints were placed on glass cover slips and photographed under a model 1M7100 bright-field inverted microscope (Zeiss, Jena, Germany) with a mounted Hitachi HV-D30 CCD camera (Hitachi, Tokyo, Japan). Stomatal images were later analyzed to determine aperture size using the ImageJ software (http://rsb.info.nih.gov/ij/) fit-line tool. A microscopic ruler (Olympus) was used for size calibration.

**Measurement of leaf area**

Total leaf area was measured using a model Li 3100 leaf area meter (Li-Cor, Licor Biosciences, Lincoln, Nebraska, USA).

**Measurement of stomatal pore area in detached epidermis**

Abaxial epidermal strips were peeled and the detached layer was incubated in stomatal opening buffer (Wigoda et al., 2006) for 2 h in the light (400 µmol m⁻² sec⁻¹). The strips were then transferred to fresh stomatal opening buffer, with or without ABA. After 40 min, the strips were placed on glass cover-slips and
photographed under a bright-field inverted microscope and images were analyzed as above.

Detection of $\text{H}_2\text{O}_2$ in guard cells
Abaxial epidermal strips were peeled and floated on stomatal opening buffer (Wigoda et al., 2006) for 2 h in the light (400 µmol m$^{-2}$ sec$^{-1}$). The strips were then transferred to stomatal opening buffer supplemented with 50 µM H2DCF-DA (Sigma-Aldrich, St Louis, MO, USA) for 10 min and then transferred to glass cover-slips containing ABA dissolved in stomatal opening buffer (or mock). Images were taken using a fully motorized epifluorescence inverted microscope (Olympus-IX8, Cell-R, Olympus) equipped with a 12-bit CCD camera: Orca-AG (Hamamatsu, Hamamatsu, Japan). The images were later analyzed using ImageJ software. Fluorescence was visualized with an excitation wavelength of 485 nm at low power (1.5%) and an emission wavelength of 530 nm.

Isolation of guard cells
Guard cells from tomato leaves were isolated according to Araújo et al. (2011) with some modifications. Briefly, 20 g of fully expanded leaves without the veins were ground twice in a blender in 100 ml cold distilled water, each time for 1 min. The blended mixture was poured onto a 200-µm mesh (Sefar AG, Heiden, Switzerland) to remove broken mesophyll and epidermal cells. The remaining epidermal peels were rinsed thoroughly with deionized water. The peels were then transferred into 10-ml buffer (Araújo et al., 2011) containing the enzyme CELLULYSIN cellulase from *Trichoderma viride* (Calbiochem, La Jolla, CA, USA) and digested for 1 h at a shaking speed of 150 rpm. This enzymatic treatment digests pavement cells, but not guard cells (Wang et al., 2011). The digested material was poured again onto a 200-µm mesh placed in a tube and rinsed thoroughly with digestion buffer (without the enzyme). To remove residues of buffer and cell particles, the tubes were centrifuged at 4°C for 5 min at 2200 rpm. Samples of digested epidermal strips were stained with neutral red, and cell vitality was examined microscopically (Supplemental Figure 4).

Relative water content (RWC) determination
Leaf RWC was measured as follows: fresh leaf weight (FW) was measured immediately after leaf detachment. Leaves were then soaked for 8 h in 5 mM CaCl₂ at room temperature in the dark, and the turgid weight (TW) was recorded. Dry weight (DW) was recorded after drying the leaves at 70°C. RWC was calculated as \((FW - DW)/(TW - DW) \times 100\) (Sade et al., 2009).

**Stomatal conductance and relative water content (RWC)**

Stomatal conductance was determined using a SC-1 Leaf Porometer (Decagon Devices, Pullman, WA, USA). VWC was measured using the 5TM soil moisture and temperature sensor, combined with the ‘ProCheck’ interface reader (Decagon Devices, Pullman, WA, USA).

**Whole-plant transpiration measurements**

Whole-plant transpiration rates were determined using an array of lysimeters placed in the greenhouse (Plantarry 3.0 system, Plant-DiTech, Rehovot, Israel http://www.plant-ditech.com), as described in detail by Halperin et.al. (2017). Briefly, plants in 4L pots were grown under semi-controlled temperature conditions (20-28°C day and 12-16°C night), natural day length and light intensity of approximately 1000 µmol m⁻² s⁻¹. Each pot was placed on a temperature-compensated load cell with digital output (Vishay Tedeahuntleigh, Netanya, Israel) and sealed to prevent evaporation from the surface of the growth medium. The weight output of the load cells was monitored every 3 min. Daily plant transpiration rate (weight loss between predawn and sunset) was calculated based on the difference in weight between the two data points and was normalized to plant weight at this time.

**GFP analysis**

GFP signals in guard cells were detected with a model SP8 confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany), with a 488 nm excitation laser line and a 505-525 nm emission filter.

**Hormone treatments**

GA₃ and ABA (Sigma-Aldrich, St. Louis, MO, USA) were applied to plants by spraying. To evaluate the sensitivity of guard cells to ABA, epidermal peels
were incubated for 30 min in a solution containing different concentrations of ABA and then the stomata were examined microscopically.

**ABA analysis**

ABA extraction and analysis were carried out as previously described (Lashbrooke et al., 2016). Briefly, 40-100 mg of ground frozen plant tissue was extracted at -20°C with methanol/water/formic acid containing stable isotope-labeled internal standards of ABA. Abscisates were purified by solid phase extraction (SPE) and detected by ultra-performance liquid chromatography-electrospray tandem mass spectrometry (UPLC-ESI-MS/MS) (Waters, Milford, MA, USA) operated in MRM mode. Quantification was performed against an external calibration curve using analyte/internal standard peak ratios.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: **PRO**- Solyc11g011260; **TUBULIN**- Solyc04g077020; **SIP5CS1**- Solyc06g019170; **SIRAB18**- Solyc02g084850; **SIPYR1**- Solyc06g061180; **SIPYL2-1**- Solyc12g095970; **SIPYL2-2**- Solyc08g065410; **SIPYL4-1**- Solyc06g050500; **SIPYL4-2**- Solyc10g085310; **SIPYL4-3**- Solyc10g076410; **SIPYL6-1**- Solyc03g095780; **SIPYL6-2**- Solyc05g052420; **SIPYL6-3**- Solyc09g015380; **SIPYL-8-1**- Solyc01g095700; **SIPYL8-2**- Solyc03g007310; **SIPYL9-1**- Solyc08g082180; **SIPYL9-2**- Solyc12g055990; **SIPP2C**- Solyc07g062970; **SIOST1**- Solyc01g108280; **SIROH1**- Solyc08g081690.

**SUPPLEMENTAL DATA**

**Supplemental Figure 1.** Stomatal size and density in M82 and pro plants.

**Supplemental Figure 2.** Overexpression of rgaΔ17 in tomato plants reduces leaf size and increases stomatal density.

**Supplemental Figure 3.** Overexpression of the tomato stable DELLA (S-della), proΔ17, reduces stomatal pore area and transpiration.
**Supplemental Figure 4.** PRO and pro∆17 expression in guard cells.

**Supplemental Figure 5.** Expressing pro∆17 under the control of the FIL promoter reduces leaf size and increases stomatal density.

**Supplemental Figure 6.** Abaxial leaf epidermal tissues of control M82, pro and sit plants.

**Supplemental Figure 7.** qRT-PCR analysis of proΔ17 in sit.

**Supplemental Figure 8.** Stomata in leaf no. 4 of M82, sit, 35Spro:proΔ17 and 35Spro:proΔ17 in the sit background.

**Supplemental Figure 9.** Stomatal response of FILpro>>GFP-proΔ17 to ABA treatment.

**Supplemental Figure 10.** H2O2 accumulation (DCF fluorescent signal) in M82, pro and 35Spro:proΔ17 guard cells following ABA application.

**Supplemental Table 1.** Primers used in this study.

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**AUTHOR CONTRIBUTIONS:** D.W., I.N. and N.O designed the research plan; I.N., H.S. I.P. performed the research; A.A contributed analytic tools; I.N., H.S., I.P. A.A. and D.W. analyzed data; I.N., N.O. and D.W. wrote the paper.
REFERENCES


yield production: is the tonoplast aquaporin SlTIP2;2 a key to isohydric to anisohydric conversion? New Phytol. 181: 651–61.


Figure 1. The tomato DELLA loss-of-function pro mutant exhibits rapid water loss under water-deficit conditions. M82 and pro were grown until they produced 6 leaves before irrigation was stopped.

(A) Representative plants grown under continuous irrigation (+irrigation) or after 4 days of limited water conditions (-irrigation).

(B) Average leaf relative water content (RWC) of control M82 and pro plants grown with or without irrigation for 4 days. Values are means of five replicates (five leaflets taken from the fourth leaf below the apex from five different plants) ± standard error (SE). Each set of letters above the columns represents significant differences between respective treatments (Tukey–Kramer HSD, \( P < 0.05 \)).

(C) Stomatal conductance \( (g_s) \) of the fourth leaf below the apex, as measured, with a porometer, at 10:00 AM. Values are means of 8 measurements from 8 plants ± SE.

(D) Whole-plant transpiration \( (E) \) over the course of 12 h (06:00 AM to 06:00 PM). M82 and pro plants were placed on the lysimeter system and pot (pot + soil + plant) weight was measured every 3 min. To eliminate the effect of plant size on transpiration rate, the rate of plant water loss was normalized to plant weight. Values are means of 8 plants ± SE.

(E) Stomatal pore area (\( \mu m^2 \)) of the fourth leaf below the apex of irrigated M82 and pro plants, measured at 10:00. Values are means of approximately 100 measurements (stomata) ± SE. Asterisk (in C and E) denotes a significant difference (Student's t test, \( P < 0.05 \)).
Figure 2. Overexpression of the Arabidopsis S-della gene, rgaΔ17, in tomato plants improves water status under water-deficit stress. Control M82 and transgenic 35S_pro:rgaΔ17#4 (rgaΔ17) plants were grown until they produced 5 expanded leaves before irrigation was stopped.

(A) Representative plants grown under continuous irrigation or for 5 or 10 days without irrigation. After 14 days without irrigation, plants were rehydrated and their recovery was monitored.

(B) Average leaf RWC in control M82 and rgaΔ17 plants, grown with or without irrigation for 5 days. Values are means of five replicates (five leaflets taken from the fourth leaf below the apex from five different plants) ± SE.

(C) Whole-plant transpiration (E) over the course of 12 h (06:00 AM to 06:00 PM). M82 and rgaΔ17 plants were placed on the lysimeter system and pot weight was measured every 3 min. The rate of plant water loss was normalized to plant weight. Values are means of 8 plants ± SE.

(D) Stomatal pore area of the third leaf below the apex of irrigated control and 35S_pro:rgaΔ17 plants, measured at 10:00 AM. Values are means of approximately 100 measurements (stomata) ± SE. Asterisk (in B and D) denotes a significant difference (Student's t test, P < 0.05).

(E) Stomatal conductance (gs) of the fourth leaf below the apex under water-deficit conditions in M82 and rgaΔ17 plants. At the same time, soil relative volumetric water content (VWC) was measured using the EC-5 soil moisture sensor. Measurements were taken once a day at noon, starting on the first day after the cessation of irrigation. Numbers above (for M82) and below (for rgaΔ17) the lines indicate the day of measurement. Values are means of 8 measurements taken from 8 different plants ± SE.
Figure 3. Reducing the leaflet size by FIL<sub>pro</sub>▷GFP-proΔ17 has no effect on water loss. Control and transgenic FIL<sub>pro</sub>▷GFP-proΔ17 were grown until they produced 5 expanded leaves before irrigation was stopped. 

(A) Representative plants grown under continuous irrigation or subjected to 5 days of without irrigation. 

(B) Fourth leaf below the apex of control M82 and FIL<sub>pro</sub>▷GFP-proΔ17. Bar = 2 cm. 

(C) Average leaf RWC in control M82 and FIL<sub>pro</sub>▷GFP-proΔ17 plants grown with irrigation or subjected to 5 days of water-deficit conditions. Values are means of five replicates (five leaflets taken from the fourth leaf below the apex from five different plants) ± SE. Each set of letters above the columns represents significant differences between respective treatments (Tukey–Kramer HSD, P < 0.05). 

(D) Mean stomatal pore area of the fourth leaf below the apex of irrigated control and FIL<sub>pro</sub>▷GFP-proΔ17. Values are means of approximately 100 measurements (stomata) ± SE.
Figure 4. ABA is required for the enhanced DELLA-dependent stomatal closure.

(A) Representative M82, sit, 35S<sub>pro</sub>:proΔ17 (proΔ17) and 35S<sub>pro</sub>:proΔ17 plants in the sit background (proΔ17 sit) grown under a normal irrigation regime (+irrigation) or without irrigation for 7 days. After 7 days without irrigation, the plants were rehydrated and recovery was assessed after 10 days.

(B) Representative stomata of the lines described in A. Images (microscopic analysis) of imprints of the abaxial leaf epidermal layer of the fourth leaf below the apex collected at 11:00 AM from irrigated plants.

(C) Stomatal pore area of the fourth leaf below the apex measured at 11:00 AM. Values are means of approximately 100 measurements (stomata) ± SE. Each set of letters above the columns represents significant differences between respective treatments (Tukey–Kramer HSD, P < 0.05).
Figure 5. DELLLA activity promotes the response of guard cells to ABA. (A) ABA content of the fourth leaf below the apex of M82, pro and 35Spro:proΔ17 (proΔ17) plants grown with irrigation or subjected to water-deficit conditions. For the water-deficit treatment, leaves from all lines were collected when soil VWC reached 40%, and all were collected at the same time of day. Values are means of three replicates, taken from 3 plants ± SE. Each set of letters above the columns represents significant differences between the respective treatments (Tukey–Kramer HSD, P < 0.05).

(B) Epidermal strips from leaf no. 4 of M82, pro and 35Spro:rgaΔ17 were first incubated in stomatal opening buffer and then treated with 0, 10 or 100 µM ABA for 40 min and stomatal pore area was microscopically assessed. Values are means of approximately 100 measurements (stomata) ± SE. Percentage of stomatal pore area with respect to the mock treatment is presented for each line above the bars.

(C) Epidermal strips, taken from leaf no. 4 of M82 and 35Spro:proΔ17 (proΔ17) were incubated under light in stomatal opening buffer for 2 h and then treated with 10 µM ABA (or mock) for 40 min before stomatal pore area was microscopically assessed. Values are means of approximately 100 stomata ± SE. Asterisk denotes a significant difference (Student's t test, P < 0.05).

(D) Expression of ABA-responsive genes in isolated guard cells. qRT-PCR analyses of SlP5CS1 (Solyc06g019170) and SlRAB18 (Solyc02g084850) expression. RNA was extracted from isolated M82 and 35Spro:proΔ17 guard cells. Values are means of three biological replicates (each contains RNA extracted from guard cells isolated from five leaves of independently grown plant) ± SE. Asterisk denotes a significant difference (Student's t test, P < 0.05). The experiment was repeated twice and yielded similar results.

(E) Quantification of H2O2 accumulation (DCF fluorescent signal) in guard cells of M82, pro and 35Spro:proΔ17. Epidermal strips were taken from leaf no. 4 of M82, 35Spro:proΔ17 and pro, immersed for 10 min in 50 µM H2DCF-DA and then transferred to 10 µM ABA solution; the fluorescent signal was detected microscopically every 30 seconds after the application of ABA. The intensity of the fluorescent signals was quantified using ImageJ software. Values are means of 5 different images (each contains approximately 15 stomata) ± SE. The experiment was repeated 3 times and yielded similar results each time.

(F) Stomatal response to sorbitol treatment. Epidermal strips, taken from leaf no. 4 of M82, pro and 35Spro:proΔ17 plants were incubated under light in stomatal opening buffer for 2 h and then transferred to 50 µM sorbitol solution (or mock) for 30 min, before stomatal pore area was analyzed microscopically. Values are means of approximately 100 measurements (stomata) ± SE.
Figure 6. Expressing S-della in guard cells only promotes stomatal sensitivity to ABA and reduces water loss under water-deficit conditions.

(A) Leaf no. 3 of M82 and KST1pro>>GFP-proΔ17 plants. Bar = 2 cm.
(B) Microscopic analysis of the GFP signals in KST1pro>>GFP-proΔ17 leaves. Bar = 20 µm.
(C) Irrigated (+Irrigation) and water-deficit-treated (-Irrigation for 7 days) M82 and KST1pro>>GFP-proΔ17 plants.
(D) Average leaf RWC in M82 and KST1pro>>proΔ17 grown with irrigation or subjected to 7 days without irrigation. Values are means of five replicates (five leaflets taken from the fourth leaf below the apex from five different plants) ± SE. Each set of letters above the columns represents significant differences between respective treatments (Tukey–Kramer HSD, P < 0.05).
(E) Stomatal pore area in leaf no. 3 of M82 and KST1pro>>proΔ17, measured at 11:00 am. Values are means of approximately 100 measurements (stomata) ± SE. Asterisk denotes a significant difference (Student's t test, P < 0.05).
(F) Epidermal strips (leaf no. 4) were incubated under light in stomatal opening buffer for 2 h and then treated with 10 µM ABA or mock for 40 min before stomatal pore area was microscopically assessed. Values are means of approximately 100 measurements (stomata) ± SE.
Figure 7. DELLA activity promotes the expression of two ABA-receptor genes in guard cells. (A) qRT-PCR analyses of all putative tomato ABA-receptor genes in M82 and 35Spro:proΔ17 guard cells. RNA was extracted from isolated guard cells. Values are means of three biological replicates (each contains RNA extracted from guard cells isolated from five leaves of independently grown plant) ± SE. Asterisk denotes a significant difference (Student's t test, P < 0.05). The experiment was repeated twice and yielded similar results. nd- not detected
(B) qRT-PCR analyses of SlPYR1 and SlPYL8-2 expression in M82 and pro guard cells. RNA was extracted from isolated guard cells. Values are means of three biological replicates (each contains RNA extracted from guard cells isolated from five leaves of independently grown plant) ± SE. Asterisk denotes a significant difference (Student's t test, P < 0.05). The experiment was repeated twice and yielded similar results.
(C) qRT-PCR analyses of SlPP2C, SlOST1 and SlRBOH (NADPH OXIDASE) expression in M82 and 35Spro:proΔ17 guard cells. RNA was extracted from isolated guard cells. Values are means of three biological replicates (each contains RNA extracted from guard cells isolated from five leaves of independently grown plant) ± SE. The experiment was repeated twice and yielded similar results.

Figure 8. Hypothetical model of the mechanism by which DELLA promotes plant tolerance to water deficit stress. Under transient water deficit, GA biosynthesis is suppressed, leading to DELLA accumulation. The accumulated DELLA in guard cells promotes, directly or indirectly, the transcription of two ABA receptor genes (SlPYR1 and SlPYL8-2). It is possible that the increased expression of these two receptor genes is responsible for the enhanced sensitivity to ABA, which leads to rapid stomatal closure. Under prolonged drought, GA levels are low and DELLA accumulates to high levels, leading to growth suppression. Both stomatal closure and growth suppression protect plants from short and long drought episodes. Dashed line indicates the hypothetical link suggested by our study.