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Uncovering DELLA-Independent Gibberellin Responses by Characterizing New Tomato *procera* Mutants

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Gibberellin (GA) regulates plant development primarily by triggering the degradation/deactivation of the DELLA proteins. However, it remains unclear whether all GA responses are regulated by DELLAs. Tomato (*Solanum lycopersicum*) has a single DELLA gene named *PROCERA* (*PRO*), and its recessive *pro* allele exhibits constitutive GA activity but retains responsiveness to external GA. In the loss-of-function mutant *pro*$^{\Delta GRAS}$, all examined GA developmental responses were considerably enhanced relative to *pro* and a defect in seed desiccation tolerance was uncovered. As *pro*, but not *pro*$^{\Delta GRAS}$, elongation was promoted by GA treatment, *pro* may retain residual DELLA activity. In agreement with homeostatic feedback regulation of the GA biosynthetic pathway, we found that *GA20oxidase1* expression was suppressed in *pro*$^{\Delta GRAS}$ and was not affected by exogenous GA_3. In contrast, expression of *GA2oxidase4* was not affected by the elevated GA signaling in *pro*$^{\Delta GRAS}$ but was strongly induced by exogenous GA_3. Since a similar response was found in *Arabidopsis thaliana* plants with impaired activity of all five *DELLA* genes, we suggest that homeostatic GA responses are regulated by both DELLA-dependent and -independent pathways. Transcriptome analysis of GA-treated *pro*$^{\Delta GRAS}$ leaves suggests that 5% of all GA-regulated genes in tomato are DELLA independent.

INTRODUCTION

The phytohormone gibberellin (GA) regulates numerous developmental processes throughout the plant life cycle, including seed germination, stem elongation, flowering, and fruit set (Yamaguchi, 2008). The signaling pathway from GA perception to transcriptional activation has been intensively studied over the past two decades and its major components have been identified. The nuclear DELLA proteins, a subgroup of the GRAS transcription factors family, suppress GA signaling (Locascio et al., 2013). GA binding to the soluble GIBBERELLIN INSENSITIVE DWARF1 (GID1) receptor triggers GID1 interaction with the DELLA proteins (Ueguchi-Tanaka et al., 2005; Nakajima et al., 2006; Griffiths et al., 2006), which then stimulates assembly of the DELLA proteins into an SCF E3 ubiquitin ligase complex via the GID2/SLEEPY1 F-box proteins. The SCF complex polyubiquitinates the DELLA proteins, targeting them for destruction by the 26S proteosome (Sasaki et al., 2003; Dill et al., 2004; Griffiths et al., 2006; Harberd et al., 2009; Hauvermale et al., 2012). GA, via GID1, can also reduce DELLA activity through a degradation-independent mechanism (Ariizumi et al., 2008, 2013; Ueguchi-Tanaka et al., 2008).

Despite the central role of DELLAs in GA signaling, the mechanism underlying this regulation is not fully understood. Several studies have shown that protein-protein interactions play a major role in DELLA function. DELLAs bind to various transcription factors and proteins affecting transcription, including PHYTOCHROME-INTERACTING FACTORs (PIFs), ALCATRAZ, MYC2, JASMONATE-ZIM-DOMAIN PROTEIN9, SCARECROW LIKE3 (SCL3), and TCP transcription factors (de Lucas et al., 2008; Feng et al., 2008; Arnaud et al., 2010; Gallego-Bartolomé et al., 2010; Hong et al., 2012; Yang et al., 2012; Hou et al., 2010; Zhang et al., 2011; Davière et al., 2014). The interaction between DELLA and PIFs, for example, suppresses the binding of the latter to target promoters and thus inhibits their activity. Although DELLAs lack a DNA binding domain, they possess transactivation properties (Hirano et al., 2012), and several studies have shown that DELLAs can act as coregulators when interacting with transcription factors and directly regulate gene expression (Zentella et al., 2007; Hirano et al., 2012; Yoshida et al., 2014).

The DELLA N-terminal region consists of the conserved DELLA and VHYNP motifs (Locascio et al., 2013). These motifs interact with the GID1 N-terminal arm to form the GID1-GA-DELLA complex (Murase et al., 2008). The C-terminal region of DELLAs consists of several distinct motifs comprising the GRAS domain. These motifs include two leucine heptad repeats (LHRI and LHRII) with putative nuclear localization signals, flanking a VHIID motif, forming the LHRI-VHIID-LHRII domain said to be involved in protein-protein interactions (Sun et al., 2012). Hirano et al. (2010) have shown that the SLENDER RICE1 (SLR1; the rice [*Oryza sativa*] DELLA protein) GRAS domain is also required for a stable interaction between DELLA and GID1. Recently, Sato et al. (2014) confirmed this observation and demonstrated an interaction between the purified SLR1 GRAS domain and GID1.

Arabidopsis thaliana has five DELLA proteins (Repressor of *ga1-3* [RGA], GA-INSENSITIVE [GAI], RGA-LIKE1 [RGL1], RGL2,

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and RGL3), whereas rice, barley (*Hordeum vulgare*), and tomato (*Solanum lycopersicum*) each have only one, called SLR1, SLENDER1, and PROCERA (PRO), respectively (Ikeda et al., 2001; Chandler et al., 2002; Jasinski et al., 2008; Harberd et al., 2009). The one well-studied recessive tomato *pro* allele contains a point mutation within the VHIID domain (Val [V] to Glu [E] at position 273; Bassel et al., 2008). Creating a similar mutation in *gai*, an Arabidopsis gain-of-function DELLA allele, completely abolished its growth-suppressing activity (Jasinski et al., 2008), suggesting a loss-of-function allele. The *pro* phenotype resembles wild-type plants treated with GA and includes elongated internodes, thinner leaves, and reduced lobing of the main leaflets (George Jones, 1987; Jupe et al., 1988; Van Tuinen et al., 1999; Bassel et al., 2008; Jasinski et al., 2008). Antisense suppression of *PRO* also promoted GA responses, including pollination-independent ovary growth, resulting in parthenocarpic fruit formation (Martí et al., 2007).

In striking contrast with other plants with a single DELLA, such as barley and rice, *pro* plants respond to GA treatment and the *pro* mutation does not completely suppress chemicals or mutations that inhibit GA biosynthesis (George Jones, 1987; Jupe et al., 1988; Van Tuinen et al., 1999; Bassel et al., 2008; Jasinski et al., 2008; Fleishon et al., 2011). The responsiveness of *pro* to GA might be due to an incomplete loss of DELLA function (Van Tuinen et al., 1999) or due to the activity of a DELLA-independent response pathway (Fleishon et al., 2011).

While the central role of DELLA in the regulation of GA responses is indisputable, it is not yet clear if DELLA mediates all GA responses. Recently, Yano et al. (2015) have shown that GID1-DELLA is the sole mechanism for GA regulation of gene expression in rice aleurone cells. On the other hand, results from a number of studies support the existence of a DELLA-independent GA signaling pathway. Our earlier work in Arabidopsis has suggested the existence of a cytosolic, SPINDLY-dependent, DELLA-independent GA response pathway (Maymon et al., 2009). These findings stood in line with those reported by Cao et al. (2006), who demonstrated that some GA-regulated genes are not regulated by DELLA. Moreover, GA-induced increases in cytosolic calcium concentrations, detectable within ~2 min of exposure to GA (Bush, 1996), have been suggested to occur too rapidly to be regulated by DELLA proteins, whose levels are only significantly reduced 5 to 10 min after GA treatment (Gubler et al., 2002). Furthermore, cytosolic activity of DELLA has never been detected, thereby challenging attempts to ascribe it a regulatory role in cytosol-emanating responses. Finally, application of GA to emasculated pistils of *global* (an Arabidopsis mutant that lacks the activities of all five DELLA proteins) resulted in significant promotion of their growth (Fuentes et al., 2012). This DELLA-independent response is mediated by the basic helix-loop-helix transcription factor SPATULA, which suppresses fruit growth. Despite these findings and other evidence of the existence of a DELLA-independent, GA response pathway, its significance remains unclear.

Here, we present a *pro* loss-of-function mutant tomato line named $pro^{\Delta GRAS}$. All examined GA-dependent developmental responses were much stronger in $pro^{\Delta GRAS}$ than in *pro*. In addition, roles of PRO in seed desiccation tolerance and pollen tube elongation were uncovered. The presented results suggest that while GA regulation of tomato plant development is primarily DELLA dependent, ~5% of all identified GA-regulated genes are DELLA independent. Our results indicate that feedback regulation of GA catabolism is at least partially DELLA independent.

RESULTS

Identification and Characterization of a *pro* Mutant

In a visual screen of a tomato activation tagging population, a slender elongated mutant was identified. This mutant population was produced in the dwarf Micro-Tom tomato background by a maize Ds transposon element containing an enhancer sequence (see Methods). Backcross analysis of the newly identified mutant showed a recessive mode of inheritance, suggesting a loss-of-function mutation. After introgressing the mutant into the M82 (SP^+) background by four successive backcrosses, the homozygous progeny exhibited similar slender-elongated growth. Since the mutant phenotype resembled that of *pro*, we sequenced the *PRO* gene and found a mutation likely to be caused by excision of a transposon used for activation tagging. The mutation created a stop codon downstream to the VHIID domain (position 339, Glu to stop); thus, the allele was predicted to encode a truncated protein lacking most of the GRAS domain (Figure 1A; Supplemental Figure 1). These $pro^{\Delta GRAS}$ plants were extremely slender and tall compared with M82 and the *pro* mutant. Four-week-old $pro^{\Delta GRAS}$ plants were ~3 times taller than M82 plants and twice as tall as M82 with *pro* introgressed into it (Figures 1B and 1C). The leaf phenotype of $pro^{\Delta GRAS}$ was also stronger than that of *pro*, with larger, smoother, and curlier leaflets that featured longer petioles lacking intercalary leaflets (Figure 2A). In addition, flowering time was delayed and first inflorescence emerged after the production of 8 to 10 leaves rather than 5 to 7 and 7 to 8 leaves in M82 and *pro*, respectively (Figure 2B). The stigmas of the $pro^{\Delta GRAS}$ pistils protruded above the staminal cone due to the long style (Figure 2C), and when fruits were made, they were all seedless, small, and oval (Figure 2D). Notably, the development of partenocarpic fruits in tomato can be triggered by constitutive GA signaling (Carrera et al., 2012).

Recently, new *pro* alleles were produced using a transcription activator-like effector nuclease (TALEN; Lor et al., 2014). $pro^{\Delta TALEN\text{-}2}$ plants were similar to $pro^{\Delta GRAS}$ and had stronger defects than *pro* (Supplemental Figures 2A and 2B). This includes longer stem, simpler leaves with smoother leaflets, long styles, and production of small partenocarpic fruits. When *pro*, pro^{TALEN_2}, $pro^{\Delta GRAS}$, and $pro^{TALEN\_2}/pro^{\Delta GRAS}$ plants (Supplemental Figure 3) were grown side by side for 4 weeks and their phenotypes were compared, $pro^{TALEN\_2}/pro^{\Delta GRAS}$ plants were indistinguishable from homozygous pro^{TALEN_2} and $pro^{\Delta GRAS}$ plants (Supplemental Figure 2B), indicating that both are strong alleles that are likely null.

Loss of PRO Activity Affects Fertilization and Seed Set

As lack of fertilization in $pro^{\Delta GRAS}$ and pro^{TALEN_2} flowers could stem from the long styles that prevent self-pollination (Figure 2C), $pro^{\Delta GRAS}$ flowers were hand-pollinated with $pro^{\Delta GRAS}$ pollen. Fertilization was rarely observed, suggesting a physiological barrier that prevents the fertilization process. This differs from the *pro* mutant that exhibits facultative parthenocarpy (Carrera et al., 2012). Pollination of $pro^{\Delta GRAS}$ flowers with M82 pollen grains

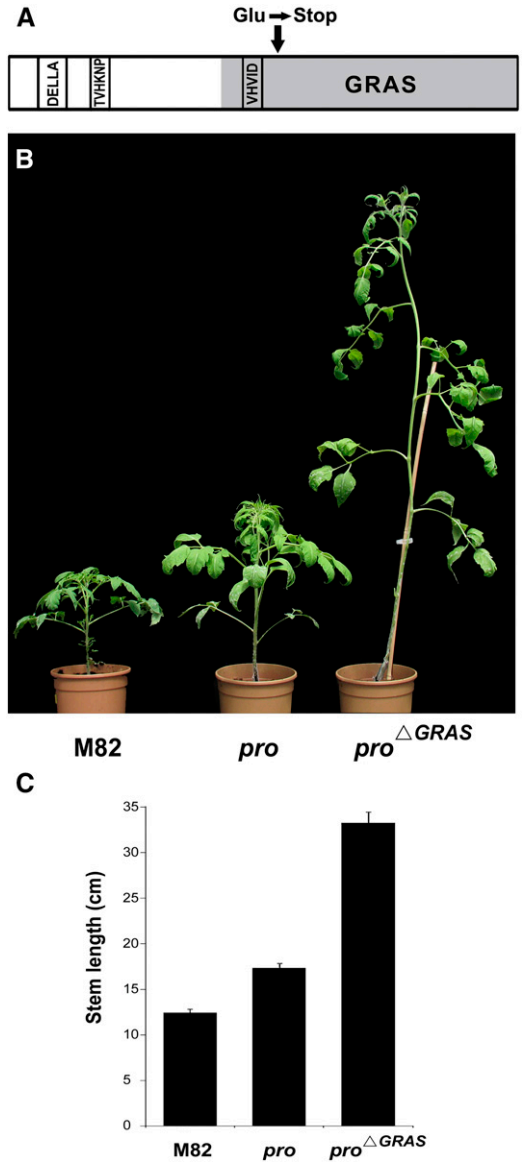

Figure 1. The *pro*$^{\Delta GRAS}$ Mutant.

(A) Schematic presentation of the PRO protein structure. The arrow indicates the position of the mutation in *pro*$^{\Delta GRAS}$ converting the amino acid Glu to a stop codon.
(B) Five-week-old M82, *pro*, and *pro*$^{\Delta GRAS}$ plants.
(C) The mean length (n = 12) ± SE of the main stem of 4-week-old M82, *pro*, and *pro*$^{\Delta GRAS}$ plants.

resulted in partial seed set (Supplemental Figure 4), suggesting that female gametophytes are fertile. Similar male sterility and female fertility were found in *pro*$^{\Delta TALEN\_2}$ (Supplemental Figure 4 and Supplemental Table 1). Scanning electron microscopy images revealed that *pro*$^{\Delta GRAS}$ anthers were thinner and smaller and contained fewer pollen grains compared with M82 and *pro* (Figure 3A). An in vitro pollen germination assay showed that pollen of M82, *pro*, and *pro*$^{\Delta GRAS}$ germinated; however, while M82 and *pro* pollen tubes continued to elongate during the 6 h of the experiment, *pro*$^{\Delta GRAS}$ pollen tubes stopped elongating shortly after germination (Figure 3B). This growth suppression of *pro*$^{\Delta GRAS}$ pollen tubes may explain the obligatory parthenocarpy observed in this mutant.

Seed Viability and Segregation Distortion in Progenies of *pro*$^{\Delta GRAS}$/+ Plants

Since homozygous *pro*$^{\Delta GRAS}$ did not produce seeds, we had to use progenies of heterozygous plants to obtain homozygous plants. When sowing these seeds after a short period of storage (2 to 5 weeks of dry storage), ~2 to 8% of the seedlings were homozygous, and not the 25% expected by the Mendelian segregation ratio (Supplemental Figure 5A). To test if PRO activity is required for embryo vitality or for embryo survival under dry storage conditions, we extracted seeds from red fruits and sowed them either immediately or after longer periods of dry storage. The expected ratio of 25% seedlings with a *pro*$^{\Delta GRAS}$ phenotype was obtained for fresh seeds. In contrast, only 8% of the seedlings from seeds that were stored for 10 d exhibited the *pro*$^{\Delta GRAS}$ phenotype, while ~18% of the seeds did not germinate (Figure 4A). After 2 months of dry storage, only 5% of the seedlings exhibited the *pro*$^{\Delta GRAS}$ phenotype. These results led us to speculate that *pro*$^{\Delta GRAS}$ seeds are intolerant to desiccation. However, it should be mentioned that when seeds were sown, *pro*$^{\Delta GRAS}$ seedlings were the first to germinate, pointing at a promoting effect of the constitutive GA signaling on germination (Supplemental Figure 5B). To further examine this phenomenon, we conducted the same experiment with *pro* and *pro*$^{TALEN\_2}$ seeds. Dry storage of *pro* seeds (5 months) did not affect their germination (Supplemental Figure 6A), while *pro*$^{TALEN\_2}$ seeds, similar to *pro*$^{\Delta GRAS}$, exhibited reduced germination after short periods of dry storage (Supplemental Figure 6B).

Abscisic Acid Responses in *pro*$^{\Delta GRAS}$ Seeds

To understand how PRO promotes desiccation tolerance, we followed the expression of desiccation-related genes by quantitative RT-PCR (qRT-PCR) analysis of RNA extracted from both M82 and the scarce fresh homozygous *pro*$^{\Delta GRAS}$ seeds. To this end, we collected pollen from a large number of *pro*$^{\Delta GRAS}$ anthers and pollinated many *pro*$^{\Delta GRAS}$ flowers that eventually produced a few homozygous seeds. We analyzed the expression of the tomato *ABA INSENSITIVE3* (*ABI3*), *LATE EMBRYOGENESIS25* (*LE25*), and *GALACTINOL SYNTHASE1* (*GOLS1*) genes, all of which are known to be regulated by abscisic acid (ABA) and to be involved in the acquisition of seed desiccation tolerance (Cohen and Bray, 1992; Downie et al., 2003; Bassel et al., 2006; To et al., 2006). In addition, we analyzed the expression of the tomato *FUSCA3-like* (*FUS3-like*) homolog, a major player in the acquisition of desiccation tolerance (To et al., 2006). All four genes exhibited significantly lower levels of expression in *pro*$^{\Delta GRAS}$ compared with M82 seeds (Figure 4B), suggesting that the machinery to induce desiccation tolerance is suppressed in *pro*$^{\Delta GRAS}$ seeds. Since ABA has a major role in the acquisition of desiccation tolerance during seed maturation (Ooms et al., 1993; Koornneef et al., 2002; Finkelstein et al., 2008), and DELLA positively regulates ABA accumulation via the transcriptional activation of *XERICO*, a RING-E3 ligase (Zentella et al., 2007; Ariizumi et al., 2013), we analyzed the

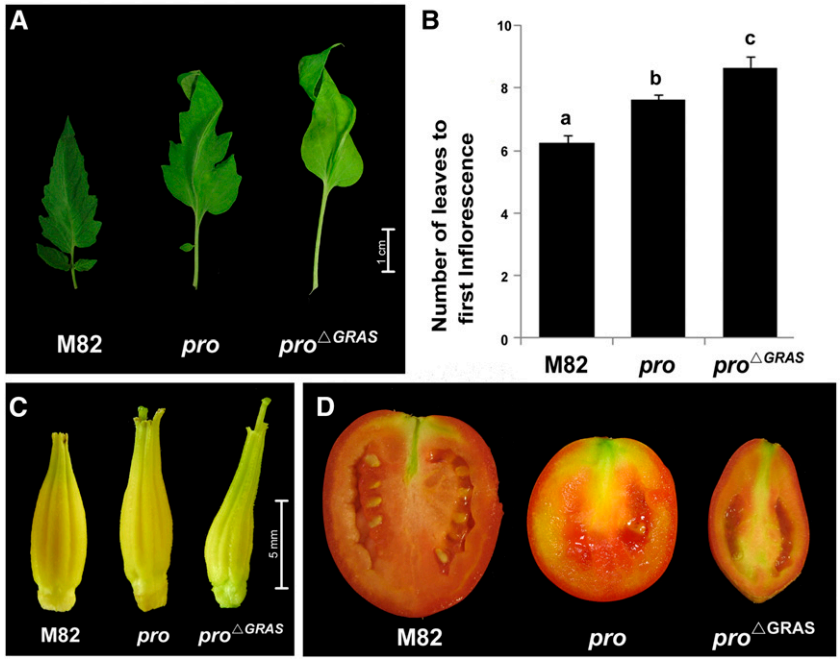

Figure 2. Phenotypic Characterization of *pro*$^{\Delta GRAS}$.

(A) First leaflet of the fifth leaf in 5-week-old plants.
(B) Mean number of leaves to first inflorescence (n = 11 plants) ± SE. Letters indicate significant differences, as determined by t test $P < 0.05$.
(C) M82, *pro*, and *pro*$^{\Delta GRAS}$ flowers before anthesis.
(D) M82, *pro*, and *pro*$^{\Delta GRAS}$ fruits.

expression of the tomato *XERICO* homolog in fresh *pro*$^{\Delta GRAS}$ seeds. *XERICO-like* expression was lower in *pro*$^{\Delta GRAS}$ compared with M82 seeds (Supplemental Figure 7), implying that the lack of desiccation tolerance in *pro*$^{\Delta GRAS}$ seeds may result from reduced ABA levels.

DELLA-Independent GA Responses

Our data suggest that the *pro*$^{\Delta GRAS}$ allele is much stronger than *pro* and may represent a null allele. Thus, we next tested whether the well-documented responsiveness of *pro* to GA (Van Tuinen et al., 1999) is due to a partial loss of DELLA function or due to the activity of a DELLA-independent GA signaling pathway (Fleishon et al., 2011). To this end, we first treated M82, *pro*, and *pro*$^{\Delta GRAS}$ seedlings with the GA biosynthesis inhibitor paclobutrazol (PAC), followed by application of GA_3. PAC treatment of M82 and of the *pro* mutant suppressed stem elongation (Figures 5A and 5B), an effect that was reversed by application of GA_3. However, PAC, GA_3, or their sequential application did not alter elongation of *pro*$^{\Delta GRAS}$ or *pro*$^{TALEN\_2}$ stems (Supplemental Figure 8). Likewise, chlorophyll content was elevated by PAC and reduced by GA_3 in M82 and *pro* but not in *pro*$^{\Delta GRAS}$ leaves (Figure 5C). These results suggest that *pro*$^{\Delta GRAS}$ and *pro*$^{TALEN\_2}$ plants are largely insensitive to GA, while *pro* plants retain some DELLA activity.

To examine the molecular responses of *pro*$^{\Delta GRAS}$ to GA, we compared the regulation of GA metabolism and catabolism genes by GA. GA homeostasis is regulated by a negative feedback loop, where high GA levels/signals suppress GA production via the inhibition of the GA biosynthetic gene *GA20oxidase* (*GA20ox*) and promote GA deactivation by the induction of the GA deactivation gene, *GA2oxidase* (*GA2ox*; Yamaguchi, 2008). M82 and *pro*$^{\Delta GRAS}$ seedlings were treated with PAC for 3 d and then treated with 0, 1, or 100 μM GA_3. Three hours after the GA treatment, RNA was extracted from young leaves and the expression levels of *GA20ox1* and *GA2ox4* were analyzed by qRT-PCR. We would like to emphasis that the names of these and other tomato GA metabolism and catabolism genes do not necessarily reflect their relatedness to the Arabidopsis genes. The accession numbers of all the tomato genes used in this study can be found in Methods. As expected, *GA20ox1* expression was promoted by PAC and suppressed by GA_3 in M82 leaves. In agreement with the constitutive GA signaling and insensitivity to GA, *GA20ox1* expression was extremely low in *pro*$^{\Delta GRAS}$ and neither affected by PAC nor by GA_3 treatment (Figure 6A). *GA2ox4* expression was low in mock-treated M82 and induced by GA_3 treatment. However, the *GA2ox4* expression level in *pro*$^{\Delta GRAS}$ remained low, similar to the level found in M82 leaves, indicating that it was not affected by the endogenous constitutive GA signal. Moreover, expression of this gene in *pro*$^{\Delta GRAS}$ was strongly induced by exogenous GA_3 (Figure 6B). As these results were unexpected, the experiment was repeated six times and similar results were obtained (Supplemental Figure 9). However, it should be noted that in some experiments, the GA induction of *GA2ox4* was stronger in *pro*$^{\Delta GRAS}$ than is M82, but not in others (Figure 6B versus Supplemental Figure 9). We next examined the impact of GA_3 treatment of *pro* leaves on the expression of these two genes. *GA20ox1* expression was low in

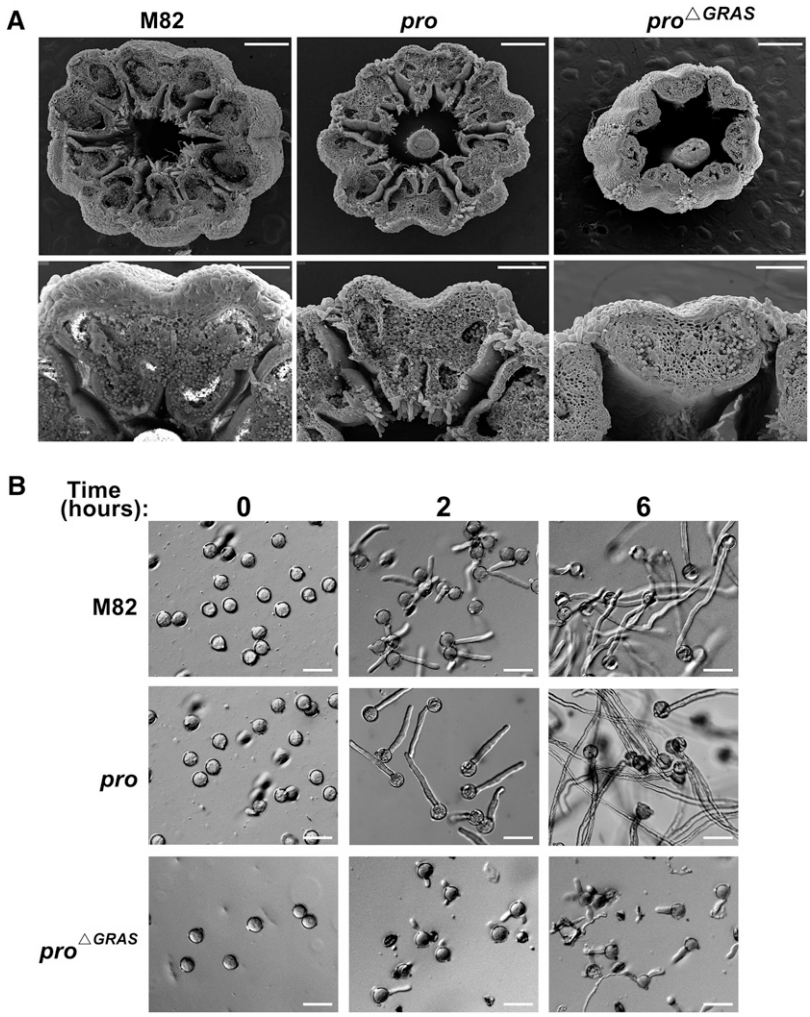

Figure 3. The Effect of *pro*$^{\Delta GRAS}$ and *pro* on Anther Development, Pollen Production, and Pollen Tube Elongation.

(A) Scanning electron microscopy images of M82, *pro*, and *pro*$^{\Delta GRAS}$ anther cones and single anthers. Flowers were detached prior to anthesis and cut widthwise. Bars in the upper panels = 500 μm; bars in the lower panels = 250 μm.

(B) Real-time observation of in vitro germination of M82, *pro*, and *pro*$^{\Delta GRAS}$ pollen. Flowers were detached at anthesis and pollen was incubated in germination solution. Germination and tube elongation were monitored for 6 h using a light microscope. Bar = 50 μm.

mock-treated *pro* (due to the constitutive GA responses) but was further inhibited by treatment with 10 μM GA_3 (Supplemental Figure 10), indicating partial PRO activity. *GA2ox4* expression, on the other hand, was not affected by the constitutive GA signaling in *pro* but was induced by exogenous GA_3 treatment. We next analyzed the expression levels of other *GA2ox* genes, *GA2ox2* and *GA2ox5*. *GA2ox2* expression was not altered by GA application to the wild-type M82; therefore, its expression was not examined in *pro*$^{\Delta GRAS}$ seedlings (Supplemental Figure 11A). The expression profile of *GA2ox5*, on the other hand, in response to GA was similar to that of *GA2ox4*, i.e., induced by exogenous GA_3 in *pro*$^{\Delta GRAS}$ (Supplemental Figure 11B).

The strong induction of *GA2ox4* by exogenous GA_3 in *pro*$^{\Delta GRAS}$ combined with the lack of effect of the constitutive endogenous GA signaling in this mutant suggest a GA response that is DELLA independent. However, it should be noted that *GA2ox4* did not respond to application of GA_3 in M82 and *pro*$^{\Delta GRAS}$, without prior exposure to PAC, and the PAC treatment itself, typically weakly promoted expression. Similar results were found previously in rice (Huang et al., 2010).

To further investigate this possible DELLA-independent GA response, we generated transgenic *rgaΔ17* tomato plants (M82 background) overexpressing the Arabidopsis DELLA RGA lacking the DELLA domain (Dill et al., 2001). The 17-amino acid deletion in RGA inhibits the degradation of the protein in response to GA and, therefore, when overexpressed, constitutively suppresses GA responses (Dill et al., 2001). We used the Arabidopsis gene to bypass possible cosuppression. *35S:rgaΔ17* tomato lines with high *rgaΔ17* expression levels (Figure 7A) and a severe dwarfism were self-pollinated and homozygous lines were generated. These lines also had small dark-green leaves, typical of tomato plants with reduced GA activity (Nir et al., 2014). Application of exogenous

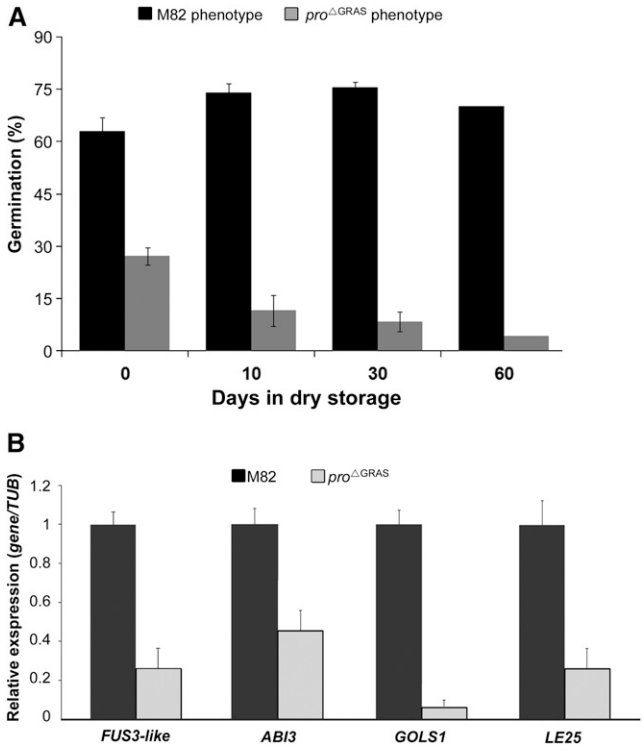

Figure 4. *pro*$^{\Delta GRAS}$ Seeds Are Sensitive to Desiccation.

(A) Seeds were harvested from heterozygous *pro*$^{\Delta GRAS}$ fruits (after self-pollination) and sown immediately thereafter, or after different periods of dry storage. Values represent the percentages of germinating seedlings with M82 (M82 and heterozygous *pro*$^{\Delta GRAS}$ seedlings) or *pro*$^{\Delta GRAS}$ (homozygous *pro*$^{\Delta GRAS}$ seedlings) phenotypes from total number of seeds. Values are the average of three replicates; each contains 50 seeds ± SE.
(B) qRT-PCR analyses of *ABI3*, *FUS3-like*, *LE25*, and *GOLS* expression in M82 and *pro*$^{\Delta GRAS}$ seeds. RNA was extracted from fresh M82 and *pro*$^{\Delta GRAS}$ homozygous seeds. Values are the average of three biological replicas ± SE.

GA_3 (100 μM) strongly promoted elongation of M82 plants, but had no effect on the elongation of *rgaΔ17* plants, suggesting insensitivity to GA (Figure 7B).

We analyzed the expression of *GA20ox1* and *GA2ox4* in *rgaΔ17* tomato plants, following PAC and GA treatments, as described above. As expected in cases of feedback regulation, *GA20ox1* expression was high in the untreated *RGAΔ17* plants (Figure 7C). The expression of this gene was suppressed by GA_3 treatment in M82 plants but was not affected in leaves of the transgenic line. On the other hand, *GA2ox4* expression was induced by GA_3 in both M82 and *rgaΔ17* leaves (Figure 7D), again suggesting that GA regulates the tomato *GA2ox4* via a DELLA-independent pathway. Notably, while in some experiments the induction of *GA2ox4* by GA_3 in *rgaΔ17* leaves was stronger than that in M82 (Figure 7D), in other experiments, we found similar response to GA_3 in the different lines (Supplemental Figure 12).

To examine whether feedback regulation of *GA2ox* by GA is DELLA independent in other species, we examined the Arabidopsis *della* pentuple mutant (*dellaP*; Park et al., 2013). The *dellaP* (*rga-28*, *gai-t6*, *rgl1-SK62*, *rgl2-SK54*, and *rgl3-3*) in the Columbia-0 (Col-0) background has impaired activity of all five *DELLA* genes and, therefore, as in *pro*$^{\Delta GRAS}$, exhibits constitutive GA signaling. Wild-type Col-0 and *dellaP* seedlings were treated with PAC (5 mg/L) once a day for 3 d followed by a single GA_3 application (10 μM). Three hours after the GA treatment, RNA was extracted from seedling shoots and analyzed for At-*GA20ox2* and At-*GA2ox4* expression. At-*GA20ox2* exhibited normal feedback regulation in the wild type but expression was unaffected by PAC or GA in *dellaP* (Figure 8A). At-*GA2ox4* was induced by GA_3 in both the wild type and *dellaP* (Figure 8B). We also tested the response of these two genes to GA_3 in flowers. To this end, seedlings were treated with PAC (5 mg/L) twice a week until flowering and then treated once with 10 μM GA_3. Three hours after the GA treatment, RNA was extracted from the flowers and analyzed for At-*GA20ox2* and At-*GA2ox4* expression. While At-*GA20ox2* exhibited normal feedback regulation in the wild type, in *dellaP* its basal expression was low and was unaffected by either PAC or GA (Figure 8C). At-*GA2ox4* was not affected by the endogenous constitutive GA signaling in *dellaP* but was induced by GA_3 in both the wild type and *dellaP* (Figure 8D). These results suggest that in Arabidopsis, the regulation of At-*GA2ox4* by GA is also DELLA independent.

To further explore DELLA-independent GA responses in Arabidopsis, we examined the expression of At-*GA2ox1* in the flowers. At-*GA2ox1* behaved as expected of a DELLA-regulated gene, i.e., high expression in *dellaP* and lack of response to GA_3 (Figure 8E). To examine whether the activation of At-*GA2ox4* by GA is initiated by the GA receptor GID1, we treated wild-type and *gid1ac* (loss of two out of the three GID1 receptor genes; Griffiths et al., 2006) seedlings with PAC (5 mg/L) once a day for 3 d followed by a single GA_3 application (10 μM). Three hours after the GA treatment, RNA was extracted and analyzed for At-*GA2ox4* expression. The lack of GID1a and GID1c activity significantly reduced the response of At-*GA2ox4* to GA_3 (Figure 8F), suggesting that this DELLA-independent GA response is initiated by GA binding to the GID1 receptors. The observed weak response of At-*GA2ox4* to GA_3 in *gid1ac* was probably mediated by GID1b.

Global Analysis of DELLA-Independent GA Responses

To understand the scope of DELLA-independent GA-regulated genes, deep sequencing (RNA-seq) was performed to RNA samples extracted from GA-treated M82 and *pro*$^{\Delta GRAS}$ plants. M82 and *pro*$^{\Delta GRAS}$ seedlings were treated with PAC (10 mg/L) once a day for 3 d followed by a single GA_3 application (100 μM). Three hours after the GA treatment, young leaves were collected, RNA was extracted, and cDNA libraries were sequenced by Illumina HiSequation 2500. A total of eight samples were analyzed, and each treatment had two biological replicates. TopHat was used to align the reads to the tomato genome SL2.50 (Trapnell et al., 2009). Counts of aligned reads per gene were obtained using HTSeq-count (Anders et al., 2015), and the DESeq2 package was used to identify genes that were differentially expressed between PAC and PAC + GA_3 treated leaves. Using a 2-fold increase or decrease cutoff (adjusted P value for multiple comparisons $\leq$0.05), we identified 81 GA-upregulated and 15 GA-downregulated genes (Tables 1 and 2; Supplemental Table 2). The majority of these genes were DELLA dependent, i.e., their expression was unaffected by GA_3 in *pro*$^{\Delta GRAS}$. These include some well-characterized GA-regulated genes, such as *GA20ox*,

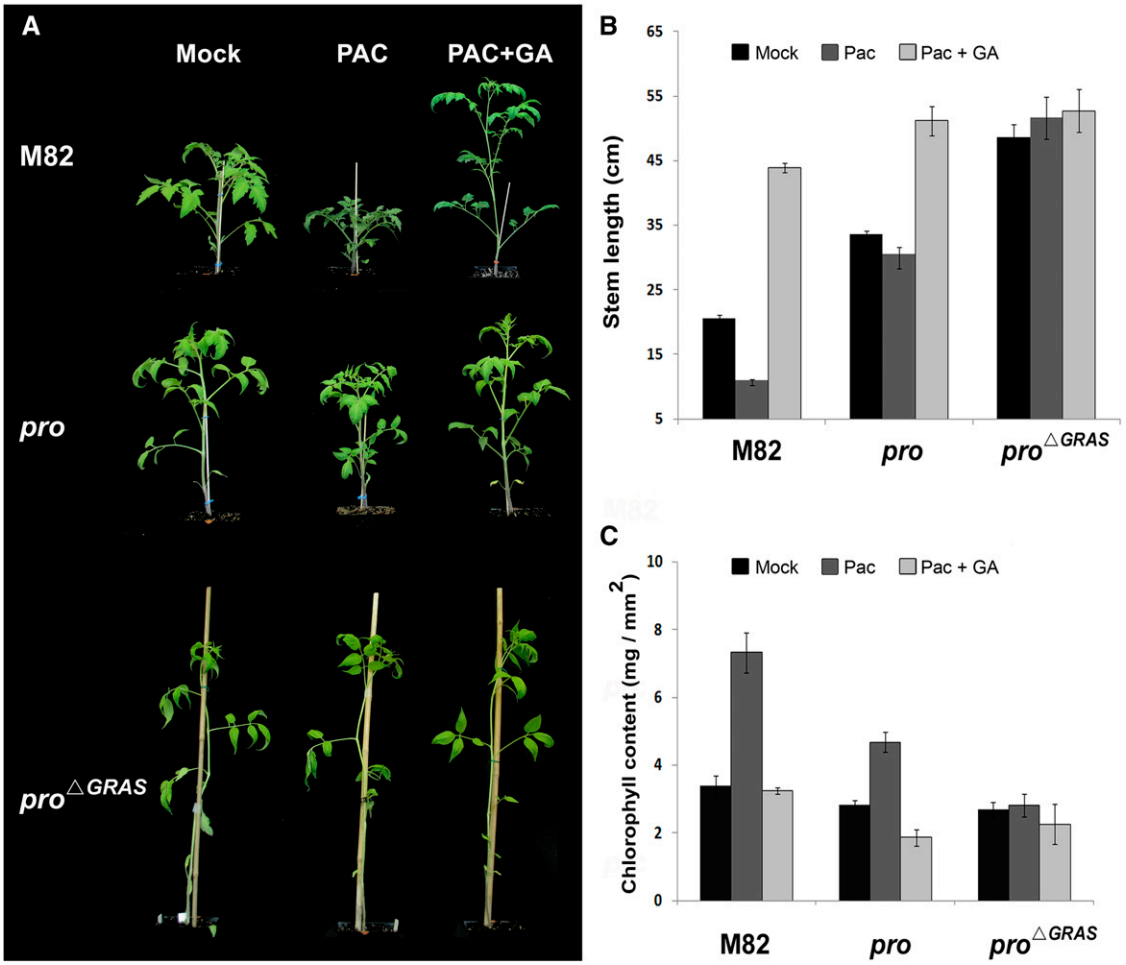

Figure 5. *pro*$^{\Delta GRAS}$ Is Insensitive to PAC and GA.

(A) Six-week-old M82, *pro*, and *pro*$^{\Delta GRAS}$ plants were treated with 10 mg/L PAC three times a week, for 2 weeks (starting at two true leaves), followed by 2 weeks of GA_3 application (100 μM, three times a week).
(B) Mean length ± SE of the main stems of the plants treated as in **(A)** (n = 8 to 11 plants).
(C) Mean chlorophyll content ± SE in the first leaflet of the forth leaf taken from 6-week-old plant treated as in **(A)** (n = 8).

GID1, *SCL*, *GAST1*, and *EXPANSINS* (Shi et al., 1992; Chen et al., 2001; Zentella et al., 2007). Five of the GA-regulated genes (four upregulated and one downregulated) were DELLA-independent, i.e., they were similarly induced or suppressed by GA_3 in M82 and *pro*$^{\Delta GRAS}$ (Table 3). It should be noted that in this experiment, all GA2ox genes were expressed at low levels and none of them was affected significantly by GA_3 in M82 or *pro*$^{\Delta GRAS}$. To confirm the results, we analyzed the expression of the identified GA-regulated DELLA-independent genes, Solyc07g064600.2 (encoding Endoribonuclease) and Solyc09g008670.2 (encoding Thr ammonia lyase) by qRT-PCR. The results confirm those of the RNA-seq and show that GA induces both in a DELLA-independent manner (Supplemental Figure 13).

DISCUSSION

The tomato genome contains a single DELLA gene, named *PRO*, and a *pro* mutant has been extensively characterized (George Jones, 1987; Jupe et al., 1988; Van Tuinen et al., 1999; Jasinski et al., 2008; Bassel et al., 2008; Carrera et al., 2012). *pro* exhibits constitutive GA activity but retains some responsiveness to the hormone, either due to incomplete loss of DELLA activity (Van Tuinen et al., 1999) or due to activity of a DELLA-independent GA response pathway (Fleishon et al., 2011). Here, we describe *pro* mutants, *pro*$^{\Delta GRAS}$ and *pro*$^{TALEN\_2}$ (Lor et al., 2014) that are likely null or close to null alleles. Our study suggests that the responsiveness of the "classic" *pro* mutant to GA is due to residual DELLA activity but also uncovers DELLA-independent GA responses.

The phenotype of *pro*$^{\Delta GRAS}$ and *pro*$^{TALEN\_2}$ plants resembles that of tomato plants treated with high doses of GA. In tomato, exogenous GA application has a dramatic effect on stem elongation. In Arabidopsis, on the other hand, application of GA or lack of DELLA activity has only a mild effect on final stem length (King et al., 2001). A strong effect is found only when the hormone is applied to GA-deficient mutants. This difference between Arabidopsis and tomato

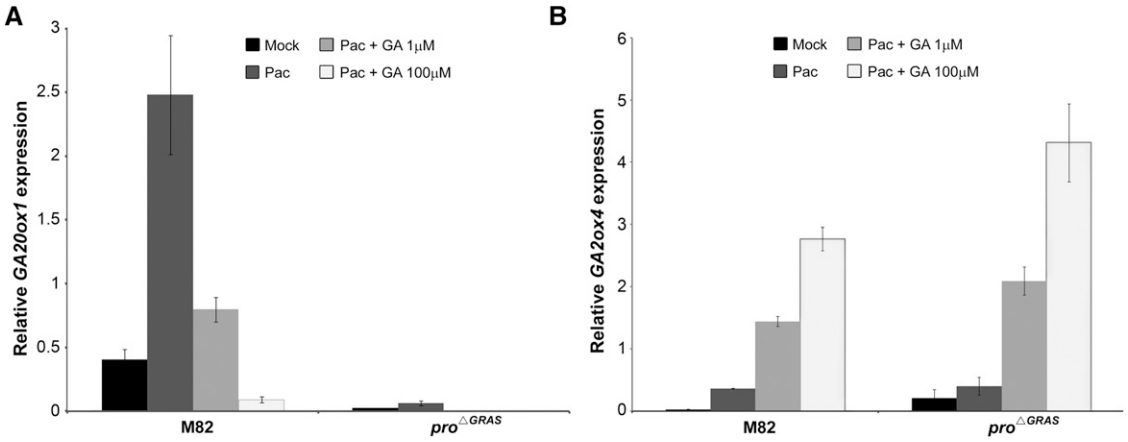

Figure 6. Regulation of *GA20ox* and *GA2ox* Expression by GA in *pro*$^{\Delta GRAS}$.

qRT-PCR analysis of *GA20ox1* **(A)** and *GA2ox4* **(B)** expression. Seedlings were treated with 10 mg/L PAC for 3 d, followed by one application of GA_3 (1 or 100 μM). RNA was extracted from young leaves and analyzed. Values (gene-to-*TUBULIN* ratios) are means of three biological replicates ± SE.

may be due to differences in basal levels of endogenous active GAs. A rapid stem elongation (bolting) in Arabidopsis occurs after the floral transition and is associated with a dramatic increase in GA level (Eriksson et al., 2006). Thus, GA activity may be saturated and the loss of DELLA or addition of exogenous GA has only a mild effect. On the other hand, the tomato stem elongates slowly but continuously throughout the life of the plant. It is possible that this slow elongation requires intermediate GA levels, below saturation; therefore, loss of PRO activity or application of high GA doses has a dramatic effect on stem elongation.

All analyzed GA-related phenotypes were more severe in *pro*$^{\Delta GRAS}$ and *pro*$^{TALEN\_2}$ plants than in *pro*, suggesting that *pro* is a "leaky" mutant, as previously proposed (Van Tuinen et al., 1999). While *pro* exhibits facultative partenocarpy (Carrera et al., 2012), *pro*$^{\Delta GRAS}$ and *pro*$^{TALEN\_2}$ did not produce seeds even after hand-pollination, suggesting obligatory partenocarpy. Previous studies suggested that the facultative partenocarpy of *pro* is due to the longer style, which prevents self-pollination (Bassel et al., 2008; Carrera et al., 2012). While the *pro*$^{\Delta GRAS}$ and *pro*$^{TALEN\_2}$ styles are longer than that of *pro*, it cannot explain the obligatory partenocarpy. Since pollination of *pro*$^{\Delta GRAS}$ and *pro*$^{TALEN\_2}$ flowers with M82 pollen resulted in an almost normal seed set, the lack of fertilization in homozygous *pro*$^{\Delta GRAS}$ or *pro*$^{TALEN\_2}$ flowers is probably due to male sterility. An in vitro pollen germination assay showed that the elongation of *pro*$^{\Delta GRAS}$ pollen tube, but not that of *pro*, is arrested shortly after germination. Previous studies in Arabidopsis and rice suggested that while GA is required for pollen tube elongation, GA concentrations higher than optimal inhibit this process (Singh et al., 2002; Chhun et al., 2007). This can explain why *pro*$^{\Delta GRAS}$ but not *pro*, inhibited pollen tube elongation. The suppression of pollen tube elongation in *pro*$^{\Delta GRAS}$ is probably not a cell-autonomous effect. If it was, homozygous seeds would not be obtained by self-pollination of heterozygous plants, since haploid *pro*$^{\Delta GRAS}$ pollen would not elongate to fertilize the *pro*$^{\Delta GRAS}$ egg cells. Thus, it is possible that the effect of *pro*$^{\Delta GRAS}$ on the ability of the pollen cells to elongate is via the supporting tissues, the connective and tapetum cell layers. Indeed, scanning electron microscopy analysis showed malformation of these tissues in *pro*$^{\Delta GRAS}$.

Tomato seeds can be considered "orthodox" seeds (Angelovici et al., 2010), since they can tolerate desiccation and can be stored in a dry state for years (Priestley et al., 1985). Our results show that homozygous *pro*$^{\Delta GRAS}$ and *pro*$^{TALEN\_2}$ seeds lose their ability to germinate shortly after harvest and cannot survive even short periods (days) of dry storage. Analysis of desiccation tolerance-related genes (*ABI3*, *FUS3*, *LE25*, and *GOLS*) in *pro*$^{\Delta GRAS}$ seeds revealed reduced expression levels, suggesting that PRO is required for activation of the machinery that acquire tolerance. The germination of *pro* seeds, on the other hand, was not affected by long dry storage, suggesting that residual DELLA activity is sufficient to acquire desiccation tolerance.

ABA plays a major role in the acquisition of desiccation tolerance as well in the induction of dormancy during the late stages of seed maturation (Ooms et al., 1993; Koornneef et al., 2002; Finkelstein et al., 2008). Previous studies have shown that DELLA regulates ABA synthesis in seeds via the transcriptional activation of the RING ubiquitin E3 ligase *XERICO*, an inducer of ABA synthesis (Zentella et al., 2007; Piskurewicz et al., 2008; Ariizumi et al., 2013). We found reduced expression of the putative tomato homolog of *XERICO* in *pro*$^{\Delta GRAS}$ seeds, suggesting that PRO increases desiccation tolerance by promoting ABA synthesis. Although desiccation tolerance is tightly associated with dormancy and both are regulated by ABA, previous studies linked DELLA activity in seeds with dormancy only (Lee et al., 2010; Ariizumi et al., 2013). Our results suggest that the loss of PRO activity suppresses both processes; while homozygous *pro*$^{\Delta GRAS}$ seeds had reduced desiccation tolerance, they germinated much faster than M82 seeds, suggesting weaker dormancy.

It is possible that the loss of seed viability during dry storage prevented the identification of strong *pro* alleles in all previous tomato mutant screenings. It is also possible that the Micro-Tom background, which has a mutation in the *DWARF* (*D*) gene, allowed the identification of this allele in our screening. *D* encodes a P450 protein involved in brassinosteroid biosynthesis (Bishop et al.,

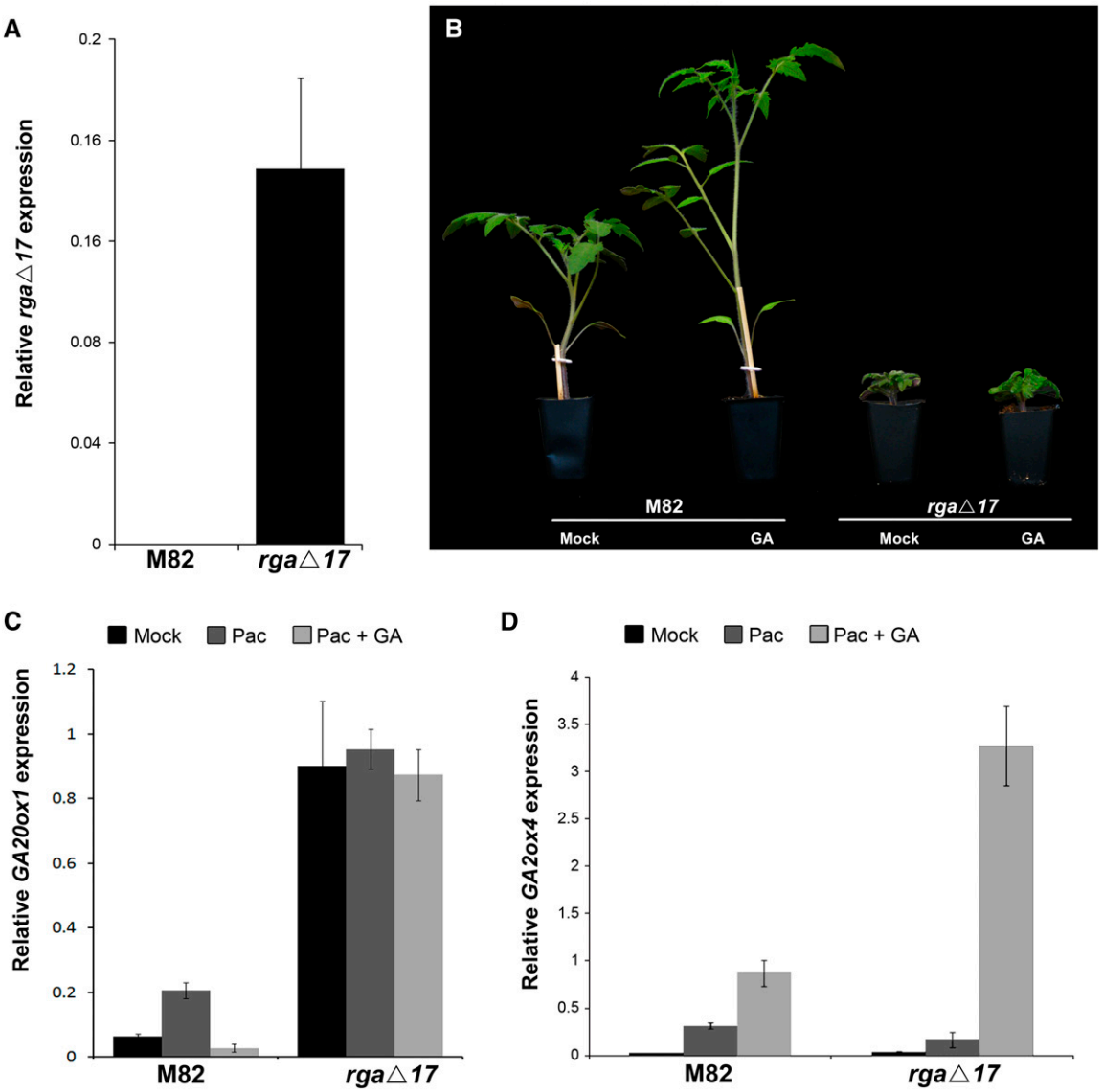

Figure 7. Regulation of Growth and Gene (*GA20ox* and *GA2ox*) Expression by GA in the Transgenic Tomato Overexpressing the Arabidopsis *RGA△17* Mutant Gene.

(A) qRT-PCR analysis of *RGA△17* expression in M82 and transgenic tomato plants. RNA was extracted from young leaves of the T2 generation. Values (gene-to-*TUBULIN* ratios) are means of three biological replicates ± SE.
(B) M82 and transgenic *RGA△17* plants treated with 100 μM GA_3 three times a week for 2 weeks.
(C) and **(D)** qRT-PCR analyses of *GA20ox1* **(C)** and *GA2ox4* **(D)** expression in tomato leaves treated with 10 mg/L PAC for 3 d or PAC for 3 d followed by one application of 100 μM GA_3. Values (gene-to-*TUBULIN* ratios) are means of three biological replicates ± SE.

1999). Since GA and brassinosteroids act synergistically (Bai et al., 2012), and the response to GA in Micro-Tom partially depends on brassinosteroids (Martí et al., 2006), it is possible the GA responses are partially suppressed in Micro-Tom, improving seed tolerance to desiccation.

Our results suggest that the reported, relatively strong response of *pro* to GA (Van Tuinen et al., 1999) is due to the "leaky" nature of the *pro* allele and not due to the activity of an alternative GA signaling pathway. In parallel, while the null mutants $pro^{\Delta GRAS}$ and pro^{TALEN_2} exhibited insensitivity of growth to GA and PAC, a DELLA-independent GA response in $pro^{\Delta GRAS}$ plants, namely, the feedback regulation of GA catabolism, was discovered. As expected, the expression level of *GA20ox1* was lower in $pro^{\Delta GRAS}$ than in M82 and was not affected by GA or PAC treatments. On the other hand, the expression of *GA2ox4* and *GA2ox5* was unexpectedly low in $pro^{\Delta GRAS}$ and was strongly induced by GA_3. These findings suggest that *GA2ox4* and *GA2ox5* do not respond to the endogenous constitutive GA signaling produced by the loss of *PRO*, but rather, are induced by exogenous GA treatment. In addition, although transgenic tomato plants overexpressing the

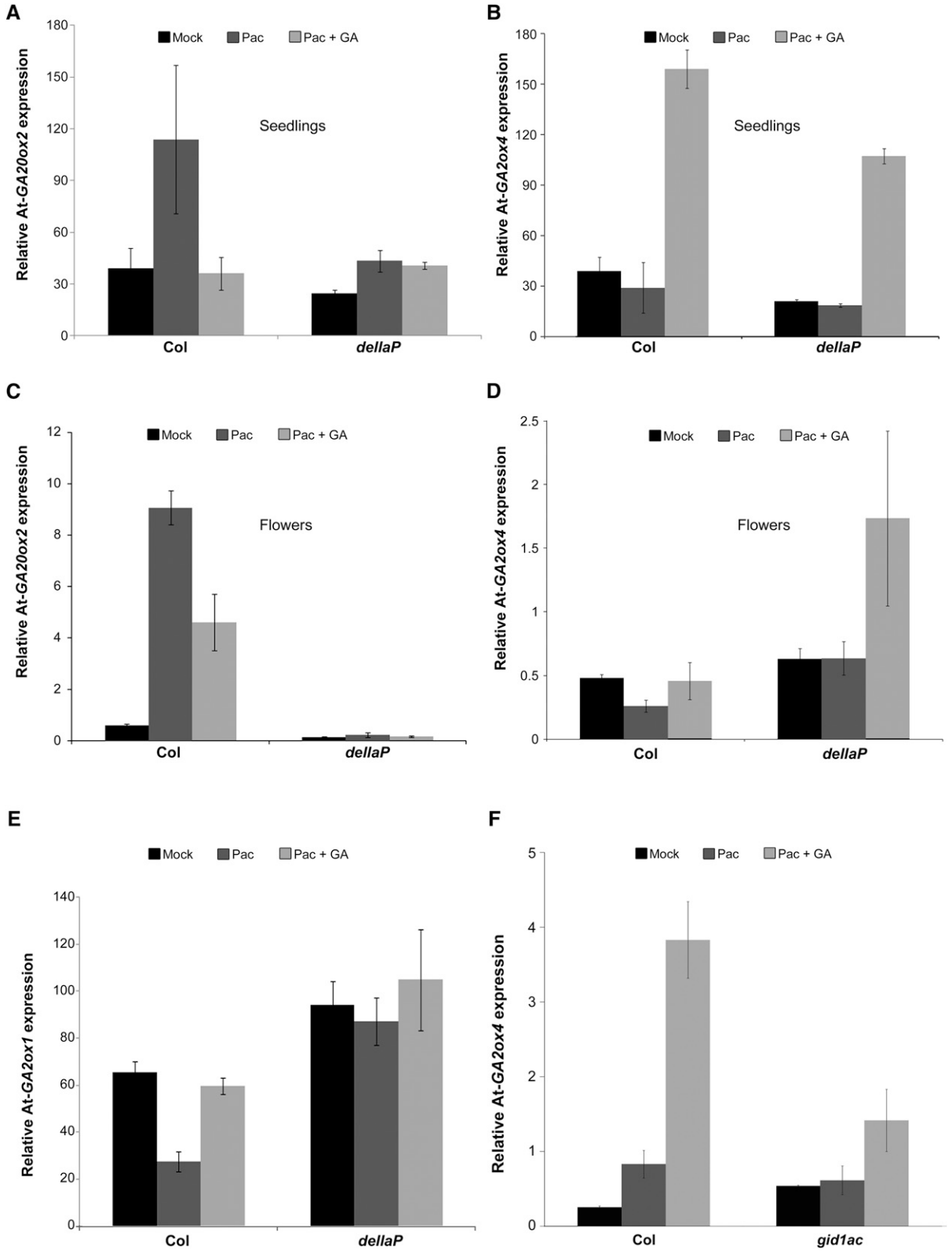

Figure 8. Regulation of Arabidopsis *GA20ox2*, *GA2ox4*, and *GA2ox1* Expression by GA in Arabidopsis.

Table 1. GA Upregulated, DELLA-Dependent Genes (Fold Change > 4)

SolyC Locus	Description	Mean Pac[a]	Mean Pac+GA[a]	Fold Change[b]	Adj. P Value[c]
Solyc05g007950.2	Ribonuclease T2	68	1068	15.78	5.62E-08
Solyc12g010800.1	BZIP transcription factor	10	106	10.29	7.15E-06
Solyc03g025380.2	Peroxidase	24	243	9.98	0.000267
Solyc03g005320.2	3-Ketoacyl-CoA synthase	60	379	6.36	1.64E-06
Solyc01g110630.2	Auxin-induced SAUR-like	18	108	5.95	0.000945
Solyc04g017720.2	GAST1	55	312	5.71	1.22E-07
Solyc12g056250.1	Glutathione *S*-transferase	341	1917	5.62	2.76E-06
Solyc07g062710.2	BZIP transcription factor	64	342	5.38	6.02E-08
Solyc04g081790.2	GDSL esterase/lipase	54	290	5.38	6.02E-08
Solyc04g016190.1	Glucosyltransferase	87	465	5.33	0.001001
Solyc03g097170.2	Cinnamoyl-CoA reductase	140	739	5.29	8.31E-07
Solyc03g078090.2	Pectinesterase	19	89	4.80	0.033812
Solyc10g005210.2	Methyladenine glycosylase	42	195	4.60	1.64E-06
Solyc10g011730.2	Arabinogalactan peptide	49	219	4.49	3.75E-05
Solyc03g006100.2	Receptor-like kinase, RLK	144	633	4.41	0.000322
Solyc08g075210.1	Acyltransferase-like protein	60	259	4.31	0.011786
Solyc03g114710.2	Glucosyltransferase	33	141	4.30	0.00217
Solyc10g052530.1	Auxin-responsive protein	544	2301	4.28	0.00546
Solyc11g069960.1	Receptor-like kinase, RLK	32	137	4.22	0.000267
Solyc04g081870.2	Expansin	467	1964	4.20	1.98E-07
Solyc02g088100.2	Expansin	297	1232	4.15	0.000293
Solyc07g008560.2	Purple acid phosphatase	25	103	4.04	0.010653

[a]Mean value of two biological replicates.
[b]Fold change is the ratio mean Pac + GA/mean Pac.
[c]Corrected P values were calculated using the Benjamini and Hochberg (1995) false discovery rate approach.

Arabidopsis gain-of-function DELLA protein RGAΔ17 were insensitive to GA in terms of growth, *GA2ox4* was strongly induced by GA_3 treatment in these plants. In summary, these findings suggest that expression of tomato *GA2ox4* is activated by GA via a DELLA-independent pathway. Similar results were found in the Arabidopsis *dellaP* mutant. While At-*GA2ox1* behaved as expected, i.e., exhibited high levels of expression in *dellaP* and insensitivity to GA_3 treatment, the expression of At-*GA2ox4* was strongly induced by exogenous GA_3 in this mutant. While numerous studies have shown that the expression of *GA20ox* is suppressed, and that of *GA2ox*, is promoted by GA (Yamaguchi, 2008), Zentella et al. (2007) suggested that At-*GA20ox* genes, but not At-*GA2ox*, are regulated directly by DELLA. The mechanism by which GA promotes *GA2ox* expression in a DELLA-independent manner is yet unknown, but our results imply that GA binding to the GID1 receptor is required. High GA activity increases plant susceptibility to various biotic and abiotic stresses (Achard et al., 2006, 2008; Nir et al., 2014) and therefore can be destructive to plants. Thus, it is possible that both DELLA-dependent and -independent induction of GA catabolism by increased GA signal evolved to ensure efficient regulation of GA homeostasis.

Our results suggest that ~5% of all tomato GA-regulated genes are DELLA-independent (Tables 1 to 3). Similarly, Cao et al. (2006) suggested that only a portion of the GA-regulated genes in Arabidopsis are DELLA dependent. In tomato, the strongest DELLA-independent induction by GA was on a ribonuclease (RNase) gene (Solyc05g007950.2, 15-fold change). A previous study in barley aleurone identified RNase as a GA-induced gene (Rogers and Rogers, 1999). Tomato GA-regulated genes include homologs of well-characterized Arabidopsis genes: GA downregulated, such as *GA20ox*, *GID1*, and *SCL*, and GA upregulated genes, such as *GASA-like* (*GAST1*) and *EXPANSIN* (Shi et al., 1992; Chen et al., 2001; Zentella et al., 2007). Thus, while many "classic" GA-associated genes are common to distantly related plants, others, e.g., bZIP transcription factor (Solyc12g010800.1, 10-fold induction), may

Figure 8. (continued).

(A) and **(B)** Seedlings of wild-type Col-0 and *dellaP* mutant Arabidopsis plants were treated with PAC (5 mg/L) once a day for 3 d followed by a single GA_3 application (10 μM). Three hours after the GA treatment, RNA was extracted from the seedlings and analyzed by qRT-PCR for At-*GA20ox2* **(A)** and At-*GA2ox4* **(B)** expression.
(C) to **(E)** Plants (wild-type Col-0 and *dellaP*) were treated with PAC (5 mg/L) twice a week until flowering and then treated once with 10 μM GA_3. Three hours after the GA treatment, RNA was extracted from the flowers and analyzed by qRT-PCR for At-*GA20ox2* **(C)**, At-*GA2ox4* **(D)**, and At-*GA2ox1* **(E)**.
(F) Wild type (Col-0) and *gid1ac* seedlings were treated with PAC (5 mg/L) once a day for 3 d followed by a single GA_3 application (10 μM). Three hours after the GA treatment, RNA was extracted and analyzed (qRT-PCR) for At-*GA2ox4* expression.
Values (gene-to-*TUBULIN* ratios) in **(A)** to **(F)** are means of three biological replicates ± SE.

Table 2. GA-Downregulated, DELLA-Dependent Genes

SolyC Locus	Description	Mean Pac[a]	Mean Pac+GA[a]	Fold Change[b]	Adj. P Value[c]
Solyc06g035530.2	Gibberellin 20-oxidase-2	79	7	−11.18	2.67E-05
Solyc03g006880.2	Gibberellin 20-oxidase-1	1244	122	−10.18	0.003222
Solyc01g008910.2	Scarecrow-like	123	15	−8.31	4.17E-05
Solyc03g119530.2	LOB domain protein 42	88	20	−4.45	0.001399
Solyc12g099900.1	GRAS family	298	68	−4.38	6.06E-06
Solyc09g009520.2	Hydrolase α/β fold	110	26	−4.32	0.00961
Solyc09g009220.2	Unknown protein	128	30	−4.20	0.005171
Solyc12g095750.1	Auxin efflux carrier	116	29	−3.99	0.015785
Solyc06g008870.2	GID1-like GA receptor	661	174	−3.81	8.98E-05
Solyc06g067950.2	Acyl-protein thioesterase	135	39	−3.50	0.048555
Solyc01g095580.2	GH3 family protein	1357	432	−3.14	0.000576
Solyc02g080510.1	Unknown protein	340	117	−2.91	0.017273
Solyc09g075590.1	Unknown protein	339	130	−2.61	0.045166
Solyc05g006420.2	ARR3	2685	1182	−2.27	0.013313

[a]Mean value of two biological replicates.
[b]Fold change is the ratio mean Pac + GA/mean Pac [value is presented as: −1(Pac + GA/Pac)].
[c]Corrected P values were calculated using the Benjamini and Hochberg (1995) false discovery rate approach.

represent tomato-specific GA responses. For the five DELLA-independent genes, we were not able to find a common theme that characterizes their specific regulation.

In summary, this work presents new tomato DELLA loss-of-function mutants. Phenotypic, physiological, and molecular analyses of these *pro* mutants uncovered DELLA-regulated processes and identified GA-regulated, DELLA-independent responses, providing a powerful tool to study GA physiology and the role of DELLA in plant biology.

METHODS

Plant Materials and Growth Conditions

Tomato (*Solanum lycopersicum*) plants were in the M82 background (*SP*$^+$). The recessive *pro*$^{\Delta GRAS}$ allele was isolated from an activation-tagging population of Micro-Tom, mutagenized with an Ac/Ds system carrying a 4×35S enhancer element in the Ds transposon (MacAlister et al., 2012). The *pro*$^{\Delta GRAS}$ line used in this study was backcrossed with M82 (SP$^+$) plants four times. *pro* (Bassel et al., 2008) and *pro*^{TALEN} (Lor et al., 2014) were in the M82 (SP$^+$) background. Plants were grown in a greenhouse under 24/20°C (day/night) at natural daylength conditions. *Arabidopsis thaliana* plants were grown in a growth room under controlled temperature (22°C) and long-day (16 h light/8 h dark) conditions. The Arabidopsis DELLA pentuple mutant (*dellaP*; Park et al., 2013) and *gid1ac* double mutant (Griffiths et al., 2006) were in the Col-0 background. Tomato seeds were harvested from ripe fruits, incubated with 10% sucrose overnight at 37°C, and then treated with 1% sodium hypochlorite followed by 1% Na_3PO_4. Seeds were stored dry at room temperature.

Molecular Cloning/Constructs and Plant Transformation

The *RGAΔ17* coding sequence (Zentella et al., 2007) was fused to the 5′ of the enhanced GFP coding sequence, in a *Kpn*I site. The GFP-*RGAΔ17* fusion was inserted to a pART7 plasmid downstream of the *35S* promoter, into *Xho*I and *Bam*HI sites, to create 35S:GFP-*RGAΔ17*. The construct was subcloned into the pART27 binary vector and was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. The construct was transferred to *S. lycopersicum* variety M82 cotyledons, using the transformation and regeneration methods described by McCormick (1991). Kanamycin-resistant T0 plants were grown in the greenhouse, and three independent transgenic lines were selected and self-pollinated to generate homozygous transgenic lines. All primer sequences are presented in Supplemental Table 3.

Hormone Treatments

Tomato seedlings with two true leaves were sprayed with PAC (10 mg/L) three times a week for 2 weeks, followed by GA_3 (Sigma-Aldrich) application (100 μM), throughout the experiment. For the analysis of GA biosynthesis gene expression, young tomato seedlings were sprayed for 3 d with PAC (10 mg/L) and on the fourth day, immersed in 10 or 100 μM GA_3 for 30 min.

Table 3. GA-Regulated, DELLA-Independent Genes

SolyC Locus	Description	Fold Change[a]	Adj. P Value[b]	Fold Change[a]	Adj. P Value[b]
		M82		*pro*$^{\Delta GRAS}$	
Solyc07g064600.2	Endoribonuclease L-PSP	3.20	0.013414	11.88	7.53E-13
Solyc09g083440.2	Proteinase inhibitor I	4.22	0.00546	5.73	0.000413
Solyc09g008670.2	Threonine ammonia-lyase	4.14	0.000293	4.03	0.001424
Solyc03g121270.2	IAA-amino acid hydrolase	2.54	0.015785	3.37	0.00038
Solyc03g117280.2	Unknown protein	−2.21	0.014012	−2.40	0.01025

[a]Fold change is the ratio mean Pac + GA/mean Pac [for fold change <1, the value is presented as: −1(Pac + GA/Pac)].
[b]Corrected P values were calculated using the Benjamini and Hochberg (1995) false discovery rate approach.

Leaves were collected after 3 h and RNA was extracted. Arabidopsis seedlings were treated with PAC (5 mg/L) once a day for 3 d followed by a single GA_3 application (10 μM) or twice a week with PAC (5 mg/L) until flowering and then immersed in 10 μM GA_3 for 30 min. Seedlings or flowers were collected 3 h after the GA treatments and RNA was extracted.

Chlorophyll Measurements

Chlorophyll was extracted from fresh leaves in acetone (100%) and spectrophotometrically measured at 645 and 663 nm (Arnon, 1949). Chlorophyll concentrations were calculated using the formula: $(20.2 \times A_{645} + 8.02 \times A_{663})/cm^2$.

RNA Extraction and cDNA Synthesis

Total RNA was isolated using the GHC-phenol chloroform method: Frozen tissues were ground and resuspended in guanidine HCl and then phenol/chloroform was added. Samples were mixed by vortexing for 30 s and after 30 min 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 samples were washed with cold 70% ethanol. For the synthesis of cDNA, we used SuperScript II reverse transcriptase (Invitrogen) and 3 μg of total RNA, according to the manufacturer's instructions.

qRT-PCR Analyses

qRT-PCR analysis was performed using the Absolute Blue qPCR SYBR Green ROX Mix (AB-4162/B) kit (Thermo Fisher Scientific). Reactions were performed using a Rotor-Gene 6000 cycler (Corbett Research). A standard curve was obtained for each gene using dilutions of a cDNA sample. Each gene was quantified using Corbett Research Rotor-Gene software. At least three independent technical repeats were performed for each cDNA sample. Relative expression of each sample was calculated by dividing the expression level of the analyzed gene by that of *TUBULIN*. Gene-to-*TUBULIN* ratios were then averaged. All primer sequences are presented in Supplemental Table 3.

Library Construction and Sequencing

Total RNA (0.5 μg) was processed using the TruSeq RNA Sample Preparation Kit v2 protocol (Illumina). Libraries were evaluated by Qubit and TapeStation. Sequencing libraries were constructed with barcodes to allow multiplexing of eight samples on one lane. Twenty to twenty-five million single-end 60-bp reads were sequenced per sample on an Illumina HiSequation 2500 V4 instrument.

Sequence Data Analysis

TopHat (v2.0.10) was used to align the reads to the tomato genome sequence SL2.50 (downloaded from the Sol genomics network http://solgenomics.net/organism/Solanum_lycopersicum/genome) (Trapnell et al., 2009). The percentage of the reads that were aligned uniquely to the genome was between 85 and 91%. Counting reads on ITAG2.4 genes (downloaded from Sol genomics network) was done with HTSeq-count (version 0.6.1p1) (Anders et al., 2015). Differential analysis was performed using DESeq2 (1.6.3) (Anders and Huber, 2010). Raw P values were adjusted for multiple testing using the procedure of Benjamini and Hochberg (1995). Genes with a false discovery rate of <0.05 and fold changes >2 were regarded as differentially expressed genes.

Expression data were submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE68018).

Genotyping $pro^{TALEN\_2}/pro^{\Delta GRAS}$ Seedlings

DNA was extracted from cotyledons of progenies of the $pro^{TALEN\_2} \times pro^{\Delta GRAS}$ crosses that exhibited elongated hypocotyls using the DNeasy Plant Mini Kit (Qiagen). To identify the pro^{TALEN_2} allele, the forward primer pro^{TALEN}_tF1 and reverse primer pro^{TAELN}_tR1 (Supplemental Table 3) were used to amplify the region encompassing the pro^{TALEN_2} deletion site (Lor et al., 2014). Each PCR reaction used 50 ng of genomic DNA template in a 50-μL volume using ExTaq polymerase (Clontech). Thermocycler conditions were set according to the manufacturer's recommendations with the annealing temperature set for 55°C and elongation time set for 1 min. pro^{TALEN_2} PCR amplicons were digested with *Sm1*I, which cuts the wild-type sequence but not the pro^{TALEN_2} mutant sequence, and 10 μL of the digestion was run on a 0.8% agarose gel. To identify the $pro^{\Delta GRAS}$ allele, we designed derived cleaved amplified polymorphic sequence (Neff et al., 1998) primers $pro^{\Delta GRAS}$_dF1 and $pro^{\Delta GRAS}$_dR1 using dCAPs Finder 2.0 (http://helix.wustl.edu/dcaps). The resulting primers produce a wild-type PRO amplicon that is digested with *Pvu*II to produce 302- and 27-bp products, while the $pro^{\Delta GRAS}$ amplicon is resistant to digestion. PCR reaction mixes and conditions are similar to the pro^{TALEN_2} PCR conditions except for the anneal temperature that was set at 65°C. $pro^{\Delta GRAS}$ PCR amplicons were digested with *Pvu*II and separated on 1.5% agarose gel.

Microscopy

Samples for scanning electron microscopy were immersed in increasing concentrations of ethanol (25% up to 100%) and critical-point dried with liquid carbon dioxide in a CPD 750 (Bio-Rad), sputter-coated with gold, and photographed with a Jeol scanning electron microscope (JSM-5410 LV).

In Vitro Pollen Germination Assay

Flowers were detached at anthesis and shaken with a pollen buzzer into a microfuge tube containing germination solution (100 g L^{-1} sucrose, 40% polyethylene glycol 4000, 0.01 M HEPES, pH 6, 2 mM boric acid, 2 mM calcium nitrate, 2 mM magnesium sulfate, and 1 mM potassium nitrate); tubes were shaken well to release the pollen grains. The final solution with the pollen grains was transferred to a slide covered with glass slip and sealed with grease. Germination and tube elongation were monitored for 6 h under a light microscope.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: *PROCERA*, Solyc11g011260; *ABI3*, Solyc06g083590; *GOLS1*, Solyc01g100830.1.1; *LE25*, Solyc10g078770.1.1; *FUS3-like*, Solyc02g094460.1.1; *XERICO-like*, Solyc07g045190.1.1; *GA2ox2*, Solyc07g056670.2.1; *GA2OX4*, Solyc07g061720.2.1; *GA2OX5*, Solyc07g061730.2.1; *GA20OX1*, Solyc03g006880.2.1; At-*GA20OX2*, AT5G51810.1; At-*GA2OX1*, AT1G78440.1; At-*GA2OX4*, AT1G47990.1; At-RGA, AT2G01570.1; *ENDORIBONUCLEASE*, Solyc07g064600.2; *THREONINE AMMONIA LYASE*, Solyc09g008670.2. In addition, sequence data and their sources are provided in Tables 1 to 3 and Supplemental Table 2.

Supplemental Data

Supplemental Figure 1. Sequence alignment of PRO from M82, *pro*, and $pro^{\Delta GRAS}$.

Supplemental Figure 2. Phenotypic characterization of pro^{TALEN_2} and $pro^{\Delta GRAS}/pro^{TALEN\_2}$ plants.

Supplemental Figure 3. Genotyping of $pro^{\Delta GRAS}/pro^{TALEN\_2}$ plants shown in Supplemental Figure 2B.

Supplemental Figure 4. Seed set in tomato fruits following hand-pollination of $pro^{\Delta GRAS}$ and pro^{TALEN_2} emasculated flowers with M82 pollen grains.

Supplemental Figure 5. $pro^{\Delta GRAS}$ seeds are sensitive to desiccation and have weak dormancy.

Supplemental Figure 6. *pro*$^{TALEN\_2}$ but not *pro* seeds are sensitive to desiccation.

Supplemental Figure 7. qRT-PCR expression analysis of the putative *XERICO* gene in tomato.

Supplemental Figure 8. *pro*$^{\Delta GRAS}$ and *pro*$^{TALEN\_2}$ are insensitive to PAC and GA_3.

Supplemental Figure 9. Regulation of *GA2ox4* expression by GA in *pro*$^{\Delta GRAS}$.

Supplemental Figure 10. Expression analyses of *GA20ox1* and *GA2ox4* in M82 and *pro*.

Supplemental Figure 11. Expression analyses of *GA2ox2* and *GA2ox5* expression.

Supplemental Figure 12. Expression analyses of *GA2ox4* in M82 and *rgaΔ17* leaves.

Supplemental Figure 13. Expression analyses (qRT-PCR) of Solyc07g064600.2 (Endoribonuclease) and Solyc09g008670.2 (Thr ammonia lyase) in M82 and *pro*$^{\Delta GRAS}$ leaves.

Supplemental Table 1. *pro*$^{TALEN\_2}$ plants are male, but not female, sterile.

Supplemental Table 2. Complete list of GA upregulated genes.

Supplemental Table 3. Primers used in this study.

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AUTHOR CONTRIBUTIONS

S.L., V.S.L., N.E.O., Y.E., and D.W. designed the research. S.L., I.N., and V.S.L. performed the research. A.A. contributed new tools. S.L., V.S.L., N.E.O., Y.E., and D.W. analyzed data. S.L., A.A., N.E.O., Y.E., and D.W. wrote the article.

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