Uncovering DELLAb-Independent Gibberellin Responses by Characterizing New Tomato procerab 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 DELLA. Tomato (Solanum lycopersicum) has a single DELLa gene named PROCERa (PR), and its recessive pro allele exhibits constitutive GA activity but retains responsiveness to external GA. In the loss-of-function mutant pro1GRAS, all examined GA developmental responses were considerably enhanced relative to pro and a defect in seed desiccation tolerance was uncovered. As pro, but not pro1GRAS, 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 pro1GRAS and was not affected by exogenous GA2. In contrast, expression of GA20oxidase4 was not affected by the elevated GA signaling in pro1GRAS but was strongly induced by exogenous GA1. 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 pro1GRAS 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; Havaermale 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; Yoshioka 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 (LHR-I and LHR-II) with putative nuclear localization signals, flanking a VHIID motif, forming the LHR-I-VHIID-LHR-II 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-INSSENSITIVE [GAI], RGA-LIKE1 [RGL1], RGL2,
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). Antisense suppression 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 proGRAS. All examined GA-dependent developmental responses were much stronger in proGRAS 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 (Figure 1A; Supplemental Figure 1). These proGRAS plants were extremely slender and tall compared with M82 and the pro mutant. Four-week-old proGRAS 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 proGRAS 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 proGRAS 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 parthenocarpic 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). proTALEN−2 plants were similar to proGRAS 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 parthenocarpic fruits. When pro, proTALEN−2, proGRAS, and proTALEN−2/proGRAS plants (Supplemental Figure 3) were grown side by side for 4 weeks and their phenotypes were compared, proTALEN−2/proGRAS plants were indistinguishable from homozygous proTALEN−2 and proGRAS 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 proGRAS and proTALEN−2 flowers could stem from the long styles that prevent self-pollination (Figure 2C), proGRAS flowers were hand-pollinated with proGRAS 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 proGRAS flowers with M82 pollen grains
A pollen tubes continued to elongate during the 6 h of the male fertility were found in that female gametophytes are fertile. Similar male sterility and resulted in partial seed set (Supplemental Figure 4), suggesting that female gametophytes are fertile. Similar male sterility and female fertility were found in proGRAS (Supplemental Figure 4 and Supplemental Table 1). Scanning electron microscopy images revealed that proGRAS 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 proGRAS germinated; however, while M82 and pro pollen tubes stopped elongating shortly after germination (Figure 3B). This growth suppression of proGRAS pollen tubes may explain the obligatory parthenocarpy observed in this mutant.

Seed Viability and Segregation Distortion in Progenies of proGRAS+ Plants

Since homozygous proGRAS 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 proGRAS phenotype was obtained for fresh seeds. In contrast, only 8% of the seedlings from seeds that were stored for 10 d exhibited the proGRAS phenotype, while ~18% of the seeds did not germinate (Figure 4A). After 2 months of dry storage, only 5% of the seedlings exhibited the proGRAS phenotype. These results led us to speculate that proGRAS seeds are intolerant to desiccation. However, it should be mentioned that when seeds were sown, proGRAS 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 proTALEN,2 seeds. Dry storage of pro seeds (5 months) did not affect their germination (Supplemental Figure 6A), while proTALEN,2 seeds, similar to proGRAS, exhibited reduced germination after short periods of dry storage (Supplemental Figure 6B).

Abscisic Acid Responses in proGRAS 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 proGRAS seeds. To this end, we collected pollen from a large number of proGRAS anthers and pollinated many proGRAS 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 proGRAS compared with M82 seeds (Figure 4B), suggesting that the machinery to induce desiccation tolerance is suppressed in proGRAS 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
expression of the tomato XERICO homolog in fresh pro\textsuperscript{GRAS} seeds. XERICO-like expression was lower in pro\textsuperscript{GRAS} compared with M82 seeds (Supplemental Figure 7), implying that the lack of desiccation tolerance in pro\textsuperscript{GRAS} seeds may result from reduced ABA levels.

DELLA-Independent GA Responses

Our data suggest that the pro\textsuperscript{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\textsuperscript{GRAS} seedlings with the GA biosynthesis inhibitor paclobutrazol (PAC), followed by application of GA3. 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\textsubscript{3}. However, PAC, GA\textsubscript{3}, or their sequential application did not alter elongation of pro\textsuperscript{GRAS} or pro\textsuperscript{TALEN,2} stems (Supplemental Figure 8). Likewise, chlorophyll content was elevated by PAC and reduced by GA\textsubscript{3} in M82 and pro but not in pro\textsuperscript{GRAS} leaves (Figure 5C). These results suggest that pro\textsuperscript{GRAS} and pro\textsuperscript{TALEN,2} plants are largely insensitive to GA, while pro plants retain some DELLA activity.

To examine the molecular responses of pro\textsuperscript{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 GA20 oxidase (GA20ox) and promote GA deactivation by the induction of the GA deactivation gene, GA2oxidase (GA2ox; Yamaguchi, 2008). M82 and pro\textsuperscript{GRAS} seedlings were treated with PAC for 3 d and then treated with 0, 1, or 100 \mu M GA\textsubscript{3}. Three hours after the GA treatment, RNA was extracted from young leaves and the expression levels of GA20ox\textsubscript{1} and GA2ox\textsubscript{4} were analyzed by qRT-PCR. We would like to emphasize 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, GA20ox\textsubscript{1} expression was promoted by PAC and suppressed by GA\textsubscript{3} in M82 leaves. In agreement with the constitutive GA signaling and insensitivity to GA, GA20ox\textsubscript{1} expression was extremely low in pro\textsuperscript{GRAS} and neither affected by PAC nor by GA\textsubscript{3} treatment (Figure 6A). GA2ox\textsubscript{4} expression was low in mock-treated M82 and induced by GA\textsubscript{3} treatment. However, the GA2ox\textsubscript{4} expression level in pro\textsuperscript{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\textsuperscript{GRAS} was strongly induced by exogenous GA\textsubscript{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 GA2ox\textsubscript{4} was stronger in pro\textsuperscript{GRAS} than is M82, but not in others (Figure 6B versus Supplemental Figure 9). We next examined the impact of GA\textsubscript{3} treatment on the expression of pro\textsuperscript{GRAS} to GA, where high GA levels/signals suppress GA production via the
mock-treated pro (due to the constitutive GA responses) but was further inhibited by treatment with 10 μM GA₃ (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 GA₃ 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 proΔGRAS 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 GA₃ in proΔGRAS (Supplemental Figure 11B).

The strong induction of GA2ox4 by exogenous GA₃ in proΔGRAS 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 GA₃ in M82 and proΔGRAS, 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 3. The Effect of proΔGRAS and pro on Anther Development, Pollen Production, and Pollen Tube Elongation.
(A) Scanning electron microscopy images of M82, pro, and proΔGRAS 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 proΔGRAS 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.
The expression of this gene was suppressed by GA3 treatment in the untreated tomato plants, following PAC and GA treatments, as described above. As expected in cases of feedback regulation, At-GA2ox4 expression was induced by GA3 in both the wild type and deltaP (Figure 8B). We also tested the response of these two genes to GA3 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 GA3. Three hours after the GA treatment, RNA was extracted from the flowers and analyzed for At-GA2ox2 and At-GA2ox4 expression. While At-GA2ox2 exhibited normal feedback regulation in the wild type, in deltaP 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 deltaP but was induced by GA3 in both the wild type and deltaP (Figure 8D). These results suggest that in Arabidopsis, the regulation of At-GA2ox 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 deltaP and lack of response to GA3 (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-function (loss of two 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 GA3 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 GA3 (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 GA3 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 proGRAS plants. M82 and proGRAS seedlings were treated with PAC (10 mg/L) once a day for 3 d followed by a single GA3 application (100 μM). Three hours after the GA treatment, young leaves were collected, RNA was extracted, and cDNA libraries were sequenced by Illumina HiSeq. 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 + GA3 treated leaves. Using a 2-fold increase or decrease cutoff (adjusted P value for multiple comparisons ≤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 GA3 in proGRAS. These include some well-characterized GA-regulated genes, such as GA20ox.
**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^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^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
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^{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^{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^{GRAS} and pro^{TALEN, 2} styles are longer than that of pro, it cannot explain the obligatory partenocarpy. Since pollination of pro^{GRAS} and pro^{TALEN, 2} flowers with M82 pollen resulted in an almost normal seed set, the lack of fertilization in homozygous pro^{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^{GRAS} but not pro, inhibited pollen tube elongation. The suppression of pollen tube elongation in pro^{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^{GRAS} pollen would not elongate to fertilize the pro^{GRAS} egg cells. Thus, it is possible that the effect of pro^{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^{GRAS}.

Tomato seeds can be considered “orthodox” seeds (Angelovic et al., 2010), since they can tolerate desiccation and can be stored in a dry state for years (Pristley et al., 1985). Our results show that homozygous pro^{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^{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; Finkeinstein 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; Arizumi et al., 2013). We found reduced expression of the putative tomato homolog of XERICO in pro^{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; Arizumi et al., 2013). Our results suggest that the loss of PRO activity suppresses both processes; while homozygous pro^{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 D W A R F (D) gene, allowed the identification of this allele in our screening. D encodes a P450 protein involved in brassinosteroid biosynthesis (Bisho et al.,
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 pro³GRAS and proTALEN_2 exhibited insensitivity of growth to GA and PAC, a DELLA-independent GA response in pro³GRAS plants, namely, the feedback regulation of GA catabolism, was discovered. As expected, the expression level of GA20ox1 was lower in pro³GRAS 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 pro³GRAS and was strongly induced by GA. 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.
Arabidopsis gain-of-function DELLA protein RGAΔ17 were insensitive to GA in terms of growth, GA2ox4 was strongly induced by GA3 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-GA2ox4 behaved as expected, i.e., exhibited high levels of expression in dellaP and insensitivity to GA3 treatment, the expression of At-GA2ox4 was strongly induced by exogenous GA3 in this mutant. While numerous studies have shown that the expression of GA2oxox is suppressed, and that of GA2ox, is promoted by GA (Yamaguchi, 2008), Zentella et al. (2007) suggested that At-GA2oxox 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; 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

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

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<tr>
<th>SolyC Locus</th>
<th>Description</th>
<th>Mean Paca</th>
<th>Mean Pac+GAa</th>
<th>Fold Changeb</th>
<th>Adj. P Valuec</th>
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<tr>
<td>Solyc05g007950.2</td>
<td>Ribonuclease T2</td>
<td>68</td>
<td>1068</td>
<td>15.78</td>
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<td>106</td>
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<td>7.15E-06</td>
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<tr>
<td>Solyc03g025380.2</td>
<td>Peroxidase</td>
<td>24</td>
<td>243</td>
<td>9.98</td>
<td>0.000267</td>
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<tr>
<td>Solyc03g005320.2</td>
<td>3-Ketoacyl-CoA synthase</td>
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<td>379</td>
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<td>1.64E-06</td>
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<td>Solyc01g110630.2</td>
<td>Auxin-induced SAUR-like</td>
<td>18</td>
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<tr>
<td>Solyc04g017720.2</td>
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<td>Solyc12g056250.1</td>
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<td>6.02E-08</td>
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<td>Solyc04g081790.2</td>
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<td>243</td>
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<td>Receptor-like kinase, RLK</td>
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<tr>
<td>Solyc04g081870.2</td>
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<td>1964</td>
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<td>Solyc02g088100.2</td>
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<tr>
<td>Solyc07g008560.2</td>
<td>Purple acid phosphatase</td>
<td>25</td>
<td>103</td>
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</table>

aMean value of two biological replicates.
bFold change is the ratio mean Pac + GA/mean Pac.
cCorrected P values were calculated using the Benjamini and Hochberg (1995) false discovery rate approach.

---

(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 GA3 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 GA3. 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 GA3 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.
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^GRAS^ allele was isolated from an activation-tagging population of Micro-Tom, mutagenized with an Ac/Δs system carrying a 4×hph enhancer element in the Δs transposon (MacAlister et al., 2012). The pro^GRAS^ line used in this study was backcrossed with M82 (SP^+) plants four times. pro^+ΔGRAS^ (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 (deltaP; 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% Na3PO4. 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 KpnI site. The GFP-RGAΔ17 fusion was inserted to a pART7 plasmid downstream of the 3SS promoter, into XhoI and BamHI sites, to create 3SS: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.

**Table 2. GA-Downregulated, DELLA-Dependent Genes**

<table>
<thead>
<tr>
<th>SolyC Locus</th>
<th>Description</th>
<th>Mean Pac^a</th>
<th>Mean Pac+GA^b</th>
<th>Fold Change^b</th>
<th>Adj. P Value^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solyc08g035530.2</td>
<td>Gibberellin 20-oxidase-2</td>
<td>79</td>
<td>7</td>
<td>–11.18</td>
<td>2.67E-05</td>
</tr>
<tr>
<td>Solyc09g006880.2</td>
<td>Gibberellin 20-oxidase-1</td>
<td>1244</td>
<td>122</td>
<td>–10.18</td>
<td>0.003222</td>
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<tr>
<td>Solyc01g008910.2</td>
<td>Scarecrow-like</td>
<td>123</td>
<td>15</td>
<td>–8.31</td>
<td>4.17E-05</td>
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<td>Solyc03g119530.2</td>
<td>LOB domain protein 42</td>
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<td>GRAS family</td>
<td>299</td>
<td>68</td>
<td>–4.38</td>
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<tr>
<td>Solyc09g009220.2</td>
<td>Hydrolylase ω/β fold</td>
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<tr>
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<td>GID1-like GA receptor</td>
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<td>–3.81</td>
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<td>Solyc06g067950.2</td>
<td>Acyl-protein thioesterase</td>
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<td>39</td>
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<td>Solyc01g095580.2</td>
<td>GH3 family protein</td>
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<td>Solyc02g080510.1</td>
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<td>Solyc05g006420.2</td>
<td>ARR3</td>
<td>2685</td>
<td>1182</td>
<td>–2.27</td>
<td>0.013313</td>
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^aMean value of two biological replicates.

^bFold change is the ratio mean Pac + GA/mean Pac [value is presented as: –1(Pac + GA/Pac)].

^cCorrected P values were calculated using the Benjamini and Hochberg (1995) false discovery rate approach.

**Table 3. GA-Regulated, DELLA-Independent Genes**

<table>
<thead>
<tr>
<th>SolyC Locus</th>
<th>Description</th>
<th>Fold Change^a</th>
<th>Adj. P Value^b</th>
<th>Fold Change^b</th>
<th>Adj. P Value^b</th>
</tr>
</thead>
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<td>Solyc07g064600.2</td>
<td>Endoribonuclease L-PSM</td>
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<td>Solyc09g038440.2</td>
<td>Proteinase inhibitor I</td>
<td>4.22</td>
<td>0.00546</td>
<td>5.73</td>
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<tr>
<td>Solyc09g008670.2</td>
<td>Threonine ammonia-lyase</td>
<td>4.14</td>
<td>0.000293</td>
<td>4.03</td>
<td>0.001424</td>
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<td>Solyc03g121270.2</td>
<td>IAA-amino acid hydrolase</td>
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<td>0.015785</td>
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<td>Solyc07g117280.2</td>
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<td>–2.21</td>
<td>0.014012</td>
<td>–2.40</td>
<td>0.01025</td>
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</table>

^aFold change is the ratio mean Pac + GA/mean Pac [for fold change <1, the value is presented as: –1(Pac + GA/Pac)].

^bCorrected 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 GA₃ application (10 µM) or twice a week with PAC (5 mg/L) until flowering and then immersed in 10 µM GA₃ 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 (Aron, 1949). Chlorophyll concentrations were calculated using the formula: (20.2 × A₆₄₅ + 8.02 × A₆₆₃)/cm².

**RNA Extraction and cDNA Synthesis**

Total RNA was isolated using the GTC-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-4162/8) 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 prepare Illumina HiSeq 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 the Sol genomics network http://solgenomics.net/organism/Solanum_lycopersicum/genome) (Trapnell et al., 2009). Differential expression was calculated 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 proTALEN_2/proGRAS Seeds**

DNA was extracted from cotyledons of progenies of the proTALEN_2 × proGRAS crosses that exhibited elongated hypocotyls using the DNeasy Plant Mini Kit (Qiagen). To identify the proTALEN_2 allele, the forward primer proTALEN_dF1 and reverse primer proTALEN_dR1 (Supplemental Table 3) were used to amplify the region encompassing the proTALEN_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. proTALEN_2 PCR amplicons were digested with SmFI, which cuts the wild-type sequence but not the proTALEN_2 mutant sequence, and 10 µL of the digestion was run on a 0.8% agarose gel. To identify the proGRAS allele, we designed derived cleaved amplified polymorphic sequence (Neef et al., 1998) primers proGRAS_dF1 and proGRAS_dR1 using dCAPs Finder 2.0 (http://helix.wustl.edu/dcaps). The resulting primers produce a wild-type PRO amplicon that is digested with PvuII to produce 302- and 27-bp products, while the proGRAS amplicon is resistant to digestion. PCR reaction mixes and conditions are similar to the proTALEN_2 PCR conditions except for the annealing temperature that was set at 65°C. proGRAS PCR amplicons were digested with PvuII 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 microtube 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, Solyc11g01260.1; AB28, Solyc06g083590.1; GQ51, Solyc01g00830.1; L25, Solyc01g080770.1; FUS3-1, Solyc02g094460.1; XRCFO1, Solyc07g045190.1; GABA2, Solyc07g056670.2; GA2OX4, Solyc07g061720.2; GA2OX5, Solyc07g061730.2; GA2OX1, Solyc03g06880.2; At-GA2OX2, AT5G51810.1; At-GA2OX1, AT1G78440.1; At-GRAS1, 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 proGRAS.
- **Supplemental Figure 2.** Phenotypic characterization of proTALEN_2 and proGRAS/proTALEN_2 plants.
- **Supplemental Figure 3.** Genotyping of proGRAS/proTALEN_2 plants shown in Supplemental Figure 2B.
- **Supplemental Figure 4.** Seed set in tomato fruits following hand-pollination of proGRAS and proTALEN_2 emasculated flowers with M82 pollen grains.
- **Supplemental Figure 5.** proGRAS seeds are sensitive to desiccation and have weak dormancy.
Supplemental Figure 6. proTALEN, 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. proGRAS and proTALEN, are insensitive to PAC and GA4.

Supplemental Figure 9. Regulation of GA2ox4 expression by GA in proGRAS.

Supplemental Figure 10. Expression analyses of GA2ox1 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 rga317 leaves.

Supplemental Figure 13. Expression analyses (qRT-PCR) of Solyc07g064600.2 (Endoribonuclease) and Solyc09g008670.2 (Thr ammonia lyase) in M82 and proGRAS leaves.

Supplemental Table 1. proTALEN, plants are male, but not female, sterile.

Supplemental Table 2. Complete list of GA upregulated genes.

Supplemental Table 3. Primers used in this study.

ACKNOWLEDGMENTS

We thank Gilgi Friedlander (INCPM unit, Weizmann Institute of Science) for help with the bioinformatic analysis. We thank Naomi Ori for valuable suggestions that improved the article. This research was supported by research grants from the U.S.–Israel Binational Agriculture Research and Development fund to D.W. and N.E.O. (Grant 4429-11) and by the I-CORE Program of the Planning and Budgeting Committee and The Israel Science Foundation (Grant 757/12) to D.W., A.A., and Y.E. The work in the laboratory of A. A. was supported by European Research Council (ERC) project SAMIT (Framework Programme 7).

AUTHOR CONTRIBUTIONS


Received October 2, 2014; revised April 14, 2015; accepted May 21, 2015; published June 2, 2015.

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George Jones, M.


Uncovering DELLA-Independent Gibberellin Responses by Characterizing New Tomato procera Mutants
Sivan Livne, Vai S. Lor, Ido Nir, Natanella Eliaz, Asaph Aharoni, Neil E. Olszewski, Yuval Eshed and David Weiss

*Plant Cell* 2015;27;1579-1594; originally published online June 2, 2015;
DOI 10.1105/tpc.114.132795

This information is current as of December 31, 2017