Characterization of a semidominant dwarfing PROCERA allele identified in a screen for CRISPR/Cas9-induced suppressors of loss-of-function alleles

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DELLA proteins were first identified as inhibitors of gibberellin (GA) signalling but have since been shown to be involved in regulation of many growth responses. GA causes responses by triggering the destruction of DELLAs (Willige et al., 2007). These proteins contain a conserved DELLA domain, which is followed by a GRAS domain. The GRAS domain which defines a family of proteins, including the DELLAs, is a conserved domain named after the first three family members: GAI (gibberellin-acid insensitive), RGA (Repressor of ga1-3), and SCR (Scarecrow). The DELLA domain encompasses the DELLA, LExLE and TVHYNPF motifs. These motifs are important for binding to the GA-bound GA receptor GID1, which is one of the initial steps in targeting the DELLAs for destruction by the 26S proteasome (Dill et al., 2001). The GA-GID1-DELLA complex interacts with the SLY1/GID2 SCF complex, which marks it for destruction by modifying it with ubiquitin. In this way, the suppression of GA signalling by DELLAs is released and GA responses are activated. A number of mutants with mutations affecting the DELLA domain that increase the abundance of DELLAs by weakening the interaction with the GA-GID1 complex are known (Ueguchi-Tanaka et al., 2007; Willige et al., 2007). Since these mutant DELLA proteins retain the ability to inhibit GA responses, the mutants are dwarfs.

Tomato has one DELLA protein called PROCERA (PRO). There is a well-studied partial loss-of-function mutant, pro, which has a single amino acid substitution, valine to glutamate, in the VHVID motif of its GRAS domain (Bassel et al., 2008; Jupe et al., 1988). Strong recessive pro loss-of-function alleles, proTALEN and proAGRS, were generated using transcription activator-like effector nucleases (TALENs) and an Ac/Ds system, respectively (Livne et al., 2015; Lor et al., 2014). Consistent with these mutations fully activating GA signalling, homozygous mutants are extremely tall, have light green leaves with smoother leaf margins, and do not respond to the GA biosynthesis inhibitor paclobutrazol (PAC) or GA treatment. Recently, a gain-of-function allele affected in the DELLAs motif that causes mild dwarfing and reduced GA sensitivity was reported (Tomlinson et al., 2018) but strong gain-of-function alleles have not been reported. Here, we report the generation and characterization of strong dwarfing alleles produced using the clustered regularly interspaced short palindromic repeats (CRISPR/Crispr-associated protein9 (Cas9) system to induce intragenic suppressor mutations of proTALEN alleles.

In the course of experiments to identify regenerating shoots in which the proTALEN loss-of-function mutation has been repaired due to CRISPR/cas9 stimulated homologous recombination, we recovered several dwarf plants with dark green serrated leaves (Figure 1a, b). The regenerated T₀ plants were dwarfs and the dwarfing alleles PROGFRA and PROGFSP from plants #8 and #9, respectively, were heritable. Sequencing confirmed that the PROGFRA and PROGFSP are intragenic suppressors of proTALEN and proTALEN, respectively and that each encodes a full-length mutant protein (Figure 1c, d). The protein encoded by PROGFSP is predicted...
to have a 12 amino acid insertion and 2 amino acid substitution affecting the LExLE motif, while the protein encoded by PROGF8 has a three amino acid deletion and an E to R substitution that affects the LExLE motif.

**Figure 1** (a) Strategy for generating proTALEN loss-of-function alleles and subsequent generation of suppressor mutations. (b) T-DNA construct used to edit proTALEN mutation sites were constructed in pTRANS220 as described by previously (Cermák et al., 2017). The gRNAs targeting proTALEN and proTALEN were constructed in pMOD_B2515 and the resulting module (B) was assembled together with modules from pMOD_A0101 (module A), and pMOD_C0000 (module C). Module C consisted of a fragment of genomic DNA spanning the proTALEN mutation site. (c) DNA and (d) protein sequence of CRISPR/Cas9 induced alleles. The gRNA binding site is underlined and PAM site is highlighted in red in the proTALEN and proTALEN sequences. The size of insertion (+) and deletion (−) compared with wild-type is indicated to the right of sequences. The conserved LExLE motif of DELLA protein is underlined in the wild-type sequence. (e) Four-week old F1 seedlings from a cross between T0 plant #8 and M82. Left to right, three tall plants that lack PROGF8 and three PROGF8 plants. (f, h) Plants were spayed to runoff every other day with 0.07% ethanol (control treatment), 50 μM GA3, 100 μM Paclobutrazol (PAC) or GA3 plus PAC and the height was measured every other day for 2 weeks. After 14 days of treatment, the height of control and GA3-treated PROGF8 plants were similar. After 14 days, PAC treated PROGF8 plants were significantly shorter than GA3-treated PROGF8 plants based on an ANOVA followed by t-test (P value = 0.0014). (g) Seven-week old PROGF8 plants after 2 weeks of treatment as in panel f. (j) Seven-week old PROGF8 plants after 2 weeks of treatment as in panel h. Scale bar, 50 mm. (k) Time course of germination of seeds giving rise to tall and dwarf seedlings from (j) 4 month-old F1 seeds from crossing T0 plant #8 with wild-type pollen and (k) seeds immediately after harvest from a selfed PROGF8 plant. Seeds that had not germinated after 10 days were scarified by removing the endosperm and seed coat adjacent to the root tip. Dash lines indicate the germination after scarification, which was scored at day 12. At least 40 seeds are used for each test, which was repeated three times. (l-o) 3D models of PROCERA (l) and PROGF8 (m) based on template 2ZSH (GA3-GID1A-GAI). Close-up view of the hydrogen bonds between LExLE motif of PROCERA and α of GID1 (n) LExLE motif of proGF8 and α of GID1 (o) based on template. The DELLA motif is highlighted in orange and TVHYNP motif in yellow. The LExLE motif in PRO and PROGF8 is highlighted in green; the insertion of PROGF8 is highlighted in red. Hydrogen bonds are indicated as dot lines. A water molecule bridging E and S is showed as a red sphere.
elevated in plants carrying DELLA gain-of-function alleles (Talón et al., 1990). To determine if the apparent GA insensitivity of PRO/PROGF8 plants is because they contain saturating levels of endogenous bioactive GA, we treated PRO/PROGF8 (Figure 1h, i) and wild-type (not shown) plants with the GA biosynthesis inhibitor paclobutrazol (PAC) or a combination of PAC and GA3. PAC treatment reduced the height of both PRO/PROGF8 and wild-type plants and the GA3 treatment reversed this effect. The effects of the treatments were much smaller for PRO/PROGF8, indicating that, while PRO/PROGF8 responds to GA, it is nearly insensitive to it.

While germination tests found that initially only one half of the newly harvested F1 seeds from a cross between the T0 plant #8 and M82 germinated without scarification were heterozygous and 10 of 11 when genotyped, 16 out of 16 dwarf plants from seeds that were scarified (not shown) indicating that the allele is semidominant. Consistent with this hypothesis, when genotyped, 16 out of 16 dwarf plants from seeds that germinated without scarification, and dwarf from seeds requiring scarification were tall and did not carry the PROGF8 allele while all of the seedlings from seeds that required scarification were PRO/PROGF8 and dwarf (not shown). When we examined the germination kinetics after 4 months of storage, seeds that germinated by day 3 gave rise to tall plants whereas all seeds that germinated after day 3 produced dwarf seedlings (Figure 1j). After 10 days, 30% of the seeds had not germinated. Following scarification, these ungerminated seeds germinated and gave rise to dwarf seedlings.

We also tested germination of fresh seeds harvested from selfed PRO/PROGF8 (Figure 1k). After 3 days, all of the seeds that produced wild-type stature seedlings had germinated (26%). The seeds that had not germinated could be divided into two groups. One group comprising 44% of the seeds germinated by day 10 and produced dwarf seedlings. The remaining seed required scarification and also produced dwarf seedlings. The observed ratios for the tall seedlings, dwarf seedlings from seed that germinated without scarification, and dwarf from seeds requiring scarification [Tall vs. Dwarf (non-scarified) vs. Dwarf (scarified)] fit a 1 : 2 : 1 ratio (three trials Chi-square range from 0.458 to 3.471, P value from 0.176 to 0.795), which suggested that the PROGF8 allele is semidominant. Consistent with this hypothesis, when genotyped, 16 out of 16 dwarf plants from seeds that germinated without scarification were heterozygous and 10 of 11 dwarf seedlings from seeds requiring scarification were homozygous for PROGF8 and the remaining plant was heterozygous. Plants from seeds that require scarification to germinate were shorter than the dwarf plants from seeds that germinated without scarification (not shown) indicating that proGF8 is also semidominant with respect to plant stature.

Molecular modelling of PRO and PROGF8 predicts that PROGF8 protein has a longer disordered region between the LExLE and TVHYNP motifs and the intervening alpha helix structure(s) are also affected (Figure 11–o). The changes in PROGF8 are predicted to weaken its interaction with the GA-bound GID1 because the stabilizing interaction between the second glutamic acid in LExLE motif and arginine and lysine in GID1 are disrupted. Weakening the interaction with GID1 likely increases the abundance of PROGF8.

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Conflict of interest

The authors declare that there is no conflict of interest.

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