

Targeted Mutagenesis of the Tomato *PROCERA* Gene Using Transcription Activator-Like Effector Nucleases^{1[W]}

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We report the successful use of transcription activator-like effector nucleases (TALENs) under the control of an estrogen-inducible promoter for targeted mutagenesis in tomato (*Solanum lycopersicum*) of the negative regulator of GA signaling *PROCERA* (*PRO*). TALEN expression was induced and plants were regenerated from cotyledons of seedlings derived from stable transgenic lines. Six of 40 regenerated plants carried *pro* alleles, and the mutations in the two lines examined were heritable. Homozygous *pro* segregants exhibited phenotypes consistent with increased GA response.

Tomato is an important agricultural crop in which significant resources are invested for breeding traits such as disease resistance and fruit shape and color (Foolad and Panthee, 2012). Mutations affecting these traits can be generated using random mutagens such as ethyl methanesulfonate and transfer DNA integration (Mathews et al., 2003; Menda et al., 2004); however, screening for the desired mutation is laborious and time consuming. A potentially more efficient method of gene disruption is targeted mutagenesis using sequence-specific nucleases, which create a double strand break in the target sequence. These breaks are then repaired either by the homologous recombination or nonhomologous end-joining pathway (Jasin and Rothstein, 2013). Nonhomologous end-joining is sometimes imprecise, resulting in deletions or insertions at the double strand break site.

TALENs are composed of a sequence-specific DNA-binding domain fused to the *FokI* nuclease domain (Christian et al., 2010; Zhang et al., 2010). Binding of TALEN monomers to the targeted gene allows the *FokI* nuclease domain to dimerize and cleave the DNA (Chen and Gao, 2013; Voytas, 2013). TALENs have been successfully used to make sequence-specific mutations in a variety of plant species, including *Arabidopsis* (*Arabidopsis thaliana*), tobacco (*Nicotiana tabacum*), rice (*Oryza*

sativa), *Brachypodium* spp., and barley (*Hordeum vulgare*; Cermak et al., 2011; Li et al., 2012; Christian et al., 2013; Shan et al., 2013a; Wendt et al., 2013; Zhang et al., 2013). More recently, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein9 (Cas9) system has also been demonstrated to be a promising genome-editing tool (Jiang et al., 2013; Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013b; Upadhyay et al., 2013). The CRISPR/Cas9 system has been shown to be an effective tool for targeted mutagenesis in transgenic tomato hairy roots (Ron et al., 2014). In an accompanying paper, CRISPR/Cas9 is shown to be useful for generating heritable mutations (Brooks et al., 2014).

DELLA proteins are negative regulators of GA signaling (Hauvermale et al., 2012). The loss of DELLA function results in increased GA response, which causes the mutants to be tall and slender with light green vegetation. Tomato has one DELLA gene called *PROCERA* (*PRO*). A *pro* mutant caused by a missense mutation has been characterized (Jones, 1987; Bassel et al., 2008; Jasinski et al., 2008; Carrera et al., 2012). Although the mutant has phenotypes corresponding to increased GA response, it is partially responsive to GA, suggesting that the mutant protein retains partial activity (Van Tuinen et al., 1999). We used TALENs to create new *pro* alleles that can be used to determine the role of *PRO* in GA signaling.

RESULTS AND DISCUSSION

TALEN pairs pTAL423/4 and pTAL425/6 were designed to target the *PRO* gene using TALE-NT (Supplemental Fig. S1; Supplemental Table S1; Doyle et al., 2012). Due to concerns about potential TALEN cytotoxicity (Christian et al., 2013), we generated transgenic tomato plants that express the TALEN pairs under the control of the estrogen-inducible XVE promoter (Zuo et al., 2000). One primary transgenic plant with TALEN pair pTAL423/4 (pTAL423/4 T0) and four with TALEN pair pTAL425/6 (pTAL425/6 T0) were generated.

Leaves of T0 plants were assayed for evidence of TALEN activity 7 d after spraying plants with β -estradiol. For this assay, *PRO* genomic DNA was PCR amplified and tested to determine if the *SmlI* restriction site located at the TALEN cleavage site was mutated (Zhang et al.,

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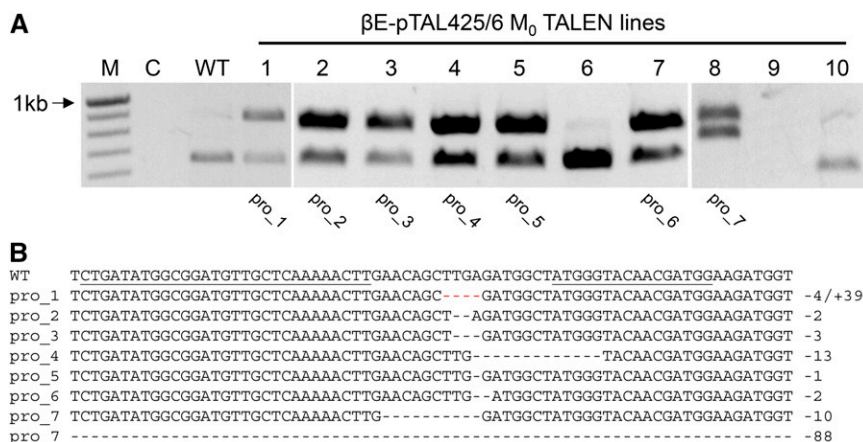


Figure 1. Characterization of TALEN-induced mutations. A, PCR screen for TALEN-induced mutations in βE-pTAL425/6 M₀ plants. Wild-type (WT) *PRO* amplicons were cleaved by *Sm1I*, producing 647- and 225-bp products, but the mutant amplicons in lines 1 to 5, 7, and 8 are not cleaved. Note that line 9 failed to amplify. The mutant line designations below the image correspond to the DNA sequences in B. M indicates the marker lane, and C indicates the no-template control. B, DNA sequence alignment of TALEN-induced mutations. Left and right TALEN-binding sequences are underlined in the wild type. The sizes of deletions (–) and insertions (+) are indicated to the right of the sequences. The red dashes indicate the location of a 39-bp insertion in *pro_1*.

2010). However, no *Sm1I*-resistant *PRO* amplicons were detected, suggesting that the TALENs were not active or that the mutation frequency was too low for detection without enrichment. Therefore, *Sm1I*-digested *PRO* amplicons were used as the template for a second round of PCR amplification, and one T₀ plant (designated as pTAL425/6_1 T₀) had *Sm1I*-resistant *PRO* amplicons. Mutations ranging in size from 2 to 168 bp were identified when these amplicons were sequenced (Supplemental Fig. S2).

We attempted to produce heritable TALEN-induced mutations by spraying the shoot apex of pTAL425/6_1 T₀ plants with β-estradiol daily for 1 week, but no TALEN-induced mutations were detected in the pTAL425/6_1 T₁ seedlings. We then sprayed unopened and opened flowers with β-estradiol daily and injected immature and mature fruits with β-estradiol, but mutations were not detected in seedlings from the treated plants. In addition, when pTAL425/6_1 T₁ seeds were germinated in the presence of β-estradiol, no mutations were detected in the T₂ seedlings.

Since the TALENs were active in somatic tissue, we attempted to regenerate plants bearing mutations from cotyledons (Supplemental Fig. S3). pTAL425/6_1 T₁ cotyledons were immersed in β-estradiol solution weekly, and plants (βE-pTAL425/6 M₀) were regenerated. Six and three plants were regenerated from tissue that received either one or two β-estradiol treatments, respectively, but none carried TALEN-induced mutations. Forty βE-pTAL425/6 M₀ plants were regenerated from tissue that had received three treatments, and seven of these plants (designated as *pro_1*–*pro_7* M₀) carried mutations (Fig. 1A). Since restriction enzyme assays do not detect all TALEN-induced mutations (Suzuki et al., 2014; Veres et al., 2014), additional plants may have carried mutations. While *pro_1* to *pro_6* each carried one mutant allele, *pro_7* carried two different mutant alleles and exhibited

characteristic *pro* mutant phenotypes, including long internode length and smooth leaf margins. Sequencing indicated that each allele was unique (Fig. 1B). The mutations in *pro_2* to *pro_7* were deletions ranging in size from 1 to

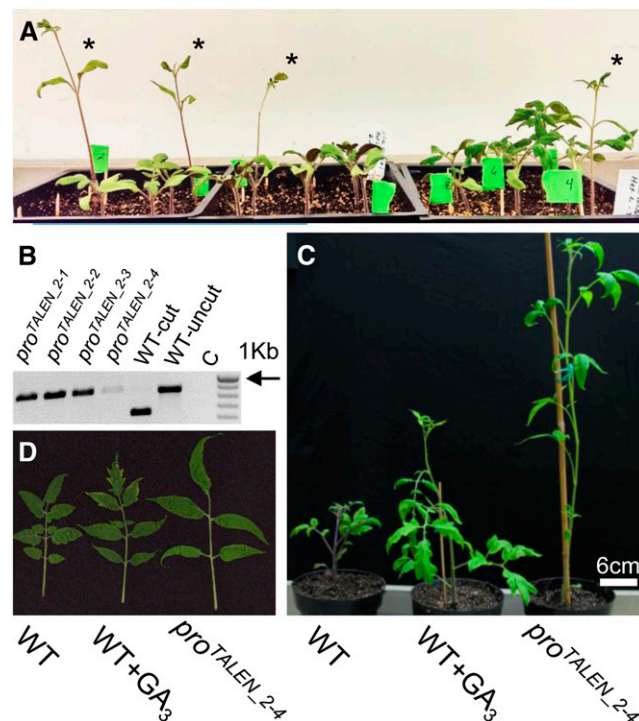


Figure 2. A, Three-week-old seedlings segregating for the *pro*^{TALEN₂} allele. Homozygous *pro*^{TALEN₂} mutants are indicated by the asterisks. B, Genotyping of the homozygous *pro*^{TALEN₂} seedlings marked in A by PCR amplification and digestion with *Sm1I*. C indicates the no-template control. C, Comparison of 8-week-old wild-type (WT) and WT+GA₃ plants with homozygous *pro*^{TALEN₂} plants. D, Third youngest fully expanded leaves of the plants shown in C.

88 bp; *pro_1* has a 39-bp insertion and a 4-bp deletion (Supplemental Fig. S4). Seven of the deletions cause frame shifts that would result in the production of a truncated PRO protein.

We then determined if the mutant alleles in the *pro_2* and *pro_1* lines (*pro*^{TALEN_2} and *pro*^{TALEN_1}, respectively) are heritable. The previously described *pro* allele is recessive (Jones, 1987); thus, if *pro*^{TALEN_2} and *pro*^{TALEN_1} are heritable, we expected one-quarter of the seedlings derived from the heterozygous *pro_2* and *pro_1* plants to exhibit a mutant phenotype. Consistent with the mutations being heritable, four of 20 *pro_2* seedlings (Fig. 2) and three of 20 *pro_1* seedlings (Supplemental Fig. S5) were tall, and genotyping indicated that they were homozygous mutants. In further experiments, 55 of 293 *pro_2* M1 seedlings were tall, and the resulting χ^2 *P* value for a 3:1 segregation ratio was 0.014, which is consistent with Mendelian inheritance of the *pro*^{TALEN_2} allele. In addition, we tested if the transgene encoding the TALEN nuclease segregated away from the TALEN-induced *pro* mutation and found that *pro*^{TALEN_2-2} and *pro*^{TALEN_2-4} M1 mutants did not have the transfer DNA insertion (data not shown).

Homozygous *pro*^{TALEN_2} plants are phenocopies of wild-type plants sprayed with GA₃ (Fig. 2, C and D). In contrast to control wild-type plants, wild-type tomato plants sprayed with 50 μ M GA₃ daily and homozygous *pro*^{TALEN_2} plants were taller and had lighter green leaves with smoother margins. These phenotypes are similar to those of the previously characterized *pro* mutant (Jones, 1987; Bassel et al., 2008; Jasinski et al., 2008; Carrera et al., 2012). The new *pro* alleles described here, and others that can be created through targeted mutagenesis, should prove useful in dissecting the role of PRO in GA signaling.

This report and the accompanying report by Brooks et al. (2014) demonstrate that both TALENs and CRISPR/Cas9 are effective tools for creating heritable mutations in tomato. The greater ease of synthesizing CRISPR/Cas9 and its higher mutagenesis frequency relative to TALEN might make it the preferred technology. However, CRISPR/Cas9 has been reported to have a higher frequency of off-target cleavage than TALENs (Fu et al., 2013; Suzuki et al., 2014; Veres et al., 2014), but progress is being made in addressing this deficiency (Fu et al., 2014).

MATERIALS AND METHODS

Materials and methods are described in Supplemental Materials and Methods S1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Locations of TALEN target sites.

Supplemental Figure S2. Sequences of TALEN-induced mutations generated in somatic cells.

Supplemental Figure S3. Strategy for regenerating pTAL425/6 TALEN-induced mutants.

Supplemental Figure S4. The coding region of *PRO* showing the sequences of *pro*^{TALEN_1} and one of the *pro_7* alleles.

Supplemental Figure S5. Six-week-old seedling segregating for the *pro*^{TALEN_1} allele.

Supplemental Table S1. TALEN Repeat Variable Diresidues sequences and TALEN target sequences.

Supplemental Materials and Methods S1. Materials and methods used.

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