An Effective Strategy for Reliably Isolating Heritable and *Cas9*-Free Arabidopsis Mutants Generated by CRISPR/Cas9-Mediated Genome Editing[1[OPEN]]

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Mutations generated by CRISPR/Cas9 in Arabidopsis (*Arabidopsis thaliana*) are often somatic and are rarely heritable. Isolation of mutations in *Cas9*-free Arabidopsis plants can ensure the stable transmission of the identified mutations to next generations, but the process is laborious and inefficient. Here, we present a simple visual screen for *Cas9*-free T2 seeds, allowing us to quickly obtain *Cas9*-free Arabidopsis mutants in the T2 generation. To demonstrate this in principle, we targeted two sites in the *AUXIN-BINDING PROTEIN1* (*ABP1*) gene, whose function as a membrane-associated auxin receptor has been challenged recently. We obtained many T1 plants with detectable mutations near the target sites, but only a small fraction of T1 plants yielded *Cas9*-free *abp1* mutations in the T2 generation. Moreover, the mutations did not segregate in Mendelian fashion in the T2 generation. However, mutations identified in the *Cas9*-free T2 plants were stably transmitted to the T3 generation following Mendelian genetics. To further simplify the screening procedure, we simultaneously targeted two sites in *ABP1* to generate large deletions, which can be easily identified by PCR. We successfully generated two *abp1* alleles that contained 1,141- and 711-bp deletions in the *ABP1* gene. All of the *Cas9*-free *abp1* alleles we generated were stable and heritable. The method described here allows for effectively isolating *Cas9*-free heritable CRISPR mutants in Arabidopsis.

The advancement of CRISPR/Cas9 genome-editing technology offers unprecedented tools to precisely edit DNA sequences in Arabidopsis (*Arabidopsis thaliana*) and other organisms (Cong et al., 2013; Feng et al., 2013, 2014; Mali et al., 2013; Gao and Zhao, 2014a). Genome editing by CRISPR/Cas9 has only three requirements: expression of the Cas9 protein, production of a guide RNA (gRNA) that complements the DNA sequences of the target gene, and the existence of an NGG protospacer adjacent motif (PAM) site in the target sequence (Cong et al., 2013; Mali et al., 2013). Cas9 is recruited to the target DNA by the gRNA molecule, which targets a specific DNA sequence by base pairing. Once at the target site, the nuclease activities of Cas9 generate a double-strand break a few base pairs upstream of the PAM site. Small deletions or insertions in the target site are generated when the double-strand break is repaired by error-prone nonhomologous end-joining DNA repair. Because of its simplicity, CRISPR/Cas9 has been widely adopted by many laboratories. Several groups have developed CRISPR vectors for editing genes in Arabidopsis (Feng et al., 2013, 2014; Mao et al., 2013; Fauser et al., 2014; Gao and Zhao, 2014b; Jiang et al., 2014; Li et al., 2014; Xing et al., 2014; Lowder et al., 2015; Ma et al., 2015; Zhang et al., 2015). Successful editing events in Arabidopsis have been widely reported. It is evident that CRISPR/Cas9-mediated gene-editing technology can successfully produce various heritable mutations in Arabidopsis. However, the majority of the reported analyses of the heredity of mutations generated by CRISPR/Cas9 did not segregate out the *CRISPR/Cas9* construct. There are two major concerns about the existence of the *Cas9/gRNA* DNA in CRISPR alleles of Arabidopsis mutants. First, it is difficult to determine whether the mutation in the T2 generation in a putative Arabidopsis mutant is actually inherited from the T1 generation or is newly produced by the *Cas9/gRNA* construct in the T2 generation. It is essentially impossible to distinguish the two possibilities if the mutation is heterozygous. This point is extremely important, because the newly produced mutation in the T2 generation is likely somatic and not heritable. Second, the prolonged existence of the *CRISPR/Cas9* construct in the mutants greatly increases the risk of

[1] This work was supported by the National Institutes of Health (grant no. R01GM114660 to Y.Z.).

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Yunde Zhao (yundezhao@ucsd.edu).

X.G., J.C., and Y.Z. designed the research; X.G., J.C., X.D., D.Z., and Y.Z. performed the experiments and analyzed the data; Y.Z. wrote the article; all authors read and approved the final article.

[OPEN] Articles can be viewed without a subscription.
www.plantphysiol.org/cgi/doi/10.1104/pp.16.00663

producing off-target mutations. Despite the many reports of successful gene-editing events in Arabidopsis, we believe that it is still an open question how efficient CRISPR/Cas9 is in generating stably heritable mutations in Arabidopsis because removal of the *CRISPR/Cas9* construct was not emphasized in previous studies. It is imperative to segregate out the *CRISPR/Cas9* construct before a mutation can be claimed heritable in Arabidopsis.

Effective isolation of targeted mutations generated by CRISPR/Cas9 requires not only reasonable editing efficiency but also an easy method to screen for the mutations. Editing events generated by CRISPR/Cas9 are normally identified by restriction enzyme digestion of PCR fragments or by in vitro digestion using purified Cas9 protein. Both methods are time consuming and laborious. Simplified screening methods are urgently needed.

Here, we report an effective strategy to reliably isolate *Cas9*-free T2 plants that contain stably heritable mutations in Arabidopsis. We added a cassette that enables the expression of the *mCherry* gene under the control of a strong promoter to the *CRISPR/Cas9* vector. The *mCherry* cassette allowed us to visually select *Cas9*-free plants in the T2 generation. We focused on the *Cas9*-free T2 plants because we hypothesized that once a mutation is identified in a *Cas9*-free T2 plant, the mutation must have been inherited from the previous generation and it will be stably transmitted to next generations. As a proof of concept, we targeted two sites in the *AUXIN-BINDING PROTEIN1* (*ABP1*) gene. We found that less than 30% of T1 plants contained detectable mutations. About 50% of the positive T1 plants were able to produce *Cas9*-free plants that harbor a mutation near the target sites. The success rates for identifying a mutation in *Cas9*-free T2 plants varied among T2 populations, but most were in the single digits. Surprisingly, the ratio between homozygous and heterozygous mutations in the T2 generation apparently failed to match the expected Mendelian segregation. We also show that screening for mutations could be greatly simplified if two gRNAs are expressed simultaneously to generate a large deletion. Our strategy of using *mCherry* and dual gRNAs led us to effectively isolate *Cas9*-free plants with the desired mutations.

RESULTS

Development of a Visual Screen for *Cas9*-Free Plants

In order to obtain stably transmissible mutations in Arabidopsis generated by CRISPR/Cas9-mediated genome-editing technology, it is imperative to segregate out the *CRISPR/Cas9* construct. Otherwise, it is very difficult to distinguish between a mutation transmitted from the previous generation and a newly generated mutation by Cas9. Traditionally, *Cas9*-free plants are identified by PCR using *Cas9*-specific primers. However, the PCR method is laborious and inefficient.

In order to quickly identify *Cas9*-free plants, we inserted an *mCherry*-expressing cassette into the *CRISPR/Cas9* vector so that *Cas9*-free plants can be visually identified using a microscope (Fig. 1A). We placed the *mCherry* gene under the control of the strong promoter *At2S3* (Kroj et al., 2003). As shown in Figure 1B, seeds harvested from the T1 plants that contained the *mCherry*-expressing cassette segregate into two groups: one group displayed strong red fluorescence and the other group had no fluorescence. Because the *mCherry* cassette and the *CRISPR/Cas9* unit are located on the same plasmid, a lack of red fluorescence is indicative of a *Cas9*-free state. Therefore, the *mCherry* cassette makes it very easy to visually differentiate the seeds with the *Cas9* transgene from those without the *Cas9* transgene (Fig. 1B).

Generation of Mutations in the *ABP1* Locus by *CRISPR/Cas9*

We previously obtained an *abp1* mutant that contains a 5-bp deletion in the first exon of *ABP1* using our ribozyme-based CRISPR technology (Gao et al., 2015). The mutation in the first exon was suggested not to be

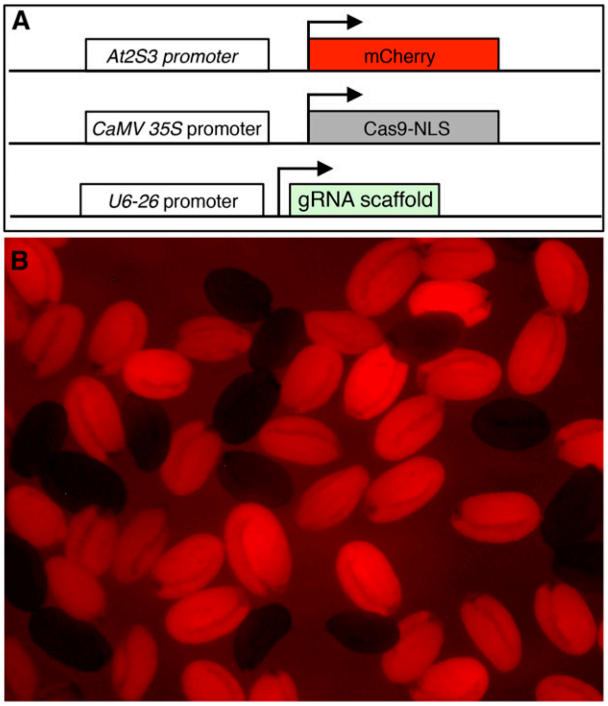

Figure 1. Design of a *CRISPR/Cas9* vector to facilitate a visual screen for *Cas9*-free Arabidopsis seeds in the T2 generation. A, Schematic representation of the new *CRISPR/Cas9* vector that contains a *Cas9* expression cassette driven by the cauliflower mosaic virus (CaMV) 35S promoter and a U6 promoter-controlled gRNA production unit. More importantly, it also expresses *mCherry* from the strong promoter *At2S3* in seeds. NLS, Nuclear localization signal. B, Visual screen for T2 seeds that no longer harbor the *CRISPR/Cas9* construct. The *Cas9*-free seeds do not produce the red fluorescence.

optimal because of the potential production of truncated proteins (Chen et al., 2015; Dai et al., 2015; Habets and Offringa, 2015; Pan et al., 2015). Here, we designed two new gRNAs to target two discrete sites in the *ABP1* gene (Fig. 2A) to test our new vector and to generate additional *abp1* alleles. The new target sites (named *CRP2* and *CRP3*; Fig. 2A) were selected in an attempt to disrupt the auxin-binding pocket in ABP1. The *CRP2* target has a *Bsa*JI restriction site near the PAM motif, and the *CRP3* target contains a *Taq*I site (Fig. 2A). The two restriction enzymes can be used to screen editing

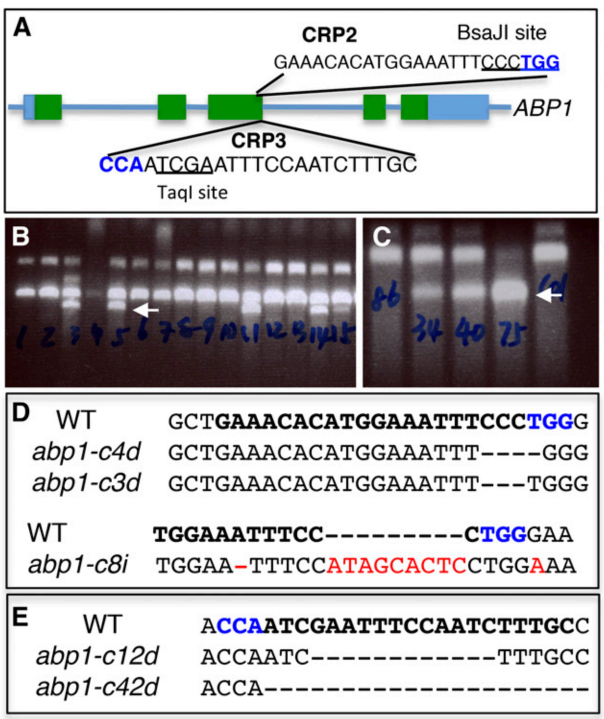

Figure 2. Generation of *abp1* mutants using the *mCherry*-containing *CRISPR/Cas9* editing vector. A, Schematic representation of the *ABP1* gene and the sequences of the selected target sites for editing *ABP1*. PAM sites (NGG or CCN) are highlighted in dark blue. *CRP2* and *CRP3* target opposite strands of the *ABP1* genomic DNA. The restriction enzyme sites used for genotyping and screening for mutations are underlined. *Bsa*JI recognizes CCNNGG, while *Taq*I cuts TCGA. Note that Cas9 usually cuts 3 bp upstream of the PAM site. Therefore, screening with *Bsa*JI is not optimal. B, Restriction digestion screen of T1 plants transformed with *CRP2/CRISPR* vector using the enzyme *Bsa*JI. Plants with mutations generate PCR bands resistant to *Bsa*JI digestion (arrow). Among the 15 samples shown, four potentially have been edited at the *ABP1* locus (3, 5, 11, and 14). C, Restriction digestion of PCR products from T1 plants that have disrupted the *Taq*I site at the *CRP3* target site. Note that sample 75 has very little wild-type (WT) DNA. The arrow points to a *Taq*I-resistant PCR band. D, Three *abp1* mutants with deletions/insertions at the *CRP2* target site. *abp1-c4d* has a 4-bp deletion, and *abp1-c3d* has a 3-bp deletion; *abp1-c8i* has a very complex mutation. E, Two editing events at the *CRP3* site that resulted in two stable *Cas9*-free *abp1* alleles. One has a 12-bp deletion and the other deletes 42 bp near the target site. Note that the 42-bp deletion is not shown in full.

events at the targets (Fig. 2, B and C). We used the cauliflower mosaic virus *35S* promoter to drive the expression of *Cas9* and used a *U6* promoter to express the specific gRNAs (Fig. 1A). We transformed the *CRISPR/Cas9* constructs into wild-type Arabidopsis Columbia-0 and screened for potential gene-editing events in T1 plants. As shown in Table I, we were able to identify T1 plants that had undergone successful editing at the two *ABP1* target sites. Interestingly, the editing efficiencies for the two target sites differed significantly. For the *CRP3* target, only 3.5% of the T1 plants (three of 86) had detectable mutations at the target site. In contrast, the mutation rate was much higher at the *CRP2* site: about 21% (seven of 33) of T1 plants had detectable mutations at the *CRP2* site. We noticed that the mutation rate at the *CRP2* site was significantly underestimated, because there are two overlapping *Bsa*JI sites at the target. In addition, Cas9 usually cuts DNA 3 bp upstream of the PAM motif, and mutations there would not disrupt the *Bsa*JI restriction site at the *CRP2* target. The exact reasons for why editing efficiencies varied greatly between the two targets are not fully understood. Recent studies have clearly shown that certain features in gRNAs greatly affected editing efficiency, and some guidelines for designing better gRNAs have been proposed (Chari et al., 2015; Liang et al., 2016). We did not obtain any apparent homozygous *abp1* T1 plants, even though plant 75 appeared to contain very little wild-type *ABP1* DNA at the *CRP3* target site (Fig. 2C).

Isolation of *Cas9*-Free and Stably Transmissible *abp1* Mutations

We harvested seeds from individual T1 plants and used a fluorescence-based visual screen to identify T2 seeds that did not contain the *CRISPR/Cas9* construct (Fig. 1B). We then germinated the *Cas9*-free T2 seeds and transplanted the seedlings to soil. We genotyped at least 48 *Cas9*-free T2 plants harvested from each T1 plant. Less than 50% (three of seven) of the *CRP2* T1 plants produced *Cas9*-free T2 plants with a mutation at the *CRP2* target site (Table I). We genotyped 95 *Cas9*-free T2 plants from the *CRP2* T1 plant 11 and obtained one plant in this population that had a mutation (Table I), which was a 4-bp deletion (*abp1-c4d*; Fig. 2D). The mutation was heterozygous. The 4-bp deletion resulted in a frame shift. In theory, *abp1-c4d* would produce a truncated ABP1 protein with the first 117 amino acid residues identical to those of wild-type ABP1. *abp1-c4d* is likely a loss-of-function mutant because it lacked the $P(X)_4H(X)_3N$ fingerprint that is important for zinc and auxin binding (Woo et al., 2002). Among the 94 *Cas9*-free T2 plants from *CRP2* T1 plant 14, three plants contained a 3-bp deletion, which were also heterozygous (*abp1-c3d*; Fig. 2D). We further identified one heterozygous plant out of 95 *Cas9*-free T2 plants from the *CRP2* T1 plant

Table I. *Editing efficiencies by CRISPR/Cas9 for different target sites*

The target sequences for *CRP2* and *CRP3* are described in Figure 2A. The *RGR* target site was described previously (Gao et al., 2015). The *RGR* sequence and design also are shown in Supplemental Figure S2. *CRP2/RGR* refers to targeting both *CRP2* and *RGR* sites simultaneously. The ratios represent the editing efficiency. For example, 7:33 refers to seven positive plants out of 33 total plants. All of the T2 plants analyzed were *Cas9* free based on a lack of red fluorescence (Fig. 1B). For the T2 plants in boldface, the number of mutant plants (both heterozygous and homozygous) from each T1 plant is shown (boldfaced indicates non-bi-allelic mutations). *abp1-c12d* and *abp1-c42d* are from the same T1 plant 75; *abp1-c2* shows an unusual segregation pattern.

Target	*CRP2*	*CRP3*	*CRP2/RGR*	*CRP3/RGR*
T1	7:33	3:86	5:61	0:92
T2	T1 plant 3, died	T1 plant 34, 0:72	T1 plant 14, 0:48	Not analyzed
	T1 plant 5, 0:72	T1 plant 40, 0:72	**T1 plant 29, 26:52 (*abp1-c2*)**	Not analyzed
	T1 plant 11, 1:95		***abp1-c2*$^{+/-}$, 13:52**	Not analyzed
	T1 plant 14, 3:94	**T1 plant 75, 8:196**	***abp1-c2*$^{-/-}$, 13:52**	Not analyzed
	T1 plant 25, 1:95	***abp1-c12d*, 7:196**	T1 plant 38, 0:72	Not analyzed
	T1 plant 30, 0:72	***abp1-c42d*, 1:196**	T1 plant 56, 2:96 (*abp1-c3*)	Not analyzed
	T1 plant 33, 0:96		T1 plant 65, 0:72	Not analyzed

25 with a complex mutation pattern (*abp1-c8i*; Fig. 2D). *abp1-c8i* harbored a 9-bp insertion, a 1-bp deletion, and a point mutation (Fig. 2D). This allele also will be useful in future studies because of a lack of the key C-terminal region of ABP1.

To determine whether the mutations identified in the *Cas9*-free T2 plants could be stably transmitted to the next generations, we genotyped 28 T3 plants generated from selfing a T2 *abp1-c3d* plant. We found that 13 plants were heterozygous, eight homozygous, and seven without the mutation, indicating that the mutation identified in a *Cas9*-free plant at the T2 stage was stably transmitted to the T3 generation in a Mendelian fashion (Supplemental Table S1). Genotyping results of the T3 plants from the other *Cas9*-free T2 mutants at the *CRP2* targets also were consistent with the expected Mendelian ratios, suggesting that once a mutation is confirmed in a *Cas9*-free T2 plant, the mutation would be stable and could be transmitted to next generations following Mendelian genetics (Supplemental Table S1).

We also analyzed the mutations generated at the *CRP3* site. Among the three T1 plants that contained mutations at the *CRP3* target, only one T1 plant produced *Cas9*-free offspring with mutations at the intended target site (Table I). Among the 196 *Cas9*-free T2 plants for the *CRP3* target we genotyped, eight plants contained mutations. Moreover, seven out of the eight plants had a 12-bp deletion at the CRP3 site and one contained a 42-bp deletion (Fig. 2E). Interestingly, among the plants with the 12-bp deletion (*abp1-c12d*), two were homozygous and five were heterozygous. The 42-bp deletion (*abp1-c42d*) was homozygous. We crossed the *abp1-c12d* homozygous T2 plants to wild-type plants and found that all of the F1 plants were heterozygous for the mutation (Supplemental Table S1). We also genotyped some T3 plants from heterozygous T2 *abp1-12d* plants. It was very clear that the mutation segregated in a Mendelian fashion (Supplemental Table S1).

Generation of Large Deletions Using Two gRNAs

We tested whether we could delete a large fragment by simultaneously expressing two gRNAs that target the sites flanking the intended deletion. If successful, such a strategy will greatly simplify the screening process for gene-editing events because mutants will yield a much smaller PCR fragment than the wild type. Another advantage of a large deletion is that such a mutation would be an unambiguous knockout. We have shown previously that a ribozyme-flanked gRNA unit (RGR) that targeted the first exon of *ABP1* successfully produced the *abp1-c1* mutant with a 5-bp deletion (Fig. 3A; Gao et al., 2015). Here, we placed the same RGR unit under the control of a *UBIQUITIN10* promoter (*UBQ10*) to produce a gRNA targeting the first exon of *ABP1* (Fig. 3A; for vector map and RGR sequences, see Supplemental Figs. S1 and S2). We made two dual gRNA constructs to delete most of the genomic DNA of *ABP1*. The first construct combined the *UBQ10*:*RGR* unit with *U6*:*CRP2*, and the other combined *UBQ10*:*RGR* with *U6*:*CRP3* (Fig. 3A). We transformed the two constructs into wild-type Arabidopsis and isolated T1 plants. For the *RGR/CRP3* construct, we genotyped 92 T1 plants, but none of them produced the expected small PCR fragment. Given the lower editing efficiency that we had observed at the *CRP3* site (Table I), the failure to generate a deletion from this construct was not a surprise. For the *RGR/CRP2* construct, we obtained five T1 plants out of 61 that produced a smaller PCR fragment than the wild type, suggesting that these two gRNAs together were able to cause the deletion of part of the *ABP1* gene.

We then screened *Cas9*-free T2 plants from the T1 plants that were positive for deletions to identify stably heritable *abp1* deletion mutations. From the five positive T1 plants generated with *RGR/CRP2*, two T1 plants produced *Cas9*-free T2 offspring that

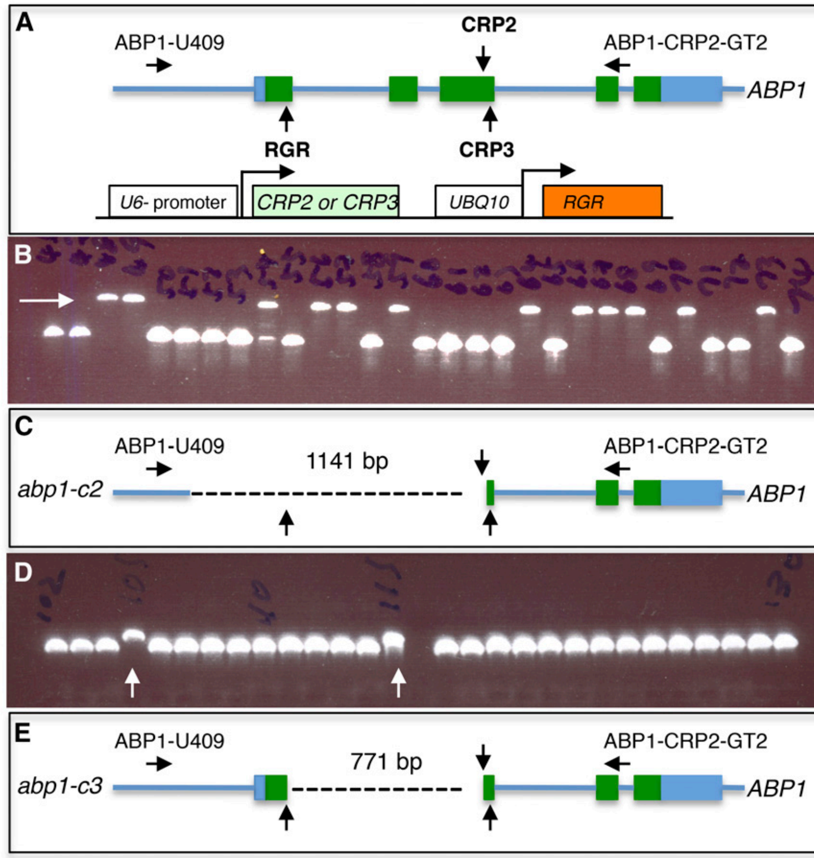

Figure 3. *CRISPR/Cas9*-mediated deletions of a large DNA fragment between two gRNA target sites in Arabidopsis. A, We produced *CRISPR* plasmids that target three sites of the *ABP1* gene. We combined the *RGR* and *CRP2* modules to delete the first three exons. We also combined the *RGR* and *CRP3* modules in another plasmid. *RGR* is controlled by the *UBQ10* promoter. Green boxes refer to *ABP1* exons. Vertical arrows point to gRNA target sites. ABP1-U409 and ABP1-CRP2-GT2 are the primer pair used in the PCR screening. The RGR sequence and design are shown in Supplemental Figure S2. B, PCR amplification using ABP1-U409 and ABP1-CRP2-GT2 primers and the genomic DNA from *Cas9*-free T2 plants generated from a single T1 plant transformed with the *RGR-CRP2* dual gRNA vector. About half of the plants contained a deletion. Note that this primer pair preferentially amplifies the small fragment and cannot differentiate homozygous from heterozygous plants. C, Schematic representation of the *abp1-c2* mutation, which is a deletion of 1,141 bp including the first three exons and 304 bp of the *ABP1* promoter. The dashed line represents the deleted region. D, Identification of a second *abp1* allele that has a large deletion. Only two plants (105 and 115) out of 96 *Cas9*-free T2 plants from a single *RGR-CRP2* T1 plant contained a deletion (arrows). E, Further sequencing analysis shows that the deletion is 711 bp, which is the exact expected size generated by gRNAs targeting *RGR* and *CRP2* sites.

contained a large deletion in the *ABP1* gene. We genotyped 52 *Cas9*-free T2 plants generated from the single T1 plant 29 (Fig. 3B). We found that 26 of the 52 T2 plants contained a large deletion at the *ABP1* locus and 13 of the mutants were homozygous for the mutation (Table I). The results apparently did not match the expected results from Mendelian genetics (χ^2 = 19.5). We further sequenced the small PCR fragment and found that the deletion was 1,141 bp (*abp1-c2*; Fig. 3C), which included part of the *ABP1* promoter and the first three exons (Fig. 3C; for the deleted sequences, see Supplemental Document S1). Interestingly, the designed deletion between the two gRNAs was only 711 bp.

We also identified two plants in the T2 generation that had a 711-bp deletion in the *ABP1* locus (*abp1-c3*) after screening 96 *Cas9*-free progeny from the T1 plant 56 (Fig. 3, D and E; Table I). One plant was homozygous and the other was heterozygous. We further tested whether the deletion mutations identified in the T2 plants could be stably transmitted to next generations by genotyping T3 plants generated from selfing and by genotyping F1 plants that resulted from a cross between the T2 mutants and the wild type. Our results demonstrated that the two deletion mutants were stable and segregated into T3 plants following the rules of Mendelian genetics (Supplemental Table S1).

DISCUSSION

We designed a new *CRISPR/Cas9* vector to generate *Cas9*-free T2 plants with targeted mutations in Arabidopsis. We successfully isolated at least two different mutations at each intended target site. The mutations in the *Cas9*-free plants are stable and are transmitted to next generations in a Mendelian fashion (Supplemental Table S1). The method described in this article (Fig. 4A) is reliable and effective.

We show that it is extremely important to focus on *Cas9*-free T2 plants in order to unambiguously identify heritable mutations generated by CRISPR/Cas9 in Arabidopsis. Identification of a targeted mutation generated from CRISPR/Cas9 is based mainly on analyses of PCR fragments digested with enzymes. The PCR usually uses genomic DNA isolated from a piece of leaf tissue as a template. Results from such assays often cannot reveal the mosaic nature of the mutations if the majority of the cells contain the mutation (Fig. 2C), thus often yielding false positives. We previously identified putative homozygous T2 plants based on restriction digestion (Supplemental Fig. S3), but in the T3 generation we found that the mutation was not heritable, because none of the *Cas9*-free T3 plants contained the mutation. However, mutations observed in *Cas9*-free T2 plants must have been transmitted from the previous generation. Because less than 25% of the T2 plants

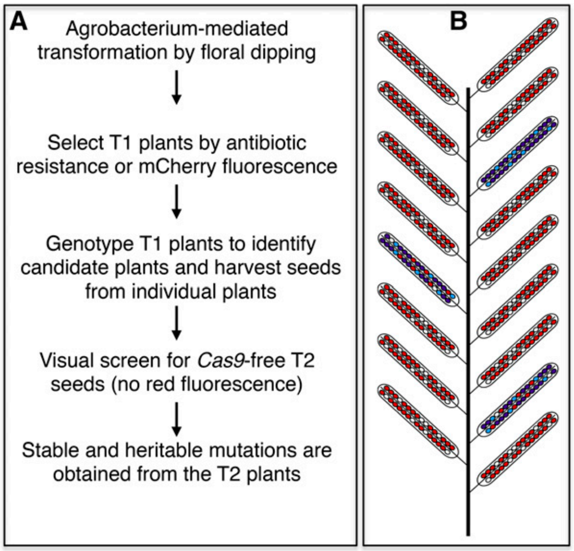

Figure 4. Reliably isolating stable and heritable targeted mutants using CRISPR/Cas9 genome-editing technology in Arabidopsis. A, Flow chart for isolating CRISPR alleles of Arabidopsis mutants. The key is to use the visual screen to quickly identify *Cas9*-free T2 seeds. Mutations in *Cas9*-free T2 plants are stably transmitted to next generations following Mendelian genetics (Supplemental Table S1). B, Schematic representation of the mosaic nature of mutations generated by CRISPR/Cas9 in T1 plants. If a founder cell for a flower is mutated, the seeds generated from that particular flower will contain heritable mutations (blue or purple). However, seeds in the majority of the siliques do not contain heritable mutations. Red refers to seeds with the *mCherry-CRISPR/Cas9* construct.

are *Cas9* free, it would be 75% more genotyping workload if we did not preselect the *Cas9*-free plants. In addition, we found that the chance of identifying a mutation in *Cas9*-free T2 plants is usually low (Table I). For example, we only identified one plant that contained a heterozygous *abp1* mutation (*abp1-c8i*) after we genotyped 95 *Cas9*-free T2 plants generated from the *CRP2* T1 plant 25 (Table I; Fig. 2D). In order to identify one mutant plant in this case, we would have to genotype at least 380 T2 plants if we did not preselect the *Cas9*-free plants. Given that less than 50% of the positive T1 plants produced *Cas9*-free plants with a mutation (Table I), the workload would be so heavy that identification of a heritable mutation in a *Cas9*-free plant becomes prohibitive if we do not preselect the *Cas9*-free T2 plants. Expression of the *mCherry* gene in seeds makes the selection of *Cas9*-free T2 plants very convenient and efficient (Fig. 1B).

We were puzzled by why the ratio between heterozygous and homozygous mutants in some cases was not 2:1. For example, the ratio was 1:1 (13:13) for *abp1-c2* in the T2 generation (Fig. 3C; Table I). Moreover, 50% of the *Cas9*-free T2 plants contained the *abp1-c2* mutation. The 1:1 ratio and the 50% of mutants observed apparently cannot be explained by Mendelian genetics, which would give 75% of plants with the mutation (homozygous and heterozygous) among the T2 plants and a 2:1 ratio between heterozygous and homozygous mutants. We realized that mutations in T1 plants are probably mosaic. If a given floral founder cell contains a mutation in the target site, seeds in the silique developed from that particular flower will have the mutation (Fig. 4B). If the mutation in the founder cell is heterozygous, seeds in the silique will segregate according to Mendelian genetics. However, if the mutation in the founder cell is homozygous, every seed in the silique will contain the homozygous mutation (Fig. 4B). When we harvest seeds from a T1 plant, seeds from all of the siliques are mixed. Because the founder cells for the majority of flowers/siliques do not contain a mutation, mutated seeds from a few siliques will be diluted, greatly decreasing the chance for identifying *Cas9*-free mutants at the T2 stage. That could explain the single-digit rate observed for identifying *Cas9*-free mutants (Table I). For the abnormal heterozygous-to-homozygous ratio, we believe that it is determined by the ratio between homozygous siliques and heterozygous siliques (Fig. 4B). Another example of the mosaic nature in T1 plants was observed in the *CRP3* T1 plant 75 (Table I). This T1 plant produced two different mutations (Table I). We think that plant 75 was not biallelic, because one mutation appeared predominantly (Table I).

Recently, it was reported that the expression *Cas9* under the control of some specialty promoters could greatly increase gene-editing efficiency in Arabidopsis (Wang et al., 2015; Yan et al., 2015; Mao et al., 2016). It was reported that homozygous mutants could be obtained in the T1 generation (Wang et al., 2015). However, the studies did not determine what percentage of *Cas9*-free T2 plants contained the edited mutations. Given the dramatically increased editing efficiency at the T1 stage when these specialty promoters were used instead of the 35S promoter, it is worth combining our *mCherry* cassette with the specialty promoter-driven *Cas9* unit to increase the efficiency for isolating *Cas9*-free heritable Arabidopsis mutations.

The generation of a large deletion by employing two gRNAs greatly simplifies the screening process (Fig. 3). We did not observe a dramatic decrease in editing efficiency when dual gRNAs were used (Table I). Another advantage is that large-deletion mutations are more likely null compared with small-deletion mutations.

In summary, CRISPR/Cas9 gene-editing technology is a powerful tool for creating targeted mutations in Arabidopsis, but it is important to identify mutations in *Cas9*-free T2 plants to ensure that the mutations observed can be stably transmitted to future generations. Our fluorescence-based visual screen facilitates the isolation of *Cas9*-free T2 seeds easily and quickly. Combined with the use of dual gRNAs, our method reliably identifies useful targeted mutations in Arabidopsis.

MATERIALS AND METHODS

Plasmids and Constructs

Two *CRISPR/Cas9* vectors were used in this study: *pHDE-35SCas9-mCherry* and *pHDE-35SCas9-UBQ10-mCherry*. The complete sequence information of the two vectors is shown in Supplemental Document S2. The maps and annotated vector sequences are shown in Supplemental Figure S1. The U6-gRNA unit was cloned into the *Pme*I site in both vectors by Gibson assembly (Gibson et al., 2008). The RGR unit was cloned into the *Mfe*I site in *pHDE-35SCas9-UBQ10-mCherry* by Gibson assembly. The RGR design and sequences are shown in Supplemental Figure S2.

Screen for Editing Events in Arabidopsis

The *CRISPR/Cas9* constructs were transformed into Arabidopsis (*Arabidopsis thaliana*) wild-type Columbia-0 through floral dipping. T1 plants were selected either by red fluorescence or on 16 μg L^{-1} hygromycin. Genomic DNA samples extracted from leaf tissues of 2-week-old T1 plants were used as templates for PCR. To screen mutations at the *CRP2* target, we used the primer pair ABP1-U409 (5′-CCTCATCACACAACAAAGTCACTC-3′) and ABP1-CRP2-GT2 (5′-CATGAGGACCTGCAGGTGTTG-3′) to amplify the *CRP2* target-containing fragment. The PCR product was digested using restriction enzyme *Bsa*JI. Putative mutations should produce a *Bsa*JI-resistant band. To genotype mutations at the *CRP3* site, we used primers ABP1-2E (5′-TTGCCAATCGTGAGGAATATTAG-3′) and ABP1-CRP2-GT2 for PCR. Then, we digested the PCR product with *Taq*I. To screen for large deletions, we conducted PCR using ABP1-U409 and ABP1-CRP2-GT2 to screen for smaller fragments.

Cas9-free T2 seeds were isolated using a dissecting fluorescence microscope equipped with an mCherry filter. We focused our PCR screening for mutants on the identified *Cas9*-free plants.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Annotation and restriction map of *pHDE-35SCas9-mCherry*.

Supplemental Figure S2. Sequence and general design of *RGR*.

Supplemental Figure S3. Failure in identifying heritable mutants among T2 offspring from a single T1 plant that had been shown to contain mutations.

Supplemental Table S1. Segregation patterns of the various *abp1* mutants generated by *CRISPR/Cas9*.

Supplemental Document S1. Molecular lesions and the deletion junctions of *abp1-c2* and *abp1-c3*.

Supplemental Document S2. DNA sequences of *pHDE-35SCas9-mCherry* and *pHDE-35S-Cas9-mCherry-UBQ10*.

ACKNOWLEDGMENTS

We thank Drs. Rui Wu and Detlef Weigel at the Max Planck Institute for Developmental Biology for suggesting the use of mCherry-based screening, for providing the *mCherry* expression cassette, and for valuable discussions.

Received April 26, 2016; accepted May 13, 2016; published May 15, 2016.

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