

A multiplex CRISPR/Cas9 platform for fast and efficient editing of multiple genes in *Arabidopsis*

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Abstract The recently developed CRISPR/Cas9 system is a promising technology for targeted genome editing in a variety of species including plants. However, the first generation systems were designed to target one or two gene loci at a time. We designed a new multiplex CRISPR/Cas9 system that allows the co-expression of six sgRNA modules in one binary vector using a simple (three steps) cloning strategy in *Arabidopsis*. The transcription of the sgRNA modules is under the control of three different RNA Polymerase III-dependent promoters. We tested the efficiency of the new multiplex system by targeting six of the fourteen *PYL* families of ABA receptor genes in a single transformation experiment. One line with mutations in all six targeted *PYLs* was identified from 15 T1 plants. The mutagenesis frequency for the six individual *PYL*

targets in the T1 lines ranged from 13 to 93 %. In the presence of ABA, the transgenic line identified as containing mutations in all six *PYL* genes produced the highest germination rate in the T2 progeny (37 %). Among these germinated seedlings, half of the analyzed plants (15/30) were homozygous mutants for at least four targeted genes and two plants (6.7 %) contained homozygous mutations in five of the targeted *PYLs* and the other targeted *PYL* had biallelic mutations. Homozygous sextuple mutants were identified in the T3 progeny and characterized together with previously described triple and sextuple *PYL* mutants. We anticipate that the application of this multiplex CRISPR/Cas9 system will strongly facilitate functional analysis of genes pathways and families.

Keywords CRISPR/Cas9 · Multiplex · Gene editing · *Arabidopsis*

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Introduction

Reverse genetics is the most popular approach for gene functional studies in plants (Alonso and Ecker 2006). Targeted gene silencing by RNA interference (RNAi) has been widely employed for loss-of-function gene studies, and for *Arabidopsis thaliana*, a vast collection of T-DNA mutant lines with known insertion sites has been available for some time (Alonso and Ecker 2006; Waterhouse et al. 1998). However, both approaches have important practical limitations. Silencing levels achieved by RNAi in individual lines are variable and the stability of silencing over several generations is not guaranteed. In addition the production of mutant collections with characterized insertion sites similar to the one existing in *Arabidopsis* is not practical in most plant species. The emergence of the

Streptococcus pyogenes derived CRISPR/Cas9 system as a powerful tool for genome editing can facilitate gene functional studies in a variety of organisms, including plants (Doudna and Charpentier 2014). The engineered CRISPR/Cas system is composed of a single-stranded guide RNA (sgRNA) for target DNA recognition via base pairing, and a CRISPR-associated protein (Cas9) for DNA cleavage ~3 bp upstream of the protospacer adjacent motif (PAM = 'NGG') (Jinek et al. 2012). In the absence of homologous DNA templates, the CRISPR/Cas9-triggered double-stranded breaks will be repaired via the error-prone non-homologous end joining (NHEJ) pathway, resulting in the generation of frame-shift mutations in the targeted genes (Cong et al. 2013; Mali et al. 2013).

Successful application of the CRISPR/Cas9 gene editing system has been reported in a variety of plant species (Brooks et al. 2014; Fauser et al. 2014; Feng et al. 2013; Jia and Wang 2014; Jiang et al. 2013; Li et al. 2013a; Mao et al. 2013; Miao et al. 2013; Nekrasov et al. 2013; Shan et al. 2013; Upadhyay et al. 2013; Xie and Yang 2013; Xing et al. 2014; Zhang et al. 2014). However, most of the first generation CRISPR/Cas9 systems used for plant gene editing usually provided only one or two docking sites for sgRNA modules, thus limiting the gene-targeting capacity of the system. New CRISPR/Cas9 systems have been designed for multiplex gene editing in plants thus allowing systematic study of gene families or metabolic pathways (Xie et al. 2015; Xing et al. 2014).

In animals, multiplex gene editing can be achieved by co-injection of in vitro transcribed sgRNAs and Cas9 mRNA into the one-cell embryo (Li et al. 2013b, c; Wang et al. 2013). However, in plants, this method is not practical due to the physical barrier provided by the cell wall. Instead, the *Agrobacterium*-mediated gene transformation method is used for delivering the CRISPR/Cas9 system into germline cells (Katavic et al. 1994). To perform multiplex gene targeting in plants, the crux of the problem is how to construct multiple sgRNA modules into one binary vector along with the expression cassette for Cas9. One solution is to mimic the native composition of type II or type III CRISPR arrays (Cong et al. 2013; Nissim et al. 2014). For such RNA precursors, the tandem repeated crRNAs or sgRNAs units are first transcribed from a RNA Pol III (Pol III) promoter and then subjected to the excision of an engineered endonuclease system to release the individual RNA components. Recently, a similar design hijacking the plant endogenous tRNA processing system for sgRNA maturation was reported in rice (Xie et al. 2015). However, a potential risk of applying these strategies is that transformation of highly repetitive DNA sequences might cause transgenic gene silencing in a variety of species (Ma and Mitra 2002).

To reduce the repetitiveness of the sgRNA cassette, we adopted a relatively conservative strategy by engineering three sgRNAs modules with different Pol III-dependent promoters. These three sgRNA modules were designed with adaptive restriction sites to allow one step assembly of multiple fragments. Using a previously developed CRISPR/Cas9 system with two separated sgRNA docking sites (Mao et al. 2013), we generated a sextuple CRISPR/Cas9 vector containing six sgRNAs. Using this upgraded CRISPR/Cas9 system, we successfully targeted six of the 14 PYR/PYL gene family members (*PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5* and *PYL8*) in a single transformation event. These six *PYR/PYL* genes encode a branch of the ABA receptors in *Arabidopsis* (Park et al. 2009). In the absence of these ABA receptors, the newly obtained *pyl* sextuple mutant exhibited an ABA-insensitive phenotype similar to the previously described sextuple mutant, which has a mixed background of both Col-0 and Ler (Gonzalez-Guzman et al. 2012). The targeting efficiency of the three sgRNA modules was studied using YFP single-stranded annealing assay (SSA) in protoplasts. Our results show that the AtU6 and At7SL promoters had relatively higher activity than the AtU3b promoter for targeted gene modification in protoplasts. The sequential order of these sgRNA modules in the expression cassettes had no effect on their gene targeting efficiency. Thus, we propose that this upgraded CRISPR/Cas9 system can be used for efficient multiplex gene editing in *Arabidopsis* with up to six sgRNAs. Future application of this system will facilitate the generation of high order mutants for the functional analysis of gene families in *Arabidopsis* and other plants.

Results

Workflow of vector construction for sextuple gene targeting in *Arabidopsis*

The workflow to produce the sextuple CRISPR/Cas9 vector for targeted gene modification is shown in Fig. 1. To construct multiple sgRNA modules for gene targeting in *Arabidopsis*, three different Pol III-dependent gene promoters (AtU6-26, AtU3b and At7SL-2) were cloned from the *Arabidopsis* genome to drive the expression of the sgRNAs. For target recognition, 20-nt guide oligo-nucleotides were synthesized with appropriate adaptors for seamless ligation with the three sgRNA modules separately. Note that the 5' adaptor sequences of those synthesized oligo-nucleotides were actually determined by the designated sgRNA promoters (Table 1). Subsequently, the three customized sgRNA modules were digested with the corresponding restriction enzymes for cloning into the Cas9 expression vector in tandem. The resulting CRISPR/

Cas9 vectors were used directly for plant transformation (for 3-gene targeting) or as an intermediate vector for further construction of the sextuple construct. In the later case, two intermediate CRISPR/Cas9 vectors with three sgRNAs each were constructed in parallel from the beginning. To clone the second sgRNA cassette, it was first amplified from its own intermediate vector by PCR, and then cloned into the construct already containing the first three sgRNAs to generate the final CRISPR/Cas9 sextuple vector.

Assemblage of a sextuple CRISPR/Cas9 vector for *PYL* gene targeting

In Arabidopsis, perception of the phytohormone abscisic acid (ABA) is mediated by proteins in the 14-member PYR/PYL family (Park et al. 2009). A PYR/PYL receptor sextuple mutant, namely *pyr1pyl1pyl2pyl4pyl5pyl8* (*pyl112458*) (all mutations except *pyl2* were in the Col-0 background) was reported to be insensitive to high ABA concentrations (Gonzalez-Guzman et al. 2012). To facilitate the screening of sextuple mutants as well as to test the gene editing efficiency of the multiplex CRISPR/Cas9 system, these six *PYR/PYL* genes were selected as targets for editing. For most of the *PYL* genes, the sgRNA target sites were chosen from the CDS region, while for *PYL8*, the sgRNA target site was located within the 5' untranslated (UTR) region (Fig. 2a). Phylogenetic analysis of *PYL/PYR* genes, reveals that the six targeted *PYL/PYR* genes can be classified into three clades and each gene shows limited sequence similarity with other members (Table S1). To predict the specificity of the sgRNAs, potential off-target sites were investigated using the Blastn algorithm with the full length guide sequences (including 3nt PAMs) as queries. In total, 24 putative off-targets were identified in the Arabidopsis genome (Exp <3.0) with all but one containing at least two mismatches. The most significant putative off-target sequence belonged to *PYL1* with a single mismatch compared to the target site for sgR-PYR1 (Table S2). The arrangement of these *PYL* targeting sgRNA modules in the CRISPR/Cas9 sextuple construct is shown in Fig. 2b. This binary vector was used for Agrobacterium-mediated transformation of Arabidopsis. In subsequent analysis, the six *PYL* genes were always presented in the order of *PYL1* > *PYR1* > *PYL4* > *PYL2* > *PYL8* > *PYL5* following the order of sgRNAs that target these genes.

Fifteen individual T1 transformants were analyzed by sequencing their *PYL* target regions. Triple mutants (six plants) and quintuple mutants (three plants) were identified as the two dominant multiple mutations in the population, while only one sextuple mutant (line #11) was detected (Fig. 3a). The mutation efficiencies for the different loci

were highly variable with *PYR1*, *PYL1* and *PYL2* showing high efficiency (>70 % of T1 plants), while for the remaining three loci the efficiency was low (13–26 %) (Fig. 3b). The combination patterns of the *PYL* mutations observed in the 15 T1 plants is summarized in Fig. 3c.

Moreover, we investigated the putative off-target site of sgR-PYR1 in the *PYL1* gene but no mutations were detected among the 15 T1 plants (Fig. S1).

Identification of *pyl* sextuple mutants in the T2 generation

To facilitate the screening of heritable *pyl* sextuple mutants, T2 seeds originating from the 15 individual T1 plants were germinated on MS medium supplemented with 1 μ M ABA. The ratios of seedlings growing in the presence of 1 μ M ABA for lines #11 and #12 were much higher than those observed for WT seeds, suggesting that the targeted *PYLs* were extensively mutated in plants derived from these two lines (Fig. 4a). To identify *pyl* sextuple mutants, 122 T2 plants (previously germinated on 1 μ M ABA) were randomly selected for analysis of targeted gene mutations by sequencing, among which 30 were from line #11. Overall, the abundance of homozygous multiple mutants were in inverse proportion to the number of simultaneously mutated gene loci. Line #11 was an exception to this rule with most of its T2 homozygotes being quadruple mutants (37 %). No sextuple homozygotes were identified (Fig. 4b). The segregation ratios of homozygous mutants for each of the six *PYL* loci in line #11 were above the population average (Fig. 4c). Analysis of the mutation patterns for the 122 analyzed T2 plants, shows that all of the 11 quadruple and the 4 quintuple homozygotes were derived from line #11 (Fig. 4d; Table S3). The zygosity type of all 30 individuals from line #11 was determined by amplifying the corresponding loci by PCR, cloning the products into plasmids and sequencing of the inserts. Analysis of the four quintuple mutants shows that two of the non-homozygous *PYL* loci were biallelic (*PYL4* locus in #11-3 and *PYL2* locus in #11-4) while the remaining two were heterozygous (*PYL8* locus in #11-24 and #11-28) (Fig. 4d).

Segregation of homozygous sextuple mutants in T3 generation

To obtain homozygous *pyl* sextuple mutants, two T2 quintuple mutant lines (#11-4 and #11-24) were grown to maturity, allowed to self-pollinate and used for further genotyping in the T3 generation. Twelve individuals were analyzed for each line. The segregation ratio of each *PYL* locus is summarized in Table 2. It was confirmed that the *PYL2* locus in #11-4 and the *PYL5* locus in #11-24 were

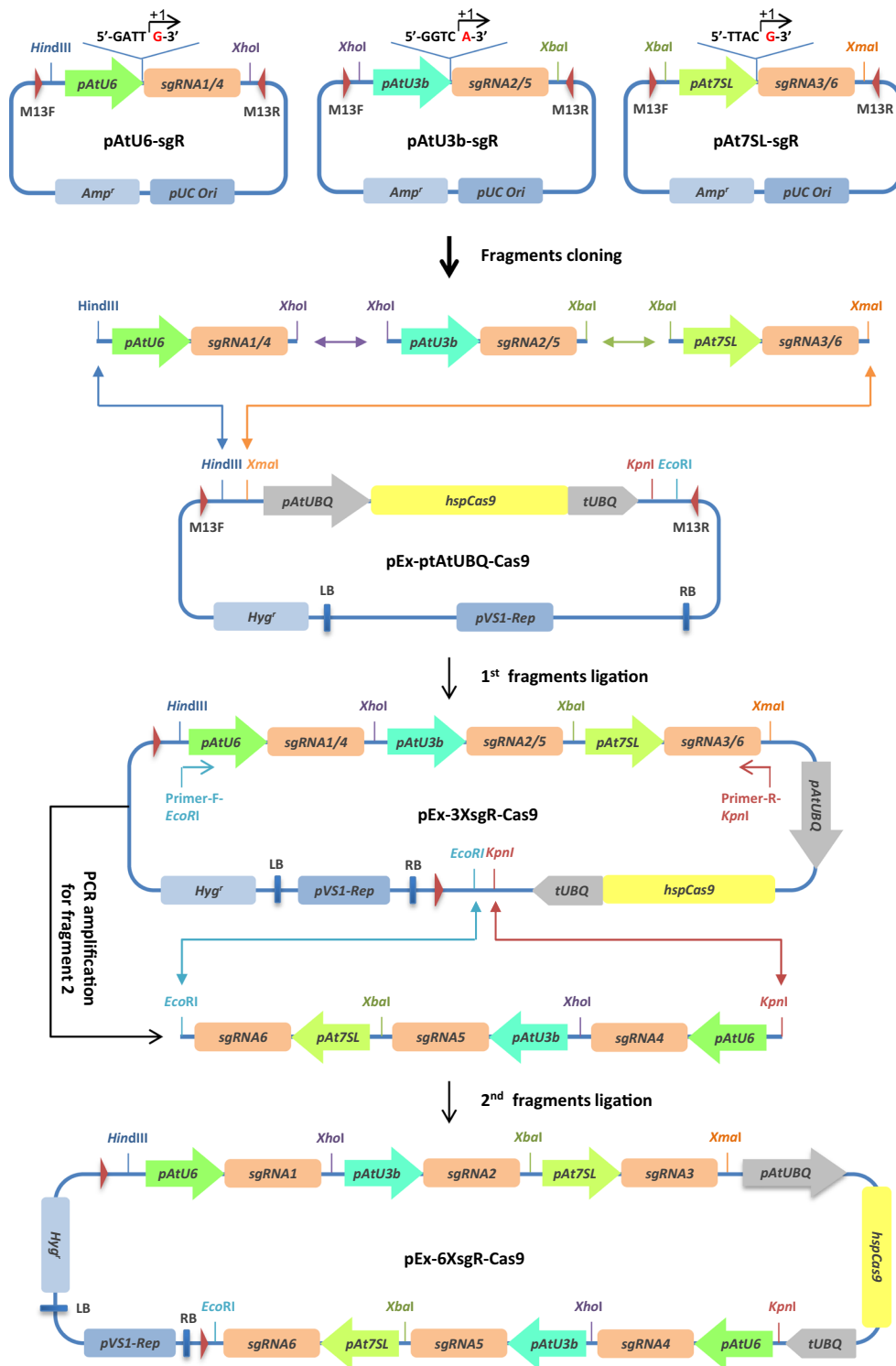


Fig. 1 Pipeline for the construction of a sextuple CRISPR/Cas9 system for multiplex gene editing in Arabidopsis. The assembly of a sextuple CRISPR/Cas9 expression vector can be accomplished in three steps. (1) Clone synthesized guide oligo-nucleotides into the sgRNA modules. For ligation with the three sgRNA scaffolds containing different Pol III promoters, a specific 5-nt adaptor should be added to the 5' end of the sense oligo-nucleotides. The sequences of the adaptors for the sense oligos are determined by their promoters. (2) Assemble the first three sgRNA modules (sgRNAs 1, 2 and 3) into the expression vector containing Cas9. The three customized sgRNA modules can be digested directly from their hosting vectors with the indicated restriction enzymes or after PCR amplification by M13F/M13R. The digested fragments are subsequently cloned into the *HindIII* and *XmaI* sites of the binary vector containing Cas9 in tandem to produce an intermediate vector with three sgRNAs. To construct the sextuple vector, a second triple intermediate vector containing sgRNAs 4, 5 and 6 is generated as described above (cloning sgRNAs 4, 5, and 6 into the *HindIII* and *XmaI* sites of the binary vector containing Cas9). (3) To clone the second sgRNA cassette into the intermediate vector, the fragment containing sgRNAs 4, 5 and 6 is amplified by PCR with primers containing *KpnI* and *EcoRI*. The amplicon is then digested with *KpnI* and *EcoRI* and cloned into the intermediate vector to generate the final CRISPR/Cas9 binary vector with six sgRNAs. The three Pol III-dependent promoters are labeled with different color. Red arrowheads indicate the M13F and M13R sites in plasmids. Blue rectangles indicate the left and right border sites of T-DNA. *HygR* hygromycin resistance gene, *pVSI-Rep* bacterial origin of replication, *pAtUBQ* promoter of *AtUBQ1* gene, *tUBQ* terminator of *AtUBQ1* gene, *hspCas9* human-codon optimized Cas9 gene

biallelic and heterozygous, respectively, while all the other targeted gene loci contained homozygous mutations as expected. In the T3 generation for both lines the presence of T-DNA insertions was detectable by PCR (data not shown) but the amplified *PYL5* wild type alleles in #11-24 were remained uncut, suggesting that the integrated CRISPR/Cas9 genes might be silenced after several generations.

Phenotypic analysis of CRISPR/Cas9-generated *PYL* sextuple mutants

The homozygous *pyl* sextuple mutants from line #11-4 (112458-C) were subjected to phenotypic analysis of root

elongation and plant development in response to ABA. The previously reported *pyr1pyl1pyl4* triple mutant (114-T) and *pyr1pyl1pyl2pyl4pyl5pyl8* (112458-T) sextuple mutant were used for comparison (Gonzalez-Guzman et al. 2012). Seedlings were grown on vertically oriented MS plates for 5 days before being transplanted to new MS plates with or without ABA supplementation at the indicated concentrations. All three *pyl* mutants were highly resistant to ABA-mediated inhibition of root growth at several ABA concentrations (5–50 μ M), but there were slight differences among them (Fig. 5a, b). For example, the 112458-T mutant had shorter roots than the 112458-C or 114-T mutant in the absence of ABA, but in the presence of high concentrations of ABA (20–50 μ M) their roots were the longest among the three mutants, suggesting that this mutant is more resistant to ABA than the other two (Fig. 5c). In addition, we noticed that 5–10 μ M ABA stimulated plant growth for *pyl* mutants, especially for the two sextuple mutants (Fig. 5c, d). We also found that the fresh weight of the 112458-C mutant was lower than that of the 112458-T mutant under all of the conditions, but their responses to ABA treatment were similar (Fig. 5d). Our results demonstrate the efficiency of the upgraded CRISPR/Cas9 system to generate multiple inheritable mutations within three generations in Arabidopsis.

In vivo evaluation of the gene targeting efficiency for the sgRNA modules

We observed that the mutagenesis frequencies for the *PYL4*, *PYL5* and *PYL8* loci were quite low; two of the *PYLs* were targeted by sgRNAs driven by the *At7SL* promoter (Fig. 3b). Previous studies in rice suggested that a low GC content in the sgRNA sequence might affect gene targeting efficiency (Zhang et al. 2014). However in this case, the GC content of the guide sequences was comparable (Fig. 2a), thus we hypothesize that the transcriptional activity of the *At7SL* promoter might be lower than the *AtU6* and *AtU3b* promoters. To compare the gene targeting efficiencies of the three sgRNA modules, they were

Table 1 Design of the sgRNA modules and their guide oligos

sgRNA module	Promoter sequences	Guide oligos
AtU6-sgRNA	5'- <u>TTTATATA</u> CAGCTAGAGTCGAAGTAGTGATT G-3'	Sense: 5'-GATTG(N) ₂₀ -3' Antisense: 5'-ACCC(N) ₂₀ C-3'
AtU3b-sgRNA	5'- <u>TATATATA</u> AACTGAGGGAGCACCATTGGTC ACG-3'	Sense: 5'-TCACG(N) ₂₀ -3' Antisense: 5'-ACCC(N) ₂₀ C-3'
At7SL-sgRNA	5'- <u>TTTATATA</u> TTACACGAACCGATCTTTGTTAC G-3'	Sense: 5'-TTACG(N) ₂₀ -3' Antisense: 5'-ACCC(N) ₂₀ C-3'

TATA box regions are indicated by underlines. The 5-nt adaptor sequences are indicated in bold. The transcriptional start sites are indicated as bold italic

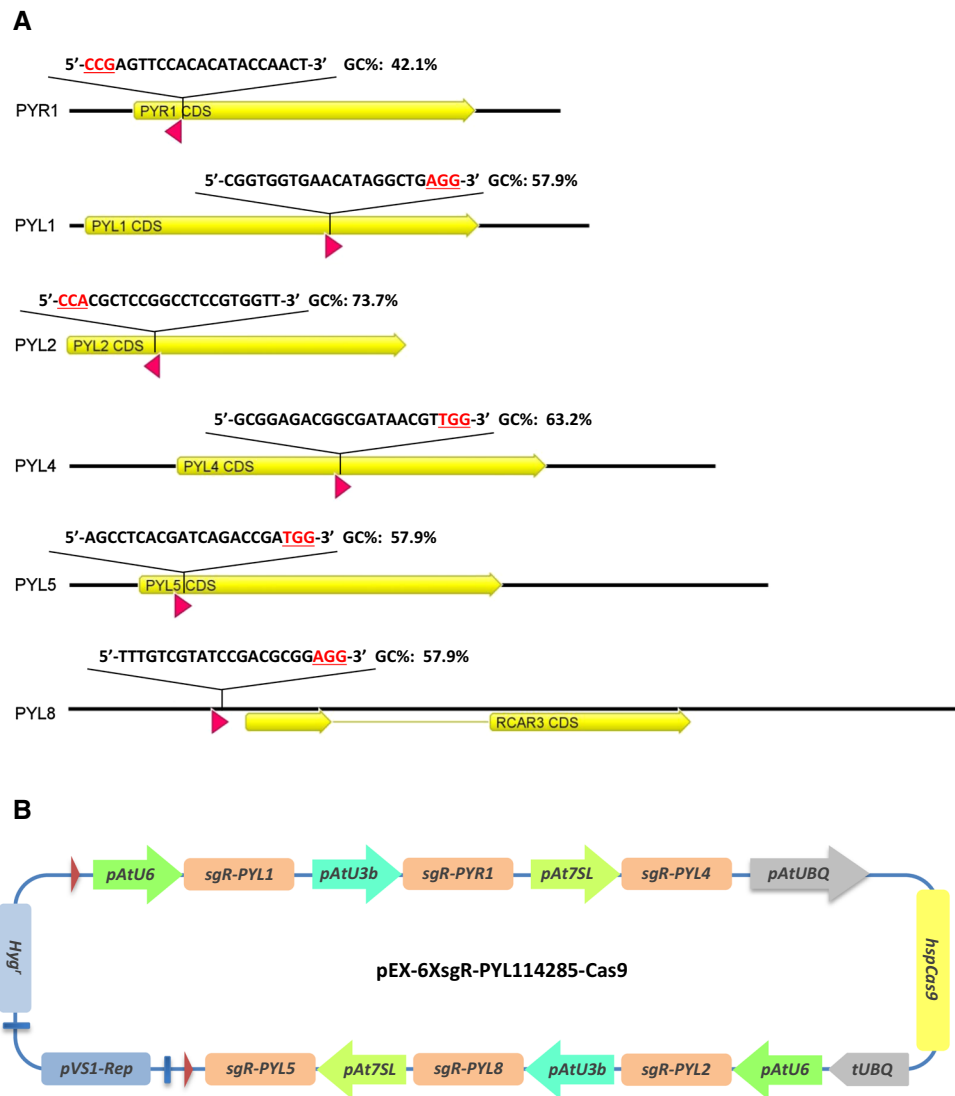


Fig. 2 Targeted gene modification of *PYL* genes using the sextuple CRISPR/Cas9 system. **a** Schematics of the *PYL* gene loci. CDS regions are shown in yellow and the mRNA transcripts are depicted in black lines. Location and the sequences of the sgRNA targets are indicated with PAM and GC % content in red. **b** Schematic of the sextuple CRISPR/Cas9 vector used to modify *PYL* genes. Red

arrowheads indicate the M13F and M13R sites in plasmids. Blue rectangles indicate the left and right border sites of T-DNA. *Hyg^R* hygromycin resistant gene, *pVSI-Rep* bacterial origin of replication, *pAtUBQ* promoter of *AtUBQ1* gene, *tUBQ* terminator of *AtUBQ1* gene, *hspCas9* human-codon optimized *Cas9* gene

customized to target the multiple recognition sites (MRS) in the YFP reporter gene (Mao et al. 2013). In protoplasts, the CRISPR/Cas9-induced DSBs in the YFP reporter gene can be restored via the SSA DNA repair pathway so that the gene targeting efficiency of the three sgRNA modules can be evaluated by counting the number of YFP positive cells (Mao et al. 2013; Zhang et al. 2012) (Fig. 6a). Two different guide sequences were used for each sgRNA module. Our results show comparable ratios of YFP positive cells in transfected protoplasts for the three sgRNA modules and not much difference was observed between the two guide sequences (Fig. 6b). However, the average fluorescence intensities of YFP positive protoplasts

in protoplasts transfected with the two AtU3b-driven sgRNAs were lower than those driven by the AtU6 or At7SL promoter (Fig. 6c). These results suggest that the AtU3b promoter was less efficient than the AtU6 and At7SL promoter for triggering DSB.

Moreover, two of the three poorly performing sgRNAs were placed in the last position of the sgRNA cassettes. We thus hypothesize that the relative position of the sgRNA module might affect its mutagenesis activity. To test this possibility, we reversed the order of the AtU6 and the At7SL modules (Fig. 7a). The *PYR1*, *PYL1* and *PYL4* gene loci were targeted in protoplast assays but no differences in mutagenesis efficiency was observed (Fig. 7b).

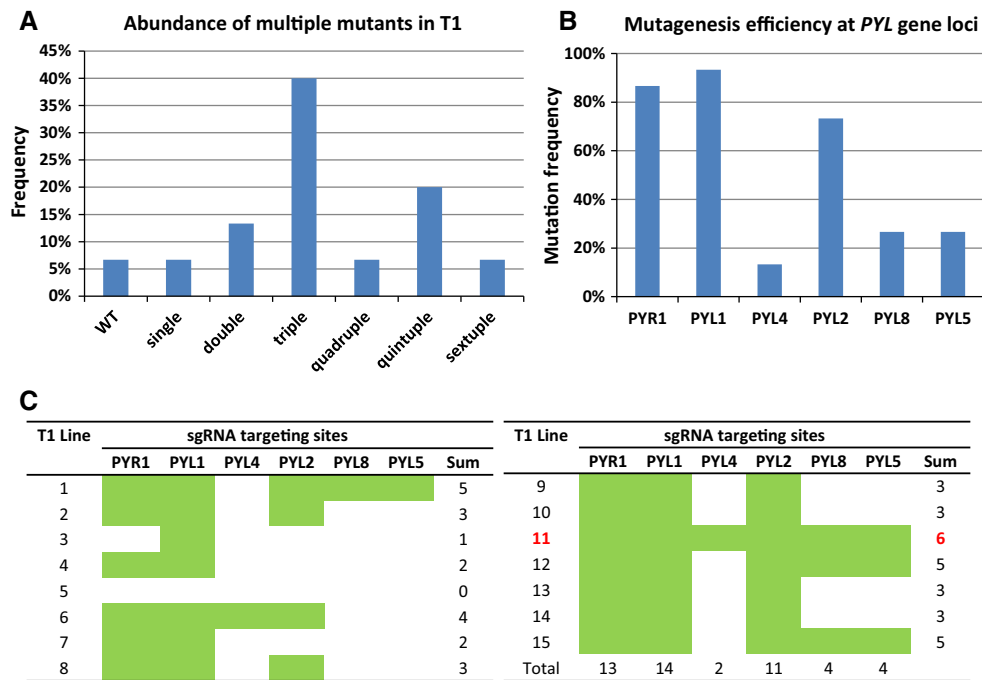


Fig. 3 Characterization of the CRISPR/Cas9-induced *PYL* gene mutations in the T1 generation. **a** Frequency distribution of the chimeric *pyl* multiple mutants. **b** Mutagenesis frequency at the six individual *PYL* gene targets. **c** Summary of the different *PYL* mutation

combinations in T1 plants. PCR products of the six *PYL* target loci in each T1 plant were sequenced for mutation detection. Samples with detected gene mutations are shown in green. The T1 line containing sextuple gene mutations is highlighted in red

Discussion

Designing an easy-to-assemble multiplex CRISPR/Cas9 system for gene editing in Arabidopsis

In this study we developed a CRISPR/Cas9 platform that allows sextuple gene editing in Arabidopsis with a single binary vector. To increase the capacity of the CRISPR/Cas9 system originally described (Mao et al. 2013), we designed a strategy to insert three sgRNA modules into a single sgRNA docking site so that the assembly of a sextuple gene editing system can be achieved in three steps using conventional molecular cloning.

It has been reported that transgenes containing three or four direct repeats can induce post-transcriptional gene silencing (PTGS) in tobacco at high frequency (80–100 %), regardless of the relative strength of the promoter used. To reduce the repetitiveness of the inserted sgRNA modules, we used three different Arabidopsis Pol III-dependent promoters to drive the expression of the individual sgRNAs. In addition, the two cloning sites for the sgRNA modules were separated by the Cas9 expression cassette. As we have shown, this multiplex CRISPR/Cas9 system was able to generate homozygous sextuple mutations in a single transformation experiment, although its efficiency might be further increased by using stronger RNA polymerase III promoters.

Factors influencing the gene targeting efficiency of the tandem sgRNA modules

Analysis of the mutagenesis efficiency for the six *PYL* loci revealed that *PYL4*, *PYL5* and *PYL8* showed relatively low efficiencies compared with *PYR1*, *PYL1* and *PYL2*. In vivo experiments in Arabidopsis protoplasts showed that, although the AtU3b promoter had slightly reduced activity, all three promoters had comparable strength (Fig. 6b, c). Unlike the AtU6 and At7SL promoters, which use a guanine “G” as the transcriptional start site, the transcriptional start site of the AtU3b promoter was an adenosine “A”. To validate the effect of this transcriptional start site in sgRNA expression, additional experiments with very strict controls will be required.

Since no obvious transcriptional differences were observed among the three RNA polymerase III promoters, we hypothesized that the sgRNA mutagenesis activity could be influenced by its relative position in the expression cassette. However, this hypothesis was also disproved since swapping the positions of the AtU6 and At7SL promoters in the sgRNA cassette did not influence their gene targeting efficiencies (Fig. 7a, b). It is therefore obvious that, in addition to the GC content and transcriptional level, the targeting efficiency of sgRNAs may be affected by other factors such as chromosome structure and the transcriptional level of the targeted genes.

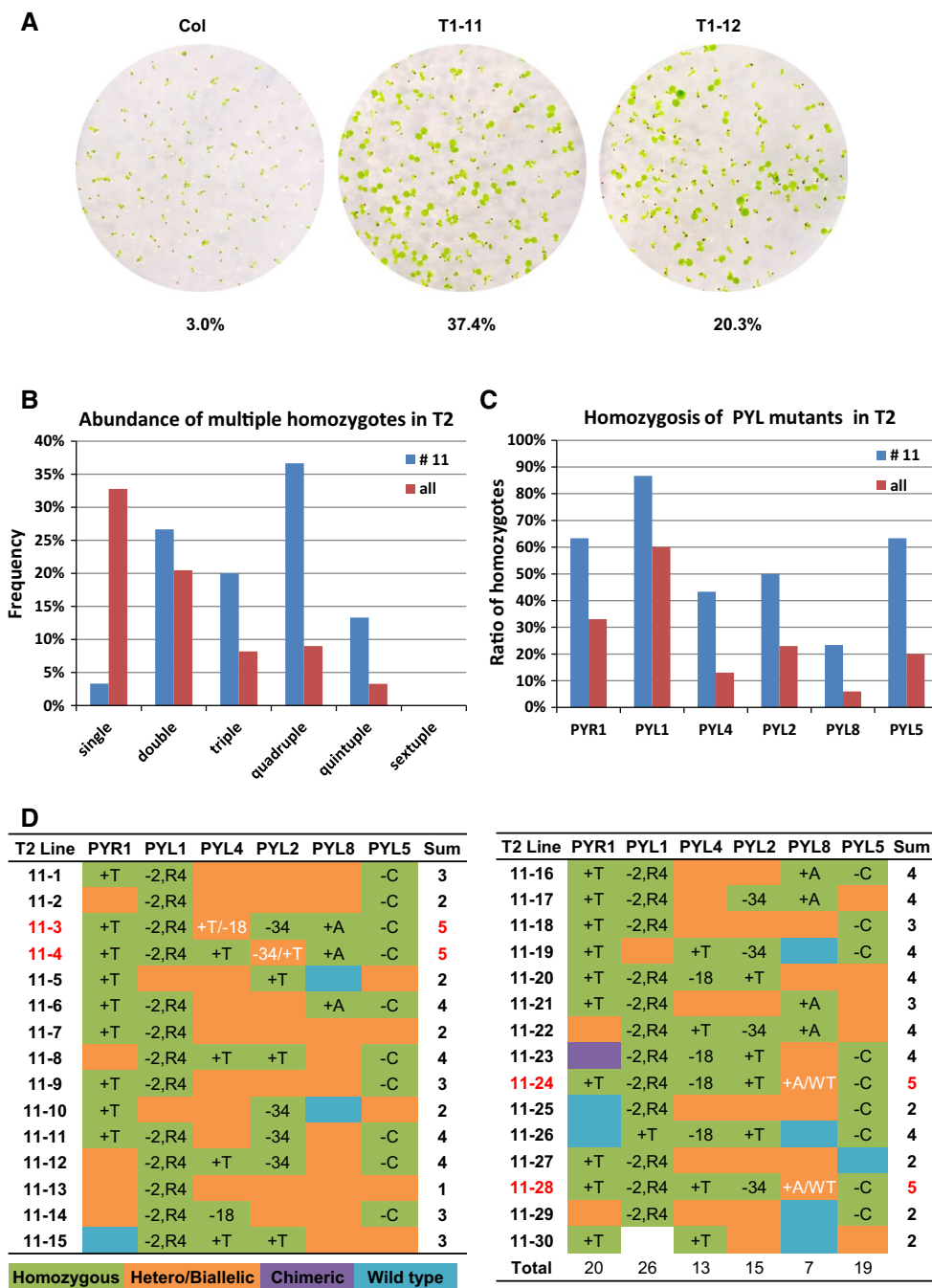


Fig. 4 Characterization of the CRISPR/Cas9 induced *PYL* gene mutations in the T2 generation. **a** Screening for ABA-insensitive T2 mutants with 1 μ M ABA. Survival ratios of the screened T2 populations are shown at the bottom. **b** Frequency distribution of homozygous *pyl* multiple mutants in the T2 population. Averages for all lines are shown in red while individual values for line #11 are shown in blue. **c** Segregation ratios of homozygous mutants for the six *PYL* genes in the T2 population. Averages for all lines are shown in red while individual values for line #11 are shown in blue. **d** Summary

of the zygosity and genotype of *PYL* mutations in the T2 population of line #11. PCR products of the six *PYL* target loci in each T2 plant were sequenced for mutation detection. The four zygosity types are shown in different colors and the four homozygous quintuple mutants are highlighted in red. Mutation types of the homozygous target sites are shown. Plus symbol denotes insertion, minus symbol denotes deletion, R symbol represents replacement, A/T/C/G nucleotide type, Number nucleotide number

Table 2 Segregation analysis of *PYL* loci in T3 generation

	<i>PYL1</i>	<i>PYL1</i>	<i>PYL4</i>	<i>PYL2</i>	<i>PYL8</i>	<i>PYL5</i>
#11-4	12 (+T)	12 (–2, R4)	12 (+T)	2 (+T):7 (Bi):3 (–34)	12 (+A)	12 (–C)
#11-24	12 (+T)	12 (–2, R4)	12 (–18)	12 (+T)	1 (+A):8 (Hetero):3 (WT)	12 (–C)

Segregation ratio of genotypes are shown as “n1 (a):n2 (b):n3 (c)”. *n*1,2,3 number of T3 individuals, **a**, **b**, **c** genotypes

WT wild type, *Bi* biallelic, *Hetero* heterozygous, homozygous mutations are shown as: *plus symbol* denotes insertion, *minus symbol* denotes deletion, *R symbol* represents replacement, *A/T/C/G* nucleotide type, *Number* nucleotide number

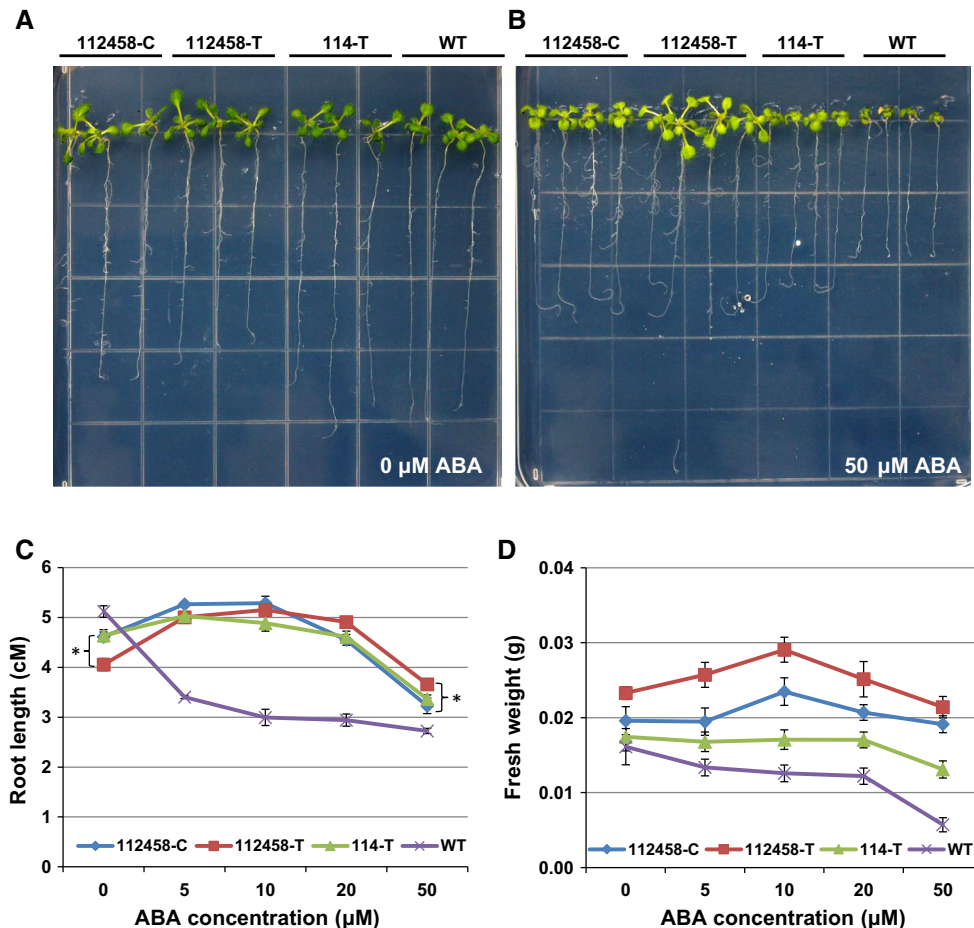


Fig. 5 Phenotype analysis of *pyl* mutants. **(a, b)** 14-day-old seedlings grown on MS0 medium without **(a)** or with the addition of 50 μM ABA 5 days after germination **(b)**. **(c)** Quantification of root length in 10-day-old seedlings grown on MS0 medium supplemented with different concentrations of ABA. Data are averages ± SE (*n* = 20).

Generation of sextuple mutants with a single transformation experiment

To evaluate the gene targeting efficiency of the multiplex CRISPR/Cas9 system, six of the 14 *PYR/PYL* gene family members were selected as targets for mutagenesis. Previous studies have shown that these six *PYR/PYL* genes have non-redundant functions in ABA perception and their sextuple mutants exhibited a dramatic ABA-insensitive phenotype

(Gonzalez-Guzman et al. 2012). Even though we obtained only 15 transgenic plants in the T1 generation, one of them was identified as containing sextuple mutations. Compared to the other lines, this sextuple mutation line produced more ABA-insensitive seedlings in the T2 generation, with all of the 11 homozygous quadruple mutants and the four quintuple mutants identified in the T2 population belonging to this line. Our results show that the selection of T1 mutated lines can be critical to increase the rate of homozygous mutants in the T2 population.

A CRISPR/Cas9-mediated YFP SSA Assay

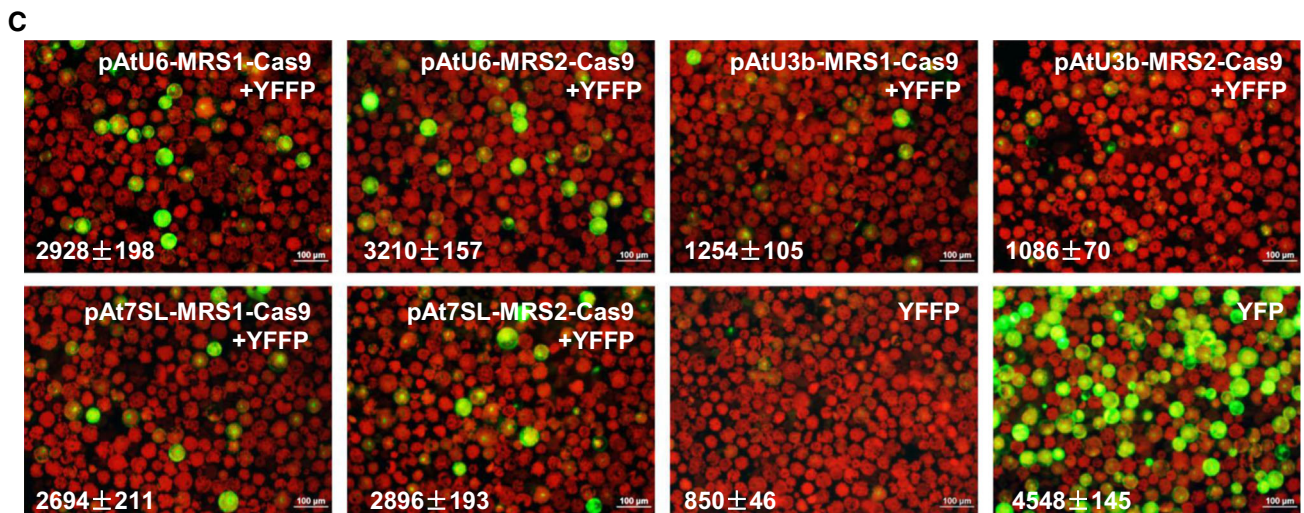
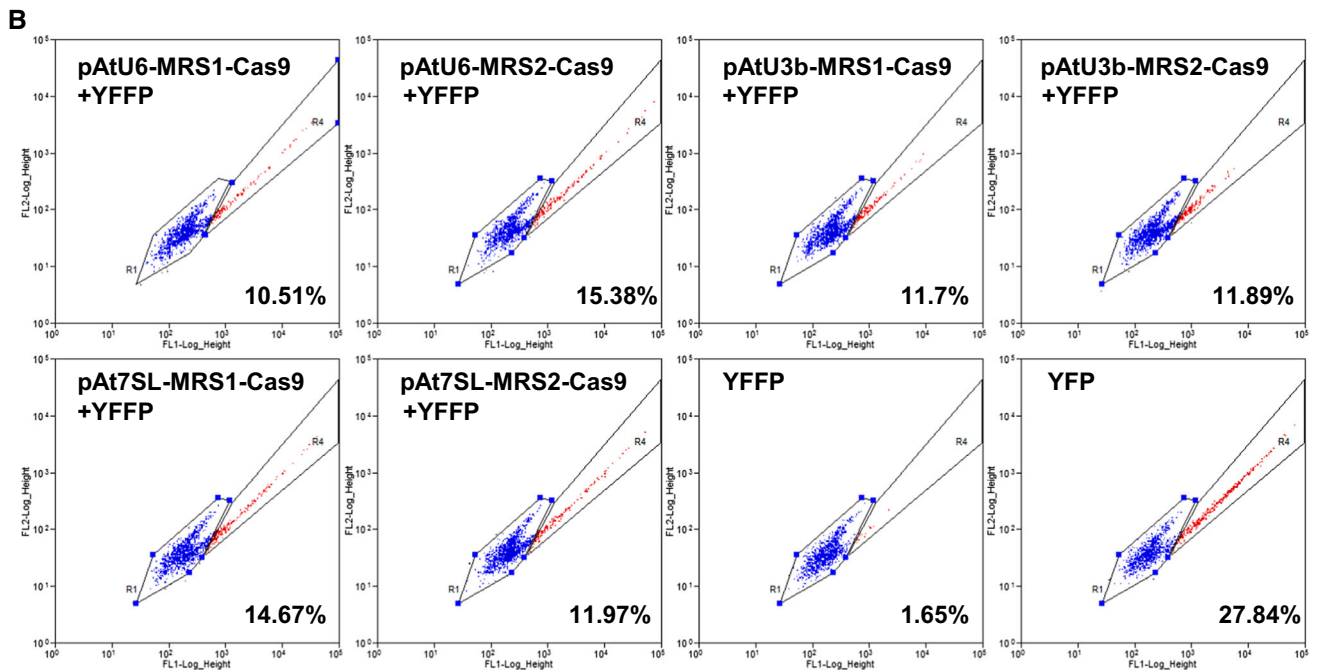
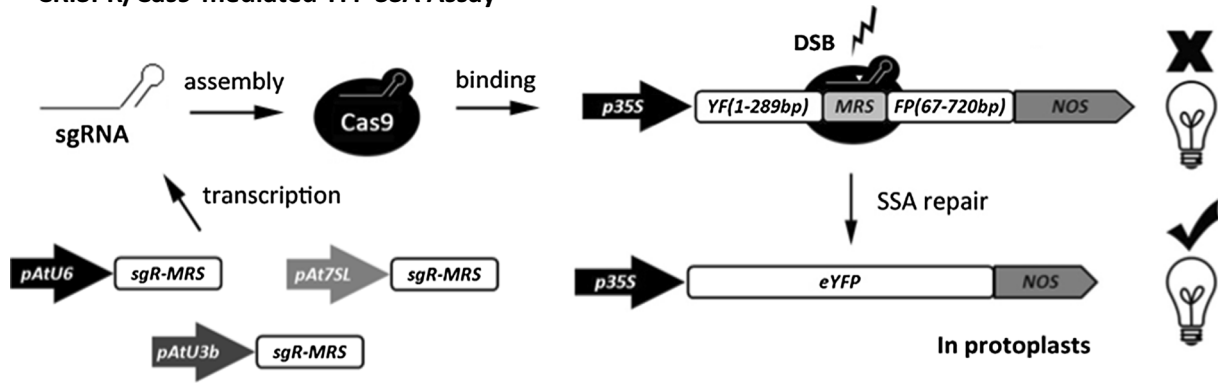


Fig. 6 Gene editing efficiency of the sgRNA modules using the YFP single-stranded anneal assay. **a** A schematic showing the mechanism of CRISPR/Cas9-mediated SSA repair of the YFP gene in protoplasts. The sgRNA transcripts derived from the three sgRNA modules are assembled into the Cas9 protein to target the MRS region in YFP gene. The CRISPR/Cas9 induced DSBs can be repaired via single-stranded anneal to restore a functional YFP gene for detection. **b** Fluorescence activated cell sorting (FACS) quantification of YFP positive cells in transfected protoplasts. The plasmids used for transfection are shown at the *top*. The ratios of YFP positive cells in each transfected population are shown at the *bottom*. **c** Fluorescence microscope images of the transfected protoplasts. The plasmids used for transfection are shown at the *top*. The fluorescence intensity of each transfected population were evaluated by FACS and labeled as average \pm SD ($n > 2000$)

Phenotype analysis of the CRISPR/Cas9-generated T3 homozygous *pyl* sextuple mutant (112458-C) performed along with two previously described *pyl* mutants (114-T and 112458-T) (Gonzalez-Guzman et al. 2012) revealed very similar, although not identical, ABA-insensitive phenotypes. Slight differences were nevertheless observed between the newly generated 112458-C mutant and the

sextuple 112458-T mutant such as the reduced fresh weight displayed by 112458-C in the absence or presence of ABA at different concentrations. These differences were most probably caused by the fact that the mutation in *PYL8* was in the 5' UTR in the CRISPR/Cas9-generated mutant. We targeted the 5' UTR of *PYL8* in an attempt to study the regulation of *PYL8* by the untranslated region.

The multiplex CRISPR/Cas9 system can facilitate functional studies targeting multiple genes and gene families

Conventional methods used to generate multiple mutants are laborious and time consuming. While crossing individual mutants to obtain double mutants is relatively easy, the difficulty increases exponentially when trying to produce higher order mutants given the normal Mendelian segregation ratios. In addition, duplication events have sometimes resulted in the presence of tandemly arranged gene repeats in close proximity within the chromosome. In these cases the affected genes have a strong genetic linkage making the

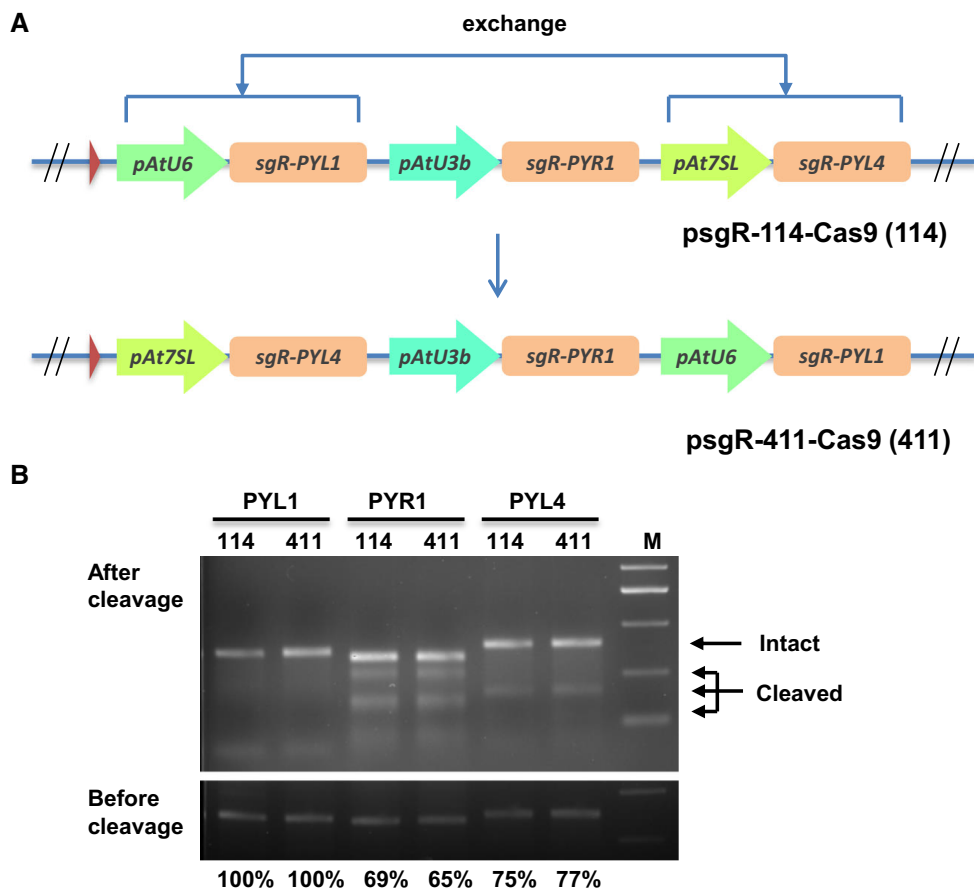


Fig. 7 Influence of the sgRNA module position on mutagenesis efficiency. **a** Schematics of two different sgRNA cassettes containing the sgRNA modules in reverse order. Both cassettes were designed to target three *PYL* genes in protoplasts. **b** Surveyor assay evaluating the

mutagenesis efficiency of the three *PYL* genes targeted by the two CRISPR/Cas9 cassettes described in **a**. The cleavage efficiencies of the PCR products were calculated using the Bio-rad image lab software and shown at the *bottom*

production of multiple mutants by crossing practically impossible. Although non-homologous multiple mutations can be generated frequently by CRISPR/Cas9, the advantage of this gene editing tool is that researchers can screen for transgenic lines showing the highest mutation frequency at most of the targeted positions. Thus, the chance of obtaining homozygous multiple mutants will be largely increased. Our easy-to-use CRISPR/Cas9 system for multiplex gene editing in Arabidopsis can prove invaluable in these situations. We expect that by co-transforming two sextuple CRISPR/Cas9 vectors it will be possible to generate duodecuple mutants.

Methods

Vector construction

To construct the pEx-ptAtUBQ-Cas9 vector, the ptAtUBQ-Cas9 module from psgR-Cas9-At was cloned into the *XmaI-KpnI* site of pCAMBIA1300 binary vector. This ptAtUBQ-Cas9 module contains a 680 bp AtUBQ1 promoter, a human code optimized Cas9 gene and a 208 bp AtUBQ1 terminator as described before (Mao et al. 2013). The three sgRNA modules were synthesized using overlapping PCR. The AtU6-26, AtU3b, At7SL-2 promoter were amplified from the Arabidopsis genome by PCR using primer pairs: AtU6-F-HindIII/AtU6-sgR-R, AtU3b-XhoI-F/AtU3b-sgR-R and At7SL-XbaI-F/At7SL-sgR-R, respectively. And their corresponding sgRNA scaffolds with overlapping sequences was also amplified by PCR using primer pairs: sgR-U6-F/sgR-XhoI-R, sgR-U3b-F/sgR-XbaI-R and sgR-7SL-F/sgR-R-XmaI, respectively. The sequences of these primers are listed in Table S4. For each sgRNA module, overlap PCR was performed using primer pairs at the remote end. The obtained products were then cloned into the *HindIII-XmaI* site of pMD18T vector (Takara, Japan) to generate pAtU6-sgR, pAtU3b-sgR and pAt7SL-sgR vector, respectively.

To construct a pEX-6XsgR-Cas9 vector for targeting PYR/PYL genes, two complementary 24-bp oligonucleotides with a 20-bp target sequence were synthesized for each PYR/PYL targets. These oligo pairs were annealed to generate a double-strand DNA with 4-bp overhangs on both ends and cloned into *BbsI* sites of the three sgRNA modules, respectively following a standard procedure (Liu et al. 2015). The sequences of the synthesized DNA oligos are shown in Table S4. In total, six sgRNA modules were generated to target the six PYR/PYL genes. They were named pAtU6-sgR-PYL1, pAtU6-sgR-PYL2, pAtU3b-sgR-PYL1, pAtU3b-sgR-PYL8, pAt7SL-sgR-PYL4 and pAt7SL-sgR-PYL8.

To assemble these sgRNA modules into the pEx-ptAtUBQ-Cas9 vector, the three sgRNA modules were first

amplified from their host vectors by PCR using primer pair M13F/M13R. The reaction mix was set up as follows:

0.4 μ L	Forward primer (M13F) (10 μ M)
0.4 μ L	Reverse primer (M13R) (10 μ M)
2 μ L	10X <i>Ex Taq</i> buffer (Mg ²⁺ plus)
1.6 μ L	dNTP mix (2.5 mM)
0.1 μ L	<i>Ex Taq</i> polymerase (5 units/ μ L) (Takara, Japan)
Up to 20 μ L	ddH ₂ O

Perform PCR using the following cycling conditions: 94 °C 2 min, (94 °C 10 s, 58 °C 15 s, 68 °C 20 s) X 35 cycles, 68 °C 5 min, keep at 12 °C.

The amplified fragments were then digested by *HindIII* and *XhoI* (for pAtU6-sgRNA), *XhoI* and *XbaI* (for pAtU3b-sgRNA) and *XbaI* and *XmaI* (for pAt7SL-sgRNA) in separate reactions at 37 °C for 2 h:

X μ L	Amplified fragments (100 ng)
5 μ L	10X NEB CutSmart Buffer
1 μ L	Restriction enzyme 1 (20 units/ μ L) (New England Biolab, USA)
1 μ L	Restriction enzyme 2 (20 units/ μ L) (New England Biolab, USA)
Up to 50 μ L	ddH ₂ O

And the binary vector pEx-ptAtUBQ-Cas9 was digested by *HindIII* and *XmaI* accordingly:

X μ L	pEx-ptAtUBQ-Cas9 (1 μ g)
5 μ L	10X NEB CutSmart Buffer
1 μ L	<i>HindIII</i> (20 units/ μ L) (New England Biolab, USA)
1 μ L	<i>XmaI</i> (20 units/ μ L) (New England Biolab, USA)
Up to 50 μ L	ddH ₂ O

The three digested sgRNA fragments were then mixed at equal volume ratio and purified together by column before assembly, while the Phenol–Chloroform extraction method was recommended for purifying the digested binary vector.

Two triplicated CRISPR/Cas9 vectors named pEx-3XsgR-PYL114-Cas9 and pEx-3XsgR-PYL285-Cas9 was assembled, respectively as follows and incubated at 16 °C for 2 h:

X μ L	Purified sgRNA mix (>50 ng)
Y μ L	pEx-ptAtUBQ-Cas9 (<i>Hind</i> III and <i>Xma</i> I digested) (>10 ng)
1 μ L	10XT4 ligase buffer
0.5 μ L	T4 DNA ligase (400 units/ μ L) (New England Biolab, USA)
Up to 10 μ L	ddH ₂ O

Transform the ligation reaction into DH5 alpha competent cells and plate cells on LB plates containing 50 μ g/mL kanamycin for selection. Positive clones were identified by colony PCR as follows:

0.4 μ L	Forward primer (AtU6-F-Kpn) (10 μ M)
0.4 μ L	Reverse primer (sgR-R-EcoRI) (10 μ M)
2 μ L	10X <i>Ex Taq</i> buffer (Mg ²⁺ plus)
1.6 μ L	dNTP mix (2.5 mM)
0.1 μ L	<i>Ex Taq</i> polymerase (5 units/ μ L) (Takara, Japan)
Up to 20 μ L	ddH ₂ O

Perform PCR using the following cycling conditions:

94 °C 5 min, (94 °C 15 s, 58 °C 20 s, 68 °C 50 s) X 35 cycles, 68 °C 5 min, keep at 12 °C. The positive PCR product is about 1.5 Kb in length.

To generate the final sextuple CRISPR/Cas9 construct, the three sgRNA modules in the pEx-3XsgR-PYL285-Cas9 vector was amplified by PCR using primer pair AtU6-F-KpnI/sgR-R-EcoRI. The reaction mix was set up as follows:

0.4 μ L	Forward primer (AtU6-F-Kpn) (10 μ M)
0.4 μ L	Reverse primer (sgR-R-EcoRI) (10 μ M)
2 μ L	10X <i>Ex Taq</i> buffer (Mg ²⁺ plus)
1.6 μ L	dNTP mix (2.5 mM)
0.1 μ L	<i>Ex Taq</i> polymerase (5 units/ μ L) (Takara, Japan)
Up to 20 μ L	ddH ₂ O

Perform PCR using the following cycling conditions:

94 °C 2 min, (94 °C 15 s, 58 °C 20 s, 68 °C 50 s) X 35 cycles, 68 °C 5 min, keep at 12 °C.

The amplified sgR-PYL285 fragments were then digested by *Kpn*I and *Eco*RI at 37 °C for 2 h:

X μ L	Amplified sgR-PYL285 fragments (100 ng)
5 μ L	10X NEB CutSmart Buffer
1 μ L	<i>Kpn</i> I (20 units/ μ L) (New England Biolab, USA)
1 μ L	<i>Eco</i> RI (20 units/ μ L) (New England Biolab, USA)
Up to 50 μ L	ddH ₂ O

And the pEx-3XsgR-PYL114-Cas9 binary vector was digested by *Kpn*I and *Eco*RI accordingly.

X μ L	pEx-3XsgR-PYL114-Cas9 (1 μ g)
5 μ L	10X NEB CutSmart Buffer
1 μ L	<i>Kpn</i> I (20 units/ μ L) (New England Biolab, USA)
1 μ L	<i>Eco</i> RI (20 units/ μ L) (New England Biolab, USA)
Up to 50 μ L	ddH ₂ O

To assemble the final pEx-6XsgR-PYL114285-Cas9 binary vector, the digested sgR-PYL285 fragments were purified by column and the pEx-3XsgR-PYL114-Cas9 binary vector was purified using the Phenol–Chloroform extraction method. The ligation reaction was set up as follows and incubated at 16 °C for 2 h:

X μ L	Purified sgR-PYL285 fragments (>50 ng)
Y μ L	pEx-3XsgR-PYL114-Cas9 (<i>Kpn</i> I and <i>Eco</i> RI digested) (>10 ng)
1 μ L	10XT4 ligase buffer
0.5 μ L	T4 DNA ligase (400 units/ μ L) (New England Biolab, USA)
Up to 10 μ L	ddH ₂ O

Transform the ligation reaction into DH5 alpha competent cells and plate cells on LB plates containing 50 μ g/mL kanamycin for selection. Positive clones were identified by colony PCR as follows:

0.4 μ L	Forward primer (tUBQ-F) (10 μ M)
0.4 μ L	Reverse primer (M13R) (10 μ M)
2 μ L	10X <i>Ex Taq</i> buffer (Mg ²⁺ plus)
1.6 μ L	dNTP mix (2.5 mM)
0.1 μ L	<i>Ex Taq</i> polymerase (5 units/ μ L) (Takara, Japan)
Up to 20 μ L	ddH ₂ O

Perform PCR using the following cycling conditions:

94 °C 5 min, (94 °C 15 s, 58 °C 20 s, 68 °C 50 s) X 35 cycles, 68 °C 5 min, keep at 12 °C. The positive PCR product is about 1.7 Kb in length.

To generate plasmids used for YFP SSA assay in protoplasts, eight sgRNA guide oligos were synthesized to form six DNA-duplexes for cloning into the three sgRNA-modules, respectively. The sequences of these DNA oligos were listed in Table S4. The obtained six sgRNA modules were then cloned into the *HindIII-XmaI* sites of psgR-Cas9-At to yield six transiently expressed vectors. They were named pAtU6-MRS1-Cas9, pAtU6-MRS2-Cas9, pAtU3b-MRS1-Cas9, pAtU3b-MRS1-Cas9, pAt7SL-MRS1-Cas9, pAt7SL-MRS2-Cas9. The YFP vector used for this study has been described before (Mao et al. 2013).

To generate the expressional vector used for targeting *PYR1*, *PYL1* and *PYL4* genes in protoplasts, the three sgRNA modules in pEx-3XsgR-PYL114-Cas9 was cloned into the *HindIII-XmaI* site of psgR-Cas9-At to yield the psgR-114-Cas9 vector. To exchange the position of pAtU6-sgR-PYR1 and pAt7SL-sgR-PYL4 in this vector, these two sgRNA modules were first amplified by PCR using primer pair At7SL-HindIII-F/sgR-R-XhoI and AtU6-XbaI-F/sgR-R-XmaI and then cloned into the same restriction sites of psgR-Cas9-At together with the *XhoI* and *XmaI* digested AtU3b-sgR-PYL1 module to produce the psgR-411-Cas9 vector.

Plant transformation and growth conditions

Agrobacterium-mediated transformation of *Arabidopsis thaliana* Columbia-0 with the binary vectors was performed using the floral dipping method as previously described (Weigel and Glazebrook 2006). Seed collected from the Agrobacterium infected plants was sterilized with 2 % sodium hypochlorite for 15 min and plated on Murashige and Skoog (MS0) medium containing 30 mg/L hygromycin plus 50 mg/L Carbenicillin to inhibit Agrobacterium growth. The resulting T1 plants were transplanted to soil after growing under long-day conditions (16 h light/8 h dark) at 22 °C for 2 weeks.

Detection of targeted gene mutations

DNA was extracted from transgenic plants by the CTAB method. Genomic regions surrounding the *PYR/PYL* target sites were amplified by PCR using primer pairs listed in Table S4. Targeted gene mutations were detected by aligning the sequencing chromatograms of these PCR products with the wild type controls. Genotyping for the heterozygous or biallelic *PYL* mutations in quintuple mutant were performed by cloning the PCR products of

those target loci into the pMD18T vector (Takara, Japan) for DNA sequencing. Segregation ratios of these *PYL* mutations were calculated by sequencing at least 24 clones.

Phenotype analysis for the *pyl* mutants

The *pyr1pyl1pyl4* triple mutant and the *pyr1pyl1pyl2pyl4pyl5pyl8* sextuple mutant have been described previously (Gonzalez-Guzman et al. 2012). Seedlings were grown on vertically oriented MS0 plates with 3 % sucrose for 5 days. Subsequently, 20 plants were transferred to new MS0 plates lacking or supplemented with ABA at a concentration of 5, 10, 20, 50 μM. Root lengths were measured by vernier caliper and the fresh weights were measured using four seedlings as a group by precision electronic autobalance 8 days after the treatment. Pictures were taken by high resolution camera 10 days after the treatment.

Transient expression of the CRISPR/Cas9 system in Arabidopsis protoplasts

Isolation of Arabidopsis mesophyll protoplasts were performed by the ‘Tape-Arabidopsis Sandwich’ method (Wu et al. 2009). Plasmids were purified using the QIAGEN Plasmid Mini Kit (Qiagen, USA) according to manufacturers’ instructions. Transfection of the Arabidopsis protoplast was performed using the PEG-calcium method (Yoo et al. 2007). For each reaction, 10 μg total plasmids were used. The transfected Arabidopsis mesophyll protoplasts were incubated in the dark at room temperature for 12–24 h.

To analyze the gene targeting activities of different sgRNA modules, YFP positive cells were measured by flow cytometry (BECKMAN COULTER MoFlo™ XDP, USA). Photographs were taken by a fluorescence microscope (IX71, Olympus, Japan) under the RFP and YFP channels. To evaluate the mutagenesis efficiency of endogenous *pyl* genes, protoplasts were transferred to a 1.5 mL Eppendorf tube and collected by centrifugation. Genomic DNA was extracted for mutation detection using the CTAB method (Springer 2010).

Author contribution statement Y. M., Z. Z., and J. -K. Z. designed research; Z. Z., Y. M., S. H, W. L, performed research; Z. Z., Y. M., J.B., and J. -K. Z. analyzed data; Y. M., J. B., and J. -K. Z. wrote the paper.

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