Multigene editing via CRISPR/Cas9 guided by a single-sgRNA seed in Arabidopsis

Summary We report that a solo single-guide RNA (sgRNA) seed is capable of guiding Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR—associated 9 (CRISRP/Cas9) to simultaneously edit multiple genes AtRPL10A, AtRPL10B and AtRPL10C in Arabidopsis. Our results also demonstrate that it is possible to use CRISPR/Cas9 technology to create AtRPL10 triple mutants which otherwise cannot be generated by conventional genetic crossing. Compared to other conventional multiplex CRISPR/Cas systems, a single sgRNA seed has the advantage of reducing off-target gene-editing. Such a gene editing system might be also applicable to modify other homologous genes, or even less-homologous sequences for multiple gene-editing in plants and other organisms.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR—associated 9 (CRISRP/Cas9) is an adaptive immune mechanism that protects bacteria and archaea from extrachromosomal DNA and viral invasions (Jinek et al. 2012). CRISPR/Cas9 generates double-stranded breaks (DSBs) under specific guidance of a single-guide RNA (sgRNA). These DSBs can then be repaired either by homologous recombination, or predominantly by non-homologous end-joining, which leads to introduction of mutations such as nucleotide substitution, insertion or deletion into the targeted DNA molecules (Jinek et al. 2012; Cong et al. 2013).

This ancient defense has been exploited for efficient genome/gene editing in organisms across kingdoms (Jinek et al. 2012; Cong et al. 2013; Fu et al. 2013; Mao et al. 2013; Gao and Zhao 2014; Ma et al. 2015; Yan et al. 2015a; Kim et al. 2016; Shen et al. 2016). Moreover, multiplex CRISPR/Cas9-based gene editing can also be simultaneously achieved through the use of different sgRNAs in animals and plants (Cong et al. 2013; Feng et al. 2014; Wang et al. 2015; Yan et al. 2015b). However, it remains to be elucidated whether multigene-editing, via CRISPR/Cas9, can be directed by a single sgRNA seed.

To address this possibility, we searched the Arabidopsis genome database and identified the AtRPL10 family that includes three homologous members, AtRPL10A (AT1G14320), AtRPL10B (AT1G26910) and AtRPL10C (AT1G66580) coding for the Ribosomal Protein Large 10 subunits. The three AtRPL10 genes reside at different loci on Arabidopsis chromosome 1 (Figure S1), sharing 81%–88% nucleotide (nt) identities, and their protein products are 95%–98% identical (Table S1). AtRPL10A and AtRPL10B are expressed in female and male reproductive organs, while AtRPL10C is restricted to pollen grains. The three multifunctional genes are involved in protein translation and plant response to viral infection and abiotic stress (Falcone Ferreyra et al. 2013; Zorzatto et al. 2015). Homozygous AtRPL10A T-DNA insertion mutation is lethal and RNA interference (RNAi) of AtRPL10B affects plant growth, although AtRPL10C knockout results in no phenotypic change (Falcone Ferreyra et al. 2010). Interestingly, genetic crosses can generate AtRPL10A, AtRPL10B or AtRPL10C heterologous double, but not triple, mutants in Arabidopsis (Falcone Ferreyra et al. 2013).

We generated an “AtRPL10 sgRNA+CRISPR/Cas9” construct in pCAMBIA1300 (Figure 1A). The AtRPL10 sgRNA consists of an identical 19 nucleotides (ATGTTGG-TATGAAGGAA) targeting the three genes. However, the protospacer adjacent motif (PAM) is AGG in AtRPL10B and AtRPL10C, but GGG in AtRPL10A (Figure 1B). A. thaliana ecotype Col-0 was transformed with the binary vector, via the floral dip method (Supplemental Materials and Methods). Four independent lines, Line 7, Line 9, Line 10 and Line 11, were generated. Transgenic T1 plants from Lines 7, 9 and 10 showed severe growth retardation and delayed flowering whereas Line 11 had slightly weaker growth compared to the wild-type Col-0 plants (Figure 1C, D). These lines showed similar phenotypes to AtRPL10B RNAi plants, but differed from AtRPL10A T-DNA insertion mutants or AtRPL10C knockout plants.

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www.jipb.net
Figure 1. Continued
To detect potential multigene-editing events in these transgenic lines, we first analyzed the sgRNA-targeted sequences using a high-fidelity PCR-RFLP (polymerase chain reaction – restriction fragment length polymorphism) assay. An Earl site is located 4–9 nucleotides upstream of the AtRPL10 sgRNA PAM sequence (Figure 1B), the region in which CRISPR/Cas9-mediated DSBs frequently occur (Jinek et al. 2012). We extracted genomic DNA from transgenic and non-transformed Col-0 plant leaf tissues and amplified the AtRPL10 target sequences using gene-specific primers (Table S2). Incomplete Earl-digestion of the resultant PCR products suggests that AtRPL10A and AtRPL10C were successfully edited in Line 9 (Figure 1E).

To further characterize multigene-editing in these transgenic lines, we cloned the PCR products into pMD19-T (Supplemental Materials and Methods). Sequencing analyses showed that nucleotide deletions and/or replacements were introduced into AtRPL10A, AtRPL10B and AtRPL10C in all transgenic lines (Figures S2–S13; Table S3; Dataset S1). However, the efficiency of multigene-editing of all target sequences was relatively higher (Figures 1F, S5–S7) although varied among AtRPL10A (8.8%), AtRPL10B (3.8%) and AtRPL10C (23.6%) in Line 9 (Table 1). Using an alternative assay, we identified seven more (four deletion and three substitution) mutations that were introduced into AtRPL10B in Line 9 (Figure 1G–I). In Line 7 (Figures S2–S4) and Line 10 (Figures S8–S10), we detected nucleotide deletions in AtRPL10A or AtRPL10C but not in AtRPL10B, while only point mutations were detected in the three AtRPL10 genes in Line 11 (Figures S11–S13). In total we sequenced 1,222 clones and identified 75 different mutations, 37 of which were a deletion of two nucleotides. There were single cases of 1nt or 4nt-deletions, and 36 cases of 1nt-substitution (Table S3). Nevertheless, multiple deletion and/or point mutations introduced by a single sgRNA seed-directed CRISPR/Cas9 were correlated with the abnormal phenotypes in the transgenic lines (Figure 1C).

Multiplex gene editing through CRISPR/Cas9 that is directed by a number of different sgRNAs has been previously reported in animals and plants (Cong et al. 2013; Feng et al. 2014; Wang et al. 2015; Yan et al. 2015a). Here, we show that a single sgRNA seed is capable of guiding CRISPR/Cas9 to edit multiple genes in Arabidopsis. Second, we demonstrate that it is possible to use CRISPR/Cas9 technology to obtain AtRPL10A/B/C triple mutants which otherwise cannot be generated by conventional genetic crossing. Third, we observed that most mutations resulting from

Figure 1. A solo single-guide RNA (sgRNA) seed directs Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (CRISPR/Cas9) to simultaneously edit three AtRPL10 homologous genes (A) Schematic of the single sgRNA seed and CRISPR/Cas9 construct in the binary vector pCAMBIA1300. Nucleotides corresponding to the sgRNA seed sequence are underlined. The AtU6-26 promoter (arrow), sgRNA and the scaffold, enhanced 35S promoter (arrow), NLS (nuclear localization signal)-tagged Cas9, hygromycin (HYG) as well as the right and left borders (RB and LB) in the binary vector are indicated. (B) Comparison of the sgRNA seed-targeted AtRPL10 gene sequences. The Earl site is underlined. The PAM sequences are highlighted in red. Nucleotide coordinates are indicated. (C) Phenotypes of transgenic plants of four independent lines. Bar = 3 cm in Line7, Line9, Line10 and Line11. Bar = 5 cm in Col-0. (D) Confirmation of plant transformation. The Cas9 gene was detected in four transgenic lines, as indicated. A BM2000 DNA ladder (Marker) as well as the size and position of the Cas9 transgene polymerase chain reaction (PCR) fragment are indicated. (E) PCR-RFLP (restriction fragment length polymorphism) assay of multiple gene-editing in four transgenic lines. Gene-specific PCR products were digested with Earl. Incomplete digestion shows three clear bands, indicating that successful editing of AtRPL10A and AtRPL10C in Line9. A BM2000 DNA ladder (Marker) was included in gel electrophoresis. (F) Sequencing analysis of multiple gene-editing in Line9. Representative sequencing show indels in AtRPL10A, AtRPL10B and AtRPL10C. The sgRNA target sequences are shown in lowercase. (G–I) PCR-RFLP and sequencing assays of AtRPL10B editing in Line9. After Earl digestion, residual DNA in the position of the red box was extracted from the agarose gel (G) and subcloned for sequencing analysis (H). A BM2000 DNA ladder (M) was included in gel electrophoresis. Sequences of 19 individual clones for AtRPL10B were aligned, and mutations with two nucleotide-deletion (red arrow) or single nucleotide-substitution (highlighted red) are indicated (I). RD stands for restriction endonuclease digestion.
the single sgRNA seed-guided CRISPR/Cas9 are 2 nt-deletion or 1 nt-substitution within the sgRNA-target sequences. This differs from a previous report that mutations induced by CRISPR/Cas9 were predominantly 1 nt-insertion and short deletions of nucleotides (Feng et al. 2014), but consistent with others (Wang et al. 2015; Yan et al. 2015b). Fourth, the different AtRPL10A/B/C-editing efficiencies (Table 1), particularly in Line 9, suggest that chromosomal locations of genes along with the contexts of their surrounding sequences, heterochromatin architectures and/or DNA/histone methylation may affect the CRISPR/Cas9 system for editing multiple homologous genes (Kleinstiver et al. 2015). Nevertheless, Line 9 may prove to be a valuable model to investigate positional effects on the ability of single sgRNA-directed CRISPR/Cas9 to target and edit multiple genes in plants. Lines 7, 10 and 11 may be also useful to explore why the single sgRNA-directed CRISPR/Cas9 to target and edit multiple genes in plants. Lines 7, 10 and 11 may be also useful to explore why the single sgRNA-directed CRISPR/Cas9 system preferably causes nucleotide substitution, rather than deletion mutations in target genes. It is interesting to note that all deletion mutations created in our transgenic lines result from removal of one, two or four nucleotides, causing frameshifts of the target genes.

Compared to conventional multiplex CRISPR/Cas systems (Fu et al. 2013), a single sgRNA seed has the advantage of reducing off-target gene editing. This approach is also applicable for the modification of other homologous genes. Moreover, considering how CRISPR/Cas9 recognizes canonical or non-canonical PAMs, such as NGG, NGA, NGCG, TTN and YTN (Kleinstiver et al. 2015; Zetsche et al. 2015; Fonfara et al. 2016), as well as how sgRNAs interact with their target sequences (Jinek et al. 2012), it should also be possible to design a single “less-stringent” sgRNA seed that may target less-homologous sequences for multigene-editing in plants and other organisms.

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**Table 1. Summary of multigene editing efficiency**

<table>
<thead>
<tr>
<th>Transgenic lines</th>
<th>AtRPL10A</th>
<th>AtRPL10B</th>
<th>AtRPL10C</th>
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<td></td>
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<td>Point mutation</td>
<td>Deletion</td>
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<td>0/99</td>
</tr>
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<td>0/101</td>
</tr>
<tr>
<td>Line 11</td>
<td>0/93</td>
<td>2/93</td>
<td>0/109</td>
</tr>
</tbody>
</table>

*The number of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (CRISPR/Cas9) edited sequences (clones) out of the total number of sequenced samples for the three AtRPL10 genes in each of the transgenic lines.*
AUTHOR CONTRIBUTIONS

Z.Y. and Y.H. designed experiments; Z.Y. and Q.C. performed all experiments; W.C. and X.Z. analyzed bioinformatics data; J.N., F.M., P.Z., M.Z., X.W. and N.S. performed research. S.J. analysed the data and helped write the paper; Z.Y. and Y.H. wrote the paper.

REFERENCES


SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.12622/suppinfo

Dataset S1. Sequences of the PCR products for the three AtRPL10 genes.

(A–B) Restriction fragment length polymorphism (RFLP) analysis of AtRPL10A (A), AtRPL10B (B) AtRPL10C (C). Sequences corresponding to the “seed” single guide RNA (sgRNA) are indicated in lowercase. The EARl digestion site (l) is indicated and its recognition sequence is underlined.

Figure S1. Physical positions of AtRPL10A, AtRPL10B and AtRPL10C in Arabidopsis chromosome 1

**Figure S2.** Triple gene editing in Line 7  
(A) AtRPL10A sequences of individual clones. Proto-spacer adjacent motif (PAM) sequence is boxed and the edited nucleotides are highlighted red.  
(B) Representative chromatograms of AtRPL10A sequences with edited nucleotides

**Figure S3.** Triple gene editing in Line 7  
(A) AtRPL10B sequences of individual clones. Proto-spacer adjacent motif (PAM) sequence is boxed and the edited nucleotides are highlighted red.  
(B) Representative chromatograms of AtRPL10B sequences with edited nucleotides

**Figure S4.** Triple gene editing in Line 7  
(A) AtRPL10C sequences of individual clones. Proto-spacer adjacent motif (PAM) sequence is boxed and the edited nucleotides are highlighted red.  
(B) Representative chromatograms of AtRPL10C sequences with edited nucleotides

**Figure S5.** Triple gene editing in Line 9  
(A) AtRPL10A sequences of individual clones. Proto-spacer adjacent motif (PAM) sequence is boxed and the edited nucleotides are highlighted red.  
(B) Representative chromatograms of AtRPL10A sequences with edited nucleotides

**Figure S6.** Triple gene editing in Line 9  
(A) AtRPL10B sequences of individual clones. Proto-spacer adjacent motif (PAM) sequence is boxed and the edited nucleotides are highlighted red.  
(B) Representative chromatograms of AtRPL10B sequences with edited nucleotides

**Figure S7.** Triple gene editing in Line 9  
(A) AtRPL10C sequences of individual clones. Proto-spacer adjacent motif (PAM) sequence is boxed and the edited nucleotides are highlighted red.  
(B) Representative chromatograms of AtRPL10C sequences with edited nucleotides

**Figure S8.** Triple gene editing in Line 10  
(A) AtRPL10A sequences of individual clones. Proto-spacer adjacent motif (PAM) sequence is boxed and the edited nucleotides are highlighted red.  
(B) Representative chromatograms of AtRPL10A sequences with edited nucleotides

**Figure S9.** Triple gene editing in Line 10  
(A) AtRPL10B sequences of individual clones. Proto-spacer adjacent motif (PAM) sequence is boxed and the edited nucleotides are highlighted red.  
(B) Representative chromatograms of AtRPL10B sequences with edited nucleotides

**Figure S10.** Triple gene editing in Line 10  
(A) AtRPL10C sequences of individual clones. Proto-spacer adjacent motif (PAM) sequence is boxed and the edited nucleotides are highlighted red.  
(B) Representative chromatograms of AtRPL10C sequences with edited nucleotides

**Figure S11.** Triple gene editing in Line 11  
(A) AtRPL10A sequences of individual clones. Proto-spacer adjacent motif (PAM) sequence is boxed and the edited nucleotides are highlighted red.  
(B) Representative chromatograms of AtRPL10A sequences with edited nucleotides

**Figure S12.** Triple gene editing in Line 11  
(A) AtRPL10B sequences of individual clones. Proto-spacer adjacent motif (PAM) sequence is boxed and the edited nucleotides are highlighted red.  
(B) Representative chromatograms of AtRPL10B sequences with edited nucleotides

**Figure S13.** Triple gene editing in Line 11  
(A) AtRPL10C sequences of individual clones. Proto-spacer adjacent motif (PAM) sequence is boxed and the edited nucleotides are highlighted red.  
(B) Representative chromatograms of AtRPL10C sequences with edited nucleotides

**Table S1.** Comparisons of AtRPL10 genes and their protein products

**Table S2.** Primers used in this study

**Table S3.** Multigene editing and their impacts on protein sequences in Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (CRISPR/Cas9) transgenic lines

Supplemental Materials and Methods