Gene targeting in Arabidopsis via an all-in-one strategy that uses a translational enhancer to aid Cas9 expression

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Gene targeting, that is precise sequence knockin or replacement, is the ultimate goal of precision genome engineering. Although homologous recombination (HR)-mediated gene targeting is widely used in many organisms, including mammals (Thomas and Capecchi, 1987) (Chandrasegaran and Carroll, 2016), the low frequencies of HR in higher plants limit its utilization for gene targeting (Fauser et al., 2012). We recently described a sequential transformation approach for efficient (5.3%–9.1%) HR-mediated gene targeting in Arabidopsis. Briefly, a construct encoding an HR donor and a single-guide RNA (sgRNA) is transformed into parental transgenic plants that stably express the Cas9 nuclease in egg cells and the early embryo (Miki et al., 2018). Given that the sequential approach requires genetically modified plants already expressing Cas9, which limits its use in diverse genetic backgrounds, we aimed to establish an efficient all-in-one method for gene targeting in the T1 generation of Arabidopsis.

Previously, we attempted an all-in-one strategy targeting the GL2 locus. We transformed Arabidopsis with an ‘all-in-one’ construct that contained: DD45pro::Cas9 (which drives Cas9 expression in egg cells and the early embryo), an HR donor sequence, and GL2 sgRNA. Because we had previously found that the DD45pro::Cas9 system in Arabidopsis led to an elevated mutation frequency in T2 populations compared with T1 (Mao et al., 2016), we screened T2 seedlings in bulk (Miki et al., 2018). However, we did not identify any heritable gene targeting events at the GL2 locus after screening 28 T2 pools (Miki et al., 2018).

Since we had observed heritable gene targeting of the ROS1 locus upon sequential transformation, we next tested whether we could use an all-in-one strategy to generate a heritable GFP knockin at this locus. We designed an all-in-one construct that contained DD45pro::Cas9, as well as the ROS1 sgRNA and HR donor from our previous report (Miki et al., 2018; Figure 1a). Given that the DD45 promoter drives Cas9 expression in the egg/early embryo, we hypothesized that gene targeting could be established in the T1 generation. We transformed this construct into the nontransgenic Col-0 accession, selected for T1 transformants that were resistant to hygromycin, and performed PCR-based genotyping to detect gene targeting events. We used two strategies for PCR genotyping: one primer set amplifies only the knockin allele (‘GT-specific’) whereas the other primer set amplifies both the endogenous and the knockin alleles (‘external’; Figure 1a).

We screened a large number of T1 transformants, given the potentially low efficiency of gene targeting (Wolter et al., 2018), and obtained two heterozygous gene targeting-positive T1 plants from 293 independent T1 transformants (Figure 1b). The GT-specific primer set gave rise to some false positive signals that were not detected by the external primer set (Figure 1b). All plants that were positive for both genotyping strategies could be confirmed by Southern blotting (Figure 1b), and all knockins were inherited in T2 plants (Figure 1c). The gene targeting efficiency of the all-in-one strategy at the ROS1 locus was 0.68% (Figure 1d), almost 10-fold lower than our previously reported sequential transformation strategy (Miki et al., 2018).

Transcriptional and translational enhancer sequences have been reported to improve Cas9 expression and, in turn, the efficiency of CRISPR/Cas9-mediated targeted mutagenesis in plants (Gasparis et al., 2018; Kusano et al., 2018). We therefore investigated whether the omega translational enhancer from tobacco mosaic virus (TMV) would improve gene targeting by enhancing Cas9 translation (Galli and Kado, 1989; Mitsuhashi et al., 1996; Figure 1a). We obtained three heterozygous gene targeting-positive T1 plants from 125 independent T1 transformants of the DD45pro::omega enhancer-Cas9 all-in-one (Figure 1e), which were heritable to the T2 generation (Figure 1f).

Figure 1 Gene targeting of the ROS1 gene by an all-in-one strategy. (a) Schematic representation of the all-in-one ROS1-GFP gene targeting T-DNA constructs (top), the relevant region within the endogenous ROS1 locus (middle) and the ROS1-GFP gene targeted locus (bottom). Orange squares represent omega enhancer, magenta hexagons represent sgRNA target sites and striped arrow represents a chimeric EC1.2/DD45 enhancer with EC1.1 promoter. The horizontal line indicates the position of a probe for Southern blot hybridization following HindIII digestion. Purple arrows represent BstUI (CCGG), green arrow represents BsmI (ACTGGG) and orange arrows represent TseI (GCWGC) restriction enzymes, respectively. (b, c) PCR genotyping and Southern blot hybridization of ROS1-GFP T1 (b) and T2 (c) by DD45pro::SpCas9 all-in-one construct. (d) Summary of gene targeting efficiencies in T1 Arabidopsis by all-in-one strategy. (e, f) PCR genotyping and Southern blot hybridization of ROS1-GFP T1 (e) and T2 (f) by DD45pro::omega enhancer-SpCas9 construct. (g) Southern blot hybridization for analysis of DNA methylation of the knockin GFP sequence.
Cas9 mediated gene targeting in Arabidopsis

(a) Schematic diagram of the donor constructs and the ROS1 gene structure. (b) Southern blot analysis of the T1 generation. (c) Southern blot analysis of the T2 generation. (d) Summary of gene targeting in T1 Arabidopsis. (e) Southern blot analysis of the T1 generation with the omega enhancer. (f) Southern blot analysis of the T2 generation with the omega enhancer. (g) Summary of gene targeting in T2 Arabidopsis.

| Promoter | Omega enhancer | Donor excision | T1 transformant | GT-positive | GT frequency |
|----------|----------------|----------------|-----------------|-------------|--------------|
| DD45     | -              | -              | 293             | 2           | 0.68%        |
| DD45     | +              | -              | 125             | 3           | 2.4%         |
| DD45     | +              | +              | 123             | 0           | 0%           |
| EC1.2-EC1.1 | +          | -              | 190             | 0           | 0%           |

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The gene targeting efficiency of the DD45pro::omega enhancer-Cas9 all-in-one strategy was 2.4%, which is at least three times higher than without omega enhancer (Figure 1d).

In addition, a previous all-in-one strategy was shown to successfully target the ALS locus in Arabidopsis. This strategy used a chimeric EC1.2/DD45 enhancer with the EC1.1 promoter to drive Cas9 expression in egg cells (Wolter et al., 2018). Therefore, we also generated an all-in-one construct that drives omega translational enhancer-Cas9 expression from a chimeric EC1.2/DD45 enhancer with the EC1.1 promoter (Figure 1a). We screened 190 independent T1 transformants of this all-in-one construct, but all the tested plants were negative (Figure 1d). These data suggest that the EC1.2/DD45 enhancer and EC1.1 promoter are not as efficient for gene targeting compared with the DD45 promoter in our system.

Another strategy that has been used to improve gene targeting efficiency in planta is to excise the donor fragment from the T-DNA by using sequence-specific nucleases (SSNs) (Wolter et al., 2018). To determine if excision of the HR donor improves the efficiency of our DD45-promoter-based all-in-one gene targeting system, we designed a construct in which the HR donor sequence was flanked by two recognition sites for the same sgRNA as the knockin sequence, we analyzed homozygous T2 GT-positive plants by cytosine DNA methylation at the homology arms of ROS1 (Figure 1a). We screened 123 independent T1 transformants, but did not obtain any positive lines with gene targeting events (Figure 1d). These data suggest that excising the HR donor template from the T-DNA does not increase but rather reduces the efficiency of the DD45 promoter-based all-in-one strategy. Our sequential transformation gene targeting strategy did not affect cytosine DNA methylation at the homology arms of homozygous ROS1-GFP T4 plants (Miki et al., 2018). In this study, we did not observe substantial changes in cytosine methylation in either the 5’ or 3’ homology arm regions in two heterozygous DD45pro::Cas9 all-in-one ROS1-GFP T1 GT plants (data not shown), confirming that gene targeting does not affect DNA methylation at the target locus (Miki et al., 2018).

To examine whether any new DNA methylation is established de novo at the knockin sequence, we analyzed homozygous T2 DD45pro::Cas9 all-in-one ROS1-GFP GT plants by cytosine methylation sensitive restriction enzymes-based Southern blotting (Figure 1a). All cytosine contexts, CG, CHG and CHH, lacked methylation (Figure 1g), suggesting that de novo methylation did not occur. In summary, our data show that heritable ROS1-GFP GT-positive plants can be obtained by an all-in-one strategy, though at a much lower frequency than with the sequential approach. Further, placing the omega translational enhancer upstream of Cas9 improves the efficiency of gene targeting by the all-in-one strategy.

Although our data further suggest that egg cell- and early embryo-specific expression of Cas9 contributes to heritable gene targeting, our all-in-one strategy using a chimeric EC1.2/DD45 enhancer with EC1.1 promoter to drive Cas9 expression in egg cells did not support efficient gene targeting. Previous experiments used the same chimeric promoter to drive Staphylococcus aureus Cas9 (Wolter et al., 2018), whereas we used Streptococcus pyogenes Cas9, which might have contributed to the lower efficiency.

Although sequential transformation gives rise to higher gene targeting frequencies, our results suggest that the DD45 promoter-based all-in-one strategy could be optimized to achieve gene targeting, so that gene targeting may be carried out at any genetic background or natural accession of Arabidopsis, or other plant species that can be transformed by floral dipping. We propose that high Cas9 protein levels in the egg cells/early embryos generate efficiently DNA double stranded breaks that promote HR-mediated gene targeting.

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### Author Contributions
D.M. and J.K.Z. designed the research; F.N.P., W.X.Z., W.J.Z. and D.M. performed the experiments; D.M. and J.K.Z. supervised the project; D.M., F.N.P., W.X.Z. and J.K.Z. wrote the paper. Correspondence and requests for materials should be addressed to D.M. or J.K.Z.

### Conflict of interest
The authors declare no competing financial interests.

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