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Brief Communication

Transient cotransformation of CRISPR/Cas9 and oligonucleotide templates enables efficient editing of target loci in *Physcomitrella patens*

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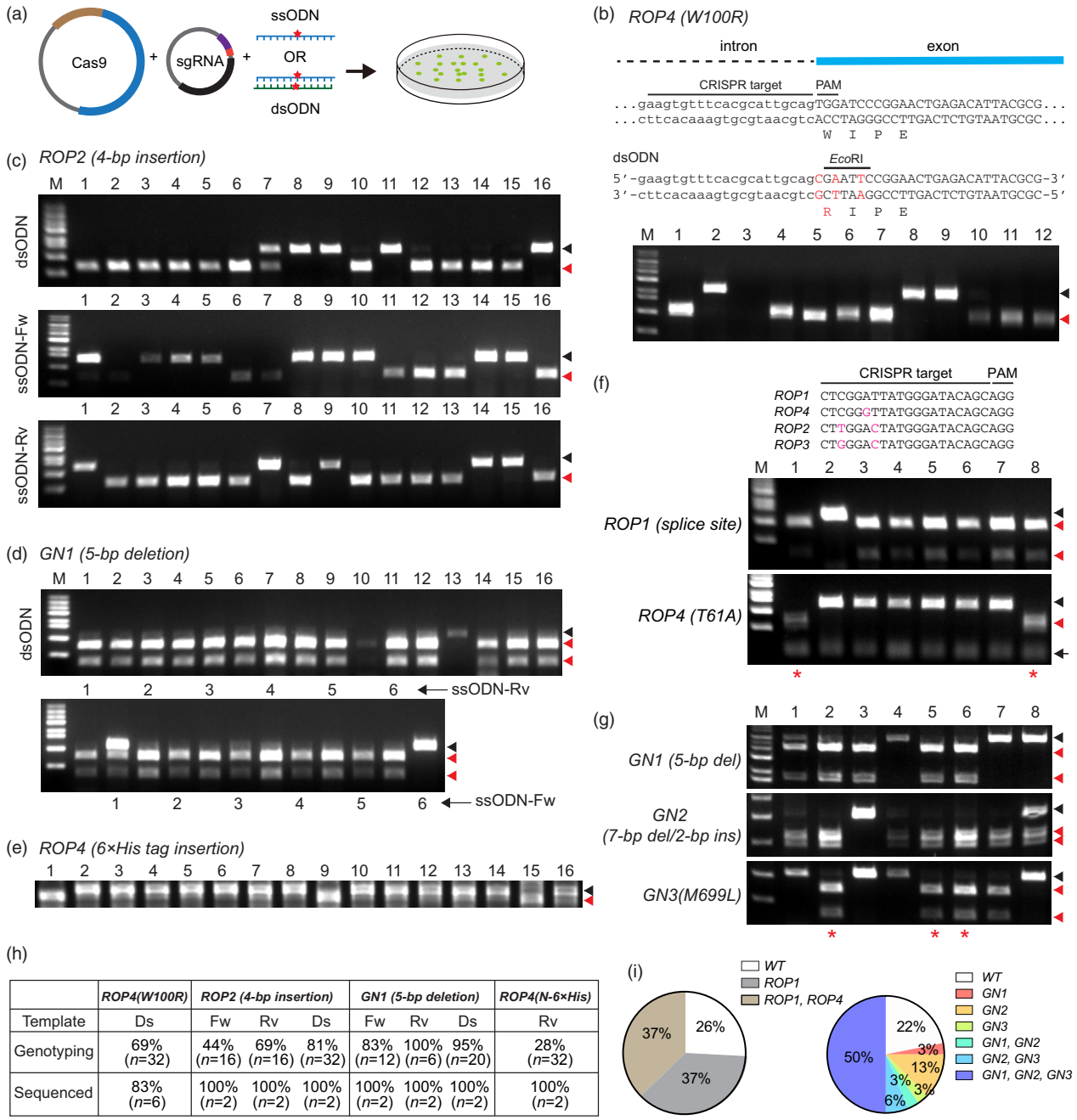
The moss *Physcomitrella patens* is a valuable system to address evolutionary changes of plants (Prigge and Bezanilla, 2010). Till date, functional studies in *P. patens* have been mainly carried out by gene knockout owing to its high rate of homologous recombination (HR) (Schaefer and Zrÿd, 1997). However, replacement of redundant genes sequentially by HR is laborious and time-consuming. Furthermore, hypomorphic mutants, another valuable resource for gene function studies, have been scarcely generated. The clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system is a powerful tool to generate mutants via targeted genome editing (Pickar-Oliver and Gersbach, 2019). In *P. patens*, CRISPR/Cas9 has been exploited to generate heritable mutants (Collonnier *et al*., 2017a; Lopez-Obando *et al*., 2016; Nomura *et al*., 2016). However, it is unclear whether hypomorphic mutations can be introduced when a template is supplied. Moreover, template-free editing can induce specific deletions at a high frequency, which may complicate the identification of out-of-frame mutations.

Oligodeoxynucleotides (ODNs) and CRISPR/Cas9 have been used in combination to achieve mutation knock-in in flowering plants (Collonnier *et al*., 2017b). We asked whether the same strategy can be applied to *P. patens*. As a proof-of-principle, we targeted *ROP4* to introduce a W100R substitution. Double-stranded ODNs (dsODNs), which were prepared by annealing equal amounts of the complementary single-stranded ODNs (ssODNs, each 250 pmol), were used as repair templates. We cotransformed the dsODNs with 10 μg Cas9 plasmid and 10 μg sgRNA vector into protoplasts using the PEG-mediated transformation protocol (Figure 1a) (Yamada *et al*., 2016). After transient resistance selection, transformants were recovered. Restriction fragment length polymorphism-based genotyping indicated that 69% of the colonies ($N = 32$) were edited (Figure 1b and h). Sequencing of six lines demonstrated that five of them were modified with correct mutations and one of them carried a 179-bp deletion.

Next, we tested whether a small insertion or deletion can be introduced. We targeted *ROP2* and Pp3c9_11830 (encoding a GNOM homologue, which we named GN1) to generate a 4-nt insertion and a 5-nt deletion, respectively. As shown in Figure 1c, d and h, 81% ($N = 32$) and 95% ($N = 20$) of the colonies targeting *ROP2* and *GN1*, respectively, were edited when dsODNs were used. Interestingly, cotransformation of CRISPR/Cas9 and ssODNs also generated edited mutants. The editing efficiency of ssODNs of *GN1* was almost comparable to that of dsODNs, whereas ssODNs were less efficient than dsODNs for *ROP2*. We interpret that since the targeting efficiency of *GN1* is extremely high, ssODNs can trigger editing as efficient as dsODNs. Sequencing of two lines from each category confirmed the correct modification of each locus (Figure 1h). To further test the potential of ssODNs in triggering genome editing and the introduction of larger insertions, we used 62-nt ssODNs to insert a 6 × His tag at *ROP4* N-terminus. Genotyping and sequencing revealed that 28% of the transformants ($N = 32$) were edited (Figure 1e and h). We repeated the transformation for *ROP2* and *GN1* without supplying Cas9. However, no edited lines were identified ($N = 96$ in total), suggesting that ODNs alone are insufficient to trigger genome editing. Taken together, these results demonstrate that CRISPR/Cas9 and ODNs are necessary for precise genome modification. Our data also indicate that both dsODNs and ssODNs can induce mutation knock-in; however, dsODNs are likely more efficient.

We next investigated the potential of multi-gene editing by one target. We used one sgRNA that targets *ROP1* and potentially, the homologous regions of *ROP2-ROP4* (Figure 1f). The dsODN templates of *ROP1* and *ROP4* were cotransformed with CRISPR/Cas9 to introduce a splice site modification and a T61A substitution, respectively. Genotyping revealed that 37% ($N = 27$) of the colonies were edited at both loci and 37% ($N = 27$) were edited at the *ROP1* locus alone (Figure 1i). Interestingly, no edited line at *ROP4* alone was identified. A plausible explanation is that the *ROP4* locus is less efficiently edited due to the mismatch between the sgRNA and target (Figure 1f). We sequenced 12 mutants, including the 10 double-edited lines. All of them were correctly edited at the *ROP1* locus and seven of the double-edited lines were correctly modified at the *ROP4* locus. We did not detect any mutation at the *ROP2* or *ROP3* loci ($N = 12$), suggesting the absence of off-targets at these sites. Taken together, our results revealed the possibility of mutation knock-in at multiple sites by a single sgRNA. However, the editing efficiency seems sensitive to target-sgRNA mismatches. This sensitivity is consistent with previous reports of the high specificity of RNA-guided nucleases (Collonnier *et al*., 2017a; Lopez-Obando *et al*., 2016; Nomura *et al*., 2016).

We then asked whether cotransformation of multiple sgRNAs and dsODN templates would enable multiplex editing. We focused on all the three *GNOM* homologues, namely *GN1* and its paralogs, Pp3c15_11320 and Pp3c22_6150, which we named *GN2* and *GN3*, respectively. We used a single vector that expressed all the sgRNAs targeting *GN1-GN3* and the dsODN templates of *GN1-GN3* to introduce frameshift mutations in *GN1*



	ROP4(W100R)	ROP2 (4-bp insertion)			GN1 (5-bp deletion)			ROP4(N-6×His)
Template	Ds	Fw	Rv	Ds	Fw	Rv	Ds	Rv
Genotyping	69% (*n*=32)	44% (*n*=16)	69% (*n*=16)	81% (*n*=32)	83% (*n*=12)	100% (*n*=6)	95% (*n*=20)	28% (*n*=32)
Sequenced	83% (*n*=6)	100% (*n*=2)	100% (*n*=2)	100% (*n*=2)	100% (*n*=2)	100% (*n*=2)	100% (*n*=2)	100% (*n*=2)

Figure 1 CRISPR/Cas9 and ODN-assisted genome editing. (a) Cotransformation of CRISPR/Cas9 and ODNs into *P. patens* protoplasts. ODNs were arbitrarily designed at the length of 42–62 nt and contained 20–23 nt homology arms. A selection marker (black) was added to the sgRNA vector. Stars indicate the mutations which introduced a restriction enzyme site for genotyping. (b) Introduction of a W100R mutation to the *ROP4* locus. Mutations are in red. Black and red arrowheads indicate the wild-type and edited bands, respectively (the same in other panels). (c) Introduction of a 4-bp insertion to the *ROP2* locus. (d) Introduction of a 5-bp deletion to the *GN1* locus. (e) Introduction of a 6 × His tag to the *ROP4* N-terminus. (f) Double editing by one CRISPR target. Mismatches are highlighted in magenta. Black arrow, non-specific bands. Star, double-edited lines. (g) Multiplex editing at the *GN1-GN3* loci. Star, triple-edited lines. (h) Summary of single-gene editing results. Ds, Fw, and Rv represent the dsODN, forward ssODN, and reverse ssODN templates, respectively. (i) Summary of double-gene and triple-gene editing results.

and *GN2*, and a M699L substitution in *GN3*. Genotyping revealed that 50% (N = 32) of the colonies were edited at all loci and 28% were edited at one or two sites (Figure 1g and i). Sequence analysis of triple- and double-edited lines confirmed the correct modification of endogenous loci. These results demonstrate that CRISPR/Cas9 and dsODN-assisted multiplex genome editing in *P. patens* is efficient and accurate.

In this study, we show that transient cotransformation of CRISPR/Cas9 and ODNs enables efficient editing of target loci in *P. patens*. This method can be used to introduce various types of mutations at single or multiple sites, which will not only facilitate multi-gene knockout, but also allow in-depth analysis of any gene, especially those that are essential for viability. As illegitimate integration occurs frequently in *P. patens*, we examined

such events by resistance check and PCR amplification. However, no integration was found in the 24 selected lines, suggesting the absence of unexpected integration.

Our results also suggested some features of the CRISPR/Cas9 and ODN-assisted editing: first, dsODNs are likely more efficient than ssODNs; second, the introduction of small insertions/deletions appears more efficient than that of complex or longer mutations. Although more studies are needed to illustrate the underlying mechanism, these features generally support the involvement of a template-target pairing mechanism during DNA repair. As ODNs alone are not sufficient to induce editing, we speculate that microhomology-mediated end joining (MMEJ), rather than homology-directed repair pathway, is responsible for DNA repair. Indeed, MMEJ is highly active in *P. patens*, which may also account for the higher efficiency of ODN-assisted genome editing than in other species (Collonnier *et al*., 2017a,b; Kamisugi *et al*., 2006). However, non-homologous end joining may also participate, as additional point mutations or deletion/insertion mutations were sometimes detected (1/6 and 3/10 in the editing experiments of ROP4(W100R) and ROP4(T61A), respectively). In our hands, 20–23-bp homology length is sufficient to trigger efficient editing. Increasing homology length might further increase editing efficiency. However, it needs more investigation. The use of CRISPR/Cas9 can cause off-target effects. Our data and previous studies suggest that Cas9 activity is sensitive to sgRNA-target mismatches in *P. patens*. For example, a single mismatch caused a relatively lower editing efficiency (Figure 1f) and no off-targets were found when more mismatches existed (Collonnier *et al*., 2017a; Lopez-Obando *et al*., 2016; Nomura *et al*., 2016). Nevertheless, without whole-genome analysis, off-targets cannot be simply ruled out. Alternatively, performing a rescue experiment will verify the observed phenotypes, if any. Furthermore, using optimized Cas proteins with higher fidelity or expanded recognition motifs will improve the ODN-assisted genome editing in the future.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

P.Y. conceived the project, performed the experiments and analysed the data. P.Y. and G.G. wrote the manuscript.

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