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# CRISPR/Cas9-Mediated Targeted Mutagenesis in the Liverwort *Marchantia polymorpha* L.

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**Targeted genome modification technologies are key tools for functional genomics. The clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonuclease Cas9 system (CRISPR/Cas9) is an emerging technology for targeted genome modification. The CRISPR/Cas9 system consists of a short guide RNA (gRNA), which specifies the target genome sequence, and the Cas9 protein, which has endonuclease activity. The CRISPR/Cas9 system has been applied to model animals and flowering plants, including rice, sorghum, wheat, tobacco and Arabidopsis. Here, we report the application of CRISPR/Cas9 to targeted mutagenesis in the liverwort *Marchantia polymorpha* L., which has emerged as a model species for studying land plant evolution. The *U6* promoter of *M. polymorpha* was identified and cloned to express the *gRNA*. The target sequence of the *gRNA* was designed to disrupt the gene encoding auxin response factor 1 (ARF1) in *M. polymorpha*. Using *Agrobacterium*-mediated transformation, we isolated stable mutants in the gametophyte generation of *M. polymorpha*. CRISPR/Cas9-based site-directed mutagenesis in vivo was achieved using either the *Cauliflower mosaic virus 35S* or *M. polymorpha EF1α* promoter to express *Cas9*. Isolated mutant individuals showing an auxin-resistant phenotype were not chimeric. Moreover, stable mutants were produced by asexual reproduction of $T_1$ plants. Multiple *arf1* alleles were easily established using CRIPSR/Cas9-based targeted mutagenesis. Our results provide a rapid and simple approach for molecular genetics in *M. polymorpha*, and raise the possibility that CRISPR/Cas9 may be applied to a wide variety of plant species.**

**Keywords:** CRISPR/Cas9 • Genome editing • Liverwort • *Marchantia polymorpha* • Targeted genome mutagenesis.

**Abbreviations:** ARF1, auxin response factor 1; Cas9, CRISPR-associated endonuclease 9; CRISPR, clustered regularly interspaced short palindromic repeats; EF, elongation factor 1α; gRNA, short guide RNA; NAA, 1-naphthalene acetic acid; NHEJ, non-homologous end joining; PAM, the requisite protospacer adjacent motif; RFLP, restriction fragment length polymorphism; snRNA, small nuclear RNA; TALEN, transcription activator-like effector nuclease.

## Introduction

Targeted genome modification technologies are key tools not only for the basic science of plants but also for crop breeding. Recently, engineered nucleases, such as zinc finger nucleases and transcription activator-like effector nucleases (TALENs), have been shown to generate sequence-specific mutagenesis in flowering plants (Osakabe et al. 2010, Zhang et al. 2010, Zhang et al. 2013). These nucleases have tandem repeats of a DNA-binding protein and an endonuclease domain of a restriction enzyme, *Fok*I (Chen and Gao 2013). *Fok*I generates double-strand breaks, which are repaired through non-homologous end joining (NHEJ) or homologous recombination to produce mutagenesis at specific loci defined by the designed DNA-binding region. Zinc finger nucleases (Shukla et al. 2009, Townsend et al. 2009) and TALENs (Christian et al. 2013, Li et al. 2012) have already been applied to genome editing in plants. However, these technologies need elaborate design and laborious work to construct vectors because of the highly repetitive DNA-binding motifs of these artificial nucleases; therefore, the application of engineered nucleases in plants has been limited.

Recently, using a prokaryote-specific adaptive immune system CRISPR (clustered regulatory interspaced short palindromic repeats)/Cas (CRISPR-associated protein) (Wiedenheft et al. 2012), a simple targeted genome modification technology, the CRISPR/Cas9 system, was reported (Cong et al. 2013, Mali et al. 2013). The CRISPR/Cas9 system requires only a single Cas9 endonuclease of *Streptococcus pyogenes*, and a short guide RNA (gRNA) that specifies the target sequence for cleavage (**Fig. 1A**)

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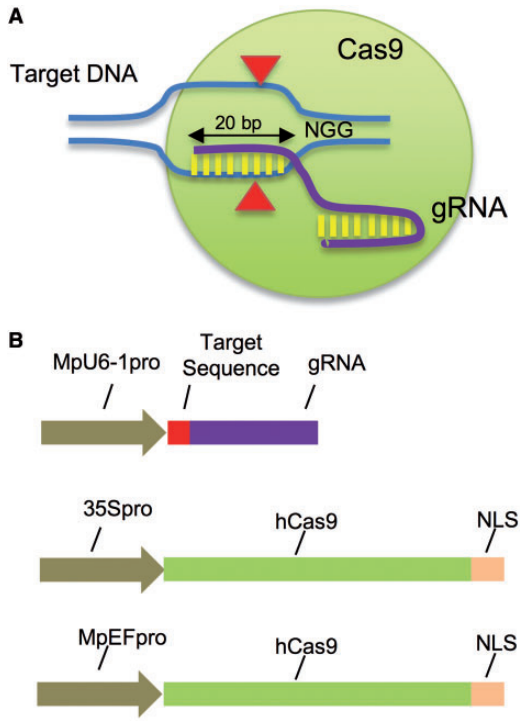


**Fig. 1** CRISPR/Cas9 system used in this study. (A) Schematic diagram of the CRISPR/Cas9 system. Cas9 (green) forms a sequence-specific endonuclease when complexed with gRNA (purple). The Cas9–gRNA complex recognizes a target-specific DNA sequence (blue) that is 23 bp in length, ending with one arbitrary nucleic acid and two guanines (NGG, PAM sequence). The 20 bp region is designable and termed the target sequence. Cas9 endonuclease tends to cleave DNA 3 bp upstream of the PAM sequence (red arrowhead). (B) Constructs used in this study. *gRNA* was expressed using the *U6* promoter isolated from *M. polymorpha*. *hCas9* fused with a nuclear localization signal (NLS) was expressed by either the *35S* promoter or the *EF1α* promoter.

(Cong et al. 2013, Mali et al. 2013). The CRISPR/Cas9 system has been successfully applied to make knockout mutants in eukaryotic organisms from human cell lines (Cho et al. 2013) to yeast (Dicarlo et al. 2013). Successful gene modifications in plants have also been reported in *Oryza sativa* (Shan et al. 2013, Xie and Yang 2013), *Triticum aestivum* (Shan et al. 2013), *Nicotiana benthamiana* (Li et al. 2013, Nekrasov et al. 2013), *Sorghum bicolor* (Jiang et al. 2013) and *Arabidopsis thaliana* (Feng et al. 2013, Mao et al. 2013). To the best of our knowledge, however, all the reports of targeted mutagenesis in plants focused on flowering plant species. Basal land plant species have not been tested.

The liverwort *Marchantia polymorpha* is an emerging model species of basal land plants. *M. polymorpha* occupies a crucial position in the evolution of land plants (Qiu et al. 2006), and has a haploid-dominant life cycle which provides advantages over diploid vascular plants for molecular genetic analysis. For rapid reverse genetics in liverworts, homologous recombination-mediated gene targeting was established (Ishizaki et al. 2013a). Homologous recombination-mediated gene targeting is beneficial for obtaining a single allele for *M. polymorpha* (Ishizaki et al. 2013a, Ishizaki et al. 2013b). CRISPR/Cas9-mediated genome editing is also an attractive technology that has the potential to induce multiple types of mutations in a single gene by NHEJ-based repair of a target genome sequence (Cong et al. 2013, Mali et al. 2013).

In the present study, we demonstrated targeted genome mutagenesis of the *ARF1* gene in the haploid generation of a basal land plant, *M. polymorpha*. The *U6* promoter of *M. polymorpha* was identified and used to express *gRNA*. Vectors for the CRISPR/Cas9 system were transformed into sporelings of liverworts using *Agrobacterium*-mediated transformation. Multiple mutant alleles of *arf1* were obtained using either the *Cauliflower mosaic virus 35S* promoter (*35Spro*) or the *M. polymorpha* elongation factor 1α promoter (*MpEFpro*) to express *Cas9*. We also isolated monoclonal mutant individuals that showed an auxin-resistant phenotype.

## Results

### Cloning the *U6* promoter from *M. polymorpha*

We first cloned the promoter sequence of *U6* small nuclear RNAs (snRNAs) in *M. polymorpha* (**Supplementary Fig. S1**). In the CRISPR/Cas9 system, *gRNA* is commonly expressed using promoters of *U6 snRNA* genes, which are RNA polymerase III-dependent promoters (Belhaj et al. 2013). It is reported that *U6* promoters cannot be replaced with those of other evolutionarily distant plant species (Wang et al. 2008). Therefore, *U6* promoter sequences of *M. polymorpha* should be used to express *gRNA*. *U6 snRNA* genes have not been identified in *M. polymorpha*; therefore, we conducted a genomic sequence search of our database and identified the coding sequences for eight putative *U6 snRNA* genes (**Supplementary Fig. S1**). The *U6 snRNA* sequences in *M. polymorpha* are highly conserved (**Supplementary Fig. S1**). All *U6 snRNA* genes deduced from genomic sequences should be expressed in liverworts, because the sequences were also identified as expressed sequence tags (data not shown). We designated the 2 kb sequence upstream of the *U6-1* coding sequence as *MpU6-1pro* and used it for *gRNA* transcription.

### Vector construction for the CRISPR/Cas9 system

A *gRNA* expression vector for *M. polymorpha* was constructed (**Fig. 1B**). *MpU6-1pro* was fused with an artificially designed *gRNA* sequence cloned from the plasmid used by Mali et al. (2013) to generate a binary vector harboring *MpU6-1pro:gRNA* (**Fig. 1B**). For expression of *Cas9* endonuclease, the human codon optimized *hCas9* (Mali et al. 2013) sequence was driven by the *35Spro* or the endogenous *MpEFpro* (**Fig. 1B**). These two promoters were both used for overexpression, as the expression patterns overlap but are slightly different from each other in liverwort (Althoff et al. 2013). *35Spro* has a weak activity in the meristematic zones but drives strong expression in

thalli. Conversely, *MpEFpro* causes strong meristematic expression (Althoff et al. 2013). *hCas9* expression vectors were designed to harbor a hygromycin-resistant gene.

To test CRISPR/Cas9-based targeted mutagenesis in liverwort, we designed the target sequence of the gRNA. As a model endogenous gene target, we chose *ARF1*, which is one of the auxin response factors and acts as a positive regulator for the auxin response in *M. polymorpha* (T. Kohchi et al. in preparation). *ARF1* is an ideal model target of CRISPR-mediated targeted mutagenesis in *M. polymorpha* because the defective gene confers an auxin-resistant phenotype that can easily be selected in medium containing auxin, as in Arabidopsis (Li et al. 2006). We designed the gRNA target sequence to disrupt the DNA region encoding a putative DNA-binding motif of ARF1. It is reported that the target sequence should have the 3 bp sequence NGG, the requisite protospacer adjacent motif (PAM) and a 20 bp arbitrary sequence (Jinek et al. 2012). We successfully identified the PAM sequence in the DNA-binding motif of *ARF1*, and the target sequence contained a recognition site for the restriction enzyme *Bcl*I 5 bp upstream of the PAM sequence (see **Fig. 3**). Using *Bcl*I, restriction fragment length polymorphism (RFLP) analysis could be applied to detect mutations in the target region.

## Isolation of *arf1* mutants generated by CRISPR/Cas9

Expression vectors of *hCas9* and *gRNA* were co-transformed into the sporelings of liverworts using an *Agrobacterium*-mediated transformation method (Ishizaki et al. 2008). Transformed cells were screened with 3 μM 1-naphthalene acetic acid (NAA)- and hygromycin-containing media. When both *hCas9* and *gRNA* were expressed in one cell, the target sequence of the *ARF1* locus of the cell would be disrupted and a cell with such mutations would survive in positive selection using NAA-containing media. In four independent experiments, NAA-resistant plants were obtained using either *35Spro* or *MpEFpro* (**Table 1**).

To confirm disruption of the target sequence of *ARF1*, we conducted RFLP analysis of DNA extracted from thalli of NAA-resistant plants (**Fig. 2**). PCR products (1,700 bp) without mutations in a *Bcl*I recognition site would be cleaved into 1,000 bp and 700 bp fragments by digestion with *Bcl*I. RFLP analysis indicated that some of the plants that survived NAA had mutations in the target site (**Fig. 2**). In applying CRISPR/Cas9 in flowering plants, it was reported that the digested PCR products and the undigested PCR products could be detected simultaneously in the same leaf, because the activity of the CRISPR/Cas9 system in plant cells is not uniform (Feng et al. 2013). However, we did not detect such mixed PCR products from individual samples, suggesting that the NAA-resistant liverworts were not chimeric. Next, direct DNA sequencing analyses were conducted for all NAA-resistant lines (**Fig. 3**; **Supplementary Fig. S2**). We found that five lines out of 45 NAA-resistant $T_1$ transgenic plants had mutations in the target site (**Table 1**). These five mutants showed an undigested pattern with RFLP analysis (**Fig. 2**). The other 40 NAA-resistant $T_1$ transgenic plants had no mutation in the target site. The NAA-resistant phenotypes of these lines were lost when these thalli were transplanted into new media containing 3 μM NAA. These results suggest that the 40 candidates with no mutation were false-positive lines.

**Table 1** Summary of CRISPR/Cas9-based targeted mutation assay for the *ARF1* locus in *M. polymorpha*

| Promoter for *hCas9* | Sequenced | Mutations detected | Mutant name |
|---|---|---|---|
| *35Spro* | 13 | 1 | #1 |
| *35Spro* | 11 | 1 | #7 |
| *MpEFpro* | 1 | 1 | #10 |
| *MpEFpro* | 20 | 2 | #13, #15 |

Each row shows an independent experiment. Numbers of individuals are shown. Lines in which a mutation was detected were reproducibly resistant to NAA medium.

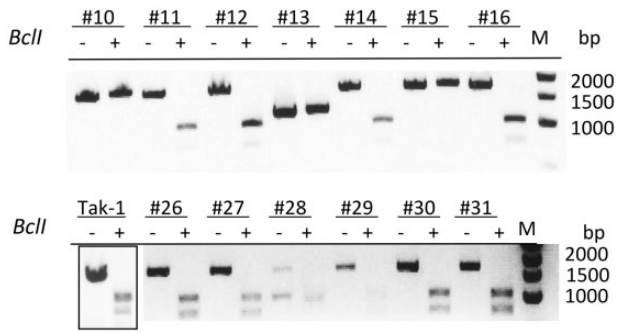


**Fig. 2** RFLP analysis of the *ARF1* locus of NAA-resistant thalli. The expected PCR product size is 1,700 bp, and has a unique *Bcl*I site in the CRISPR/Cas9 target region. M; size marker. Tak-1 was used as the wild type.

In these five mutants, multiple *arf1* mutant alleles were obtained (**Fig. 3**). Three types of deletion mutants were identified: a 3 bp deletion, a 6 bp deletion and a 30 bp deletion, all of which occurred 3 bp upstream of the PAM sequence (**Fig. 3**; **Supplementary Fig. S2**). These results correlate with Cas9 endonuclease cleavage of DNA at preferentially 3 bp upstream of the PAM sequence in vitro (Jinek et al. 2012). Each mutant had a deletion in the amino acid sequence. In addition, a mutation with a 39 bp insertion and a 569 bp deletion was found in mutant #13. This mutant had both a deletion and an insertion of the amino acid sequence. These results indicate that the CRISPR/Cas9 system using *Agrobacterium*-mediated stable transformation can generate NHEJ-based targeted genome modification in the DNA-binding motif of *ARF1* in liverwort.

## Stability of isolated *arf1* mutants

We tested the stability of the mutations generated by CRISPR/Cas9. *M. polymorpha* reproduces asexually by means of bud-like structures called gemmae that originate from single initial

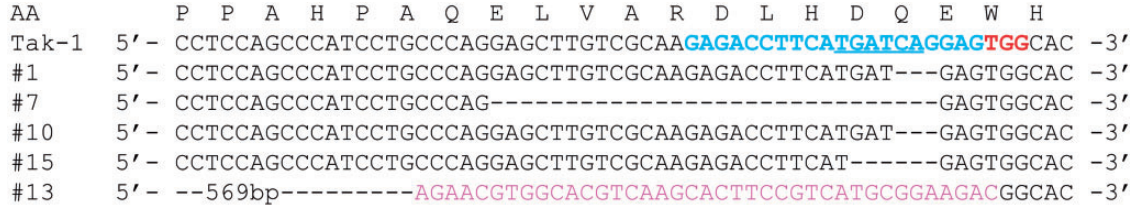


**Fig. 3** Mutations detected by sequencing analysis. The amino acid (AA) sequence encoded by *ARF1* is shown at the top. The wild-type (Tak-1) sequence is shown with the PAM sequence highlighted in red and the target sequence in blue. The *Bcl*I recognition site is underlined. Dashes, deleted bases; pink bases, insertions. #1–15 show the mutants obtained in this study. *hCas9* is driven by *35Spro* in mutants #1 and #7; and by *MpEFpro* in mutants #10, #13 and #15. Mutant #13 has a 569 bp deletion from the PAM sequence.

cells (Barnes and Land 1908, Ishizaki et al. 2012). Therefore, isogenic lines can be established through gemmae. We tested the NAA sensitivity of the gemmae of the mutants generated by the CRISPR/Cas9 system (**Fig. 4**). Gemmae of all the mutants showed an NAA-resistant phenotype, while the wild-type plants showed an NAA response by producing a large number of rhizoids (**Fig. 4**). Isogenic lines derived from $T_1$ gemmae were termed the $G_1$ generation. Sequencing analyses of the $G_1$ generation thalli of mutant #1 showed that the $G_1$ plants of mutant #1 had the same mutation as that of the $T_1$ generation (seven individuals were tested, data not shown). These results indicated that $G_1$ plants generated by the gemmae initial single cells of $T_1$ plants are stable. Gemmae derived from the $G_1$ generation of #1 plants (called $G_2$ plants), and those derived from $G_2$ plants, also had a NAA-resistant phenotype and the same mutation as the $T_1$ thalli (**Supplementary Fig. S3**). These results show that mutations generated in $T_1$ liverworts are fixed through production of subsequent gemmae. Gemmae produced by asexual reproduction of liverworts are useful for making monoclonal mutants using CRISPR/Cas9.

## Discussion

In the present study, we demonstrated CRISPR/Cas9-mediated targeted genome mutagenesis in *M. polymorpha*. Five mutants with targeted mutations in the *ARF1* locus were isolated. *Agrobacterium*-mediated transformation of *M. polymorpha* generates approximately 1,000 stable transformants per one transformation (Ishizaki et al. 2008). In the co-transformation of *M. polymorpha* with two different *Agrobacterium* strains, we observed that >50% of the transformants obtained by a single drug selection contained transgenes from two constructs (data not shown). From the viewpoint of transformation efficiency, hundreds of transformants that express both *hCas9* and *gRNA* would be obtained in one experiment; however, only five mutants were generated in total (**Table 1**). Accordingly, it is essential that the efficiency of CRISPR/Cas9-based targeted mutagenesis methods in *M. polymorpha* is improved.

The amount of gRNA in transformed cells is an important factor for efficient CRISPR/Cas9-based targeted mutagenesis (Wang et al. 2013). We identified *U6* promoters in *M. polymorpha* (**Supplementary Fig. S1**), and used only *MpU6-1pro* to express *gRNA* in liverworts. Therefore, it should be possible to make the CRISPR/Cas9 system more efficient using other *MpU6* promoters. If there were expression specificities in the *MpU6* promoters, tissue-specific mutagenesis could be possible. Mutants were reproducibly obtained using either *35Spro* or *MpEFpro* (**Table 1**). These results indicate that the differences in the expression patterns of *35Spro* and *MpEFpro* are not critical for CRISPR/Cas9-mediated targeted mutagenesis in the case of *ARF1*. We used NAA-based positive selection in addition to hygromycin selection to obtain transformants; therefore, the difference in efficiency between the *35S* promoter and the *MpEF* promoter remains to be investigated. The expression pattern of *Cas9* should be an important factor; for example, *Cas9* driven by a germline-specific promoter greatly improved the efficiency of mutant isolation in *Drosophila melanogaster* (Kondo and Ueda 2013). There is still scope for further promoter optimization of the CRISPR/Cas9 system in *M. polymorpha*.

The amounts of DNA of *hCas9* transformed into cells are also important factors for targeted gene mutagenesis (Cho et al. 2013). To achieve a higher expression level of *Cas9*, codon usage optimization of *Cas9* for *Marchantia* is one of the most practical improvements. In addition, *Agrobacterium*-mediated transformation could deliver less DNA than direct transformation methods, such as electroporation and microinjection. Improving transformation technology is an attractive target for making the CRISPR/Cas9 system more efficient in liverworts.

The CRISPR/Cas9 system makes double-strand breaks in 'off-target' sequences at a higher frequency than TALEN, another genome editing technology (Hsu et al. 2013); therefore, improvements in its specificity are also required. In particular, as *M. polymorpha* L. is not yet fully sequenced, it has the potential to overlook off-target sites. For reducing off-target effects, double nicking methods by nickase-type Cas9, which were recently reported (Ran et al. 2013), should be established in liverworts.

Unexpectedly, direct DNA sequencing analysis of each mutant showed no heterogeneity in the $T_1$ generation (**Figs. 2**, **3**; **Supplementary Fig. S2**), suggesting that these $T_1$ mutants were not chimeric. This result is different from results

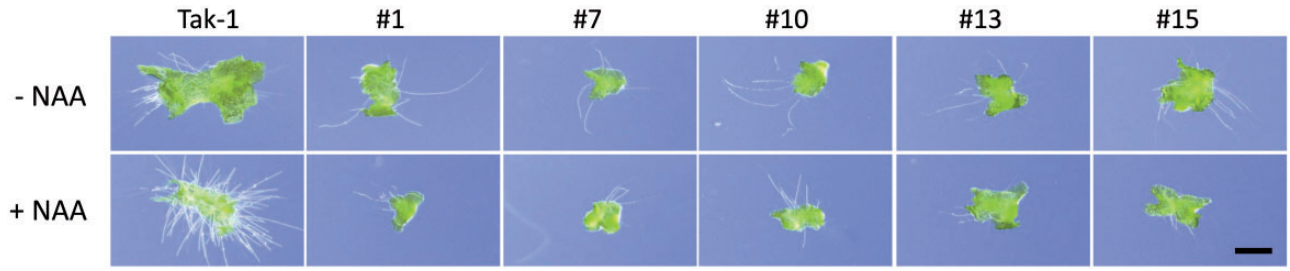


**Fig. 4** Gemmalings of the *arf1* mutants obtained using CRISPR/Cas9. Gemmae were incubated with (lower) or without (upper) 3 μM NAA for 4 d. An NAA response was observed in the +NAA Tak-1 plant. Bar = 1 mm.

for *A. thaliana*, where multiple mutations were induced by CRISPR/Cas9 in each individual (Feng et al. 2013, Mao et al. 2013). Because positive selections by NAA were conducted to screen mutants in the present study, it is possible that NAA treatments killed non-mutated cells in chimeric mutant lines and generated monoclonal mutants derived from single mutated cells. We observed that all the lines from the $G_1$ to $G_3$ generation of the mutants had an NAA-resistant phenotype and had the same mutation as the $T_1$ generation (**Fig. 4**; **Supplementary Fig. S3**). These mutants are probably monoclonal, because the gemmae originated from single initial cells (Barnes and Land 1908, Ishizaki et al. 2012). Theoretically, it should take a long time to obtain monoclonal mutants using CRISPR/Cas9-mediated mutagenesis in diploid dominant flowering plants (Mao et al. 2013); however, our report shows that isogenic mutants can easily be obtained in the haploid generation of *M. polymorpha* using asexual reproduction.

Liverworts have a sister relationship to all other groups of land plants. Accordingly, *M. polymorpha* is a model species for analyzing the evolution of the regulatory systems of land plants (Bowman et al. 2007). To uncover the evolution of the regulatory system in land plants, methods need to be established for the functional analysis of genes in *M. polymorpha*. In this study, we showed that CRISPR/Cas9 is a simple and rapid method for generating mutants with different alleles in liverworts.

The CRIPSR/Cas9 system has other applications in liverworts. CRISPR/Cas9 permits the recovery of multiple alleles in a single experiment. This is advantageous compared with homologous recombination (Ishizaki et al. 2013a), in which independent experiments with different constructs are needed to obtain multiple alleles. In addition, the low frequency of homologous recombination (about 2%) in *M. polymorpha* makes it difficult to generate plants carrying mutations in multiple genes in one step (Ishizaki et al. 2013a). CRISPR/Cas9-mediated targeted mutagenesis has the potential to overcome this problem: the CRISPR/Cas9 system has been successfully applied to make multiple knockout mutants in mice (Wang et al. 2013). Moreover, the CRISPR/Cas9 system might be applied to generate knock-in plants (tags or fluorescent reporter fusions in genomic loci) and conditional-mutant plants (e.g. using the Cre/loxP system), as has been done in animals (Wang et al. 2013).

Combining our results and CRISPR/Cas9 applications in flowering plants (Belhaj et al. 2013), we believe that the methodology described here could be applied to other land plants, including non-model species. Our results suggest that CRISPR/Cas9 systems have great potential application for a wide variety of land plants, and will permit functional molecular genetic studies of non-model plant organisms.

## Materials and Methods

### Accessions, growth conditions and transformation of *M. polymorpha*

*Marchantia polymorpha* Takaragaike-1 (Tak-1, male accession) and Takaragaike-2 (Tak-2, female accession) were used as wild types (Ishizaki et al. 2008). *M. polymorpha* was cultured asexually under 50–60 μmol $m^{-2}$ $s^{-1}$ continuous white light at 22°C. $F_1$ spores generated by crossing Tak-2 and Tak-1 were used for transformation. Formation of sexual organs was induced by far-red irradiation, as described previously (Chiyoda et al. 2008). Mature sporangia were collected 3–4 weeks after crossing, air-dried for 7–10 d and stored at −80°C until use. *Agrobacterium*-mediated transformation of $F_1$ sporelings was performed as described previously (Ishizaki et al. 2008). Transformants were selected on half-strength B5 agar medium containing 1% agar, 10 mg $l^{-1}$ hygromycin (Wako Pure Chemical Industries), 100 mg $l^{-1}$ cefotaxime (Claforan; Sanofi-Aventis) and 3 μM NAA (Nacalai Tesque).

### Vector construction

Gateway Cloning Technology (Life Technologies) was used to construct *MpU6-1pro:gRNA_ARF1*, *35Spro:hCas9* and *MpEFpro:hCas9*. The search for *M. polymorpha U6* gene homologs was performed using transcriptome data and genome databases for *M. polymorpha* and the on-going Joint Genome Institute genome-sequencing project (http://www.jgi.doe.gov/). The complete sequence of the *MpU6-1* promoter used in this study is deposited in the DNA Data Bank of Japan DDBJ (AB902572). To generate *MpU6-1:gRNA_ARF1*, a 2 kb promoter region of *MpU6-1* was amplified from Tak-1 genomic DNA using primers Mp-U6_38003_F, 5′-CACCTATTCATTCA AAAGAGATTTTTAAAGATC-3′ and MpU6_gRNA_ARF1_R,

5′-CTCCTGATCATGAAGGTCTCGAGAGGCTGGGTGCAAC-3′. *gRNA_ARF1* was amplified from Plasmid 41824: gRNA_Cloning Vector (Addgene) using a primer set of gRNA_ARF1_F, 5′-GAGA CCTTCATGATCAGGAGGTTTTAGAGCTAGAAATAGC-3′ and gRNA-R3, 5′-TAGAAAAAAAGCACCGACTCGGTG-3′. Amplified fragments were fused by PCR, cloned into pENTR/D-TOPO (Life Technologies) and used to generate a binary vector harboring *MpU6:gRNA_ARF1*. *hCas9* was amplified from Plasmid 41815: hCas9 (Addgene) using a primer set of cacc-Cas9-F, 5′-CACCAT GGACAAGAAGTACTCCATTGG-3′ and Cas9-stop-R, 5′-TCACAC CTTCCTCTTCTTCTTGGGG-3′, cloned into pENTR/D-TOPO and used to generate binary vectors harboring *35Spro:hCas9* and *MpEFpro:hCas9*.

### RFLP

DNA was extracted from the thalli of plants that survived NAA treatment. The *ARF1* target locus was PCR amplified using primers ARF1_Seq_F3, 5′-GCCGATGTGCATATACCCAGCTATC CCAGT-3′ and ARF1_Seq_R3, 5′-ATGTTATATCCTCGGTTGA TTCTCGTACGA-3′, and subjected to a *Bcl*I restriction enzyme reaction. PCR products were analyzed by agarose gel electrophoresis.

## Supplementary data

**Supplementary data** are available at PCP online.

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## Disclosures

The authors have no conflicts of interest to declare.

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