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Homologous recombination-mediated gene targeting in the liverwort *Marchantia polymorpha* L.

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The liverwort *Marchantia polymorpha* is an emerging model organism on account of its ideal characteristics for molecular genetics in addition to occupying a crucial position in the evolution of land plants. Here we describe a method for gene targeting by applying a positive/negative selection system for reduction of non-homologous random integration to an efficient *Agrobacterium*-mediated transformation system using *M. polymorpha* sporelings. The targeting efficiency was evaluated by knocking out the *NOP1* gene, which impaired air-chamber formation. Homologous recombination was observed in about 2% of the thalli that passed the positive/negative selection. With the advantage of utilizing the haploid gametophytic generation, this strategy should facilitate further molecular genetic analysis of *M. polymorpha*, in which many of the mechanisms found in land plants are conserved, yet in a less complex form.

The emergence of land plants from an aquatic ancestor was one of the most critical evolutionary events for life on Earth. The most recent molecular phylogenetic analyses strongly support the sister relationship of the charophycean green algae to land plants, and liverworts to all other extant land plants[1,2]. Therefore, liverworts are considered to be a key group to understand the genetic basis of the critical innovations that allowed green plants to evolve from an aquatic ancestor and to adapt to the terrestrial environment[3].

Marchantia polymorpha is a dioecious liverwort species. The haploid generation is dominant in the life cycle, which provides advantages over diploid vascular plants for genetic analysis. *M. polymorpha* reproduces not only sexually but also asexually by means of bud-like structures called gemmae, which allows rapid propagation of isogenic biomass for molecular and biochemical experiments. Furthermore, an ongoing *M. polymorpha* genome sequencing project under the Community Sequencing Program at the Joint Genome Institute indicates that many of the biological mechanisms found in other land plants are conserved in a less complex form (http://www.jgi.doe.gov/sequencing/why/CSP2008/mpolymorpha.html)[4,5]. Thus, *M. polymorpha* is an emerging model organism, occupying a critical evolutionary position, with which to study specific molecular and cellular developmental processes in detail[4–8].

Gene targeting mediated by homologous recombination (HR) is a powerful tool for functional analysis by reverse genetics. This technique is well established not only in bacteria and unicellular eukaryotes, but also in some multicellular model organisms such as fly[9] and mouse[10]. However, gene targeting by HR is still difficult in most plants[11,12], with the exception of the moss *Physcomitrella patens*, in which gene targeting by HR[13] is highly efficient. In many other plants, integration of a transgene by HR occurs in the order of 10^{-3} to 10^{-6} compared with random integration (RI)[14–18], and the overwhelming occurrence of RI of transgenes by non-homologous end-joining relative to targeted HR is a major obstacle for efficient gene targeting in plants. To overcome this problem, a reproducible gene-targeting procedure was developed in the monocot rice using a positive (the hygromycin-resistance gene, *hpt*)/negative (the diphtheria toxin A fragment gene, *DT-A*) selection system[12,19]. DT-A exerts cellular toxicity by ADP-ribosylation of elongation factor 2 upon protein synthesis[20]. A large number of stable transformants (10^4 to 10^5) must be generated to obtain gene-targeted lines because of the high background of the positive/negative selection system, and a highly efficient transformation system is a critical factor for the success of such a gene targeting system[21].

We recently developed a simple and rapid *Agrobacterium*-mediated transformation system for *M. polymorpha*[22]. Hundreds of stable transformants per sporangium can be obtained using sporelings (immature thalli developed from spores). The large number of transformants obtained per experiment using *M. polymorpha* is

advantageous for gene targeting based on a positive/negative selection system. We selected *NOPPERABO1* (*NOP1*) as a model target gene for evaluation of HR efficiency, because a visible but non-selective phenotype is shown when the gene is mutated. Twenty lines of 930 obtained hygromycin-resistant (Hm^r) transformants showed the expected phenotype, and their *NOP1* loci were successfully disrupted by HR. This gene-targeting strategy opens the door for systematic functional genomics in the basal land plant *M. polymorpha*, which has a haploid-dominant life cycle.

Results

Effectiveness of the positive/negative selection. To efficiently detect rare events of HR in *M. polymorpha*, we employed a strong positive/negative selection system developed for rice[12]. We modified the rice targeting vector pINA134 to construct pJHY-TMp1, in which the hygromycin phosphotransferase (*hpt*) gene was driven by the promoter of the *M. polymorpha* elongation factor 1 alpha (*EF1a*) gene instead of the rice *Actin1* promoter (Fig. 1a). A control vector, pJHY-CMp1, carried the same *hpt* cassette as pJHY-TMp1 in the T-DNA region but lacked the *DT-A* genes (Fig. 1a). To evaluate the efficiency of the negative selection by the *DT-A* gene in *M. polymorpha*, we compared the transformation efficiency of pJHY-CMp1 and pJHY-TMp1. Each sporangium contained 2–3 $\times$ 10^5 spores in *M. polymorpha*, and 10–20% of the spores germinated after imbibition in our laboratory conditions. With pJHY-TMp1, only a few dozen Hm^r transformants per sporangium survived the positive/negative selection, whereas over 1,000 Hm^r transformants per sporangium were obtained using pJHY-CMp1 (Table 1). These results indicated that positive/negative selection with the *hpt* and *DT-A* genes was applicable to *M. polymorpha*.

Targeted gene knockout by homologous recombination. As a model gene to target we chose *NOP1*, which is predicted to encode a plant U-box (PUB) type E3 ligase and acts as a critical positive regulator for air-chamber development in *M. polymorpha* (Ishizaki et al. in preparation). We selected *NOP1* for the first trial of gene targeting in *M. polymorpha* because the defective gene confers an obvious morphological phenotype that can easily be assessed by visual observation but does not offer any selective disadvantages. The *NOP1* gene comprises four exons, of which exon 2 carries the ATG initiation codon. Our targeting strategy was to disrupt *NOP1* by replacing a 116 bp portion of exon 2 with an expression cassette for antibiotics resistance. The targeting vector, pKI406, was a pJHY-TMp1 derivative in which *hpt-ΔEn* is flanked by the 3.6 kb *NOP1* upstream region containing the promoter region, first intron and 5′ untranslated region and the 3.5 kb *NOP1* coding regions including two intron regions (Fig. 1b and c). HR between the *NOP1* sequences was expected to result in substitution of the 116 bp sequence in the *NOP1* exon 1 with *hpt-ΔEn*, without integrating the flanking *DT-A* genes (Fig. 1d).

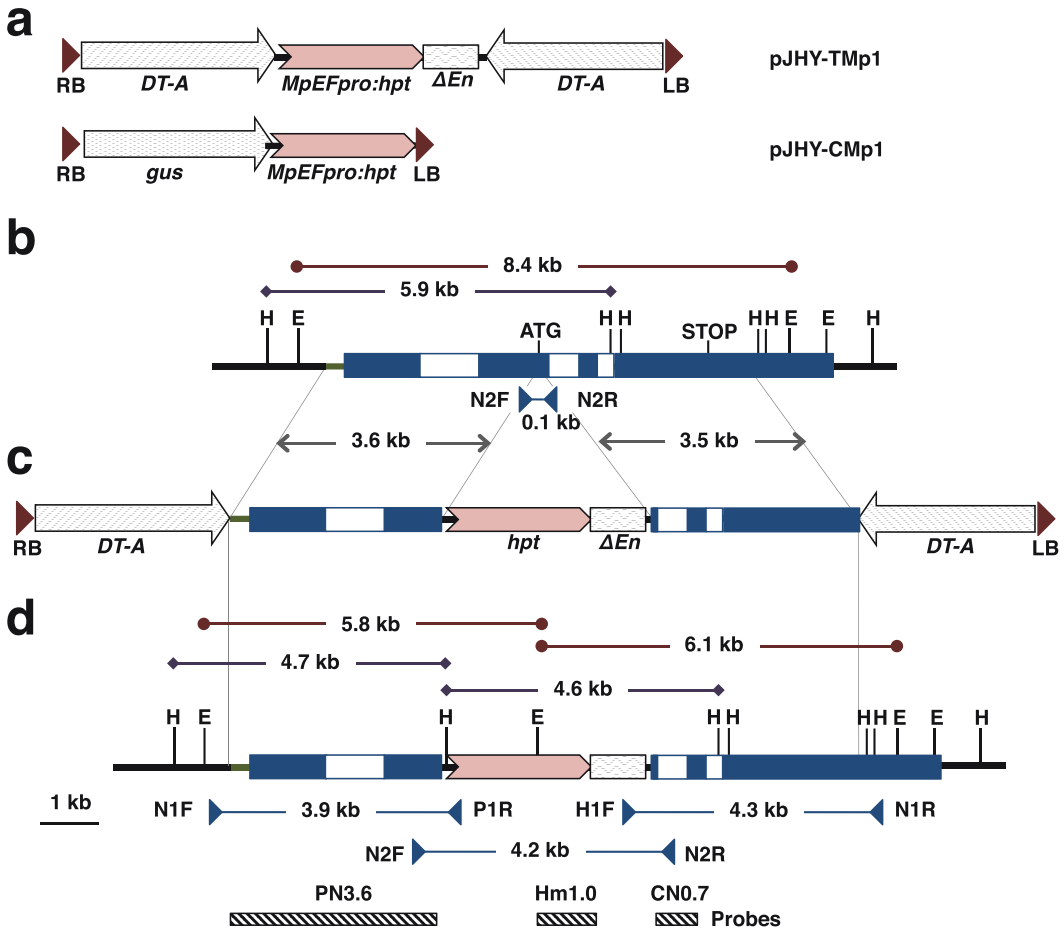

Figure 1 | Strategy for targeted disruption of the *NOP1* locus and analysis of homologous recombination events. (a) Structure of pJHY-TMp1 and pJHY-CMp1. Only the structure between the left and right borders (LB and RB, respectively) of each vector is shown. *DT-A*, Diphtheria toxin gene; *hpt*, hygromycin phosphotransferase gene; Δ*En*, 3′ part of the maize *En* element; *gus*, β-glucuronidase gene. (b) Schematic representation of the genomic structure of the wild-type *NOP1* gene region. (c) Structure of pKI406. (d) Structure of the *NOP1* locus disrupted by HR. The exons and introns of the *NOP1* gene are indicated by blue and open boxes, respectively. The green bar represents the *NOP1* flanking region carried by pKI406. Primers are indicated by triangles. Recognition sites of *Eco*RI and *Hin*dIII are indicated by vertical lines with the letters E and H, respectively.

Table 1 | Numbers of Hm^r plants obtained by positive/negative selection and targeted events at the *NOP1* locus

Experiment	Vector	Number of Hm^r plants	Plants with the *nop1* phenotype
A[1)]	pJHY-CMp1	1424	0
B[1)]	pJHY-CMp1	1168	0
C[1)]	pJHY-CMp1	1312	0
D[1)]	pJHY-TMp1	7	0
E[1)]	pJHY-TMp1	6	0
F[1)]	pJHY-TMp1	22	0
G[2)]	pKI406	276	4
H[2)]	pKI406	172	4
I[2)]	pKI406	195	7
J[2)]	pKI406	220	3
K[2)]	pKI406	67	2

[1)]Each transformation was performed using a sporangium, and transformation efficiency per sporangium was estimated.
[2)]Transformation was performed using 4 sporangia.

When pKI406 was used for transformation, an average of 186 Hm^r thalli were obtained from each of five independent transformation experiments using four sporangia (Table 1). Of 930 Hm^r transformants in total, 20 independent lines (#1 to #20) showed impaired air-chamber formation (Fig. 2) in the T_1 generation, which was indistinguishable from that of the original *NOP1* loss-of-function mutant (Ishizaki et al. in preparation). The loss-of-air-chamber phenotype in line #1 (Fig. 2b) was rescued by introduction of the *NOP1* gene (Fig. 2c), which indicated that the phenotype was caused by targeted disruption of *NOP1*. To examine if the expected HR had occurred in all of the 20 lines, we employed genomic PCR with the three pairs of primers shown in Figure 1D. In all of the lines, but not in the wild type, fragments of the size expected for the HR sites (3.9 kb and 4.3 kb) were amplified by the primer pairs N1F/P1R and H1F/N1R, respectively (Figs. 1d and 2d), suggesting the occurrence of correct crossovers at both sides and eliminating the possibility of one-sided invasion. Furthermore, only a 4.2 kb fragment containing *hpt-ΔEn* was amplified in the 20 lines, whereas a 0.5 kb fragment was amplified from the wild-type *NOP1* locus (Fig. 2d), indicating successful modification of the targeted *NOP1* locus.

To confirm that HR had occurred as expected, Southern blot analysis was performed using genomic DNA of the wild type and two lines, #5 and #9, randomly selected from among the 20 lines. In the *Eco*RI digests of the two selected lines, only 5.8 and 6.1 kb fragments corresponding to the disrupted *NOP1* locus (Fig. 1d), but not the 8.4 kb wild-type fragment (Fig. 1b), were detected (Fig. 3a). A similar result was obtained for the *Hin*dIII digests (Fig. 3b). These results indicated that *hpt-ΔEn* was integrated only into the *NOP1* locus in the haploid genome of the two lines examined.

Assuming the *NOP1* locus was disrupted also in the remaining 18 lines, the frequency of HR between pKI406 and the *NOP1* locus was approximately 2% of the Hm^r transformants that survived the positive/negative selection (Table 1).

Evaluation of gene targeting efficiency using vectors containing homologous arms of various lengths and positions. In order to optimize the design of targeting constructs, a series of vectors containing homologous arms of various lengths and inter-arm distances were constructed for comparison. The targeting vectors, pKI406-1kb and pKI406-2kb, contained shorter homologous arms, 1 and 2 kb, respectively, than those of the original vector, pKI406 (Fig. 4). The distances between the homologous arms of pKI406-d1kb and pKI406-d3kb, 1 and 3 kb, respectively, were larger than 0.1 kb of pKI406 (Fig. 4). When pKI406-1kb and pKI406-2kb were used for transformation, less than 0.015% and 0.7%, respectively, of resulting Hm^r transformants showed the *nop1* phenotype, whereas 3.2% showed the *nop1* phenotype with pKI406 (Table 2). Similarly, 1.0% and 0.1% of resulting Hm^r transformants showed the *nop1* phenotype with pKI406-d1kb and pKI406-d3kb, respectively (Table 2). These results suggest that, in practice, the length of

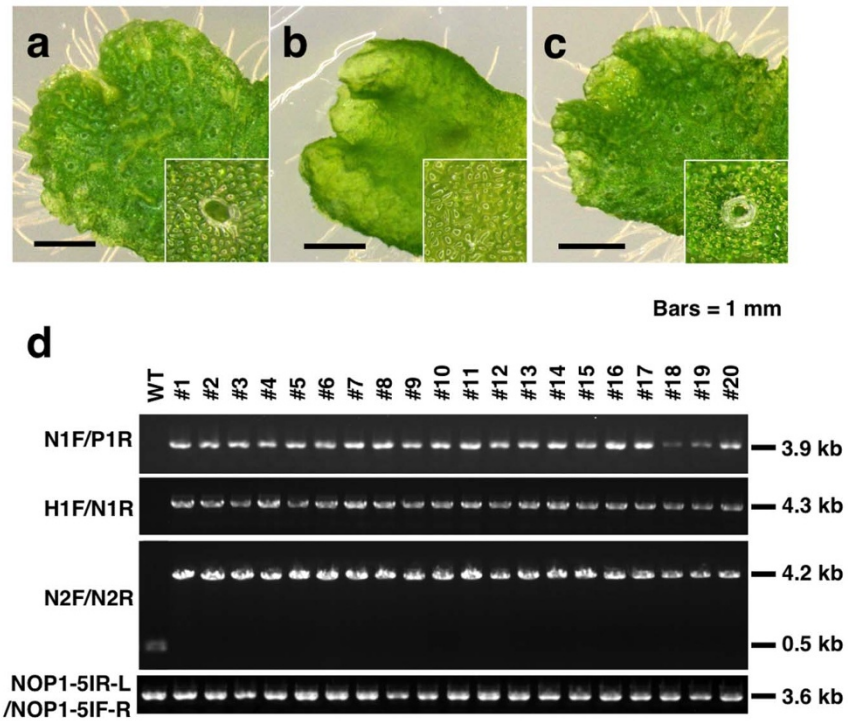

Figure 2 | Phenotype and genotype of targeted transformants. (a–c) The dorsal surface of the thallus of the wild type (a), transformant #1 (b), and the complemented transformant #1 in which the *NOP1* genomic fragment was introduced. (c). Nineteen additional lines (#2 to #20) showed the same phenotype as #1. (d) Genotyping of the *nop1* lines. The positions of the primers used for PCR analysis are shown in Figure 1.

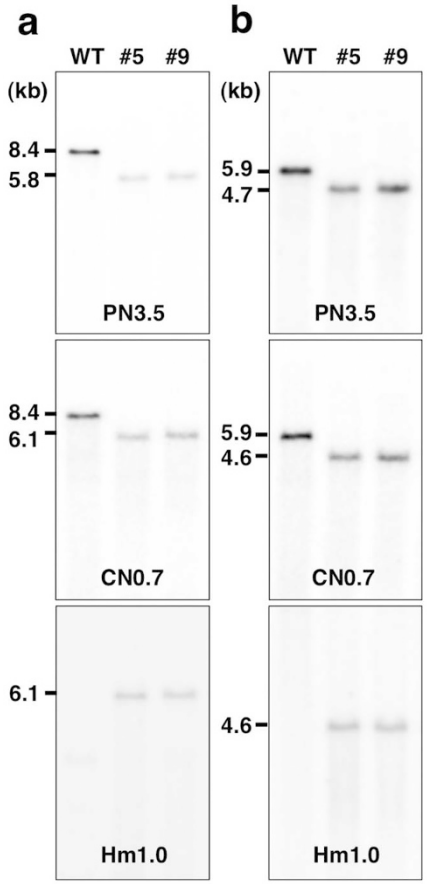

Figure 3 | Southern blot analysis of the *NOP1* locus and the integrated *hpt* sequence in the wild type and the two independent Hm^r lines that showed impaired air-chamber formation. *Eco*RI (a) and *Hin*dIII (b) digests. The probes used and the restriction map of this locus are illustrated in Figure 1.

homologous arms in the targeting vector should be larger than 2 kb, and the distance between homologous arms should not exceed 1 kb for gene targeting.

Discussion

We established a reproducible procedure for gene targeting in the liverwort *M. polymorpha* by application of a strong positive/negative selection system developed for rice[12]. The observed targeting frequency in *M. polymorpha*, as determined from the ratio of the number of targeted transformants resulting from HR to the number of transformants resulting from RI of the Hm^r marker of pJHY-CMp1, was estimated to be 7.7×10^{-4} (Table 1), which is comparable to the frequencies previously reported (10^{-3} to 10^{-6})[14–16]. Therefore, high-yield *Agrobacterium*-mediated transformation by which thousands of transformants can be generated makes it feasible to isolate transformants resulting from HR. In *M. polymorpha*, it is possible to introduce a fragment of DNA, with a frequency sufficient for isolation of rare HR transformants, by the use of only a few sporangia. We recommend, in practice, to start with 10–20 sporangia to obtain a sufficient number of candidates for gene-targeting constructs. The whole procedure from initiation of sporeling culture to identification of targeted transformants took only 5–6 weeks. In practice, further culture for several weeks is required to establish isogenic lines (the G_1 generation) that originate from single gemma-initial cells.

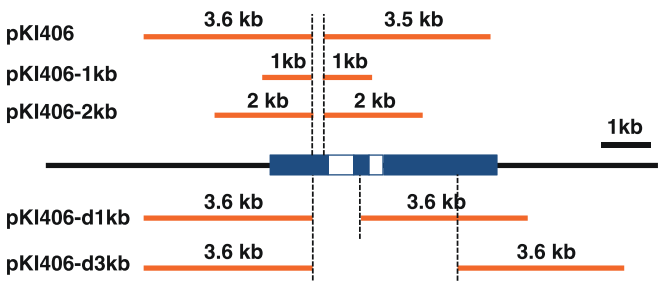

Figure 4 | Constructs for optimization of gene-targeting in *M. polymorpha*. The exons and introns of the wild-type *NOP1* gene are represented by blue and open boxes, respectively. Positions and length of homologous arms cloned in the binary vectors are indicated as orange bars.

The positive/negative selection reduced the number of false positives, and up to 3% of the selected transformants turned out to be correctly targeted as examined by genomic PCR and Southern blot analysis (Figs. 2 and 3). In the similar gene targeting system established in rice, the majority of the surviving calli with the positive/negative selection contained truncated T-DNA molecules mediated by border-independent RI, whereas the most efficient RI of T-DNA mediated by border-associated RI would result in the death of transformed calli caused by the intact *DT-A* gene[19]. There is also a possibility of the event called ectopic gene-targeting (EGT), which is also generated by HR-promoted crossover between the transgene and the target sequence, and subsequent RI of the resulting recombinant molecule[19]. The target gene remains intact in both RI and EGT. In the case of the present study on *M. polymorpha*, the *NOP1* locus is expected to be intact in the remainder of the Hm^r transformants because they did not show the expected phenotype, and the *hpt* cassette would be integrated either by border-independent RI or EGT.

The length of homologous arms and the inter-arm distances were critical factors for successful gene-targeting in *M. polymorpha* (Fig. 4 and Table 2). Similarly, in mammalian cells and the moss *P. patens*, an increase in the length of homologous arms resulted in a dramatic increase in targeting efficiency[23,24]. The gene targeting is regarded to occur via double crossovers in the *hpt*-flanking homologous regions on the vector[19]. A longer homologous arm would be more effective to search for the target locus and form a crossover structure. A Short inter-arm region would enhance the occurrence of crossovers at both homologous arms. As the distance between homologous arms should not exceed 1 kb for gene targeting in practice, it is generally difficult to design a knockout construct that will eliminate the entire protein coding region in a target gene. Therefore, in *M. polymorpha* the recommended design of a construct for targeted gene knockout is to interrupt gene structure with the *hpt* cassette. The position of the short inter-arm region should be located at an ATG codon to cancel the translation, or at a domain critical for the function of the target gene.

In *M. polymorpha*, gene-targeted or knockout transformants can be established immediately after transformation of gametophytes owing to their haploid status. Gene-targeted transformants can be readily screened by PCR or visual examination of their phenotypes in the T_1 generation. The method described here is, in principle, applicable to any gene because it does not involve gene-specific selection. Indeed, we have succeeded with application of this strategy to disrupt the other endogenous genes (unpublished data). However, disruption of some endogenous genes would lead to lethality in a haploid system, thus generating no targeted plants. In this respect, development of a conditional knockout strategy based on an inducible site-specific recombination system, such as Cre/lox[25] with inducibility, will expand the potential of this gene-targeting strategy in *M. polymorpha*.

Gene targeting mediated by HR should be useful not only for generation of knockouts but also for modification of targeted sequences and fusion of tags by knock-in. The liverwort *M. polymorpha* has

Table 2 | Evaluation of targeting efficiency for *NOP1* locus using vectors containing various homologous arms

Experiment	Vector	Number of Hm^r plants[1)]	Number of plants with *nop1* phenotype	Targeting efficiency
A	pKI406	163	7	
B	pKI406	210	5	3.2% (12/373)
C	pKI406-1kb	231	0	
D	pKI406-1kb	273	0	
E	pKI406-1kb	175	0	<0.015% (0/679)
F	pKI406-2kb	372	2	
G	pKI406-2kb	194	2	
H	pKI406-2kb	250	2	0.7% (6/816)
E	pKI406-d1kb	333	4	
F	pKI406-d1kb	319	4	
G	pKI406-d1kb	316	2	1.0% (10/968)
H	pKI406-d3kb	408	0	
E	pKI406-d3kb	397	1	
F	pKI406-d3kb	203	0	0.1% (1/1008)

[1)]Transformation was performed using 4 sporangia.

substantial potential as a model system for plant biology on account of its critical phylogenetic position in the evolution of land plants, its conserved yet less complex developmental pattern and responses to plant growth factors and environmental stimuli, and its gametophyte-dominant life cycle, which renders genetic analyses less complicated. HR-mediated gene targeting together with the ongoing genome sequencing project should accelerate functional analysis of genes in *M. polymorpha* and shed light on the evolution and diversity of regulatory systems in land plants.

Methods

Plant material and growth conditions. Male and female accessions of *M. polymorpha*, Takaragaike-1 and Takaragaike-2, respectively, were maintained asexually[22]. F_1 spores generated by crossing Takaragaike-1 and Takaragaike-2 were used for transformation. Formation of sexual organs was induced by far-red irradiation as described previously[26]. Mature sporangia were collected 3–4 weeks after crossing, air-dried for 7–10 d, and stored at −80°C until use.

Plants were cultured using half-strength Gamborg's B5 medium[27] containing 1% agar under 50–60 μmol photons m^{-2} s^{-1} continuous white fluorescent light at 22°C unless otherwise defined.

Vector construction for gene targeting. The vectors pJHY-CMp1 and pJHY-TMp1 were derivatives of pRIT1 and pINA134[12], respectively. To generate pJHY-CMp1 and pJHY-TMp1, the rice *Actin I* promoter driving *hpt* expression in pRIT1 and pINA134 was replaced with an endogenous promoter of the *M. polymorpha* elongation factor 1α (*EF1a*) gene. To generate the *NOP1*-targeting vector, pKI406, the 5′- and 3′-homologous arms (3.6 kb and 3.5 kb, respectively) were amplified from Takaragaike-1 genomic DNA by PCR using KOD FX Neo (Toyobo Co., Ltd., Osaka, Japan) with primer pairs NOP1-5IF-L/NOP1-5IF-R and NOP1-3IF-L/NOP1-3IF-R, respectively. The PCR-amplified 5′- and 3′-homologous arms were cloned into the *Pac*I and *Asc*I sites, respectively, of pJHY-TMp1 with the In-Fusion HD cloning kit (Clontech, Mountain View, CA).

In order to generate a series of *NOP1*-targeting vectors with homologous arms of different lengths and inter-arm distances, 5′- and 3′-homologous arms were amplified by PCR using the following primer pairs and cloned into the *Pac*I and *Asc*I sites of pJHY-TMp1, respectively, as in the construction of pKI406. For pKI406-1kb, the 5′- and 3′-homologous arms (1.1 and 1.2 kb, respectively) were amplified using primer pairs NOP1-5IF-L-1kb/NOP1-5IF-R and NOP1-3IF-L/NOP1-3IF-R-1kb, respectively. For pKI406-2kb, the 5′- and 3′-homologous arms (2.1 and 2.0 kb, respectively) were amplified using primer pairs NOP1-5IF-L-2kb/NOP1-5IF-R and NOP1-3IF-L/NOP1-3IF-R-2kb, respectively. The 5′-homologous arms of pKI406-d1kb and pKI406-d3kb were the same as that of pKI406, and their 3′-homologous arms (3.6 kb for each) were amplified using primer pairs NOP1-3IF-L-d1kb/NOP1-3IF-R-d1kb and NOP1-3IF-L-d3kb/NOP1-3IF-R-d3kb, respectively.

Sequences of all primers used in this study are given in Supplementary Table S1.

Introduction of targeting constructs into *M. polymorpha*. *Agrobacterium tumefaciens* strain C58C1 (GV2260) was used for transformation. Transformation of *M. polymorpha* was performed as previously described[22] with minor modifications. After co-cultivation with *A. tumefaciens*, sporelings were transferred to half-strength Gamborg's B5 agar medium containing 1% agar, 10 mg l^{-1} hygromycin (Wako Pure Chemical Industries, Osaka, Japan) and 100 mg l^{-1} cefotaxime (Claforan, Sanofi-Aventis K. K., Tokyo, Japan). Independent T_1 lines were transferred to fresh selection medium, grown for a further 2 weeks, and screened for gene-targeted lines by visual examination of phenotypes and also by genotyping as described below. Isogenic lines (the G_1 generation) were established from T_1 lines using gemmae that arose from single cells as described previously[7]. Plants grown from gemmae of G_1 lines (the G_2 generation) were used for further confirmation of targeted disruption of the *NOP1* locus.

Complementation of *NOP1* targeted lines. For complementation a binary vector, pMpGWB301 (manuscript in preparation), harbouring a mutated *ALS* gene (*mALS*) that confers chlorosulfuron resistance was used. For construction of a plasmid containing the *NOP1* genomic fragment, the promoter and coding region were amplified by PCR using the primer sets ProL/ProR and gCDSL/gCDSR, respectively. These PCR fragments were cloned into pENTR/D-TOPO (Life Technologies, Carlsbad, CA) to generate pAM101 for the promoter and pAM102 for the coding region of the *NOP1* genomic fragment, respectively. The *Avr*II-*Asc*I DNA fragment of pAM101 was subcloned into the *Avr*II-*Asc*I site of pAM102 to generate the plasmid pAM103, which contained the *NOP1* genomic fragment from 5.7 kb upstream of ATG to 0.2 kb downstream of the 3′UTR. The resultant *NOP1* cassette was subcloned into pMpGWB301 using LR clonase (Life Technologies) in accordance with the manufacturer's protocol. The resulting plasmid was transformed into regenerating thalli of the *NOP1* targeted line #1 by the method described previously[28]. Selection was conducted with 0.5 μM chlorosulfuron (DuPont, Wilmington, DE).

Genotyping. Small pieces (3 × 3 mm) of thalli were taken from individual plants and crushed with a micro-pestle in 100 μl buffer containing 100 mM Tris-HCl, 1 M KCl, and 10 mM EDTA (pH 9.5). Sterilized water (400 μl) was added to each tube and 1 μl aliquot of the extract was used as a template for PCR using KOD FX Neo DNA polymerase (Toyobo). The PCR conditions were 96°C for 2 min, followed by 35 cycles of 98°C for 10 s, 68°C for 4 min, and final extension at 72°C for 5 min. Primers used are shown in Supplementary Table S1.

Southern blot analysis. Total DNA for Southern blot analysis was extracted from approximately 5 g fresh weight of tissue with a cetyltrimethylammonium bromide (CTAB) method[29] with modifications. The tissue was frozen in liquid nitrogen and disrupted with a mortar and pestle. Disrupted cells were extracted with 10 ml CTAB buffer (1.5% CTAB, 75 mM Tris-HCl [pH 8.0], 15 mM EDTA, and 1.05 M NaCl) for 20 min at 56°C. The extract was mixed thoroughly with an equal volume of chloroform–isoamylalcohol (24 : 1, v/v) for 20 min at room temperature. After centrifugation at 4,000 × *g* for 20 min at 20°C, the aqueous phase was transferred to a fresh plastic tube and mixed with an equal volume of chloroform–isoamylalcohol (24 : 1, v/v) for 20 min at room temperature. After centrifugation at 4,000 × *g* for 20 min at 20°C, the aqueous phase was mixed slowly with 1.5 volume of CTAB precipitation buffer (1% CTAB, 50 mM Tris-HCl [pH 8.0], and 10 mM EDTA). After centrifugation at 10,000 × *g* for 30 min at 20°C, the precipitate was dissolved in 1 M sodium chloride supplemented with RNaseA (final 10 μg ml^{-1}) and incubated for 30 min at 37°C. DNA was precipitated with ethanol and dissolved in TE buffer.

For Southern blot analyses, 2 μg genomic DNA was digested overnight with *Eco*RI and *Hin*dIII at 37°C. The DNA was fractionated by electrophoresis on a 0.8% (w/v) agarose gel and blotted onto a positively charged nylon membrane Biodyne A (PALL, Port Washington, NY). The probes PN3.6 (3,624 bp), Hm1.0 (1,000 bp), and CN0.7 (700 bp) were amplified by PCR with the primer pairs NOP1-5IF-L/NOP1-5IF-R, Hm1.0-F/Hm1.0-R, and CN0.7-F/CN0.7-R, respectively. The blotted membranes were hybridized in Church hybridization buffer[30] at 65°C with the probes labelled with [α-^{32}P] dCTP by random priming using the Random Primer Labeling Kit Ver. 2

(Takara, Shiga, Japan). Washing and analysis of the blots was performed as described previously[26].

1. Turmel, M., Otis, C. & Lemieux, C. The mitochondrial genome of *Chara vulgaris*: insights into the mitochondrial DNA architecture of the last common ancestor of green algae and land plants. *Plant Cell* **15**, 1888–1903 (2003).
2. Qiu, Y. L. *et al*. The deepest divergences in land plants inferred from phylogenomic evidence. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 15511–15516 (2006).
3. Bowman, J. L., Floyd, S. K. & Sakakibara, K. Green genes-comparative genomics of the green branch of life. *Cell* **129**, 229–234 (2007).
4. Zobell, O., Faigl, W., Saedler, H. & Munster, T. MIKC* MADS-box proteins: conserved regulators of the gametophytic generation of land plants. *Mol. Biol. Evol.* **27**, 1201–1211 (2010).
5. Ueda, M. *et al*. Composition and physiological function of the chloroplast NADH dehydrogenase-like complex in *Marchantia polymorpha*. *Plant J.* **72**, 683–693 (2012).
6. Tougane, K. *et al*. Evolutionarily conserved regulatory mechanisms of abscisic acid signaling in land plants: characterization of ABSCISIC ACID INSENSITIVE1-like type 2C protein phosphatase in the liverwort *Marchantia polymorpha*. *Plant Physiol.* **152**, 1529–1543 (2010).
7. Ishizaki, K., Nonomura, M., Kato, H., Yamato, K. T. & Kohchi, T. Visualization of auxin-mediated transcriptional activation using a common auxin-responsive reporter system in the liverwort *Marchantia polymorpha*. *J. Plant Res.* **125**, 643–651 (2012).
8. Okumura, M. *et al*. Characterization of the plasma membrane H^+-ATPase in the liverwort *Marchantia polymorpha*. *Plant Physiol.* **159**, 826–834 (2012).
9. Rong, Y. S. & Golic, K. G. Gene targeting by homologous recombination in Drosophila. *Science* **288**, 2013–2018 (2000).
10. Capecchi, M. R. Altering the genome by homologous recombination. *Science* **244**, 1288–1292 (1989).
11. Puchta, H. Gene replacement by homologous recombination in plants. *Plant Mol. Biol.* **48**, 173–182 (2002).
12. Terada, R., Urawa, H., Inagaki, Y., Tsugane, K. & Iida, S. Efficient gene targeting by homologous recombination in rice. *Nat. Biotechnol.* **20**, 1030–1034 (2002).
13. Schaefer, D. G. & Zryd, J. P. Efficient gene targeting in the moss *Physcomitrella patens*. *Plant J.* **11**, 1195–1206 (1997).
14. Paszkowski, J., Baur, M., Bogucki, A. & Potrykus, I. Gene targeting in plants. *EMBO J.* **7**, 4021–4026 (1988).
15. Risseeuw, E., Offringa, R., Franke-van Dijk, M. E. & Hooykaas, P. J. Targeted recombination in plants using *Agrobacterium* coincides with additional rearrangements at the target locus. *Plant J.* **7**, 109–119 (1995).
16. Hanin, M. *et al*. Gene targeting in *Arabidopsis*. *Plant J.* **28**, 671–677 (2001).
17. Hanin, M. & Paszkowski, J. Plant genome modification by homologous recombination. *Curr. Opin. Plant Biol.* **6**, 157–162 (2003).
18. Iida, S. & Terada, R. A tale of two integrations, transgene and T-DNA: gene targeting by homologous recombination in rice. *Curr. Opin. Biotechnol.* **15**, 132–138 (2004).
19. Terada, R., Johzuka-Hisatomi, Y., Saitoh, M., Asao, H. & Iida, S. Gene targeting by homologous recombination as a biotechnological tool for rice functional genomics. *Plant Physiol.* **144**, 846–856 (2007).
20. Pappenheimer, A. M., Jr. Diphtheria toxin. *Annu. Rev. Biochem.* **46**, 69–94 (1977).
21. Terada, R., Asao, H. & Iida, S. A large-scale *Agrobacterium*-mediated transformation procedure with a strong positive-negative selection for gene targeting in rice (Oryza sativa L.). *Plant Cell Rep.* **22**, 653–659 (2004).
22. Ishizaki, K., Chiyoda, S., Yamato, K. T. & Kohchi, T. *Agrobacterium*-mediated transformation of the haploid liverwort *Marchantia polymorpha* L., an emerging model for plant biology. *Plant Cell Physiol.* **49**, 1084–1091 (2008).
23. Hasty, P., Rivera-Perez, J. & Bradley, A. The length of homology required for gene targeting in embryonic stem cells. *Mol. Cell. Biol.* **11**, 5586–5591 (1991).
24. Kamisugi, Y., Cuming, A. C. & Cove, D. J. Parameters determining the efficiency of gene targeting in the moss Physcomitrella patens. *Nucleic Acids Res.* **33**, e173 (2005).
25. Sauer, B. Manipulation of transgenes by site-specific recombination: use of Cre recombinase. *Methods Enzymol.* **225**, 890–900 (1993).
26. Chiyoda, S., Ishizaki, K., Kataoka, H., Yamato, K. T. & Kohchi, T. Direct transformation of the liverwort *Marchantia polymorpha* L. by particle bombardment using immature thalli developing from spores. *Plant Cell Rep.* **27**, 1467–1473 (2008).
27. Gamborg, O. L., Miller, R. A. & Ojima, K. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* **50**, 151–158 (1968).
28. Kubota, A., Ishizaki, K., Hosaka, M. & Kohchi, T. Efficient *Agrobacterium*-mediated transformation of the liverwort *Marchantia polymorpha* using regenerating thalli. *Biosci. Biotechnol. Biochem.* **77**, 167–172 (2013).
29. Murray, M. G. & Thompson, W. F. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **8**, 4321–4325 (1980).
30. Church, G. M. & Gilbert, W. Genomic sequencing. *Proc. Natl. Acad. Sci. U. S. A.* **81**, 1991–1995 (1984).

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Author contributions

K.I., Y.J.-H., S. Iida and T.K. designed the research, interpreted the data and wrote the paper. Binary vectors for gene targeting were designed and constructed by Y.J.-H. and S. Iida. Gene targeting experiments were performed by K.I. and S. Ishida.

Additional information

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