

## Tagged Mutagenesis and Gene-trap in the Moss, *Physcomitrella patens* by Shuttle Mutagenesis

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### Abstract

The moss, *Physcomitrella patens* has been used as a useful material in many fields, because of its simple body plan, ease of gene targeting, and other reasons. Although many mutants have been reported, no method to isolate the corresponding genes was reported. We developed a gene tagging and gene-trap system in *P. patens* by using the shuttle mutagenesis technique, which has been used in the budding yeast. In 5264 tagged lines, 203 mutants with altered developmental or morphological phenotypes were obtained. In 129 of 4757 gene-trap lines,  $\beta$ -glucuronidase (GUS) activity was detected in some tissue. Although multiple copies of a tag were detected in many tagged lines by Southern analyses, most copies are likely integrated at the same locus according to PCR analyses.

**Key words:** tagging; gene-trap; *Physcomitrella patens*; homologous recombination; shuttle mutagenesis

### 1. Introduction

Comparing the developmental mechanisms in model organisms is indispensable for understanding the diversity and evolution of development. Furthermore, to understand the general principles of development, it is necessary to study different organisms that have special advantages for developmental studies.<sup>1</sup> Many land plants, ranging from angiosperms to bryophytes, have been studied as model plants. Some mosses have been used to study physiology, genetics, and developmental biology.<sup>2-5</sup> Of these, *Physcomitrella patens* has received special attention in the last few years, since a gene-targeting technique ascribed to its high rate of homologous recombination was established.<sup>6,7</sup>

A number of *P. patens* mutants that alter their developmental or physiological traits have been described,<sup>8-11</sup> but there are no methods to isolate the genes corresponding to the mutations. *Agrobacterium*- or transposon-mediated gene-tagging methods are useful for cloning mutated genes in organisms for which genetic maps are not available,<sup>12,13</sup> such as *P. patens*. However, neither infection with *Agrobacterium* nor transposition of an endogenous or exogenous transposon has been reported

in *P. patens*. Therefore, to establish tagged mutants of *P. patens*, we focused on shuttle mutagenesis, which has been successfully used in the budding yeast, *Saccharomyces cerevisiae*.<sup>14-16</sup> This method involves three steps, as shown in Fig. 1. Its essence is the replacement of a *P. patens* genomic sequence with tagged *P. patens* DNA sequences by homologous recombination. The high rate of homologous recombination in *P. patens* is similar to that in the budding yeast,<sup>7</sup> which should make it possible to use this method.

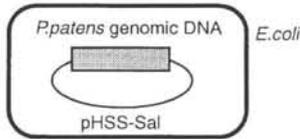
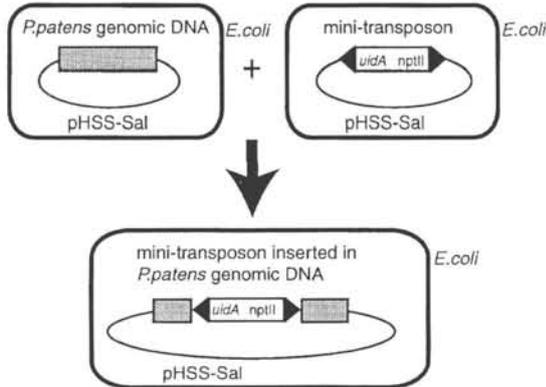
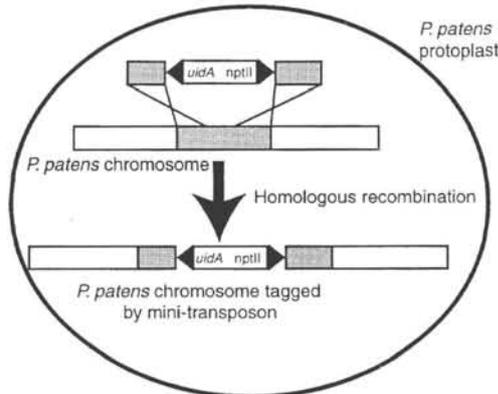
### 2. Materials and Methods

#### 2.1. Plant material and culture conditions

*Physcomitrella patens* (Hedw.) Bruch & Schimp subsp. *Patens* Tan<sup>17</sup> collected in Gransden Wood, Huntingdonshire, UK<sup>18</sup> was used as the wild-type strain. Protone-mata of *P. patens* were grown in 9-cm Petri dishes on BCDATG medium. BCD medium contains 1 mM MgSO<sub>4</sub>, 10 mM KNO<sub>3</sub>, 45  $\mu$ M FeSO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub> [pH 6.5 adjusted with KOH], and trace element solution (alternative TES<sup>19</sup>; 0.22  $\mu$ M CuSO<sub>4</sub>, 0.19  $\mu$ M ZnSO<sub>4</sub>, 10  $\mu$ M H<sub>3</sub>BO<sub>3</sub>, 0.10  $\mu$ M Na<sub>2</sub>MoO<sub>4</sub>, 2  $\mu$ M MnCl<sub>2</sub>, 0.23  $\mu$ M CoCl<sub>2</sub>, and 0.17  $\mu$ M KI). BCDATG medium is BCD medium supplemented with 1 mM CaCl<sub>2</sub>, 5 mM diammonium (+)-tartrate, and 0.5% (w/v) glucose, and so-

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(1) Generation of *P. patens* genomic library in *E. coli*(2) Insertion of mini-transposon to *P. patens* genomic DNA in *E. coli*(3) Transformation of *P. patens* with *P. patens* genomic DNA tagged by mini-transposon

**Figure 1.** Schematic diagram of the shuttle mutagenesis of *Physcomitrella patens*. The procedure for shuttle mutagenesis consists of three steps: (1) generating a *P. patens* genomic library in *E. coli*, (2) inserting mini-transposons into the genomic library in *E. coli*, and (3) transforming *P. patens* with the genomic library containing mini-transposon inserts. The overall effect is the insertion of the mini-transposons into the *P. patens* genome.

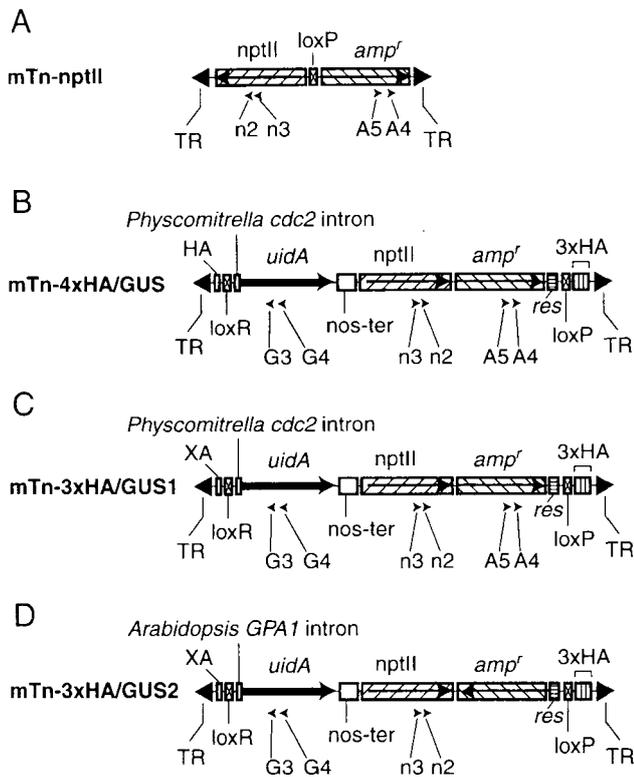
lidified with 0.8% (w/v) agar (A-9799, SIGMA) (D. Cove, personal communication). The solidified medium was covered with a layer of cellophane (Futamura Chemical Industries Co., Ltd., Nagoya, Japan) to facilitate collection of protonemata from the medium. The dishes were incubated at 25°C under continuous light (40  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ ). For vegetative propagation, the protonemata were collected every 5 days, and were ground with a Polytron homogenizer (Kinematica, Littau, Switzerland)

at a speed setting of 4. The ground protonemata were soaked on the BCDATG medium using the same conditions described above.

2.2. Transformation of *P. patens*

The transformation procedure followed Schaefer<sup>20</sup> with minor modifications. Protoplasts were prepared using vegetatively propagated protonemata cultured for 4–5 days.

The room temperature was kept at 20°C during the transformation experiments. Collected protonemata were incubated in 8% (w/v) mannitol and 2% (w/v) Driserase (Kyowa Hakko Kogyo Co., Ltd.) for 30 min at 25°C in the dark with gentle mixing every 5 min. The protoplasts were filtered through a 50  $\mu\text{m}$  nylon mesh, collected by centrifugation at 180 $\times$ g with a swinging rotor, and suspended in 8% (w/v) mannitol. This washing procedure was repeated two more times. After counting the density of protoplasts with a hemocytometer, the protoplasts were suspended in MMM solution (8.3% mannitol, 0.1% [w/v] MES-KOH [pH 5.6], and 15 mM  $\text{MgCl}_2$ ) at  $1.6 \times 10^6$  cells/mL. Thirty microliters of DNA solution in TE (10 mM Tris-HCl [pH 8.0], and 1 mM EDTA) were aliquoted in a polypropylene tube (Falcon 2057; Becton Dickinson Labware). Then 300  $\mu\text{L}$  of the protoplast suspension and 300  $\mu\text{L}$  of PEG/T solution (28.5% polyethylene glycol 6000 in 7.2% [w/v] mannitol, 0.1 M  $\text{CaCl}_2$ , and 10 mM Tris-HCl [pH 8.0]) were added. The mixed suspension was incubated at 45°C for 5 min, and then cooled at 20°C for 10 min in a water bath. The protoplasts were diluted stepwise to 8 mL with protoplast liquid culture medium (5 mM  $\text{Ca}(\text{NO}_3)_2$ , 1 mM  $\text{MgSO}_4$ , 45  $\mu\text{M}$   $\text{FeSO}_4$ , 0.18 mM  $\text{KH}_2\text{PO}_4$  [pH 6.5 adjusted with KOH], the alternative TES, 5 mM ammonium tartrate, 6.6% [w/v] mannitol, and 0.5% [w/v] glucose), poured into a 6-cm Petri dish, and kept in the dark at 25°C for 1 day. The protoplasts were collected by centrifugation at 180 $\times$ g, and suspended in 8 mL of protoplast regeneration medium for the top layer (PRM/T; BCD medium supplemented with 5 mM ammonium tartrate, 10 mM  $\text{CaCl}_2$ , 0.8% [w/v] agar, and 8% [w/v] mannitol). This medium was warmed to 45°C to prevent jelling. The protoplasts suspended in PRM/T were poured into four 9-cm dishes that contained a bottom layer of protoplast regeneration medium (PRM/B), which was covered with cellophane. PRM/B contains BCD medium supplemented with 5 mM ammonium tartrate, 10 mM  $\text{CaCl}_2$ , 0.8% (w/v) agar, and 6% (w/v) mannitol. After 4 days incubation under continuous light, the cellophane with the PRM/T containing regenerated protoplasts was transferred to BCDAT medium containing 50 mg/L G418 (Life Technologies) to select transformants. BCDAT medium is BCD medium containing 1 mM  $\text{CaCl}_2$ , 5 mM ammonium tartrate, and 0.8% (w/v) agar. The plants that survived 3 weeks on the selection medium were transferred to BCDATG medium,



**Figure 2.** Mini-transposons used for mutagenizing *Physcomitrella patens*. The figure shows a schematic representation of the mini-transposons constructed. Each mini-transposon contains a NPTII expression cassette (nptII) and an ampicillin-resistance gene (*amp<sup>r</sup>*). The arrows within nptII and *amp<sup>r</sup>* indicate their directions. mTn-4xHA/GUS, mTn-3xHA/GUS1, and mTn-3xHA/GUS2 each contain an intron, the *uidA* coding region (thick arrow), and the nopaline synthase polyadenylation signal (*nos-ter*). The intron in mTn-4xHA/GUS and mTn-3xHA/GUS1 is the intron from the *P. patens cdc2* gene (K. Fujiwara, personal communication), and the intron in mTn-3xHA/GUS2 is the *Arabidopsis GPA1* intron with triple donor and acceptor sites.<sup>27</sup> Arrowheads show the location of the primer annealing sites used for inter-mini-transposon PCR.

incubated for 1 week, and re-inoculated onto the selection medium. Those plants that grew on the selection medium as fast as on the non-selection medium were stored as stable transformants.

### 2.3. Construction of mini-transposons

#### (1) mTn-nptII

The NPTII expression cassette (nptII), which contains the cauliflower mosaic virus (CaMV) 35S promoter,<sup>21</sup> the *nptII* gene,<sup>22</sup> and a fragment containing the CaMV polyadenylation signal<sup>23</sup> corresponding to 7482-131 of the CaMV genome,<sup>24</sup> was excised from plasmid pMBL5 (provided by D. Cove) as an *Xho*I-*Kpn*I fragment, and cloned into the *Sma*I site of pBluescript SKII+ (Stratagene). This plasmid was designated pTN3. A *Bam*HI-*Hind*III fragment containing nptII was excised

from pTN3 and cloned between the *Bam*HI and *Hind*III sites of the mini-transposon on the pTn plasmid<sup>15</sup> to make the pTn-nptII plasmid. The mini-transposon containing nptII (mTn-nptII; Fig. 2A) was transposed to the F derivative pOX38 as previously described,<sup>15</sup> and the modified F derivative with the mini-transposon was named pOX38::mTn-nptII.

#### (2) mTn-4xHA/GUS, mTn-3xHA/GUS1, and mTn-3xHA/GUS2

The mTn-4xHA/GUS mini-transposon (Fig. 2B) was made by exchanging the *lacZ*, *URA3*, and *tet<sup>r</sup>* genes on the mTn-4xHA/*lacZ*<sup>25</sup> mini-transposon with the  $\beta$ -glucuronidase (GUS) gene *uidA*,<sup>26</sup> nptII, and the *amp<sup>r</sup>* gene, respectively. The plasmid containing mTn-4xHA/*lacZ* on pHSS6<sup>14</sup> was kindly provided by M. Snyder. The mini-transposon mTn-4xHA/*lacZ* contains in order: an HA tag, a loxR site, a *lacZ* gene, a *URA3* gene, a *res* site, and three HA tags, between the terminal inverted repeats. The region from the *res* site to the loxR site, which contains the pHSS6 vector, was amplified with primers having restriction sites at their 5' ends (loxR-r, ATCGATATCgctataacttctgatagcag; res-f, CTAGTCGACCACGTGcgtaacca; the capitalized nucleotides are synthetic restriction sites) and self-ligated as pTn-4xHA. The plasmid pTn-4xHA has *Eco*RV, *Sal*I, and *Pma*CI sites between the loxR and *res* sites. An intron-GUS-nptII fragment prepared in a separate plasmid was cloned between the *Eco*RV and *Sal*I sites, and the *amp<sup>r</sup>* gene was cloned into the *Pma*CI site. The intron-GUS-nptII fragment and the *amp<sup>r</sup>* gene were prepared as follows. A DNA fragment containing the first intron of the *P. patens cdc2* gene (kindly provided by K. Fujiwara) was inserted between the *Xba*I and *Bam*HI sites of pBI221,<sup>26</sup> bordering the *uidA* gene. The *uidA* gene along with the CaMV 35S promoter, *cdc2* intron, and nopaline synthase polyadenylation signal (*nos-ter*) were excised from the plasmid as an *Eco*RI-*Hind*III fragment, and cloned between the *Eco*RI and *Hind*III sites of pTN3, which contains nptII. An *Xba*I-*Xho*I fragment containing the *cdc2* intron, *uidA*, *nos-ter*, and nptII was excised, and cloned into pTn-4xHA between the *Eco*RV and *Sal*I sites. The *amp<sup>r</sup>* gene corresponding to coordinates 758-1948 of pUC18 (accession no. L08752) was amplified by PCR and cloned into the *Pma*CI site. The mTn-4xHA/GUS mini-transposon was constructed on pHSS6 plasmid.<sup>14,25</sup> This mini-transposon was transposed to pOX38 as described,<sup>25</sup> and the modified F derivative with the mTn-4xHA/GUS mini-transposon was named pOX38::mTn-4xHA/GUS.

The mTn-3xHA/GUS1 and mTn-3xHA/GUS2 mini-transposons (Fig. 2C, D) were made by modifying the mTn-3xHA/*lacZ* mini-transposon<sup>25</sup> using the procedures described above. The mTn-3xHA/GUS1 mini-transposon contains the *P. patens cdc2* intron, as does the mTn-4xHA/GUS mini-transposon, while the mTn-

**Table 1.** *Escherichia coli* strains used in this study.

Strain	Relevant genotype	Reference or supplier
XL2 blue MRF'	$\Delta(mcrA) \Delta(mcrCB-hsdSMR-mrr)endA1$	Stratagene
DH5 $\alpha$	<i>hsdRhsdM</i> <sup>+</sup>	Life Technologies
RDP146	<i>hsdR</i> <sup>+</sup>	15
RDP146/pLB101	<i>hsdR</i> <sup>+</sup> , transposase	15
RDP146/pOX::mTns	mini-transposon on F factor derivative	this study
NS2114Sm	Sm <sup>r</sup> , $\lambda$ -cre	15
NG135	Sm <sup>r</sup> , resolvase	25

3xHA/GUS2 mini-transposon contains the *Arabidopsis thaliana* *GPA1* intron with triple acceptor sequences.<sup>27</sup>

#### 2.4. Construction of the mutagenized genomic library

The *Escherichia coli* strains used in this study and their relevant genotypes are summarized in Table 1. Chemically competent cells of XL2 blue MRF' (Stratagene) and DH5 $\alpha$  (Life Technologies, Inc.) were prepared according to Inoue et al.<sup>28</sup> Preparation of electrocompetent cells of RDP146/pLB101 and electroporation using an EasyjecT Prima (EquiBio Ltd., Kent, UK) electroporator were performed according to the manufacturer's instructions.

*P. patens* genomic DNA was isolated from protone-mata by the CTAB method.<sup>29</sup> The genomic DNA was partially digested with *Sau*3AI, and run on 0.6% (w/v) agarose gels (SeaKem GTG, FMC BioProducts) in 1 $\times$ TAE buffer. The fragments 3–6 kb long were recovered by the electroelution method,<sup>30</sup> and their ends were partially filled with dATP and dGTP. These fragments were ligated with the *Sal*I-digested pHSS-Sal vector<sup>16</sup> partially filled with dCTP and dTTP. The ligation solution was used to transform *E. coli* XL2 blue MRF'. Plasmids extracted from XL2 blue MRF' were subsequently used to transform *E. coli* DH5 $\alpha$ . Plasmids extracted from DH5 $\alpha$  were further used for the transformation of *E. coli* RDP146/pLB101. The *E. coli* RDP146/pLB101 containing the *P. patens* genomic DNA fragments was mated with *E. coli* RDP146/pOX38::mTn-nptII to form *E. coli* RDP146/pLB101/pOX38::mTn-nptII and to mutagenize the *P. patens* genomic library with the mTn-nptII mini-transposon, which was then mated with *E. coli* NS2114Sm.<sup>15</sup> To mutagenize the genomic library using other mini-transposons, *E. coli* NG135 was used instead of NS2114Sm.<sup>15,25</sup> The mutagenized library recovered from NS2114Sm or NG135 was amplified in *E. coli* XL2 blue MRF', because NS2114Sm and NG135 are *endA*<sup>+</sup> strains. The plasmids extracted from *E. coli* XL2 blue MRF' were digested with *Not*I, extracted with phenol and chloroform, precipitated with ethanol, and diluted with TE. The concentration of DNA was adjusted to 1.0  $\mu$ g/ $\mu$ l.

#### 2.5. Histochemical detection of GUS activity

The histochemical GUS activity was assayed according to Jefferson et al.<sup>31</sup> with slight modifications. The mutated lines were cultured for 14 days on BCDATG or BCDAT medium. The tissues were fixed in a solution of 0.3% (v/v) formalin, 5.45% (w/v) mannitol, and 0.2% (w/v) MES-KOH (pH 5.6) for 30 min at room temperature, washed 3 times with 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.0), infiltrated for 30 min in a substrate solution (50 mM NaH<sub>2</sub>PO<sub>4</sub> [pH 7.0], 0.5 mM 5-bromo-4-chloro-3-indolyl  $\beta$ -D-glucuronide [X-Gluc, Wako Pure Chemical Industries, Osaka, Japan], 0.5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 0.5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, and 0.3% [v/v] Triton X-100), and then incubated at 37°C for 36–48 hr for staining. After the incubation, the tissues were fixed in 5% (v/v) formalin for 10 min, and soaked in 5% (v/v) acetic acid for 10 min. Then the tissues were dehydrated through an ethanol series. Finally, the stained tissues were observed under a stereomicroscope and a light microscope. Images of the stained tissues were digitized with a CCD Camera (FUJIX HC-300Z, Fuji Film).

#### 2.6. Southern hybridization

Two micrograms of genomic DNA was digested with an appropriate restriction enzyme, run on a 0.7% (w/v) agarose gel (SeaKem GTG), and transferred to a positively charged nylon membrane (Hybond N<sup>+</sup>, Amersham-Pharmacia Biotech). An AlkPhos Direct kit (Amersham-Pharmacia Biotech) was used for labeling DNA probes, hybridization, and detection.

#### 2.7. Northern hybridization

Total RNA was isolated from each strain as described by Hasebe et al.<sup>32</sup> Poly(A)<sup>+</sup> RNA was further purified using oligo-dT magnetic beads (DynaBEADS, Dynal, Oslo, Norway). One microgram of poly(A)<sup>+</sup> RNA was electrophoresed on a denaturing agarose gel (1% [w/v] SeaKem GTG agarose, 1 $\times$ MOPS buffer, and 6.7% [v/v] formaldehyde), and transferred to a Hybond N<sup>+</sup> membrane by the downward capillary transfer method<sup>33</sup> using 10 $\times$ SSC. The membrane was fixed with 0.05 M NaOH, washed with 1 $\times$ SSC, and dried at 80°C for 20 min. The

hybridization was performed in Church buffer<sup>31</sup> at 65°C. The probes were radiolabeled using a Random Primer DNA Labeling Kit ver. 2.0 (Takara) with [ $\alpha$ -<sup>32</sup>P] dCTP, and detected by autoradiography.

### 2.8. PCR to detect the region between the mini-transposon.

To amplify the inter-mini-transposon regions, the primer sets A5 (5'-TTAATAGACTGGATGGAGCGGATAAAGTT-3') and n3 (5'-ACGGAAGGAAGGAGGAGACAAGGAAGGAT-3') or A4 (5'-TAGACTGGATGAGGCGGATAAAGTTGCAG-3') and n2 (5'-AGGAGGAAGACAAGGAAGGATAAGGTTGCCA-3') were used for the mTn-nptII mini-transposon. A5 and G4 (5'-ATTGACCCACACTTTGCCGTAATGAGTGAC-3') or A4 and G3 (5'-TCTTGTAACGCGCTTTCCACCAACGCTGA-3') were used for the mTn-4xHA/GUS and mTn-3xHA/GUS mini-transposons. Each PCR reaction contained 1  $\mu$ L of 10 ng/ $\mu$ L genomic DNA, 2  $\mu$ L of ExTaq Buffer, 1  $\mu$ L each of two 10  $\mu$ M primers, 2  $\mu$ L of 2 mM dNTP, 13  $\mu$ L of water, and 0.1  $\mu$ L of 5 unit/ $\mu$ L ExTaq DNA polymerase (Takara). Each PCR cycle consisted of 94°C for 20 sec and 68°C for 15 min. The cycle was repeated 20 times, preceded by 5 min at 94°C, followed by 5 min at 68°C. A 50-fold dilution of the first PCR product was used as the template for the second PCR, which was carried out using the same conditions, except for the sample of TN1 mutant which was carried for 15 cycles.

## 3. Results and Discussion

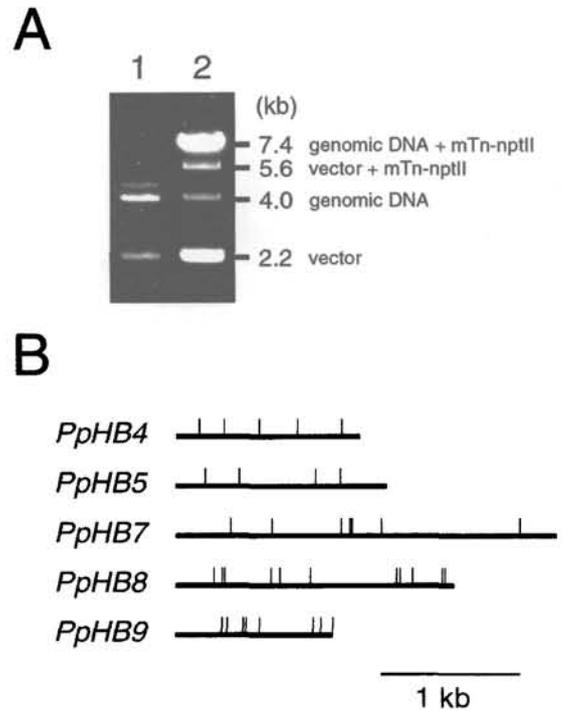
### 3.1. Generation of *P. patens* genomic library in *E. coli*

A *P. patens* genomic DNA library with insert sizes from 3 to 6 kb was constructed with the pHSS-Sal<sup>16</sup> plasmid vector, which is suitable for shuttle mutagenesis using *E. coli*. The size range of the inserted fragments is appropriate for efficient cloning using the pHSS-Sal vector and for homologous recombination of *P. patens*.

Since *P. patens* genomic DNA is thought to contain methylated bases like other eukaryotic genomes, we used the *mcrA*<sup>-</sup> *mcrCB*<sup>-</sup> *mrr*<sup>-</sup> *E. coli* strain, XL2-blue MRF' (Stratagene). Since we ultimately introduced the library into an *hsdR*<sup>+</sup> *E. coli* strain, either NG135 or NS2114Sm, the library was passed through an *hsdM*<sup>+</sup> *E. coli* strain, DH5 $\alpha$ .

### 3.2. Mini-transposons

In shuttle mutagenesis of the budding yeast, the *LEU2* or *URA3* genes are used as a selection marker.<sup>16,25</sup> To facilitate the selection of *P. patens* tagged mutants, we generated mini-transposons with nptII that confers G418 resistance (Fig. 2). For gene-trap, mini-transposons containing the *uidA* gene, which encodes  $\beta$ -glucuronidase



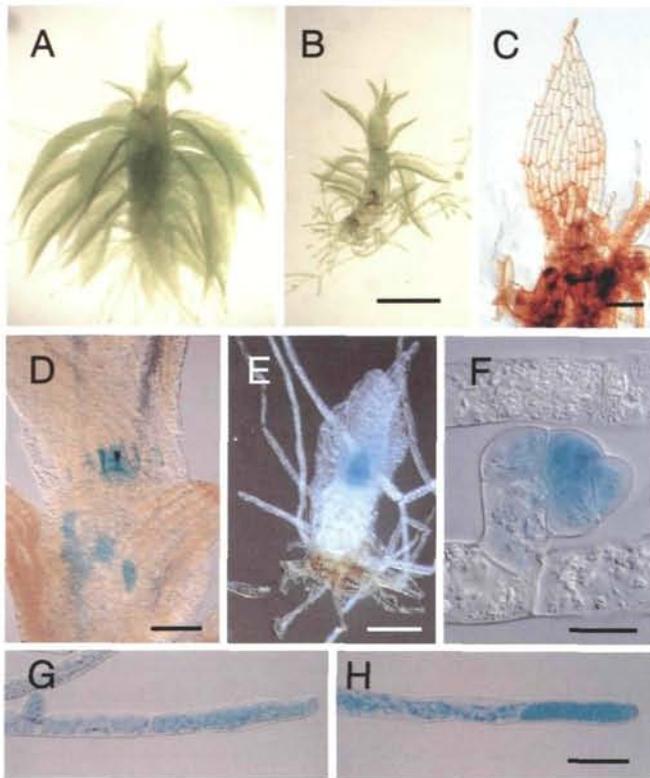
**Figure 3.** Transposition of mini-transposons on plasmids. A, the mini-transposon mTn-nptII was transposed to a 4-kb genomic clone. Lane 1, plasmids extracted from *E. coli* containing the 4-kb clone and pLB101 before the transposition were digested with *Not* I. The 4.4-kb band is pLB101. Lane 2, plasmids extracted from a population of *E. coli* containing a plasmid with a mini-transposon tag derived from the plasmid in lane 1. B, the mini-transposon mTn-nptII was transposed to each *PpHB* gene. Horizontal line indicates the region of the genomic clone of each *PpHB* gene. Vertical bars show the distribution of the insertion points of the mini-transposons.

(GUS),<sup>31</sup> were used as an expression marker instead of *lacZ*, which is used in the yeast system.

*P. patens* genes have introns like other plants, while most yeast genes lack introns. Either the *Arabidopsis* *GPA1* intron<sup>27</sup> or the *P. patens* *cdc2* intron was fused to the 5' end of the *uidA* gene, so that the expression of a tagged *P. patens* gene could be detected when the mini-transposon was inserted in the intron or coding region.

### 3.3. Transposition of mini-transposons to *P. patens* genomic DNA cloned in pHSS-Sal plasmid in *E. coli*

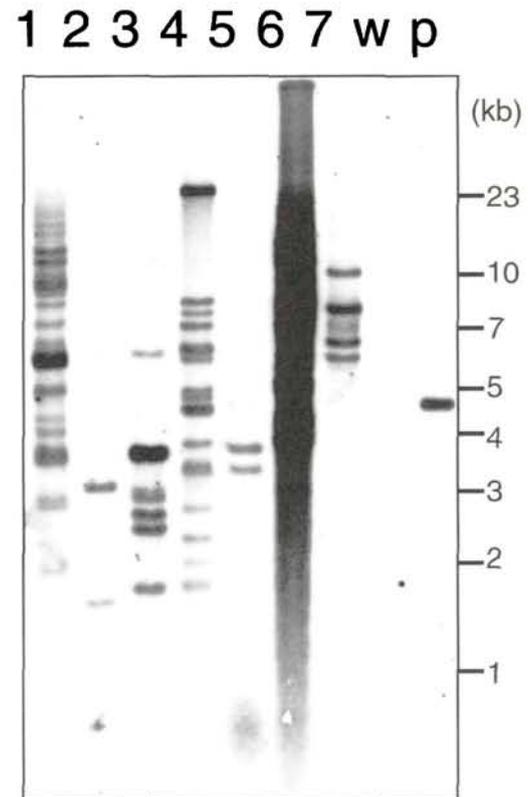
The donor strain containing the mini-transposon on the F derivative was mated with the recipient strain containing the *P. patens* genomic DNA library, and the mini-transposon was transposed to the *P. patens* genomic DNA. To test the efficiency of transposition by this system, eight randomly selected genomic clones of 2 to 4 kb were mutagenized with mTn-nptII. Since there are *Not* I sites at the boundaries between the pHSS-Sal vector and the inserted *P. patens* genomic DNA fragment, plasmids extracted from each mutagenized pop-



**Figure 4.** Example of mutant strains and gene-trap lines. A, transformant morphologically indistinguishable from the wild-type (19980725084-3). B (TN1) and C (TN2), morphological mutants obtained by shuttle mutagenesis. D-H, histochemical staining of gene-trap lines. D, YH330, mucilage hairs are stained. E, YH78, young leaves are stained. F, YH229, a young bud is stained. G and H, YH206, the chloronema (G) and caulonema (H) are stained. Bar in B = 0.5 mm for A and B. Bars in C and D = 0.1 mm. Bar in E = 0.2 mm. Bar in F = 20  $\mu$ m. Bar in H = 50  $\mu$ m for G and H.

ulation were digested with *Not* I, and separated on an agarose gel. In each clone, four bands were detected: vector, genomic DNA fragment with the inserted mini-transposon, vector with the inserted mini-transposon, and genomic DNA fragment (Fig. 3A). The first two bands were brighter than the others, indicating that most of the mini-transposons were inserted into the *P. patens* genomic DNA fragment.

To obtain a low-redundancy tagged mutant library, mini-transposons should be randomly transposed to genomic DNA. To examine where the mini-transposons were inserted in a genomic DNA fragment, the mTn-nptII mini-transposon was transposed to *P. patens* HD-Zip genes, which we cloned previously (accession numbers: AB28075, AB28076, AB28078, AB28079, and AB28080). The junction of the mTn-nptII mini-transposon and the HD-Zip genomic sequence was determined, and the insertion points were confirmed to be randomly distributed throughout the genomic sequences

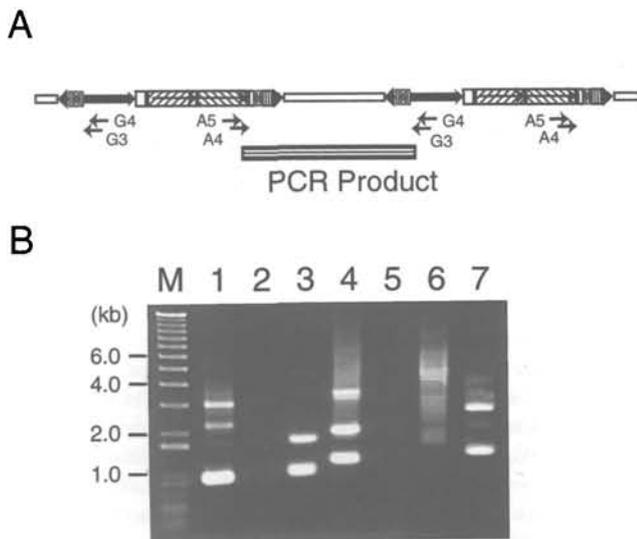


**Figure 5.** Southern hybridization of mutagenized strains. Two micrograms of genomic DNA from transformants was digested with *Eco*RI, and detected using pTn-nptII probe at 55°C. Lane 1-5, mutagenized with mTn-nptII; lane 6, 7 mutagenized with mTn-3xHA/GUS1; lane w, wild-type; lane p, 30  $\mu$ g of pTn-nptII digested with *Xho* I. The strains are as follows: 1, 19980725073-2; 2, 19981029021; 3, 1998102902132; 4, TN2; 5, 19981029034; 6, TN1; and 7, 19980928037.

(Fig. 3B). Of 53 insertion points from five analyses, 40 points (75%) were located in the HD-Zip genes and the rest were in the pHSS-Sal vector.

### 3.4. High transformation efficiency was achieved using shuttle mutagenesis.

The *P. patens* genomic library constructed using the pHSS-Sal vector was mutagenized with each mini-transposon. The mutagenized *P. patens* genomic DNA in pHSS-Sal was digested with *Not* I, and used to transform *P. patens* by the polyethylene glycol-mediated method.<sup>20,35</sup> Approximately 30% of the transformants were stable and selected after 1 week of relaxation on the non-selection medium. We typically obtained 187 $\pm$ 116 stable transformants with 30  $\mu$ g of the tagged *P. patens* genomic DNA. When we transformed *P. patens* with linearized plasmids which do not contain a sequence homologous to *P. patens* genomic DNA, the number of stable transformants obtained was reduced to 20 $\pm$ 7. The difference in the transformation efficiency concurs with a previous study that showed that the homologous recombina-



**Figure 6.** Inter-mini-transposon PCR of *P. patens* mutagenized with mini-transposons. Inter-mini-transposon regions were amplified with primers oriented to the distal of mini-transposon using genomic DNA extracted from each transformant as a template. A, a drawing showing how the inter-transposon regions are thought to be amplified. The structure of each transposon is shown in Fig. 2. The open boxes between and outside the mini-transposon indicate genomic DNA regions. The arrows indicate the primer annealing sites. B, amplified PCR products. Lane 1-5, mutagenized with mTn-nptII; lane 6, 7 mutagenized with mTn-3xHA/GUS1; lane M, 1 kb plus DNA ladder (Life Technologies). The strains are as follows; 1, 19980725073-2; 2, 19981029021; 3, 1998102902132; 4, TN2; 5, 19981029034; 6, TN1; 7, 19980928037.

nation rate is ten times higher than the non-homologous recombination rate.<sup>7</sup>

### 3.5. Phenotype of transformants

In 1358, 2136, 71, and 1699 lines mutagenized with mTn3-nptII, mTn-4xHA/GUS, mTn-3xHA/GUS1, and mTn-3xHA/GUS2 respectively, 203 mutants (3.9%) with different developmental and morphological phenotypes from the wild-type were obtained. Two examples are shown in Fig. 4. The line TN1 (Fig. 4B) produces smaller gametophores than the wild-type (Fig. 4A). The protonemata and gametophores of line TN2 (Fig. 4C) turn brownish, and the gametophores stop growing when they form up to two leaves (Fig. 4C). The leaves are also brown and pigmented, and chloroplasts do not develop.

### 3.6. Southern analysis of transformants

Genomic DNA was extracted from 15 arbitrary lines with no significant phenotype and 10 lines with altered morphology, and the number of tags inserted in the *P. patens* genome was estimated by Southern hybridization using the pTn-nptII plasmid as a probe. Some examples are shown in Fig. 5. The probe hybridized to multiple

fragments digested with *Eco*RI in most lines, suggesting that multiple mini-transposon tags were integrated in the genome.

To see how closely together the integrated mini-transposons were located, the regions between the mini-transposons were amplified by PCR with primers oriented in the distal direction of the mini-transposon (Fig. 6A). Multiple PCR products ranging from 0.9 to 6 kb were detected (Fig. 6B), indicating that multiple copies of the mini-transposon were inserted nearby. This result is concordant with previous reports that multiple copies of transformed DNA were integrated in a single site as tandem repeats.<sup>7,35</sup>

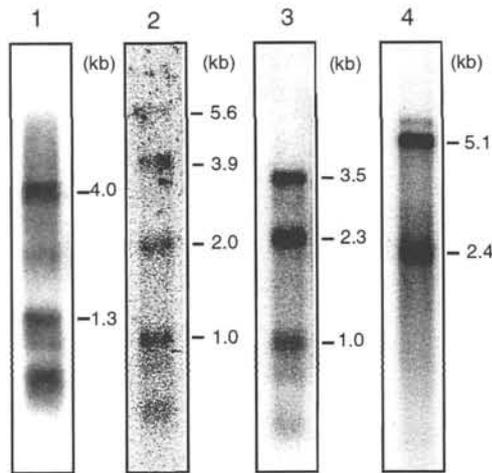
Each plasmid can contain only one mini-transposon in *E. coli*, because the 38 bp boundary sequence of Tn3 present in the mini-transposon prohibits transposition of additional mini-transposons to the same plasmid.<sup>14</sup> *Not* I digestions of the plasmid extracted from *E. coli* after transposition verified the existence of a single mini-transposon in each plasmid (Fig. 3). Therefore, the multicopy array of the mini-transposon tags is likely formed in the *P. patens* cells, and not in *E. coli*. We obtained a preliminary series of clones of the products of inter mini-transposon PCR using the TN1 genomic DNA (Fig. 6) as a template. The sequence analyses of all 16 different clones revealed that all the clones contained part of the same *P. patens* genomic DNA clone (data not shown), suggesting that many transposons are likely integrated nearby in the *P. patens* genome.

This is the first report of generating tagged mutants in the moss, *Physcomitrella patens*. This library will enable the isolation and identification of the corresponding genes of mutants.

### 3.7. GUS expression in transgenic lines

GUS activity was detected histochemically in 129 of 4757 (2.7%) lines mutagenized using the mTn-3xHA/GUS2 mini-transposon. No background staining was observed in any tissue of wild-type *P. patens*. Some examples are shown in Fig. 2. The line YH330 showed specific staining in mucilage hairs (Fig. 4D), which are multicellular hairs that form at the base of a leaf. The lines YH78, YH229, and YH206 showed GUS expression in young leaves (Fig. 4E), in the apical cell of very young buds (Fig. 4F), and in protonema, including chloronema (Fig. 4G) and caulonema (Fig. 4H), respectively. These and other lines will be useful for isolating genes expressed in specific tissues and cells.

Northern hybridization using the *uidA* gene as a probe detected transcripts of the *uidA* gene in the lines shown in Fig. 4. Transcripts with different sizes were detected in different lines (Fig. 7), suggesting that some genes are fused with the *uidA* gene. There are only a few transcripts in each line, and the 5' RACE method will be useful for isolating *P. patens* genes fused to the *uidA*



**Figure 7.** Northern blot analyses of the gene-trap lines shown in Fig. 4 using the *uidA* gene probe. Each lane contains 0.5–1  $\mu$ g of poly(A)<sup>+</sup> RNA. Lane 1, YH330; lane 2, YH78; lane 3, YH229; and lane 4, YH206.

gene.

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