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TECHNICAL ADVANCE

Establishment of gene-trap and enhancer-trap systems in the moss *Physcomitrella patens*

Yuji Hiwatashi[1,2], Tomoaki Nishiyama[1], Tomomichi Fujita[1] and Mitsuyasu Hasebe[1,2,*]
[1]*National Institute for Basic Biology, Okazaki 444-8585, Japan, and*
[2]*Department of Molecular Biomechanics, School of Life Science, The Graduate University for Advanced Studies, Okazaki 444-8585, Japan*

Received 20 April 2001; revised 19 June 2001; accepted 25 June 2001
*For correspondence (fax: +81 564 55 7546; email: mhasebe@nibb.ac.jp)

Summary

Because of its simple body plan and ease of gene knockout and allele replacement, the moss *Physcomitrella patens* is often used as a model system for studies in plant physiology and developmental biology. Gene-trap and enhancer-trap systems are useful techniques for cloning genes and enhancers that function in specific tissues or cells. Additionally, these systems are convenient for obtaining molecular markers specific for certain developmental processes. Elements for gene-trap and enhancer-trap systems were constructed using the *uidA* reporter gene with either a splice acceptor or a minimal promoter. Through a high rate of transformation conferred by a method utilizing homologous recombination, 235 gene-trap and 1073 enhancer-trap lines were obtained from 5637 and 3726 transgenic lines, respectively. The expression patterns of these trap lines in the moss gametophyte varied. The candidate gene trapped in a gene-trap line YH209, which shows rhizoid-specific expression, was obtained by 5′ and 3′ RACE. This gene was named *PpGLU*, and forms a clade with plant acidic α-glucosidase genes. Thus, these gene-trap and enhancer-trap systems should prove useful to identify tissue- and cell-specific genes in *Physcomitrella*.

Keywords: *Physcomitrella*, moss, gene-trap, enhancer-trap, glucosidase, glycoside hydrolase.

Introduction

The simple morphology of mosses makes them useful systems for studies of plant physiology and developmental biology (reviewed by Cove *et al.*, 1997; Reski, 1998; Schumaker and Dietrich, 1998). As haploids, mosses form a stem–leaf-like structure called a gametophore. This structure originates from a single apical cell at the gametophore apex, and its leaf is composed of a single layer or a few layers of cells that originate from a single leaf primordial cell. In gametophores, the development of multicellular organs, such as leaves and stems, results from the differentiation of a single cell, after asymmetric cell division of the apical cell occurs. Additionally, as in higher plants, gametophyte development is regulated by phytohormones (e.g. auxin and cytokinin) and by intracellular messengers (e.g. Ca^{2+}), thus providing an opportunity to analyse the actions of these substances in a simple system (reviewed by Schumaker and Dietrich, 1997).

Among mosses, *Physcomitrella patens* has been of particular interest recently owing to development of the gene targeting technique (Kammerer and Cove, 1996; Schaefer and Zrÿd, 1997; Schaefer, 2001), which enables directed gene disruption and allele replacement (reviewed by Mengiste and Paszkowski, 1999). The ability to target gene disruption in *P. patens* is attributed to a high rate of homologous recombination that has not been observed in other land plants (Kammerer and Cove, 1996; Schaefer, 2001). This technique has been used successfully to identify the functions of certain nuclear-encoded genes (Girke *et al.*, 1998; Girod *et al.*, 1999; Strepp *et al.*, 1998).

Gene-trap and enhancer-trap systems are useful tools for finding novel genes and unidentified regulatory elements based on the expression pattern of a reporter gene randomly integrated into the genome (reviewed by Bellen, 1999; Springer, 2000). In the gene-trap system, a reporter

gene lacking promoter sequences is inserted randomly into the genome, and genes expressed in a tissue-specific manner are detected. Tissue-specific enhancers and promoters are obtained by the enhancer-trap system by cloning genomic sequences in front of a reporter gene fused to a weak promoter.

Previously, we employed shuttle mutagenesis (Ross-Macdonald *et al.*, 1997; Seifert *et al.*, 1986) to insert transposon-tagged DNA fragments randomly in the *P. patens* genome and established a gene-tagging system (Nishiyama *et al.*, 2000). Using this system, we also demonstrated that the splice acceptor-fused reporter gene was activated *in vivo* in certain tagged lines, indicating that shuttle mutagenesis is potentially useful in gene-trap and enhancer-trap systems. In this study, we constructed one gene-trap and two enhancer-trap elements for *P. patens* in addition to the previously reported gene-trap element. The utility of each element to generate large numbers of gene-trap and enhancer-trap lines was examined. Using these elements, we obtained 235 gene-trap and 1073 enhancer-trap lines from 9363 transgenic *P. patens* plants. In order to test the usefulness of the lines, we demonstrated that a gene trapped using these elements can be easily identified.

Results

Gene-trap and enhancer-trap elements

Each gene-trap and enhancer-trap element was introduced into *P. patens* by two methods: (1) non-homologous integration, and (2) homologous integration using shuttle mutagenesis (Nishiyama *et al.*, 2000; Seifert *et al.*, 1986). For the former method, protoplasts of *P. patens* were transformed with each trap element lacking any *P. patens* genomic DNA sequence (Schaefer *et al.*, 1991), and trap elements were integrated into the *P. patens* genome by non-homologous recombination. The latter method employed homologous recombination to integrate a trap element into the *P. patens* genome and had a transformation efficiency approximately 10-fold higher than non-homologous integration (Schaefer, 2001). Shuttle mutagenesis consists of three main steps: (1) generation of a *P. patens* genomic DNA library in *Escherichia coli*, (2) introduction of a mini-transposon including each trap element to the *P. patens* genomic DNA in *E. coli*, and (3) transformation of *P. patens* protoplasts with *P. patens* genomic DNA containing a mini-transposon insertion.

The gene-trap and enhancer-trap elements used in this study are shown in Figure 1. The plasmids NHI-GT (non-homologous integration, gene-trap) and NHI-ET (non-homologous integration, enhancer-trap) were used for non-homologous integration, and HI-GT (homologous integration, gene-trap) and HI-ET (homologous integration, Enhancer-Trap) were used for homologous integration. Nishiyama *et al.* (2000) constructed HI-GT under the name of mTn-3xHA/GUS2; the other elements were constructed in this study. The NPTII expression cassette (nptII) (Nishiyama *et al.*, 2000), containing the CaMV 35S promoter (Odell *et al.*, 1985), *ntpII* gene (Beck *et al.*, 1982) and CaMV polyadenylation signal (Guerineau *et al.*, 1990), was used as a marker for selection of transformed *P. patens*. Each element contains the NPTII expression cassette, resulting in resistance to G418 (Schaefer *et al.*, 1991). The *amp*^{r} gene was used as a selection marker in *E. coli*. The *uidA* gene, encoding β-glucuronidase (GUS) (Jefferson *et al.*, 1987), was used as the reporter gene. The fourth intron of *Arabidopsis GPA1*, the alpha subunit of the G-protein gene (Sundaresan *et al.*, 1995), was fused to the 5′ end of the reporter gene in the gene-trap elements (NHI-GT and HI-GT). This construction enables detection of gene expression when the gene-trap element inserts in either intron or exon sequences (Sundaresan *et al.*, 1995). Gene expression is limited to the correct orientation of the inserted element and the site of insertion with regard to the reading frame.

The enhancer-trap elements (NHI-ET and HI-ET) contain a short core sequence (–46 to +1) of the CaMV 35S

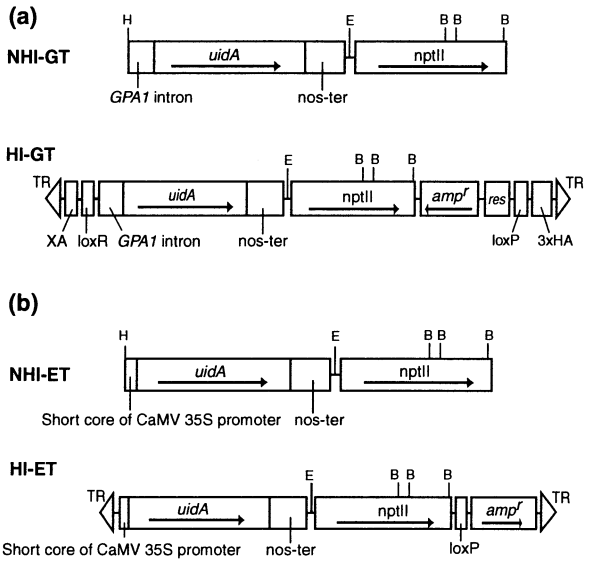

Figure 1. Schematic diagrams of gene-trap (a) and enhancer-trap (b) elements.
Each element contains the *uidA* coding region (*uidA*), the nopaline synthase polyadenylation signal (nos-ter), and an NPTII expression cassette (nptII). Gene-trap elements contain the *Arabidopsis GPA1* intron (Sundaresan *et al.*, 1995). Enhancer-trap elements contain a short core promoter (–46 to +1) of the CaMV 35S promoter. HI-GT and HI-ET are mini-transposons containing 38 bp terminal repeats of the Tn3 transposon (TR), a loxP site (loxP), and an ampicillin-resistance gene (*amp*^{r}). A factor Xa protease cleavage site, a loxR site, a Tn3 *res* site and three HA epitopes are depicted as XA, loxR, *res* and 3xHA, respectively. Arrows within *uidA*, nptII and *amp*^{r} indicate their directions. *Eco*RI (E), *Bam*HI (B) and *Hin*dIII (H) sites are shown.

promoter (Benfey *et al.*, 1989) fused to the 5′ end of the *uidA* gene. To test whether the CaMV 35S core promoter functions as a weak promoter in *P. patens*, its promoter activity was assayed using *P. patens* protoplasts into which each of the following gene- or enhancer-trap elements were transiently introduced: NHI-GT, NHI-GT lacking the *GPA1* intron (pdeltaGT3), NHI-ET and pCaMV35S. NHI-GT and pdeltaGT3 were used to examine whether the *GPA1* intron confers enhancer activity in *P. patens*. In pCaMV35S, the short core promoter of NHI-ET was replaced with a longer region (–800 to +1) of the CaMV 35S promoter. All elements were cloned into pBluescript SKII+ (Stratagene, La Jolla, CA, USA). Protoplasts were prepared from protonemata and transformed by the polyethylene glycol (PEG)-mediated method (Schaefer *et al.*, 1991). After 48 h of incubation in the dark at 25°C, GUS activity was measured (Table 1). The levels of GUS activity of pdeltaGT3 and NHI-GT were markedly lower than those of NHI-ET and p35CaMV35S. The GUS activity of NHI-ET, containing the short core promoter, was approximately 10-fold lower than that of pCaMV35S, but approximately fivefold higher than those of NHI-GT and pdeltaGT3.

Table 1 GUS activity of protoplasts transiently transformed with gene-trap and enhancer-trap elements

Constructs[a]	GUS activity[b] (pmol 4MU min^{-1} mg^{-1} protein)
No DNA	0.05 ± 0.01
NHI-GT	1.89 ± 2.58
pdeltaGT3	1.63 ± 0.26
NHI-ET	11.12 ± 5.60
pCaMV35S	128.98 ± 8.75

[a]The schematic diagrams of NHI-GT and NHI-ET are shown in Figure 1. The pdeltaGT3 is a construct lacking a *GPA1* intron of NHI-GT. pCaMV35S contains a longer CaMV 35S promoter (–800 to +1) instead of the core promoter (–46 to +1) present in NHI-ET.
[b]Mean ± SD (n = 3). 4MU, 4-methyl-umbelliferone

Comparison of non-homologous and homologous integration methods

We compared the efficiency of integration of the trap elements into the *P. patens* genome using the non-homologous and homologous integration methods. *P. patens* transformants were divided into two categories: unstable and stable. Unstable transformants are resistant to antibiotics on the primary selection medium, but become sensitive on secondary selection after two weeks of culturing on non-selective medium. Stable transformants can grow on both primary and secondary selection media. Using non-homologous integration with 30 μg of NHI-GT or NHI-ET, we obtained 5 ± 3 (n = 4) and 7 ± 6 (n = 4) stable transformants, respectively. Homologous integration, using the HI-GT or HI-ET mini-transposons and the shuttle mutagenesis method, resulted in 94 ± 47 (n = 4) and 102 ± 48 (n = 4) stable transformants, respectively.

Expression patterns of the uidA *gene in trap lines and integration complexity in the trap lines*

All transformants were screened for GUS activity and it was detected histochemically in 8 of 267 stable transformants (3%) carrying NHI-GT, 18 of 146 stable transformants (12%) carrying NHI-ET, 227 of 5370 stable transformants (4%) carrying the HI-GT mini-transposon,

Table 2 Expression patterns of the *uidA* gene in gene-trap and enhancer-trap lines

	Non-homologous integration		Homologous integration	
Stained tissue	Gene-trap	Enhancer-trap	Gene-trap	Enhancer-trap
Gametophyte[a]	8 (100%)	18 (100%)	227 (100%)	1055 (100%)
Protonema				
Chloronema	5 (63%)	8 (44%)	49 (21%)	471 (44%)
Caulonema	3 (38%)	8 (44%)	24 (11%)	165 (16%)
Gametophore				
Bud	1 (13%)	1 (6%)	13 (6%)	55 (5%)
Leaf	5 (62%)	12 (67%)	110 (49%)	330 (31%)
Stem	1 (13%)	3 (17%)	17 (8%)	31 (3%)
Rhizoid	2 (25%)	2 (11%)	28 (12%)	57 (5%)
Apex[b]	2 (25%)	1 (6%)	14 (6%)	24 (2%)
Axillary hair	6 (75%)	12 (67%)	149 (66%)	884 (84%)

[a]Numbers of lines exhibiting GUS staining in listed tissues are given. The number in gametophyte is set as 100%. As most lines showed GUS-positive staining in several different tissues, the sum of percentages is more than 100%.
[b]Apex indicates the number of lines showing GUS staining in an apical cell and leaf primordial cells.

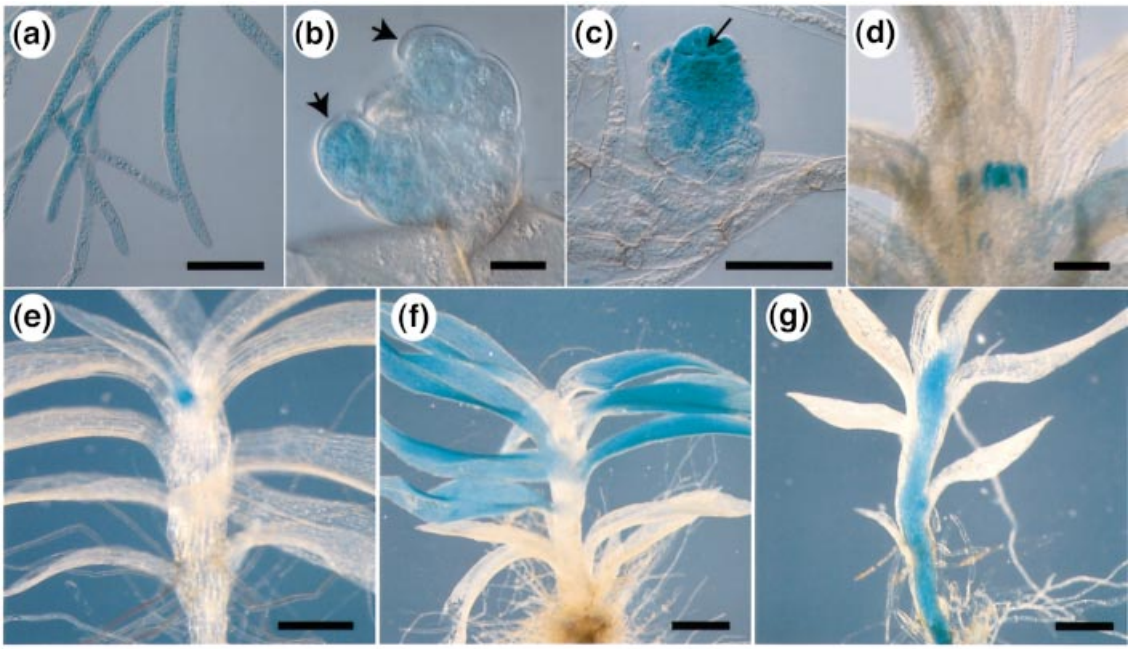

Figure 2. Histochemical GUS activity in representative trap lines.
(a) Chloronemal cells of gene-trap line YH261. (b) A bud of enhancer-trap line ET77. Leaf primordial cells (arrows) are predominantly stained. (c) A bud of gene-trap line YH727. An apical cell (arrow) and its surrounding cells are stained. (d) Apical portion of a gametophore in gene-trap line YH8. Axillary hairs that differentiate at the adaxial base of the leaf are stained. (e) A gametophore of enhancer-trap line ET63. The apical portion of the gametophore is predominantly stained. (f) A gametophore of gene-trap line YH560. Leaves are stained. (g) A gametophore of enhancer-trap line ET21. A stem is stained. LinesYH261, YH727, YH8 and YH560 were generated using HI-GT, lines ET63 and ET21 were generated using HI-ET, and line ET77 was generated using NHI-ET. Bars in (a), (c) and (d) = 100 μm, bar in (b) = 20 μm, and bars in (e), (f) and (g) = 300 μm.

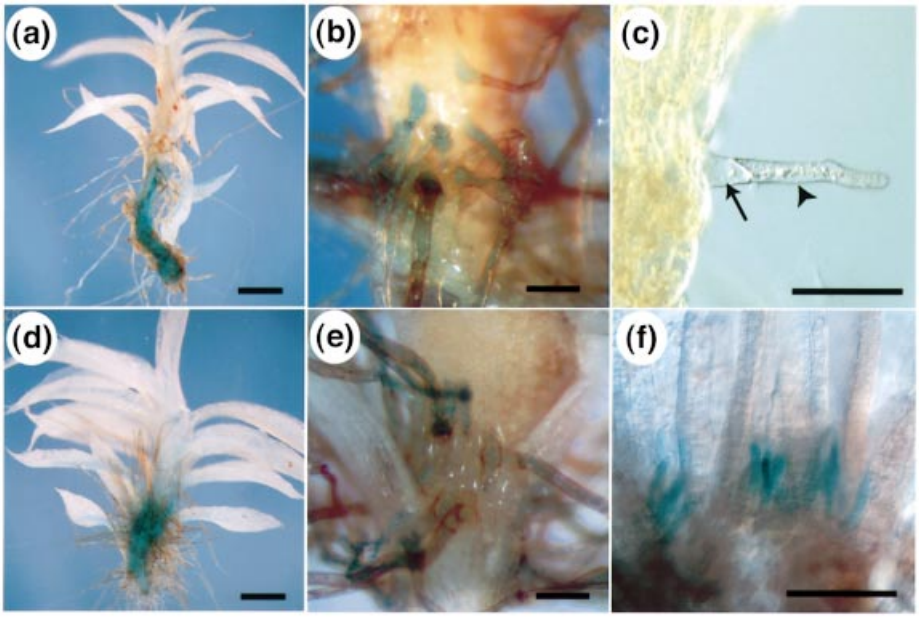

Figure 4. Histochemical detection of GUS activity in the gene-trap line YH209 (a–c) and the transformant line 0404-1 (d–f), in which the 209-8SC DNA fragment replaces its homologous site. (a) A gametophore of YH209. (b) A magnified view of the basal part of the YH209 gametophore, demonstrating staining of the pigmented rhizoid basal cells and rhizoid cells. (c) A young rhizoid of YH209. A basal cell (arrow) and a rhizoid cell (arrowhead) that lack pigmentation are not stained. (d) A gametophore of the 0404-1 line. (e) A magnified view of the basal part of the line 0404-1 gametophore showing that rhizoid basal cells and rhizoid cells are stained. (f) A magnified view of the apical portion of line 0404-1, showing stained axillary hairs. Bars in (a) and (d) = 500 μm, bars in (b) and (e) = 200 μm, bars in (c) and (f) = 100 μm.

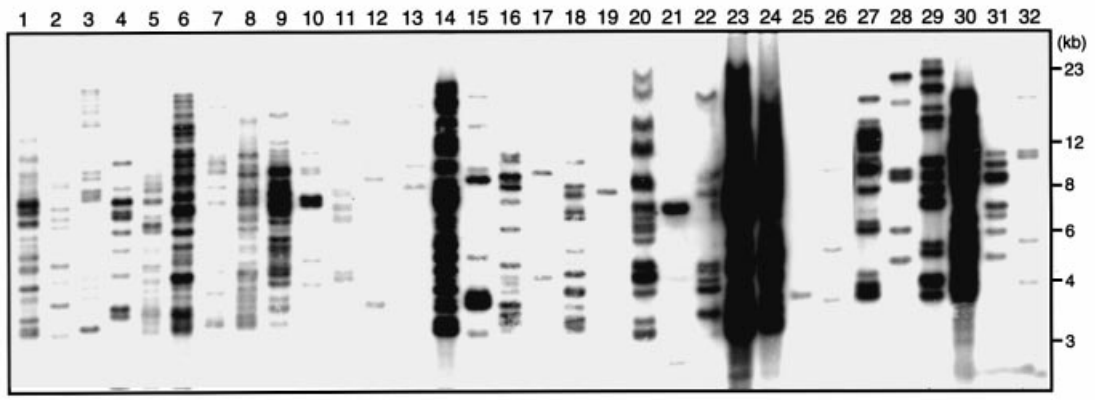

Figure 3. Estimation of the copy number of the *uidA* gene in gene-trap lines.
Genomic DNA from 32 gene-trap lines was digested with *Eco*RI and hybridized with a *uidA*-specific probe. Lanes 19 and 25 show a single band, indicating that these lines contain a single copy of the *uidA* gene.

and 1055 of 3580 stable transformants (30%) carrying the HI-ET mini-transposon. GUS expression patterns in protonemata and gametophores varied (Table 2); representative lines are shown in Figure 2.

To estimate the copy number of each trap element in a trap line, we performed genomic Southern analysis of gene-trap lines generated using the HI-GT mini-transposon. Genomic DNA preparations from 32 randomly selected lines were digested with *Eco*RI. The mini-transposon contains one *Eco*RI site (Figure 1). A PCR-amplified fragment of the mini-transposon *uidA* gene, from start to stop codons, was used as a *uidA*-specific probe. Based on hybridization patterns, the copy number of the mini-transposon in each trap line was estimated. Thirty trap lines contained multiple insertions, and two lines contained only an insertion of a single copy (nos 19 and 25 in Figure 3).

Analysis of one gene-trap line, YH209

To assess the feasibility of isolating a trapped gene, one line, YH209, generated using HI-GT, was studied further. In this line, GUS activity was observed in the basal part of the gametophore (Figure 4a). At high magnification, GUS activity could be seen predominantly in mature rhizoid cells proximal to the stem (Figure 4b). A rhizoid is a slender, multicellular filament attached to the stem of a gametophore. Rhizoid differentiation begins when an epidermal cell of a gametophore stem forms a protrusion, into which a nucleus moves. Then, cell division forms a rhizoid basal cell and a rhizoid cell (Figure 4c). The rhizoid cell divides several times to form filamentous cells. When the rhizoid is composed of four or less cells, the cells are colourless. When the rhizoid consists of more than approximately four cells, the basal cell and several rhizoid cells proximal to the stem have a brown pigmentation. GUS activity was observed in the pigmented rhizoid basal cell and in two or three pigmented rhizoid cells attached to the basal cell (Figure 4b), although pigmented rhizoid cells were not always stained. GUS activity was not observed in colourless rhizoid cells (Figure 4c).

Genomic DNA isolated from the line YH209 was digested with *Bgl*II and hybridized with the *uidA* probe. Four *Bgl*II fragments were detected (data not shown), indicating that the line YH209 contains at least four copies of the HI-GT mini-transposon. Hybridization of poly(A)+ RNA from the line YH209 with the *uidA*-specific probe detected a broad band of approximately 2.4 kb (Figure 5a). The size of *uidA* mRNA from the start to the stop codon is 1.9 kb long with a 0.1 kb 3′ untranslated region, indicating that a part of *P. patens* chromosomal genes was fused to the *uidA* gene.

We performed 5′ RACE, using *uidA* gene-specific primers and cDNA derived from gametophore poly(A)+ RNA of the line YH209, and obtained two candidate DNA fragments, 209-7 and 209-13 (Figure 5b). Fragment 209-7 contained 1041 bp of *P. patens* DNA adjacent to the 5′ end of the HI-GT mini-transposon. Fragment 209-13 contained 433 bp of *P. patens* DNA adjacent to the 5′ end of a partial HI-GT mini-transposon that begins with the second acceptor site of the *GPA1* intron. The 804 bp fragment generated by *Hin*dIII and *Xho*I digestion of *P. patens* DNA in 209-7 was used as a probe for colony hybridization screening of the tagged genomic library used for *P. patens* transformation. Sequencing of a positive clone, containing an 11.6 kb mini-transposon-tagged fragment named 209-8SC, revealed that mTn-3xHA/GUS2 separated a 5.6 kb *P. patens* genomic DNA fragment into 2.2 and 3.4 kb fragments (Figure 5b). The *P. patens* DNA fragments found in both the 209-7 and 209-13 clones were present in the 2.2 kb fragment (Figure 5b).

To determine whether the 209-8SC fragment integrated into homologous or non-homologous sites in line YH209,

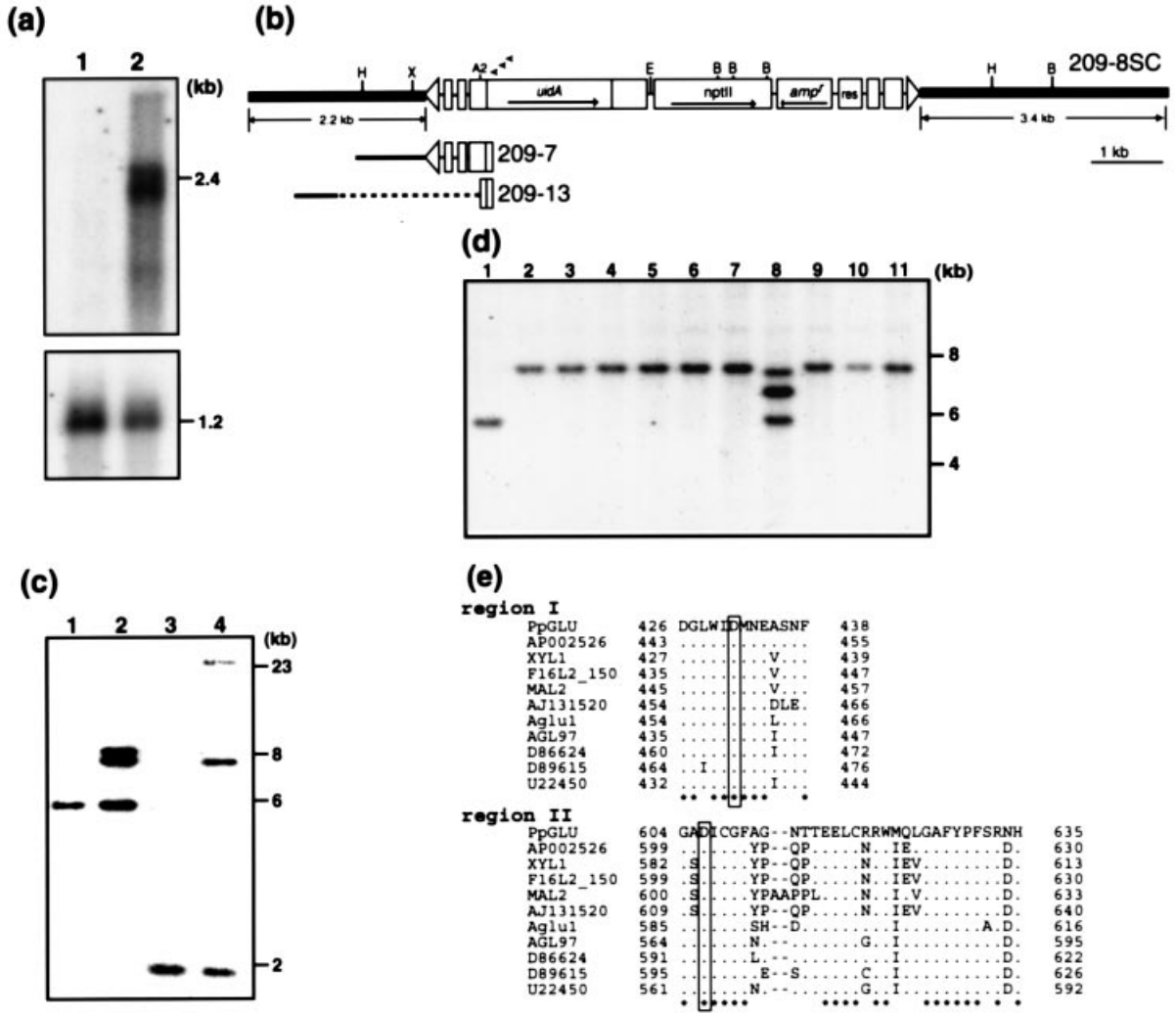

Figure 5. Molecular analysis of gene-trap line YH209, generated by the HI-GT mini-transposon.
(a) Northern analysis of wild-type (lane 1) and YH209 (lane 2). Blots were probed with a *uidA* gene-specific probe (upper panel) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a control (lower panel). (b) Schematic representation of a transposon-tagged genomic fragment (209-8SC) and two 5′ RACE products (209-7 and 209-13). Thick lines represent *P. patens* genomic DNA fragments. Locations of the *uidA* gene-specific primers used for 5′ RACE are indicated with black arrowheads. A2 indicates the second acceptor site of the *GPA1* intron. For the 5′ RACE products, the 1041 and 433 bp cDNA fragments corresponding to the genomic region in 209-8SC are indicated as black lines in 209-7 and 209-13, respectively. A dotted line in the 209-13 fragment shows the region that may be alternatively spliced out, unlike in the 209-7 fragment. *Eco*RI (E), *Bgl*II (B), *Hin*dIII (H) and *Xho*I (X) sites are shown. (c) Genomic Southern analysis of wild-type and YH209. Genomic DNA from wild type (lanes 1 and 3) and YH209 (lanes 2 and 4) was digested with *Bgl*II (lanes 1 and 2) or *Hin*dIII (lanes 3 and 4). The 804 bp DNA fragment of the 209-7 product was used as a probe. (d) Southern analysis of wild-type (lane 1) and stable transformants (lanes 2–11) with the 209-8SC fragment. Genomic DNA was digested with *Bgl*II and probed with the 804 bp DNA fragment of 209-7. Bands of lanes 2–11 were shifted, indicating that the wild-type sequence was homologously replaced with the 209-8SC fragment in these lines. Multiple 209-8SC fragments were integrated in the line of lane 8. Lane 6 corresponds to line 0404-1, shown in Figure 4(d–f). (e) Alignment of amino acid sequences in the conserved signature regions I and II of the glycoside hydrolase family 31 (Frandsen and Svensson, 1998). Asterisks indicate identical amino acids in all members and the putative catalytic site is boxed. Identical amino acid residues to PpGLU are shown as dots and gaps as dashes, respectively. Accession numbers of the aligned genes are: *PpGLU* (AB057452), *XYL1* (AF087483), F16L2-150 (CAB82818), *MAL2* (AJ277244), *AGLU1* (AF014806) and *AGL97* (AF118226). Other accession numbers show the following: AP002526, EST of *Oryza sativa*; AJ131520, α-xylosidase of *Tropaelim majus*; D86624, α-glucosidase of *Spinacia oleacea*; D89615, α-glucosidase of *Beta vulgaris*; U22450, α-glucosidase of *Hordeum vulgare*.

P. patens genomic DNA digested with *Bgl*II or *Hin*dIII was hybridized with the 804 bp DNA fragment of 209-7. A single band was detected in wild-type genomic DNA digested with either enzyme, while two extra bands were detected in digests of line YH209 genomic DNA (Figure 5c). *Bgl*II and *Hin*dIII do not cut within the 804 bp fragment used as a probe. Thus, we conclude that two copies of 209-8SC integrated into non-homologous sites.

To determine whether the GUS activity of the line YH209 reflects the endogenous expression pattern of a gene

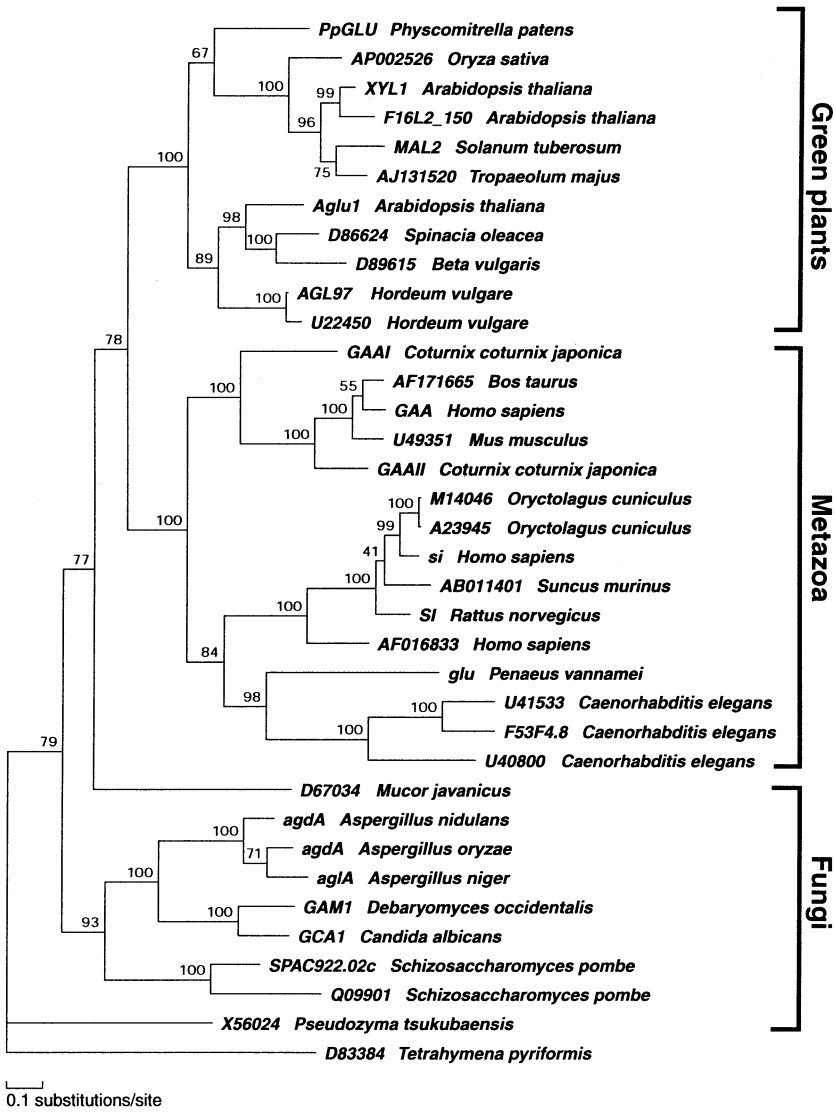

Figure 6. Phylogenetic analysis of *PpGLU*. Maximum-likelihood tree of 36 genes, rooted by the *Tetrahymena* gene. Local bootstrap values are indicated on or below branches. Horizontal branch length is proportional to the estimated evolutionary distance. Accession numbers of genes are *PpGLU* (AB057452), *XYL1* (AF087483), *F16L21-50* (AL162459), *MAL2* (AJ277244), *Aglu1* (AF014806), *AGL97* (AF118226), *GAAI* (AB000967), *GAA* (Y00839), *GAAII* (AB006754), *SI* (L25926), *si* (X63597), *glu* (AJ250828), *F53F4.8* (Z77663), *agdA* of *Aspergillus nidulans* (AF208225), *agdA* of *Aspergillus oryzae* (D45179), *aglA* (D45356), *GAM1* (M60207), *GCA1* (AF082188), *SAPC9922.02c* (Q09901).

encoded on 209-8SC, we introduced 209-8SC to *P. patens* by homologous recombination. Genomic DNA was extracted from 10 stable transformants, digested with *Bgl*II, and hybridized with the 804 bp probe fragment. The band hybridizing to the wild-type DNA was shifted in nine out of 10 lines, clearly indicating that 209-8SC is integrated in a homologous site in these nine lines (Figure 5d). GUS activity in the nine lines was examined (Figure 4d,e). The observed expression patterns were similar to those of the line YH209, except for additional activity at axillary hairs (Figure 4f).

The 1041 bp *P. patens* DNA fragment of 209-7 contained an expected start codon, as deduced from comparisons with the α-xylosidase and acidic α-glucosidase genes. To clone the 3′ region of the 1041 bp DNA fragment, 3′ RACE was performed, and a 2198 bp fragment was obtained. According to the nucleotide sequence of the 1041 bp DNA fragment and the 3′ RACE product, primers were designed to match the 5′ and 3′ ends of the fragments and were used to amplify the entire cDNA. The isolated cDNA was sequenced and found to encode a putative protein of 916 amino acids with high similarity to members of the glycoside hydrolase family 31 (Frandsen and Svensson, 1998). The gene was named *Physcomitrella patens α-glucosidase-like gene (PpGLU)*. The protein encoded by *PpGLU* contains two conserved signature regions of the glycoside hydrolase family 31, including the putative catalytic aspartic acid residues (Frandsen and Svensson, 1998) (Figure 5e).

To determine the relationship between *PpGLU* and other glycoside hydrolase family 31 genes, a phylogenetic analysis was performed. A BLAST search identified 85

genes with high similarity (*E* value $< 1 \times 10^{-5}$), and the sequences were aligned with *PpGLU*. After exclusion of short or redundant sequences, the phylogeny was inferred using 175 amino acid sites of 64 sequences by the maximum-likelihood method (data not shown). A distinct clade of eukaryotic genes with 100% local bootstrap support was found; in this clade, 12 land plant, 15 metazoan, and nine fungal genes each formed a clade. The *PpGLU* gene was positioned in the land plant clade with acidic α-glucosidase. To obtain better resolution, further analysis of the relationships within the plant gene clade was performed using 442 amino acid sites of the 36 eukaryotic sequences, with the *Tetrahymena pyriformis* gene (accession number D83384) as an outgroup. After local rearrangements starting from the neighbor-joining tree and the five most parsimonious trees, which contained 3517 steps, a tree with a log likelihood of $-18\,911.96 \pm 476.56$ was obtained as the maximum-likelihood tree (Figure 6). In this tree, flowering plant α-glucosidase genes were divided into two clades with high local bootstrap support (89 and 100%). Each clade included both eudicot and monocot genes. *PpGLU* was positioned at the base of a clade that included the *Arabidopsis* α-xylosidase gene *XYL1* (Sanpedro *et al.*, 2001) and the potato α-glucosidase gene *MAL2*, with 67% local bootstrap support.

Discussion

Large-scale generation of trap lines by the homologous integration method

Gene-trap and enhancer-trap elements were constructed for both non-homologous and homologous integration methods (Figure 1). Transient GUS assays of enhancer-trap elements indicated that the core sequence of the CaMV 35S promoter has weaker promoter activity than the longer form and that the former version of the promoter is suitable for an enhancer-trap system in *P. patens*. Transformation efficiencies of non-homologous and homologous integration methods were compared; homologous integration methods generated approximately 10-fold more stable transformants than non-homologous methods. The results indicate that homologous integration using shuttle mutagenesis is more suitable for generating large numbers of trap lines. Molecular analysis of one gene-trap line, YH209, revealed that the DNA fragment 209-8SC did not insert into its homologous site. In addition to the line YH209, three other gene-trap lines were preliminarily analysed, of which all had tagged genomic DNA at non-homologous sites. This was unexpected, since the rate of homologous recombination is 10-fold higher than the non-homologous recombination rate (Schaefer, 2001), and homologous DNA fragments are expected to insert in their homologous sites. Previous analysis of *Physcomitrella* mutant lines generated by shuttle mutagenesis suggested that arrays of inserted DNA fragments are formed in *Physcomitrella* cells (Nishiyama *et al.*, 2000). Extra-chromosomal homologous recombination may explain how the large fragments were formed and why the 209-8SC DNA fragment was inserted into non-homologous sites in the line YH209. Shuttle mutagenesis results in tagging of *Physcomitrella* genomic DNA fragments by mini-transposons. Typically, one DNA fragment is tagged by a single mini-transposon, and the insertion site is random (Nishiyama *et al.*, 2000). Thus, in a tagged genomic library, there are multiple fragments derived from the same *Physcomitrella* DNA fragment; however, each is tagged in a different position by the mini-transposon. When these differently tagged DNA fragments containing the same *Physcomitrella* DNA sequences are introduced into a protoplast, two of these fragments themselves may undergo homologous recombination to form a DNA fragment having two copies of the mini-transposon and a DNA fragment without any mini-transposon. Furthermore, DNA fragments containing different *Physcomitrella* genomic DNA sequences may be subject to homologous recombination based on homology in the mini-transposon. Successive homologous recombination of these two fragments will generate a large DNA fragment consisting of an array of homologous and non-homologous *P. patens* genomic sequences and multiple copies of the mini-transposon. Finally, this large DNA fragment may be integrated into the *P. patens* genome by homologous recombination based on the homology of the terminal *P. patens* sequence of the large DNA fragment, but the internal part of the large DNA fragment may contain different *P. patens* DNA sequences from the terminal sequence. Formation of a large DNA fragment before integration into the genome by extra-chromosomal ligation has been reported in rice (Kohli *et al.*, 1998). Homologous recombination is expected to be more frequent than ligation in *P. patens*. If extra-chromosomal ligation occurred frequently, we would have observed multiple insertions of the 209-8SC fragment.

In addition to the high transformation rate achieved, integration in a non-homologous site provides the advantage of trapping a gene or enhancer without disrupting its original site. A gene-trap element does not need to be integrated at the homologous site to drive expression of the reporter gene as long as regulatory elements are present in the tagged genomic DNA fragment that are necessary and sufficient to regulate expression of the reporter gene. This is likely to be important when using a moss system. *P. patens* has an autotrophic haploid generation that differs from the epiphytic seed plant haploid generation, and the majority of its life cycle is haploid. Thus, a greater number of lethal transformants may result

from disruption of a gene or regulatory element responsible for cell viability. Integration by non-homologous recombination will lower the possibility of such deleterious effects.

GUS expression patterns of trap lines

We generated 235 and 1073 of gene-trap and enhancer-trap lines, respectively. GUS staining of these lines revealed that genes or enhancers with various expression patterns were trapped (Table 2 and Figure 2). It is noteworthy that axillary hairs were stained in 155 of 235 (66%) gene-trap lines and 896 of 1073 (84%) enhancer-trap lines, much higher percentages than for other cells or tissues (Table 2). Axillary hairs develop at the adaxial base of a leaf and consist of a few cells arranged linearly. These hairs are reported to produce mucilage in some mosses (Ligrone, 1986), although this has not been examined in *P. patens*. It is possible that a gene expressed in axillary hairs or its enhancer element harbours a hot spot for the integration of trap elements. Alternatively, it is possible that many genes are expressed in axillary hairs compared to other cells and tissues. Our previous genomic Southern analyses (Nishiyama *et al.*, 2000) indicated that DNA fragments insert into different sites, supporting the latter explanation. However, we cannot formally exclude the alternative possibility. Further analyses of the expression patterns of *P. patens* genes and analyses of trap lines will provide insights into this issue.

Identification of a trapped gene, PpGLU

As we demonstrated in the analysis of the line YH209, a trapped gene can be identified efficiently by 5′ RACE, using *uidA*-specific primers, despite the presence of multiple insertions in a trap line. The isolation of the trapped gene would be more difficult if the reporter gene was expressed at all insertion sites. The isolated trapped gene *PpGLU* encodes a member of the glycoside hydrolase family 31 (Frandsen and Svensson, 1998). In our tree, *PpGLU* clusters with flowering plant acidic α-glucosidase genes (Figure 6). Two different functions have been assigned to plant acidic α-glucosidases. Acidic α-glucosidases of barley (accession number U22450,Tibbot *et al.*, 1998; *AGL97* [AF118226], Frandsen *et al.*, 2000) and spinach (accession number D86624,Sugimoto *et al.*, 1995) are involved in the degradation of seed starch. Additionally, acidic α-glucosidase activity has been found in fractions that include cell walls (Beers *et al.*, 1990; Klis, 1971; Monroe *et al.*, 1999; Parr and Edelman, 1975; Yamasaki and Konno, 1987, 1992), although the respective genes have not been cloned. Based on their cell wall-specific localization, Taylor *et al.* (2000) speculated that acidic α-glucosidases are involved in xyloglucan oligosaccharide metabolism within cell walls, although possible substrates were not specified. Rhizoids contain reduced chloroplasts, and their starch granules are not visible. Thus, a rhizoid is not likely to be a photosynthetic organ, suggesting that the protein encoded by *PpGLU* is indeed involved in xyloglucan oligosaccharide metabolism rather than starch degradation. *PpGLU* is expressed in mature, pigmented rhizoid cells, including the rhizoid basal cell. However, the gene is not expressed in these cells before they mature. The metabolism of the pigment is unknown, and it is difficult to speculate on the relationship between pigmentation and *PpGLU* function.

Many genes have been isolated based on similarity to known genes, using PCR or hybridization techniques, and characterized in *P. patens* (Andreeva and Kutuzov, 1999; Girke *et al.*, 1998; Girod *et al.*, 1999; Hara *et al.*, 2001; Krogan and Ashton, 2000; Leech *et al.*, 1993; Sakakibara *et al.*, 2001; Strepp *et al.*, 1998). However, no methods have been developed to identify genes based on their spatial expression patterns. In addition to the example of the line YH209, we have identified two other genes specifically expressed in meristem tissue. In conclusion, the gene-trap and enhancer-trap systems established in this study should be useful to isolate genes with specific expression patterns.

Experimental procedures

Culture conditions, transformation, and shuttle mutagenesis

The wild-type strain of *Physcomitrella patens* ssp. *patens* (Ashton and Cove, 1977) was used, and culture conditions were as described previously (Nishiyama *et al.*, 2000). For the non-homologous recombination method, plasmids containing a trap element were linearized with *Hin*dIII, purified by phenol/chloroform extraction and PEG precipitation, and resuspended in TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) to a concentration of 1 μg μl^{-1}. PEG-mediated transformation and shuttle mutagenesis of *P. patens* were performed as described previously (Nishiyama *et al.*, 2000). BCDAT medium (Nishiyama *et al.*, 2000) was supplemented with 20 mg l^{-1} G418 (Gibco-BRL, Rockville, MD, USA) for transformant selection.

Construction of gene- and enhancer-trap elements and mini-transposons

The NHI-GT and NHI-ET plasmids were constructed using pTN3, the pBluescript SKII+ plasmid (Stratagene) equipped with the NPTII expression cassette (Nishiyama *et al.*, 2000). As a splice acceptor in NHI-GT, the fourth intron of the *Arabidopsis* G protein gene, *GPA1*, was PCR-amplified using the int-F primer (5′-GCAAGCTTAGGGATGAAAACGGTCG-3′) and the int-R primer (5′-GGACCTGCATATAACCTGCATATAAC-3′), and the pWS32 plasmid as template (Sundaresan *et al.*, 1995). A fragment containing the CaMV 35S promoter, *uidA* gene and nos terminator was excised from pBI221 (Clontech Laboratories, Palo Alto, CA, USA) as an *Eco*RI/*Hin*dIII fragment, and cloned into the *Eco*RI and *Hin*dIII sites of pTN3. This plasmid was designated pCaMV35S. pCaMV35S

was digested with *Hin*dIII and *Sma*I to remove the CaMV 35S promoter, blunt-ended, and religated to make pdeltaGT3. To construct NHI-GT, the PCR-amplified *GPA1* intron was digested with *Hin*dIII to cut one end of the product, and then ligated into *Hin*dIII/*Sma*I-digested pCaMV35S. A short core sequence (–46 to +1) of the CaMV 35S promoter was amplified by PCR using the 35S mini-F primer (5′-CTAAGCTTCGCAAGACCCTTCCTC-3′) and the GUSseq primer (5′-TCACGGGTTGGGGTTTCTAC-3′) and pBI221 as template. To make NHI-ET, the resulting PCR fragment was digested with *Hin*dIII and *Sma*I and ligated into the *Hin*dIII/*Sma*I-digested pCaMV35S.

The HI-ET mini-transposon was constructed using the pTn plasmid (Seifert *et al*., 1986). A synthetic adapter containing *Hin*dIII, *Sac*II, *Kpn*I, *Cla*I and *Sal*I cleavage sites was inserted between the *Hin*dIII and *Sal*I site of pTn to make pTn3-5.

A *Cla*I/*Sac*II fragment containing the CaMV 35S promoter core sequence, *uidA* gene, nos terminator and NPTII expression cassette was excised from NHI-ET and ligated into the *Cla*I and *Sac*II sites of pTn3-5, to produce HI-ET. HI-ET was moved to the F derivative, pOX38, as described by Seifert *et al*. (1986).

DNA and RNA extractions

For DNA extraction, vegetatively propagated protonemata were cultured on BCDATG medium (Nishiyama *et al*., 2000) under continuous light for 2 weeks, and then kept in the dark for 2 days. For RNA extraction, vegetatively propagated protonemata were grown on BCDATG medium for 13 days under continuous light. Tissue was ground to a powder in liquid nitrogen. Genomic DNA was extracted with a Nucleon Phytopure plant and fungal DNA extraction kit (Amersham-Pharmacia Biotech, Bucks., UK), and further purified by the CTAB method (Murray and Thompson, 1980). Total RNA was extracted according to Hasebe *et al*. (1998), and purified with ISOGEN-LS (Wako Pure Chemical, Osaka, Japan). Poly(A)$^+$ RNA was isolated using a DynaBeads mRNA purification kit (DYNAL, Oslo, Norway).

Southern and Northern analyses

For Southern analysis, 2 μg of *P. patens* genomic DNA were digested, separated on 0.7% (w/v) SeaKemGTG agarose (BME, Rockland, ME, USA), and transferred to a Hybond N+ nylon membrane (Amersham-Pharmacia Biotech). For Northern analysis, 0.5 μg of poly(A)+ RNA was separated on 1.0% (w/v) agarose gels containing formaldehyde/MOPS and transferred to Hybond N+ nylon membrane. The *uidA* DNA fragment was PCR-amplified with the 35S mini-F (5′-CTAAGCTTCGCAAGA CCCTTCCTC-3′) and NOSter (5′-ATGTTTGAACGATCGGGGAAAT-3′) primers using NHI-ET as a template. The amplified fragment was purified from an agarose gel using GENECLEAN III (BIO101, Vista, CA, USA) and labelled using a Random Primer DNA labelling kit version 2.0 (Takara, Tokyo, Japan). The 804 bp *Hin*dIII/*Xho*I fragment of the 209-7 DNA fragment was also excised and labelled as described. For both Southern and Northern analyses, hybridization was performed in 0.5 M Church's phosphate buffer (0.5 M Na_2HPO_4 adjusted to pH 7.2 with H_3PO_4; Church and Gilbert, 1984) at 65°C. Northern blots were re-probed with *P. patens* glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) to control for loading; the *Pst*I–*Eco*RI fragment of the pPpGapC plasmid (provided by R. Kofuji, Kanazawa University, Kanazawa, Japan) was excised and labelled, and hybridization was performed as described above.

RACE

For 5′ RACE of the YH209 gene-trap line, total RNA was treated with RNase-free DNAase I (Gibco-BRL), and first-strand cDNA was synthesized with the 5′ RACE system using SuperScript II reverse transcriptase (Gibco-BRL) and the GUS R4 primer (5′-ATTGAC-CCACACTTTGCCGTAATGAGTGAC-3′). The synthesized cDNA was amplified with the anchor primer supplied by Gibco-BRL and the GUS R3 primer (5′-TCTTGTAACGCGCTTTCCCACC-AACGCTGA-3′) using ExTaq DNA polymerase (Takara). PCR cycle conditions consisted of 5 min at 94°C, then 25 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 3 min, followed by 10 min at 72°C. Further PCR was carried out with the UAP primer (5′-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3′) supplied by Gibco-BRL and the 5′GUS1 primer (5′-CAUCAUCAUCAUTTG-GCGATCCAGACTGAATG-3′). PCR conditions were as in the previous reaction, except the annealing temperature was lowered to 59°C. PCR products were purified with SUPREC-02 (Takara), and cloned into pIMAz2. pIMAz2 was constructed by PCR amplification of pGEM3z (Promega, Madison, WI, USA) using the prIMAz2-2-primer (5′-AUGAUGAUG AUGCCTCTAGAG-TCGACCTGCAGGCA-3′) and the prIMAz2-1+ primer (5′-UAGUAG-UAGUAGCCCGGGTACCGAGCTCGAA-3′) to add the sequences complementary to CAUCAUCAUCAU and CUACUACAUCAU to each end. The PCR fragment amplified with the UAP and 5′GUS1 primers was annealed with pIMAz2 using uracil DNA glycosylase (Gibco-BRL).

For 3′ RACE, to obtain the 3′ region of the 1041 bp DNA fragment, cDNA was synthesized from 2 μg of total RNA using SuperScript II reverse transcriptase with the adaptor primer. PCR was performed using this cDNA as template with the UAP primer and the 209-7p1 primer (5′-GGACATCAGAGAAGGAGGCG-3′). PCR cycle conditions consisted of 3 min at 94°C, 30 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 3 min. Additional PCR was carried out with the UAP primer and the 209-7p2 primer (5′-CGCATGGAGTGTTGATGC-3′). PCR conditions were as in the previous PCR, except that the annealing temperature was 59°C. Finally, the entire cDNA was amplified with YH209LF190 (5′-CGGAGCGGTGGATTTTTTCTC-3′) and YH209LR3030 (5′-GGATGAACACCCACTCAAGTACC-3′) primers. PCR conditions were the same as in the previous PCR. The PCR product was isolated from an agarose gel using GENECLEAN III and cloned into pGEM-T (Promega).

Phylogenetic analysis

Related protein sequences were identified by performing a BLASTP search at NCBI against the nr dataset. The amino acid sequences were aligned using CLUSTAL W, version 1.81 (Thompson *et al*., 1994; optimized for a multiprocessor by SGI, Amphitheater, CA, USA) obtained at http://www.sgi.com/chembio/resources/clustalw/parallel_clustalw.html, and revised manually. To search for the maximum-likelihood (ML) tree, we used neighbour-joining (NJ) and most-parsimonious (MP) trees as the starting trees for a local rearrangement search (Adachi and Hasegawa, 1996). ML distances under the JTT model (Jones *et al*., 1992) were calculated using ProtML (Adachi and Hasegawa, 1996). MP trees were searched by the tree bisection reconnection algorithm using PAUP* version 4.0 beta 6 (Swofford, 2001) on a PowerPC-based Macintosh. The local bootstrap probability of each branch was estimated by the resampling-of-estimated-log-likelihood (RELL) method (Hasegawa and Kishino, 1994; Kishino *et al*., 1990).

GUS assay

GUS staining was performed as described by Nishiyama *et al.* (2000). GUS transient assays in protoplasts were performed according to Jefferson *et al.* (1987) with minor modifications. Protoplasts that were cultured for 48 h after PEG-mediated transformation were collected by centrifugation (180 ***g***), and suspended in extraction buffer (50 mM NaH_2PO_4 (pH 7.4), 1 mM EDTA, 0.1% v/v Triton X-100, 0.1% w/v N-lauroyl sarcosine and 10 mM 2-mercaptoethanol). Protoplasts were lysed by three freeze/thaw cycles and then centrifuged (14 000 ***g***). The supernatant was recovered as a crude extract. GUS enzyme assays were performed in triplicate in microtitre plates. GUS activities were determined using the substrate 4-methylumbelliferone glucuronide (MUG, Wako Chemical), and the fluorescent product (4-methyl-umbelliferone, 4MU) was quantified using a CORONA 130F fluorescence plate reader (Corona Electric, Ibaraki, Japan), based on fluorescence with an excitation wavelength of 360 nm and emission wavelength of 460 nm. Protein content was determined using a BioRad Protein Assay kit (BioRad Laboratories, Hercules, CA, USA). Specific GUS enzyme activities were calculated as pmol MU produced per min of reaction per mg protein.

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