Agrobacterium-Mediated Transformation of the Haploid Liverwort Marchantia polymorpha L., an Emerging Model for Plant Biology

Kimitsune Ishizaki, Shota Chiyoda, Katsuyuki T. Yamato and Takayuki Kohchi *
Graduate School of Biostudies, Kyoto University, Kyoto, 606-8502 Japan

Agrobacterium-mediated transformation has not been practical in pteridophytes, bryophytes and algae to date, although it is commonly used in model plants including Arabidopsis and rice. Here we present a rapid Agrobacterium-mediated transformation system for the haploid liverwort Marchantia polymorpha L. using immature thalli developed from spores. Hundreds of hygromycin-resistant plants per sporangium were obtained by co-cultivation of immature thalli with Agrobacterium carrying the binary vector that contains a reporter, the β-glucuronidase (GUS) gene with an intron, and a selection marker, the hygromycin phosphotransferase (hpt) gene. In this system, individual gemmae, which arise asexually from single initial cells, were analyzed as isogenic transformants. GUS activity staining showed that all hygromycin-resistant plants examined expressed the GUS transgene in planta. DNA analyses verified random integration of 1–5 copies of the intact T-DNA between the right and the left borders into the M. polymorpha genome. The efficient and rapid Agrobacterium-mediated transformation of M. polymorpha should provide molecular techniques to facilitate comparative genomics, taking advantage of this unique model plant that retains many features of the common ancestor of land plants.

Keywords: Agrobacterium-mediated transformation — Bryophyte — Hepaticae — Liverwort — Marchantia polymorpha — T-DNA.

Abbreviations: GUS, β-glucuronidase; TAIL-PCR; thermal asymmetric interlaced-PCR.

Introduction

The emergence of land plants from an aquatic ancestor that initiated during the mid-Ordovician and lower Silurian (480–430 million years ago) was one of the major evolutionary events for the life on planet earth. The latest molecular phylogenetic analysis using three different complementary data sets, including a multigene super matrix, a genomic structural character matrix and a chloroplast genome sequence matrix, strongly supports the sister relationship of liverworts to all extant land plants, and rejects alternative topologies (Qiu et al. 2006). Therefore, liverworts are a key group in comparative genomics to address fundamental questions in plant biology, such as the genetic basis of the key innovations that allowed land plants to evolve from aquatic ancestors and adapt to life on land, and the developmental genetic changes responsible for alterations in body plan within land plants (Bowman et al. 2007).

Marchantia polymorpha is a widely spread dioecious liverwort species, and has been extensively studied by developmental biologists since the 19th century (LaRue and Narayananswami 1956, Benson-Evans 1964, Hohe and Reski 2005). The haploid gametophytic generation dominates over the diploid sporophytic generation during its life cycle, which is obviously advantageous for functional genetic analyses. Marchantia polymorpha can propagate not only sexually, but also by asexual bud-like structures called gemmae, allowing rapid proliferation of isogenic biomass for molecular and biochemical experiments. The organellar genomes and the Y chromosome of M. polymorpha were the first to be sequenced in plants (Ohyama et al. 1986, Oda et al. 1992, Yamato et al. 2007), and a M. polymorpha whole-genome shotgun sequencing project has been initiated under the Community Sequencing Program at the Joint Genome Institute (DOE-JGI: http://www.jgi.doe.gov/sequencing/why/CSP2008/mpolymorpha.html). Thus M. polymorpha is being developed as a model plant occupying a critical evolutionary position to study specific molecular and cellular developmental processes in detail.

As an essential component of functional genomics, transformation techniques have been developed in M. polymorpha. Cells of young thalli grown from gemmae can be readily transformed by particle bombardment (Takenaka et al. 2000). The use of immature thalli developed from spores significantly improved the performance of transformation by particle bombardment (Chiyoda et al. 2008). In M. polymorpha, genetic transformation has facilitated functional analyses of genes via overexpression and gene silencing (Kajikawa et al. 2003a, Kajikawa et al. 2003b, Kajikawa et al. 2008). Transformation has also led to mutant isolation, including a line that constitutively enters the reproductive phase of development (Yamaoka et al. 2004). However, physical DNA delivery often generates a large number of independent

*Corresponding author: E-mail, tkohchi@lif.kyoto-u.ac.jp; Fax, +81-75-753-6127.
insertions and extremely complex transgene rearrangements (Kohli et al. 2003), creating an obstacle for further genetic analysis of such mutants.

In contrast, Agrobacterium-mediated genetic transformation has significant advantages over direct transformation, such as higher efficiencies of transformation, integration of DNA fragments with defined ends reducing the frequency of rearrangements, introduction of fewer copies of transgene into the host genome, transfer of larger segments of DNA and easier manipulation (Kohli et al. 2003). Due to these advantages, Agrobacterium-mediated transformation has become a method of choice for gene transfer in angiosperms and gymnosperms. Although there is a brief report using Agrobacterium to transform a M. polymorpha suspension-cultured cell line (Nasu et al. 1997), Agrobacterium-mediated transformation has not been successful in pteridophytes, bryophytes and algae to date (Gelvin 2003, Hohe and Reski 2005). Here we present a protocol for Agrobacterium-mediated transformation of the haploid liverwort, M. polymorpha, using immature thalli developed from spores, and demonstrate stable integration and expression of transgenes in M. polymorpha. This technique is based on a conventional Agrobacterium strain and binary vector, which is convenient for most Agrobacterium users.

Results

Stable transformation of M. polymorpha using A. tumefaciens

The choice of tissues for Agrobacterium infection is probably the most important factor for successful transformation of M. polymorpha as in the case of other plants (Hiei et al. 1994, Cheng et al. 1997). For example, very few, if any, transformants were obtained after co-cultivation of M. polymorpha thalli, young or mature, with Agrobacterium (M. Takemura personal communication). Therefore, we established a system for preparation of surface-sterilized sporangia of M. polymorpha (Chiyoda et al. 2008), making it possible to grow spores on axenic medium. Immature thalli that develop from spores are undergoing rapid cell divisions and were thus thought to be suitable for Agrobacterium infection.

Spores of M. polymorpha were germinated and grown into immature thalli, for 7 d in liquid culture under white light (Fig. 1A, B). At this time the immature thalli consisted of small clumps of cells. The 7-day-old immature thalli (Fig. 1B) were co-cultivated with Agrobacterium in the presence of 3,5-dimethoxy-4-hydroxyacetophenone (acetosyringone) for 2 d, and transferred directly to selective M51C agar medium after washing three times with M51C liquid medium on a 50 μm nylon mesh. Incubation of immature thalli with Agrobacterium harboring the binary plasmid pIG121Hm (Ohta et al. 1990) led to the formation of hygromycin-resistant plantlets (Fig. 1C), whereas Agrobacterium carrying no binary plasmid did not (Fig. 1D). Hygromycin-resistant thalli with rhizoids became distinct 10 d after transfer to the selection agar.
medium (Fig. 1E), whereas hygromycin-sensitive plantlets developed into chlorotic cell clumps (Fig. 1F). To avoid potential chimerism of hygromycin-resistant thalli, isogenic lines were obtained from gemmae which arise asexually from single initial cells in cupules, and used for further analysis (Barnes and Land 1908, Hughes 1971).

To monitor transgene expression in planta, histochemical β-glucuronidase (GUS) activity assays were performed with the wild type and 10 independent lines randomly selected from hygromycin-resistant plants. All the selected hygromycin-resistant lines stained blue (Fig. 1G), while wild-type cells did not (Fig. 1H). As we used an intron–GUS reporter gene that does not express detectable GUS activity in Agrobacterium cells (Ohta et al. 1990), these results indicate stable expression of the transgene in the hygromycin-resistant plants.

We observed the inheritance of hygromycin resistance and GUS activity by asexual progeny after at least three rounds of subculture through gemmae, confirming stable transmission of the transgenes in the asexual progeny. We also observed the inheritance of hygromycin resistance from transgenic lines to progeny (spores) after crossing (data not shown).

Factors that influence transformation efficiency

To optimize conditions for Agrobacterium infection of M. polymorpha, we first investigated the effect of the duration of immature thalli pre-culture on transformation efficiency. One sporangium contained ~1 x 10^5 spores whose germination rate evaluated on an agar plate was 35–45%. Spores were germinated and grown for different periods of time and then co-cultivated with Agrobacterium. The duration of pre-culture had significant effects on the transformation efficiency of M. polymorpha (Fig. 2A). No hygromycin-resistant plantlets were obtained with 0- and 3-day-old cultures. Hygromycin-resistant plantlets were obtained from 5 to 10 d pre-cultures, peaking at 7 d (600–800 per sporangium; Fig. 2A). The transformation efficiency diminished after 14 d of pre-culture, and, therefore, the optimal length of pre-culture is 5–7 d.

Acetosyringone, a phenolic compound produced by wounded plant cells, can induce the transcription of the virulence genes of Agrobacterium (Gelvin 2000). To determine the optimum concentration of acetosyringone, transformation efficiency was compared using 7-day-old immature thalli co-cultivated with Agrobacterium in the presence of different concentrations of acetosyringone. No hygromycin-resistant plantlets were recovered from immature thalli incubated with Agrobacterium without acetosyringone (Fig. 2B). Transformation efficiency was dramatically reduced when the acetosyringone concentration was ≥1 mM, and the maximum transformation efficiency was achieved at 100 μM acetosyringone.

Co-cultivation is an important step in the transformation process, as T-DNA is transferred and incorporated into the host’s genomic DNA during co-cultivation. To optimize the duration of co-cultivation, transformation efficiency was compared during different periods of
co-cultivation (Fig. 2C). The number of hygromycin-resistant individuals obtained increased dramatically from 12 to 24 h of co-cultivation, and the transformation efficiency steadily increased from 24 h up to 72 h of co-cultivation.

Integration of T-DNA into the genome

To investigate whether the T-DNA of binary vector pIG121Hm (Fig. 3A) was integrated into M. polymorph genome DNA and to estimate the number of insertion sites, Southern blot analyses were performed (Fig. 3B). DNA from randomly selected hygromycin-resistant thalli that exhibited GUS activity and from wild-type (male and female) thalli was digested with either EcoRI or BamHI, and allowed to hybridize with the hygromycin phosphotransferase gene (hpt) probe. All seven randomly selected hygromycin-resistant thalli (line #1–#7) show positive hybridization signals with the transgene probe. Since the T-DNA of pIG121Hm has a single EcoRI site (Fig. 3A), the EcoRI fragments of distinct sizes indicate different individual genomic insertion sites (Fig. 3B). The number of fragments allows estimation of the number of integration events in each thallus line, with the number of integrated copies varying from one to five. A single hybridization signal, which is of the same size as the BamHI fragment of vector pIG121Hm, was detected with the BamHI-digested genomic DNA of all examined hygromycin-resistant thalli (Fig. 3B), suggesting no rearrangement or fragmentation had occurred to at least the gus–hpt region of T-DNA upon transformation.

The junction sequences between the M. polymorph genomic DNA and T-DNA were amplified from selected transformants by thermal asymmetric interlaced-PCR (TAIL-PCR) (Liu et al. 1995), and nucleotide sequences were determined (Fig. 4). The sequences of seven right boundaries and six left boundaries from transformants are shown in Fig. 4, together with the sequences of the T-DNA borders of pIG121Hm. The sequences flanking the T-DNA insertion sites revealed no significant similarity to each other or to the T-DNA border sequences. The truncations of integrated T-DNAs range from 22 to 29 bp from the right border sequences, and from 3 to 50 bp from the left border sequences (Fig. 4).

Genomic PCR using primer sets for sex chromosome-specific DNA markers of M. polymorph (Fujisawa et al. 2001) was performed to investigate the sex genotype of the resultant transformants (Fig. 5). Transformant lines #1, #2 and #3 showed amplification with the primer set for the X-chromosome-linked DNA marker, rhf73, but no amplification with the primer set for the Y-chromosome-linked DNA marker, rbm27, indicating they are female individuals. Transformant lines #4, #5, #6 and #7 showed amplification with rbm27, but no amplification with the primer set for rhf73, indicating they are male individuals. These PCR results show no obvious bias of transformation in sex ratios. Taken together, both female and male individuals can be obtained from a single transformation procedure, since sporangia of M. polymorph contain both female and male spores following meiosis.

Discussion

Agrobacterium-mediated gene transfer is the method of choice for the genetic transformation of flowering plants (Gelvin 2003), and has been applied to other eukaryotic species, ranging from yeast, to fungi to human cells (Kunik et al. 2001, Lacroix et al. 2006). Nasu et al. (1997) previously reported Agrobacterium-mediated transformation of M. polymorph suspension-cultured cells, but no intact transgenic plants were obtained. Here we have established an efficient and reproducible Agrobacterium-mediated transformation protocol for M. polymorph using immature thalli. In our protocol, immature thalli developed from spores are directly infected by Agrobacterium, with neither a protoplast preparation nor a regeneration step being required to obtain intact transgenic plants.
Furthermore, just a single step of subcultivation on selective solid medium is sufficient to obtain transformants, as compared with a two-step selection procedure, a selection with solid medium following selection in liquid medium, in the particle bombardment-mediated transformation to young thalli grown from gemmae (Takenaka et al. 2000).

The use of immature thalli developed from spores resulted in a high transformation efficiency (Figs. 1, 2). The developmental stage was crucial for transformation efficiency (Fig. 2A), suggesting that different cell types might have different competencies for Agrobacterium infection. The requirement for acetosyringone during the co-cultivation step suggests that virulence gene activity induced by acetosyringone (Gelvin 2000) is essential for Agrobacterium-mediated T-DNA transfer into M. polymorpha cells.

Southern blot analyses confirmed that the hygromycin-resistant thalli indeed had insertions of the hpt gene in their genomes. The hybridization patterns differed among the transformants, strongly indicating that T-DNAs were randomly integrated in the M. polymorpha genome. The EcoRI-digested DNA fragments that hybridized to the hpt probe (Fig. 4B) clearly did not originate from contaminating Agrobacterium used in the transformation because the intact vector would have a single band of defined size upon EcoRI digestion, 15 kb from pIG121Hm. No significant rearrangements of DNA, e.g. deletions of part of the T-DNA, were observed in the Southern blot analyses. The numbers of hybridizing fragments in EcoRI-digested genomic DNA indicate that multiple copies of the transgene can be integrated in the genome.

Analyses of the sequences flanking the right and left ends of the integrated T-DNA revealed small truncations of both ends (Fig. 4). The sizes of the truncations varied more on the left end than on the right end, as has been reported for other plants (Yadav et al. 1982, Hiei et al. 1994). Analyses of the M. polymorpha genomic sequences flanking the integrated T-DNAs did not show any extensive homology with each other, suggesting that T-DNA integration in M. polymorpha, as in other organisms, is sequence independent. This result suggests that T-DNA transfer from Agrobacterium to M. polymorpha occurs by a very similar, if not an identical, molecular mechanism to that in angiosperms (Tzfira and Citovsky 2006). One of the T-DNA flanking sequences matched an M. polymorpha expressed sequence tag (EST; accession No. BJ864633), indicating that an expressed gene was tagged by T-DNA (data not shown). A number of reports in Arabidopsis and rice have suggested that integration of Agrobacterium T-DNA into the plant genome occurs preferentially in promoter or transcriptionally active regions (Alonso et al. 2003, Chen et al. 2003, Sallaud et al. 2004, Schneeberger et al. 2005); however, a recent study suggests that these observations may have been biased due to the selection pressure requiring transgene expression (Kim et al. 2007). Thus, T-DNA integration into gene-rich or transcriptionally active regions might be observed in M. polymorpha transformants following hygromycin selection.

T-DNA integration can occur throughout the plant genome, and Agrobacterium-mediated transformation has been used as a mutagenic tool for plant genetics.

### Fig. 4 Sequence analysis of T-DNA–genomic DNA junctions

Sequences of the junctions in transformants are shown below the sequences of the T-DNA boundaries of pIG121Hm. Sequences presumably originating from M. polymorpha genomic DNA are shown in lower case letters. Bases that are identical to those from T-DNA are shown in upper case letters. Lines #1, #4, #5, #6 and #7 are the same as those in Fig. 3.

<table>
<thead>
<tr>
<th>Right boundary</th>
<th>T-DNA</th>
<th>Right 25 bp repeat</th>
<th>outside T-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIG121Hm</td>
<td>. TAAACATCA ATCGTGTTGAC ACGATA TATG GCGGTA A CCAAGAC A GACGTTTA</td>
<td>Δ23 bp</td>
<td>tgaaccttcgcttcagcctc</td>
</tr>
<tr>
<td>#1</td>
<td>. TAAACATCA ATCGTGTTG</td>
<td>Δ23 bp</td>
<td>tctgtagacagtcacactc</td>
</tr>
<tr>
<td>#4</td>
<td>. TAAACATCA ATCGTGTTG</td>
<td>Δ24 bp</td>
<td>aagctccttcgcttcagc</td>
</tr>
<tr>
<td>#5</td>
<td>. TAAACATCA ATCGTGTTG</td>
<td>Δ29 bp</td>
<td>cgccttccttcgcttcagc</td>
</tr>
<tr>
<td>#6</td>
<td>. TAAACATCA ATCGTGTTG</td>
<td>Δ22 bp</td>
<td>gatccggaggttagcataagc</td>
</tr>
<tr>
<td>#7</td>
<td>. TAAACATCA ATCGTGTTG</td>
<td>Δ22 bp</td>
<td>atatcctttccccgtagcactc</td>
</tr>
<tr>
<td>#8</td>
<td>. TAAACATCA ATCGTGTTG</td>
<td>Δ22 bp</td>
<td>gatccggaggttagcataagc</td>
</tr>
<tr>
<td>#9</td>
<td>. TAAACATCA ATCGTGTTG</td>
<td>Δ22 bp</td>
<td>gatccggaggttagcataagc</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Left boundary</th>
<th>T-DNA</th>
<th>Left 25 bp repeat</th>
<th>outside T-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIG121Hm</td>
<td>. TTAAGGGCTGCA ATTTGTTTAC ACGACTA ATATG GCGGTAC CCAAGAC A GACGCTCC</td>
<td>Δ50 bp</td>
<td>aacacattgaagattgtct</td>
</tr>
<tr>
<td>#1</td>
<td>. TTAAGGGCTGCA ATTTGTTTAC</td>
<td>Δ11 bp</td>
<td>tctgaagactgagcttcag</td>
</tr>
<tr>
<td>#4</td>
<td>. TTAAGGGCTGCA ATTTGTTTAC</td>
<td>Δ8 bp</td>
<td>tacagcatgaggtgtacct</td>
</tr>
<tr>
<td>#6</td>
<td>. TTAAGGGCTGCA ATTTGTTTAC</td>
<td>Δ13 bp</td>
<td>gctaacatgtgaatgattgg</td>
</tr>
<tr>
<td>#7</td>
<td>. TTAAGGGCTGCA ATTTGTTTAC</td>
<td>Δ13 bp</td>
<td>gctaacatgtgaatgattgg</td>
</tr>
<tr>
<td>#8</td>
<td>. TTAAGGGCTGCA ATTTGTTTAC</td>
<td>Δ13 bp</td>
<td>gctaacatgtgaatgattgg</td>
</tr>
<tr>
<td>#9</td>
<td>. TTAAGGGCTGCA ATTTGTTTAC</td>
<td>Δ13 bp</td>
<td>gctaacatgtgaatgattgg</td>
</tr>
</tbody>
</table>
Agrobacterium-mediated transformation of liverwort were developed under 40
(Feldmann 1991, Alonso et al. 2003). Utilizing our method reported here, it appears feasible to produce thousands of independent transformants in a small number of experiments. Integration of the T-DNA into a gene in a haploid genome allows immediate emergence of a mutant phenotype in primary transformants. Indeed, we have isolated several morphological mutants (Supplementary Fig. S1). The tendency for intact T-DNA insertions with defined ends in Agrobacterium-mediated transformation makes it possible to obtain flanking DNA fragments of T-DNA from transformants by TAIL-PCR (Liu et al. 1995). In contrast to random integration of T-DNA in M. polymorpha (Schaefer and Zryd 1997). Although several attempts at gene targeting have not been successful yet in M. polymorpha, a gene-targeting procedure successfully used in rice, with a strong positive/negative selection following high-throughput Agrobacterium-mediated transformation (Terada et al. 2002), would be applicable for M. polymorpha using the method reported here.

Materials and Methods
Plant material and preparation of surface-sterilized sporangia
Male, accession Takaragaike-1 (formally called 'male E line'), and female, accession Takaragaike-2 (formally called 'female E line'), M. polymorpha were aseptically maintained and propagated through gemmae growth as previously described (Okada et al. 2000, Takenaka et al. 2000). Sexual organs of female and male liverwort were developed under 40 μmol photons m⁻² s⁻¹ continuous white fluorescent light supplement with 15 μmol photons m⁻² s⁻¹ far-red light using light-emitting diodes (LEDs; MIL-IF18, Sanyo Electric, Osaka, Japan; peak emission at 734 nm, a half-bandwidth of 13 nm). Mature sporangia were collected 3–4 weeks after crossing. Sporangia were surface-sterilized for 2 min with 0.2% sodium hypochlorite solution containing 0.1% Triton-X100 and washed three times with sterilized water. They were dried by adding a few pieces of ~2 mm diameter silica gel enclosed in a tube, and stored at 4°C until use.

Southern blot analysis
Genomic DNA was isolated from 3-week-old thalli as previously described (Takenaka et al. 2000). For Southern blot analyses, 3 μg of genomic DNA was digested overnight with EcoRI or BamHI at 37°C. DNA was fractionated by electrophoresis on a 0.8% (w/v) agarose gel, and blotted onto positively charged nylon membranes. The 689 bp Ncol–BamHI fragment excised from pIG121Hm was used as a probe to detect the hpt gene. Blotted membranes were hybridized in Church hybridization buffer (Church and Gilbert 1984) at 65°C with the hpt probes labeled

Fig. 5 Sexual genotyping for hygromycin-resistant transformants. Genomic PCR using total DNAs of male (lane M) and female (lane F) plants, hygromycin-resistant transformants as templates, and primers designed for the Y-chromosome-linked DNA marker, rbm27, and for the X-chromosome-linked DNA marker, rhf73. Lines #1–#7 are the same as those in Fig. 3.

Agrobacterium strain, plasmid and culture
Agrobacterium tumefaciens strain GV2260 (Deblaere et al. 1985) harboring binary vector pIG121Hm (Ohta et al. 1990) was used for all the experiments. Plasmid pIG121Hm contains the GUS (uidA) gene with an intron of the castor bean catalase gene (Ohta et al. 1990) and the hpt gene as a selective marker within the T-DNA region. Each gene was under the control of a cauliflower mosaic virus 35S promoter. For transformation experiments, Agrobacterium cultures were started from a single colony, and grown in 5 ml of LB medium at 28°C with agitation for 2 d. The bacterial cells were harvested by centrifugation, re-suspended in M51C medium supplemented with 100 μM acetoxyringsone (Sigma-Aldrich, St Louis, MO, USA), unless otherwise described, to give an A600 of 2, and incubated for a further 6h before co-cultivation with M. polymorpha cells.

Agrobacterium-mediated transformation of M. polymorpha
Sporangia of surface-sterilized sporangia of M. polymorpha were suspended by pipeting into sterilized water (100 μl to a sporangium which contains ~100,000 sporangia). Transformation was performed as follows, unless otherwise described. A 100 μl aliquot of spore suspension was transferred into 25 ml of M51C medium (Takenaka et al. 2000) in a 100 ml flask, and cultured under continuous white light (40 μmol photons m⁻² s⁻¹) at 22°C for 7 d with agitation at 130 r.p.m. Then, 1 ml of Agrobacterium culture prepared as described above was added to the immature thalli liquid culture supplemented with 100 μM acetoxyringsone and co-cultivated at 22°C with agitation at 130 r.p.m. for 48 h. Cells were collected on a 50 μm square grid nylon mesh, and washed three times with 20 ml of M51C medium. Cells were transferred to M51C agar medium containing 1.4% agar, 10 mg l⁻¹ hygromycin (Wako Pure Chemical Industries, Osaka, Japan) and 100 mg l⁻¹ cefotaxime (Cliforans®, Sanofi-Adventis K. K., Tokyo, Japan). Cells were incubated under continuous white light of 40 μmol photons m⁻² s⁻¹ at 22°C.

Histochemical assay for GUS enzyme activity
Histochemical assays for GUS activity were performed according to Jefferson et al. (1987) with some minor modifications. Gemmae from wild-type (Takaragaike-1) and 10 randomly selected hygromycin-resistant thalli transformed by pIG121Hm were incubated on M51C solid medium at 22°C for 2 weeks prior to GUS assays. After 5 min of vacuum infiltration with the GUS assay solution containing 50 mM sodium phosphate buffer (pH 7.2), 0.5 mM potassium-ferrocyanide, 0.5 mM potassium-ferriyana-nide, 10 mM EDTA, 0.01% Triton X-100 and 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (X- Gluc: Wako Pure Chemical Industries, Osaka, Japan), each thallus was incubated at 37°C for 4 h. To enhance the contrast of GUS staining, chlorophyll was removed with 70% ethanol. GUS-expressing cells were detected as blue color observed under a dissection microscope.

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with [α-32P]dCTP by random priming. Washing and analyses of blots were performed as described elsewhere (Chiyoda et al. 2007).

**TAIL-PCR and sequencing of TAIL-PCR products**

TAIL-PCR amplification was performed according to Liu et al. (1995) with minor modifications using ExTaq DNA polymerase (TAKARA BIO INC., Shiga, Japan) and the PCR cycle listed in Supplementary Table S1. Primary PCR was performed using approximately 100 ng of genomic DNA as a template. The following nested primers for the T-DNA were designed: TL1, GAAACACTCAACCTATCTCG; TL2, GA TCAAAACAGATTTTCGCTGC; TL3, CCACCCCAGTACA TAAAACACGT; TR1, CTGCCGTCATCCATCTGT; T R2, GGAACTCAGTGAGACATTGT; and TR3, GCTCA TTAAACTCAGAAAAAC. Four arbitrary degenerate primers were used: AD1, NTCGA(G/C)(A/T)TGTT; AD2, NGTCA G(A/C)(A/T)GANA(T/A)GATTG; and AD4, GTNCGA (G/C)(A/T)CANAGA; and AD5, (A/T)GTGNAG (A/T)ANCANAGA; and AD6, (A/T)GTT.

TAIL-PCR products of tertiary reactions were analyzed using 1% agarose gel electrophoresis. Distinct bands were excised from the gel and purified using a QIAquick Gel Extraction kit (QIAGEN, Hilden, Germany). Purified products were cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA, USA) and then sequenced using a BigDye Terminator kit (Applied Biosystems, Foster City, CA, USA).

**Genomic PCR**

PCR analyses for sex chromosome-specific DNA markers, rbm27 and rhs73, were carried out using primer sets described previously (Fujisawa et al. 2001).

**Supplementary material**

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

**Funding**

The Ministry of Education, Culture, Sports, Science and Technology of Japan, Grant-in-Aid for Scientific Research of Priority Areas ‘Organelle Differentiation as the Strategy for Environmental Adaptation in Plants’ (No. 19039018 to K.I.); the Japan Society for the Promotion of Science, Grant-in-Aid for Young Scientists (Start-up) (No. 19870011 to K.I.)

**Acknowledgments**

Plasmid pG121Hm was kindly provided by K. Nakamura. We thank J. L. Bowman and M. Takemura for discussions and comments on the manuscript.

**References**


(Received March 26, 2008; Accepted May 31, 2008)