Efficient and High-Throughput Vector Construction and Agrobacterium-Mediated Transformation of Arabidopsis thaliana Suspension-Cultured Cells for Functional Genomics

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We established a large-scale, high-throughput protocol to construct Arabidopsis thaliana suspension-cultured cell lines, each of which carries a single transgene, using Agrobacterium-mediated transformation. We took advantage of RIKEN Arabidopsis full-length (RAFL) cDNA clones and the Gateway cloning system for high-throughput preparation of binary vectors carrying individual full-length cDNA sequences. Throughout all cloning steps, multiple-well plates were used to treat 96 samples simultaneously in a high-throughput manner. The optimal conditions for Agrobacterium-mediated transformation of 96 independent binary vector constructs were established to obtain transgenic cell lines efficiently. We evaluated the protocol by generating transgenic Arabidopsis T87 cell lines carrying individual 96 metabolism-related RAFL cDNA fragments, and showed that the protocol was useful for high-throughput and large-scale production of gain-of-function lines for functional genomics.

Keywords: Agrobacterium-mediated transformation — Arabidopsis thaliana — Functional genomics — Gateway cloning system — High-throughput — Suspension-cultured cells.

Abbreviations: CaMV, cauliflower mosaic virus; GST, glutathione S-transferase; GUS, β-glucuronidase; Hm, hygromycin; Km, kanamycin; MEPM, meropenem; NAA, 1-naphthaleneacetic acid; RAFL cDNA, RIKEN Arabidopsis full-length cDNA; SATase, serine O-acetyltransferase.

Introduction

Transgenic approaches are crucial for understanding gene function in molecular biological research. Recent advances in DNA array technology allow researchers to find a set of genes that function coordinately in the biological process of interest (Gachon et al. 2005). Therefore, there is increasing demand for the analysis of multiple mutant lines showing a loss or gain of function in order to clarify the roles of genes in the coordinated process. Large-scale T-DNA insertion lines have been produced as genetic resources for loss-of-function mutation analyses. Efforts have been devoted to locating the position(s) of the T-DNA within individual genes in these lines, and such defined tagged lines have been contributing significantly to gene discovery in Arabidopsis research (Alonso et al. 2003). However, when the gene of interest has functionally redundant paralogous genes in the genome, no phenotype is expected in the loss-of-function lines. Such cases are often seen, as multigene families are prevalent in the Arabidopsis genome (Arabidopsis Genome Initiative 2000). In these cases, gain-of-function mutant lines have complementary roles for functional analysis of these genes. As resources for gain-of-function mutations, transgenic plants carrying random insertion(s) of a strong promoter sequence on chromosomes, called activation tagging lines, have been generated at large scales (for a review, see Tani et al. 2004).

However, the use of these lines is limited to a certain extent as the position of the promoter insertion on chromosomes has not been located in most lines. Alternatively, large sets of full-length cDNA clones have become available from several plants such as Arabidopsis (Seki et al. 2002), rice (Kikuchi et al. 2003), poplar (Nanjo et al. 2004) and tomato (Tsugane et al. 2005). These full-length cDNA clones made it feasible to generate gain-of-function transgenic plants which carry the cDNAs driven by a strong promoter, using ordinary vector construction and transformation protocols.

Plant suspension-cultured cells, maintained in sterile medium under artificial environments, are suitable for gene function analysis as well as for physiological study. These cells are advantageous for collecting large amounts of uniform cell samples that are grown under strictly controlled conditions, such as of temperature and biotic/abiotic stress treatment, facilitating obtaining reproducible results. The Nicotiana tabacum BY-2 cell line (Nagata et al. 1992) is one of the most used cell lines for various types of such research. However, genomic information about the

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species is still limited only to expressed sequence tags (ESTs) (Matsuoka et al. 2004). Arabidopsis is a model plant for a wide range of plant research because of its unique characteristics, i.e. small plant size, short generation time and small genome size (Meinke et al. 1998), and its genome has been completely sequenced (Arabidopsis Genome Initiative 2000). Among several established Arabidopsis cell lines, the suspension-cultured cell line T87 is unique in photosynthetic ability under light irradiation (Axelos et al. 1992). Therefore, T87 cells have been widely used for genetic analyses (e.g. Callard et al. 1996, Uno et al. 2000, Takahashi et al. 2001, Stolc et al. 2005). Thus, the cells are suitable as a host for generating transgenic lines. However, a high-throughput protocol for large-scale production of transgenic cultured cell lines has not been established yet.

In this study we established a high-throughput protocol for vector construction and transformation of Arabidopsis suspension-cultured T87 cells. As a gene source, we chose cDNA clones from the RIKEN Arabidopsis full-length (RAFL) cDNA clone set (Seki et al. 2002). We designed the high-throughput procedure for introduction of RAFL cDNA fragments into binary vectors. The condition designed the high-throughput procedure for introduction of RAFL cDNA clone set (Seki et al. 2002). We validated we chose cDNA clones from the RIKEN Arabidopsis suspension-cultured T87 cells. As a gene source, the protocol for vector construction and transformation of Arabidopsis suspension-cultured T87 cells has not been established yet. The condition for Agrobacterium-mediated transformation with individual 96 binary vector constructs into Arabidopsis T87 cells was optimized for high-throughput processing. We validated the protocol by generating transgenic cell lines that over-express each of the 96 full-length cDNA clones encoding metabolism-related genes. We discuss the usefulness of the protocol for large-scale production of individual gain-of-function lines for functional genomics.

Results

High-throughput vector construction

To prepare binary vector constructs for individual genes in a high-throughput manner, we took advantage of RAFL cDNA clones (Seki et al. 2002) and the Gateway cloning system (Walhout et al. 2000) (Fig. 1). As RAFL cDNA fragments were cloned between distinct SfiI recognition sequences, 5'-GGCCAAATCGGCC-3' and 5'-GGCCCTTATGGCC-3', in vectors (Seki et al. 1998) and the recognition sequences are rarely found in the Arabidopsis genome, SfiI digestion excises, in most cases, single cDNA fragments. Even if SfiI recognition sites exist in the middle of the cDNA, proper cloning into the entry vector is expected in most of the cases, although such cases are rare (see below and Discussion). Once a target DNA fragment is cloned in the entry vector of the Gateway system, the fragment can be transferred to destination vectors via recombination in a single step. Therefore, we designed a new entry vector, named pRAFLENTR, which carries the same SfiI recognition sites as in RAFL cDNA vectors between the recombination site sequences attL1 and attL2.
After Agrobacterium B5 medium for 1–3 d (Fig. 2). Interestingly, the cells
wereacetic acid (NAA) before Agrobacterium

High-throughput transformation for Arabidopsis T87 cells

We established a procedure for efficient Agrobacterium-mediated transformation of Arabidopsis suspension-cultured T87 cells (Fig. 1). First, we replaced the micronutrient, vitamins and buffer of JPL medium (Jouannean and Pèaud-Lenoël 1967), which was established by modifying MS medium (Murashige and Skoog 1962) and has been used for maintaining the T87 cell line (Axelos et al. 1992), by one-third strength MS micronutrient, full-strength MS vitamins and 2-(N-morpholino)ethanesulfonic acid (MES), respectively. The modification allows easy preparation of the medium, named mJPL3, by using commercially available pre-mixed stock chemicals without altering the major properties of T87 cells, such as fast growth and uniform small cell clumps. We looked for suitable transformation conditions for T87 cells cultured in mJPL3 medium. We inoculated Agrobacterium tumefaciens EHA101 carrying the binary vector pIG121-Hm [carrying uidA under the cauliflower mosaic virus (CaMV) 35S promoter] into T87 cells at early- to mid-log phases (1–5 d of pre-culture), which are generally known to be suitable for efficient transformation (An 1985). However, no transgenic calli were obtained under the conditions tested (Fig. 2). Alternatively, we cultured the cells in B5 medium (Gamborg et al. 1968) supplemented with 1 μM 1-naphthaleneacetic acid (NAA) before Agrobacterium inoculation (pre-culture) and following 2 d co-cultivation. Hygromycin-resistant (Hm') calli were obtained reproducibly 2–3 weeks after Agrobacterium inoculation from cells pre-cultured in B5 medium for 1–3 d (Fig. 2). Interestingly, the cells

pre-cultured for 2 d exhibited the highest transformation efficiency (~500 Hm' calli per 10 ml of culture) among the culture conditions tested, whereas 1 and 3 d pre-culture yielded only <10 and ~200 Hm' calli, respectively. In the 1–4 d co-culture period tested, co-cultivation for 2 d in combination with 2 d pre-culture was optimal to obtain the highest transformation efficiency (data not shown). Expression of uidA, transferred from the binary vector pIG121-Hm, in the transgenic calli was confirmed by 5-bromo-4-chloro-3-indolyl-β-D-gluconic acid (X-gluc) staining (data not shown).

For high-throughput processing, we simplified the step of cell washing after Agrobacterium co-cultivation, which is laborious and time-consuming in transformation procedures. Most transformation protocols include three or more cell washings to suppress overgrowth of Agrobacterium during subsequent culture periods. In fact, overgrowth of Agrobacterium, which leads to plant cell death, was occasionally observed in our experiments, when the inoculated T87 cells were washed only once and
and found that 150 transgenic Hmr calli per 10 ml of culture transformation without cell washing after co-cultivation was possible by omitting the washing process. We tested time in the whole process. Further simplification could be efficiency (Fig. 3), which resulted in saving of labor and obtain transgenic cells without reducing the transformation efficiency (Ogawa and Mii 2007). By using MEPM, a single cell washing was enough to antibacterial activity and low phytotoxicity (Ogawa and Mii 2007).

Effects of washing of Agrobacterium co-cultivation with Arabidopsis T87 cells (0.05 g wet weight) were pre-cultured in 10 ml of B5 medium supplemented with 1 μM NAA for 2 d, inoculated with A. tumefaciens EHA101 (pIG121-Hm) and then co-cultured for 2 d. T87 cells were washed 0–3 times with mJPL3 medium, plated on selection medium, and then cultured for 2 weeks. The numbers of Hmr calli obtained are shown as the mean ± SD of three independent experiments.

Carbenicillin or timentin was used for elimination of Agrobacterium during culture. Such overgrowth was seen even if a high concentration of the antibiotic (50–200 mg l⁻¹) was used. Thus, we replaced the antibiotic by meropenem (MEPM) at 25 mg l⁻¹, which possesses high antibacterial activity and low phytotoxicity (Ogawa and Mii 2007). By using MEPM, a single cell washing was enough to obtain transgenic cells without reducing the transformation efficiency (Fig. 3), which resulted in saving of labor and time in the whole process. Further simplification could be possible by omitting the washing process. We tested transformation without cell washing after co-cultivation and found that 150 transgenic Hmr calli per 10 ml of culture were still obtained (Fig. 3), which would be enough for most experimental purposes.

In addition to simplification of the washing process, we simplified the step of Agrobacterium inoculation and co-cultivation. Most transformation protocols have used Agrobacterium cells after washing and resuspending with fresh co-cultivation medium (e.g. supplementation with acetosyringone). However, our experiments showed that direct addition of overnight-grown stationary phase Agrobacterium to pre-cultured T87 cells followed by further co-cultivation produced as many transgenic calli as the conventional protocols. Concentration of Agrobacterium cells in ratios from 1/100 to 1/1,000 in T87 suspension culture only slightly affected the transformation efficiency (data not shown).

The transgenic T87 calli obtained by the protocol were transferred onto a semi-solidified mJPL3 plate containing the antibiotics MEPM (for elimination of Agrobacterium) and Hm (for selection of plant cells), and maintained by 3- to 4-week intervals of subculture. To re-establish the suspension culture for large-scale preparation of transgenic cells, 0.5 g wet weight of 4-week-old calli were suspended in 100 ml of liquid mJPL3 medium and subcultured at biweekly intervals as described in Materials and Methods.

Evaluation of the high-throughput protocol

We evaluated the high-throughput protocol conditions in this study using 96 metabolism-related RAFL cDNA clones (Supplementary Table S1). The 96 clones were processed simultaneously and the fragments were introduced into the binary vector pGWB2 downstream of the CaMV 35S promoter. The processes from vector construction to transformation of Agrobacterium were independently repeated three times using the same starting materials. All plasmid constructs produced were analyzed by restriction enzyme digestion to check for proper cloning of the fragments in the vector. Except for pda08414 (At4g35300, sugar transporter-like protein), all clones produced proper constructs at least once in three time trials (Supplementary Table S1). The pda08414 clone was cloned successfully in another set of trials. The results suggest that bulk treatment using multiwell plates causes cloning failure of some clones in a single trial, but most can be cloned in other rounds of trials, unless the cloned constructs are incompatible with Escherichia coli. Actually, in our trials of >800 RAFL clones, the clones that were not successfully processed in the first round were included in the next round of trials and most of them (>99%) were successfully processed in the second- or third-round trials (unpublished data). All of the binary vectors carrying the RAFL cDNA fragments were successfully introduced into Agrobacterium in single trials.

We prepared 96 transformation-ready Agrobacterium strains carrying individual binary vectors, and the strains were divided into four sets, each of which contains 24 strains. Each set of Agrobacterium was independently subjected to transformation trials of Arabidopsis T87 cells. In these trials, 94 Agrobacterium strains (97.9%) gave >100 Hmr calli. Although the remaining two strains gave <20 Hmr calli, these two strains also produced >100 Hmr calli in the second or third round of trials. The current protocol allows one person (3 h maximum a day) to treat 24 different Agrobacterium strains for inoculation to T87 cells within a week (four working days), or one person (6 h maximum a day) to treat 96 strains within 2 weeks, following the 2 weeks of the selection period. Usually, we maintained 10–20 independent transgenic calli for further analyses, as the expression levels of transgenes varied among transgenic lines (described below). Practically, although some Agrobacterium strains did not produce sufficient numbers of calli, the simple and robust transformation protocol established in this study allow us to re-try
transforming those strains in the next round of our transformation routine, and we could finally obtain sufficient numbers of calli for most of the strains.

We examined the expression levels of transgenes in transgenic cells by Northern blot analysis. A total of 584 transgenic T87 lines, which contained 31 randomly selected vector constructs and 14–20 transgenic T87 lines generated from each construct, were subjected to expression analysis. Fig. 4A and 4B shows examples of two of 31 transgenes, glutathione S-transferase (GST; At1g02920, pda04245) or (B) a serine O-acetyltransferase (SATase; At5g56760, pda00783), both under a CaMV 35S promoter, were analyzed. Wild-type T87 cells (WT) were also included. Different numbers given in the figure indicate independent cell lines.

Fig. 4 Northern blot analysis of total RNA extracted from transgenic Arabidopsis T87 cell lines. Cell lines that were transformed with either (A) a glutathione S-transferase (GST; At1g02920, pda04245) or (B) a serine O-acetyltransferase (SATase; At5g56760, pda00783), both under a CaMV 35S promoter, were analyzed. Wild-type T87 cells (WT) were also included. Different numbers given in the figure indicate independent cell lines.

When compared with the endogenous expression level, 526 (90.1%) of 584 lines tested exhibited largely increased expression of the transgenes. In some cases, the expression levels in transgenic cells were apparently lower than the endogenous level, suggesting the occurrence of post-transcriptional gene silencing, which is often seen in transgenic plants when the transgene is driven by a strong constitutive promoter such as CaMV 35S (Jorgensen 2003). We further examined protein expression levels of SATase transgenic lines. Nine independent transgenic lines showing high transgene expression levels were selected for Western blot analysis. Signals indicating 34 kDa SATase proteins were detected in all nine transgenic lines examined, and these signals were apparently stronger than signals in wild-type T87 cells (Fig. 5). These results show that the protocol employed for transformation is suitable for production of large-scale gain-of-function lines.

**Discussion**

Here we established an efficient high-throughput protocol for production of vector constructs and subsequent Agrobacterium-mediated transformation of Arabidopsis suspension-cultured T87 cells with the constructs. We took advantage of RAFL cDNA clones, the Gateway cloning system and multiple-well plates for high-throughput binary vector construction. The protocol provides a means for producing a large-scale population of transgenic cell lines each of which carries a single transgene controlled under the proper promoter. The cDNA clones provided by RIKEN BioResource Center comprise 15,295 clones that cover 460% of expressed genes, which belong to diverse gene families (Seki et al. 2002). The number of RAFL clones is still increasing (20,997 clones at October 2007). Therefore, the RAFL cDNA clone collection is useful for gene function analyses in various research areas. The cell lines that overexpress RAFL cDNA fragments controlled under a strong promoter serve as gain-of-function lines. As the Gateway cloning system is versatile, once pRAFLENTR vector constructs with RAFL fragments are prepared, they can be transferred into various destination vectors other than pGWB2, such as for gene silencing experiments, in a single step. The protocol described in this report allows laboratories that are equipped with ordinary molecular biology instruments to produce several hundred transgenic cell lines within a few months by a single person. Thus, the transgenic cell lines produced using the protocol will contribute to accelerating Arabidopsis functional genomics studies. Moreover, the high-throughput vector construction protocol can also be applied to full-length cDNA clones of other plant species such as rice (Kikuchi et al. 2003) and tomato (Tsugane et al. 2005), as some (rice) or all (tomato) cDNA fragments are cloned in the SfiI site.
Transferring full-length cDNA fragments into the Gateway entry vector pRAFLENTR from RAFL clones using the restriction enzyme SfiI would be less problematic, even if the fragments contain multiple SfiI sites. If a single SfiI site exists inside a RAFL clone and SfiI digestion produces 3-nt long 3' overhang sequences that differ from those of the SfiI sites of pRAFLENTR, such as pda07581 (At5g58778), the two divided fragments can be cloned properly into pRAFLENTR with enough efficiency, as shown in the case of pda07581. If the overhang sequence produced is the same as one of the overhang sequences of the SfiI sites of pRAFLENTR, the cloning step produces two possible clones, which carry the fragment in the proper or reverse orientation. Restriction fragment analysis of such clones may determine which one is the proper one. Our search for SfiI sites in 15,295 RAFL clones revealed that 71 clones had single SfiI sites, but no clones had more than two sites. In these clones, all of the 3-nt long 3' overhang sequences differed from those of the SfiI sites of pRAFLENTR. Therefore, it is expected that these RAFL clones can be cloned properly into pRAFLENTR with our protocol. Another search showed that the frequency of the occurrence of SfiI sites in all Arabidopsis expressed sequences (0.3 × 10^-5) is much less than that expected in random nucleotide sequences (1.5 × 10^-5). Thus, it would be very rare to encounter cDNA clones that have more than two SfiI sites, and it is still advantageous to use SfiI sites as the cloning sites.

Gallego et al. (1999) have reported Agrobacterium-mediated transformation of T87 cells. However, the transformation protocol would need laborious and time-consuming manipulations when large-scale production of transgenic cell lines is required, and the protocol uses B5-based AT medium for culture of the T87 cells, instead of JPL medium which has been used as the original medium for the T87 cells (Axelos et al. 1992). We developed modified JPL medium, named mJPL3 medium, which is easier to prepare than JPL medium. The choice of culture medium for T87 cells, B5 medium or mJPL3 medium, affected the conditions and appearance of suspension-cultured cells. It has been shown that transformation of T87 cells was successful when B5-based medium was used for routine subculture and transformation steps (Gallego et al. 1999), while we failed to obtain transgenic cells when mJPL3 medium was used throughout the culture including the pre-culture and co-culture periods (Fig. 2). On the other hand, B5 medium tended to induce large cell clumps with a prolonged culture period, which are not suitable for re-establishment of suspension culture (see Fig. 2A), while mJPL3 maintained uniform and small cell clumps. These results implied that B5 medium is suitable for cells to enter into the transformation-competent state, while mJPL3 medium is suitable for keeping cells in a less aggregated state even with a prolonged culture period. Therefore, we used mJPL3 medium for subculture and re-establishment of suspension culture, and B5 medium for pre-culture and co-cultivation. Our transformation protocol could also be utilized successfully in other laboratories for T87 cells cultured in the original JPL medium (Dr. S. Takahashi, Tohoku University, Dr. D. Ohta, Osaka Prefectural University, and Dr. Kanamaru, Kobe University, personal communication).

Transgenic suspension-cultured cells have advantages over transgenic plants. First, transgenic cells are obtained within several weeks after Agrobacterium inoculation, while at least several months are needed to obtain transgenic plants. Secondly, uniform cells with the same genetic background can be obtained readily from transgenic cultured cells at a large scale. In contrast, it takes several generations to obtain Arabidopsis plants with homozygous transgenes. Thirdly, strict control of the environment of cultured cells is possible, while control of the environment of plants is not easy even in a controlled growth chamber. Our statistical analyses of the Arabidopsis transcriptome detected by microarrays showed higher reproducibility in the data obtained from three biological replicates of Arabidopsis suspension-cultured T87 cells than those from 4-week-old Arabidopsis plants grown in a well-controlled growth chamber (N.S., H.S. and D.S., unpublished data). However, transgenic suspension-cultured cells have a couple of disadvantages. First, maintenance of a large number of transgenic cell lines is laborious, while storage of seeds derived from transgenic plants is easy. Thus, we recently developed a protocol for cryopreservation of transgenic T87 cells (unpublished data). Secondly, cultured cells are not applicable to phenomena specific to differentiated tissues and organs. Taken together, transgenic cultured cell lines and plant lines will play complementary roles in accelerating functional genomics.

Recently, a system for random introduction of RAFL cDNA fragments driven by a strong promoter into plants, called the FOX hunting system, was developed (Ichikawa et al. 2006). In this system, a pool of RAFL cDNA plasmids was subjected to SfiI digestion and subsequently to ligation with a binary vector. The binary vector DNAs were pooled and introduced into Agrobacterium. Using pooled Agrobacterium strains and in planta Agrobacterium-mediated transformation, many transgenic Arabidopsis plants were generated. Several dominant phenotypes were seen in the population, indicating that the population serves as a gain-of-function mutant resource. As the chromosomal locations of transgenes in the population at a large scale have not been determined yet, genes are identified solely from the lines that exhibit phenotypes. Therefore, our approach to generate gain-of-function lines for individual
targeted genes is different and complementary to the FOX hunting system.

Using the protocol established in this study, we are currently working on generating a large population of gain-of-function T87 cell lines with >1,000 individual genes for metabolism, which will contribute to plant metabolome research. Our transformation protocol for Arabidopsis T87 cells has been successfully used by our collaborators to elucidate the functions of uncharacterized genes (Hirai et al. 2007).

Materials and Methods

DNA materials, vectors and bacterial strains

All full-length cDNA clones of Arabidopsis used in this study (RAFL cDNA clones) were obtained from RIKEN BioResource Center (Tsukuba, Japan). The list of the RAFL cDNA clones is shown in Supplementary Table S1.

We modified the Gateway donor vector pDONR201 (Invitrogen, Carlsbad, CA, USA) to accept SfiI fragments excised from RAFL cDNA clones. From a RAFL cDNA clone, pda01738, the entire cDNA fragment and SfiI recognition sequences flanking both ends of the cDNA were amplified using a set of PCR primers, RAFLB1 (5'-GGGGACAAAGTTTGTACAAAAAAGCAGGCTGGCCAAATTCGGCCCGAGCTCGAATT-C-3') and RAFLB2 (5'-GGGGACCACTTTTGTACAAGAAGGTGGTGCCCTTATGGCCGATCCAAGAGC-3') (the SfaI recognition sequences found in pda01738 are underlined). As the primers contained the recombination sites attB1 and attB2 for Gateway cloning, the PCR-amplified fragment was introduced into the donor vector pDONR201 using BP clonose (Invitrogen) at the cloning sites via recombination according to the supplier’s protocol. The resultant vector, which carried the two SfiI sites between the recombination sites attL1 and attL2, was digested with the restriction enzyme SfiI. The vector fragment with the SfiI recognition sequences at both ends, named pRAFLENTR, was purified by gel electrophoresis, and used to clone the RAFL cDNA fragments in this study.

The Gateway-based binary vector pGWB2 was obtained from Dr. Tsuyoshi Nakagawa (Shimane University, Japan).

Disarmed A. tumefaciens strain EHA101 (Hood et al. 1986) was used in the present study. A binary vector pIG121-Hm (Ohta et al. 1990; a gift from Dr. Kenzo Nakamura of Nagoya University), which has two selective marker genes, hpt and nptII, and a reporter gene, uidA, was used for experiments to establish the transformation protocol. For large-scale production of transgenic cell lines, binary vectors carrying individual genes were constructed as described below. Agrobacterium was cultured in LB medium supplemented with 50 mg l⁻¹ kanamycin (Km) and 25 mg l⁻¹ Hm at 28°C with shaking overnight before inoculation into Arabidopsis T87 cells.

High-throughput vector construction

A set of 96 RAFL cDNA plasmids (~80 ng per 17.5 µl of distilled water) was mixed individually with 2.5 µl of buffer and 5 U of the restriction enzyme SfiI (TAKARA SHUZO CO. LTD, Otsu, Japan) in a 96-well PCR plate (Thermo-Fast 96 Non-Skirted, ABgene, Epsom, UK). The plate was incubated in a thermal cycler (ASTEC, Fukuoka, Japan) at 50°C for 5 h for SfiI digestion and then at 70°C for 15 min to denature the enzyme.

A 2 µl aliquot of ligation mixture (TOYOBO, Osaka, Japan) and 1 µl (10 ng) of SfiI-digested pRAFLENTR were mixed in a new 96-well PCR plate, and the plate was incubated at 16°C for 2 h. The contents of each well, which had been kept at ~80°C, were placed on ice to thaw the frozen cells. The ligation mixtures (3 µl each) were added and mixed into 50 µl of competent cells of Escherichia coli DH5α (Inoue et al. 1990) in a 96-well PCR plate placed on ice, and the plate was further incubated for 20 min. To introduce DNA into E. coli by heat shock, the plate was heated at 42°C in a thermal cycler for 1 min, and then cooled to 4°C. The E. coli mixtures were mixed individually with 600 µl of SOC medium in a 96-well-deep-plate (2.2 ml Storage Plate, ABgene) and cultured at 37°C for 20 min. E. coli cultures (30 µl each) were spread individually over SOC agar containing 50 mg l⁻¹ Km in eight 12-well plates (3815-012, Asahi Techno Glass, Tokyo, Japan) and kept at 37°C overnight. Single colonies from the wells were inoculated in 600 µl of 2× YT medium containing 50 mg l⁻¹ Km and incubated at 37°C for 16 h. Plasmid DNAs were prepared from the cultured cells using CosMCPrep (Agencourt Bioscience, Beverly, MA, USA) according to the manufacturer’s instructions, and dissolved in 40 µl of distilled water in a 96-well plate. To check if the inserts were properly inserted into the cloning site of pRAFLENTR, 10 µl of the plasmid DNA solutions were digested with the restriction enzyme BanII in 20 µl reaction solutions in a 96-well PCR plate at 37°C for 1 h. The sizes of DNA fragments were determined by agarose gel electrophoresis and were compared with those expected from the cDNA sequences, which we determined using an in-house Perl program to calculate the fragment sizes from a set of cDNA sequences.

LR in vitro recombination was carried out using a Gateway cloning system kit according to the instruction manual. Briefly, plasmid DNA of 96 pRAFLENTRs (4 µl each) was mixed individually with 2 µl of pGWB2, 2 µl of LR clonose and 2 µl of 5× buffer in a 96-well PCR plate and incubated at 20°C for 2 h. The reaction was stopped by adding 1 µl of proteinase K and keeping it at 37°C for 30 min. The reaction mixtures (1 µl each) were used for transformation of E. coli DH5α. The procedures for E. coli transformation and plasmid DNA preparation were as described above, although both 50 mg l⁻¹ Km and 50 mg l⁻¹ Hm were used as selective antibiotics. The insertion of RAFL cDNA fragments in the vector was confirmed by checking the sizes of the fragments after PCR according to the standard protocol, followed by restriction enzyme digestion and gel electrophoresis as described above.

Transformation of A. tumefaciens EHA101 was performed by the freeze–thaw method of An et al. (1988), but with a modification for multiwell plate processing. DNA solutions (3 µl each) were mixed with the competent Agrobacterium cells (50 µl each) dispensed into a 96-deep-well plate and the mixture was frozen in liquid nitrogen. The plate was warmed at 37°C for 1 min, 300 µl of LB medium was added, and then the plate was incubated at 28°C with shaking for 1 h. The entire mixture was spread individually over 96 LB agar plates containing 50 mg l⁻¹ Km and 50 mg l⁻¹ Hm, and cultured at 28°C for 2 d. Single colonies were transferred onto fresh agar plates and further cultured at 28°C for 2 d. The plates were kept at 4°C for up to 2 months. Single clones from each vector construct were used for transformation of suspension-cultured cells. Inserts in the binary vector were confirmed by checking the sizes of the fragments by colony PCR, following restriction enzyme digestion and gel electrophoresis as described above. The clones, which were used for transformation of Arabidopsis cells, were dispensed into 96-well-formatted tubes (BIOTUBE system T101,
Sumitomo Pharma, Osaka, Japan), 20 mg l−1 JPL3 medium containing 12.5 mg l−1 of transformed calli were established by transferring cells into MS medium supplemented with 1 mg l−1 indole-3-acetic acid (IAA), 25 mg l−1 1-Methylpiperidinium (MEPM), and 5 mg l−1 1-hydroxybenzotriazole (Hm). The cells were maintained by subculture every 3–4 weeks. Suspension cultures were maintained by standard procedures as described above. A 10 ml aliquot of cell suspension was transferred into a 15 ml tube and T87 cells were cultured for a further 1–3 d. After co-cultivation, the cell suspension was transferred into a 50 ml flask and cultured under continuous illumination (0.5–20 μmol m−2 s−1) at 22 °C with rotary shaking at 120 r.p.m. Two-week-old cells were sieved through a 500 μm stainless mesh to collect fine cell clumps, and 0.5 g wet weight of cells were transferred to a 300 ml flask containing 100 ml of fresh JPL3 medium for subculture.

Two-week-old T87 cells were sieved through a 500 μm stainless mesh, collected on a 50 μm nylon mesh and then resuspended in either B5 medium (G-5893, Sigma) supplemented with 1 μM NAA and 30 g l−1 sucrose or mJPL3 medium at 0.5 g wet weight per 100 ml of medium. A 10 ml aliquot of cell suspension was transferred into a 50 ml flask and cultured under continuous illumination at 22 °C with shaking at 120 r.p.m. for 1–5 d. A 10 μl aliquot of overnight culture of Agrobacterium (200 μg ml−1 harboring a RAFL cDNA-carrying pGWB2) was directly added to a pre-cultured T87 cell suspension, and they were cultured for a further 1–3 d. After co-cultivation, the cell suspension was transferred into a 15 ml tube and T87 cells were collected by centrifugation at 200 × g for 1 min. Co-cultured T87 cells were washed with 10 ml of mJPL3 medium with shaking (60–90 r.p.m.) for 30 s. The cells were collected by centrifugation, resuspended with 3 ml of mJPL3 medium, and then spread over two plates of selection medium, which is mJPL3 medium supplemented with 25 mg l−1 MEPM. (Shimane University) for his gift of pGWB2, and Dr. M. Iwata for their technical assistance. The authors thank Dr. K. Nakamura (Nagoya University) for his gift of pIG121-Hm.

**Supplementary material**

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

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**References**


Transgenic Arabidopsis cultured cell lines


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