J. Plant Biol. 40 (3) : 206~211 (1997)

Construction of Targeting Vectors to Disrupt *PAT*1 Gene in *Arabidopsis Trp*1-100

Lim, Seonhee, Youngsoon Kim, Euiseung Lee and Hyeonsook Cheong*
Department of Genetic Engineering, Chosun University, Kwangju 501-759, Korea

To develop the gene targeting system by homologous recombination in *Arabidopsis thaliana*, we constructed two targeting vectors and showed the reliability of the scheme which is based on genetic complementation of phosphoribosylanthranilate transferase (*PAT*1) gene. The *PAT*1 gene, which is essential for tryptophan biosynthesis, was selected as a target gene because the loss of function leads to fluorescence phenotype due to the accumulation of anthranilic acid derivatives. pHS113 contains *PAT*1 gene surrounding 5' and 3' flanking portions, but the most coding region of the *PAT*1 gene is replaced by the neomycin phosphotransferase gene (*NPT*II). pHS117 consists of 1.1 kb internal fragment of genomic *PAT*1 gene following with *NPT*II gene. In this targeting strategy, *Arabidopsis PAT*1 gene can be disrupted by single-step of transformation experiment.

Keywords: *Arabidopsis thaliana*, gene disruption, gene targeting, PAT

Gene targeting *via* homologous recombination has been widely used recently. While it has become a standard method for the site directed mutagenesis of animal genome (Bollag *et al.*, 1989; Zimmer, 1992), homologous recombination in plant cells has been reported by only a few groups (Baur *et al.*, 1990; Engels and Meyer, 1992). In most cases, integrated mutant form of extrachromosomal DNA was targeted and recombinated in the plant cells so that the functional marker gene was restored (Offringa *et al.*, 1990; Peterhans *et al.*, 1990). These systems are not easily applicable to nonselectable traits. Therefore, an appropriate method needs to be designed to discriminate against nonhomologous recombination because of the low frequency of homologous recombination events in plants.

Homologous recombination of exogenous DNA permits integrative gene disruption and the replacement of genomic segments that have been altered *in vitro* (Shortle *et al.*, 1982). The gene we chose for the study of gene disruption is phosphoribosylanthranilate transferase (*PAT*1) gene (Rose *et al.*, 1992). PAT performs the second step in the biosynthesis of tryptophan in plants. An *A. thaliana* gene encoding PAT is a single copy gene defective in tryptophan mutant plants (*trp*1-100) (Last and Fink, 1988). Defect in the *PAT*1 gene leads to the accumulation of anthranilate, a fluorescent intermediate in the tryptophan pathway. Therefore, homologous recessive *pat*1 mutants are easily distinguishable with blue fluorescence under UV light. Heterozygous mutant plant (*PAT*1/*pat*1) showing wild phenotype by genetic complementation can be used to disrupt a endogenous *PAT*1 gene. In that case, disrupted *PAT*1 gene could serve as a marker to screen regenerated homozygous mutants (*pat*1/*pat*1) showing blue fluorescence. Also, *pat*1 would be proved as a useful gene that can be selected against 5-methylanthranilate (5-MA) (Last and Fink, 1988). Since the defect in the *PAT*1 gene provides the mutant cells with resistance to the 5-MA, only homozygous mutants will survive in the medium containing 5-MA after the transformation. In the scheme, transformed cells will primarily selected against 5-MA and kanamycin and then homologous mutant plants will be screened under UV light.

We are planning to establish a gene targeting system in *A. thaliana* using a phenotypic marker. To facilitate the detection of the homologous recombination event, we will target the *Arabidopsis PAT*1 gene, which is essential for tryptophan biosynthesis in the plant. Here, we propose the basic strategy for gene targeting to disrupt the *PAT*1 gene in *Arabidopsis*. We constructed two vectors (replacement and trunca-

*Corresponding author: Fax +82-62-230-6619

tion vectors) for the targeting of *PAT*1 gene by homologous recombination. Before delivering recombinant DNA into plant cells, we showed the reliability of the scheme by displaying the usefulness of *pat*1 gene as a screenable marker. So, we introduced *PAT*1 gene into *Arabidopsis trp*1-100 to alternate the fluorescenced phenotype by the genetic complementation.

MATERIALS AND METHODS

Plant Lines and Culture Conditions

Tryptophan mutant (*trp*1-100) used in this study was derived from the *A. thaliana* Columbia. A wild type (C24) was used as a control. Seeds were surface sterilized by dipping in 70% ethanol followed by incubation in a solution of 30% bleach for 30 min. The seeds were then rinsed several times with sterile water. Germination took place in a culture chamber (10 h, photoperiod 3000 lux, 17°C, 60% relative humidity).

DNA Manipulations

A genomic clone (AR1-5) that was obtained from an *A. thaliana* library (Clonetech) in EMBL3 was provided by Alan Rose (Cornell University). A 7.8 kb *Kpn*I fragment that cross-hybridized to the *PAT*1 cDNA were subcloned into the KS vector. Different lengths of partial *PAT*1 and *NPTII* DNA were introduced into pGEM or the KS vector to make targeting vector. On the other hand, *PAT*1 gene including promoter region was subcloned into a binary vector, pEND4K. The resulting plasmid, named as pHS 107, was transferred directly into *A. tumefaciens* strain LBA4404 by electroporation.

Transformation of *Arabidopsis*

Seedlings of *trp*1-100 were aseptically germinated in 0.5× MS medium with shaking for 7 days. The root explants were cut and cocultivated with *A. tumefaciens* strain containing pHS107. Shoots and roots were induced on MS media containing 50 mg/L of kanamycin. The T_1 plants were self-pollinated and finally T_2 seed were tested for the blue fluorescence on solid media containing 50 mg/L of kanamycin.

Southern Hybridization

Genomic DNA was isolated from transgenic *A. thaliana* according to Murray and Thomson (1980). Digested DNA with restriction enzymes was separated on 0.8% agarose-gel transferred to nylon membrane (Amersham). ^{32}P labelled probes were generated by nick translation and hybridization performed in 50% formamide and 5× SSPE at 68°C for 20 hr. Washing condition was at 0.1× SSPE at 68°C with several changes of 0.1× SSPE. Finally, the radioactivity was monitored on the PhosphorImager (Bio-Rad, USA).

RESULTS

Vector Construction

As strategy for gene targeting using the *PAT*1 gene, we designed two plasmid vectors with different modifications or deletions in the *PAT*1 gene. The vectors carried *NPTII* and partial *PAT*1 gene for the selection of transformants and homologous recombinants, respectively. pHS113 contains *NPTII* gene surrounded by 5' and 3' flanking sequence of *PAT*1 gene. pHS 117 comprises 1.1 kb internal fragment of genomic *PAT*1 gene and *NPTII* gene (Fig. 1).

The replacement vector, pHS113; To develope 5' flanking region for homologous recombination, 2.2 kb *Bam*HI/*Pst*I fragment from PAR118 containing *A. thaliana PAT*1 gene was cloned into KS vector and named as pHS108. Following 1.6 kb *Pst*I fragment from PAR118 was subsequently ligated into pHS108 (pHS110). pEND4K containing a Kanamycin resis-

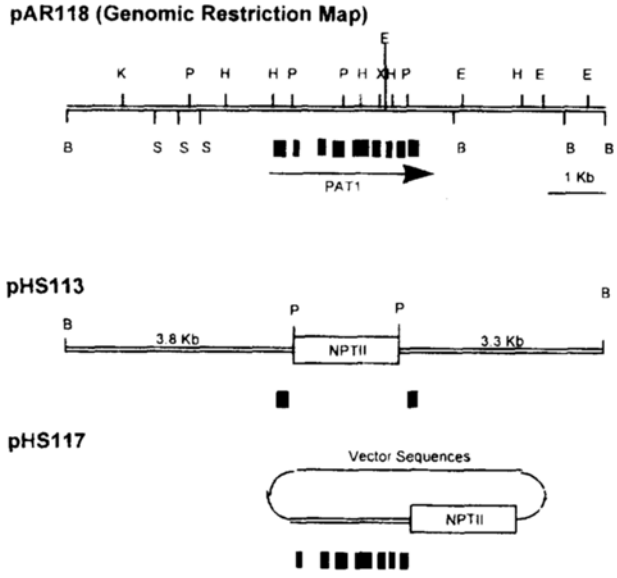

Fig. 1. Construction of gene targeting vectors, pHS113 and pHS 117, containing *NPTII* and the part of genomic DNA of *PAT*1 from *A. thaliana*.

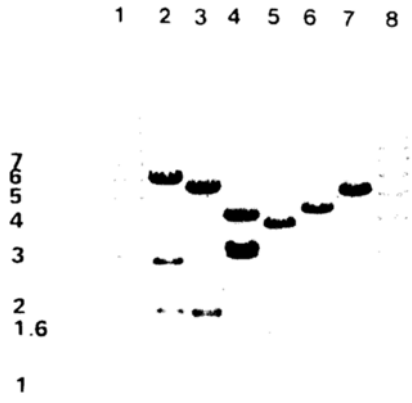

Fig. 2. Agarose gel electrophoresis of the restriction enzyme digests of pHS113 and pHS117. 1,8. BRL's 1 kb DNA ladder; 2. *Bam*H1 digest of pHS113; *Hin*dIII digest of pHS113; 4. *Xho*I/*Sal*I digest of pHS113; 5. *Eco*RI digest of pHS117; 6. *Kpn*I/*Sac*I digest of pHS117; 7. *Hin*dIII digest of pHS117.

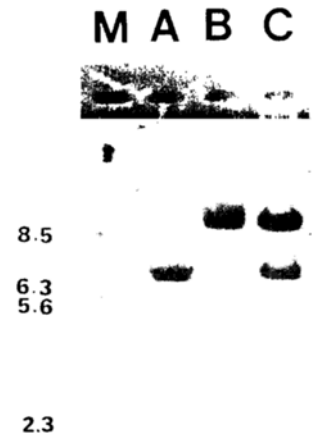

Fig. 3. Agarose gel electrophoresis of the restriction enzyme digests of pHS107. M. *Bst*EII digest of λ DNA; A. *Kpn*I fragment of *PAT*1; B. *Kpn*I digest of pEND4K; C. *Kpn*I digest of pHS107.

tance cassette (Klee *et al.*, 1985) was digested with *Eco*RI to excise 1.6 kb cassette, which was ligated into the pHS110 to create pHS111. Then, 3' flanked sequences of *PAT*1 in pAR118 were digested with *BamH*I/*Pst*I and cloned into pSP72 to develop plasmid pHS109 (Fig. 2, lanes 2-4). The 3.3 kb fragment was excised with *Sal*I/*Xho*I and finally ligated into pHS111. The resulting plasmid, pHS113, therefore contains a gene for Kanamycin resistance flanked on either side by DNA which flanks the *PAT*1 gene in the genome of *A. thaliana*.

The truncation vector, pHS117; pEND4K was digested with *Eco*RI to excise the Kanamycin resistance cassette and inserted in pGEM-7zf to create pHS112. 0.6 kb *Bcl*I/*Hin*dIII fragment and 0.5 kb *Hin*dIII fragment from pAR118 were successively ligated into KS vector. Then, 1.1 kb fragment was excised with *Sac*I/*Kpn*I and ligated into pHS112 to create pHS117 (Fig. 2, lanes 5-7). This transformation vector carried an internal fragment of the *PAT*1 gene adjacent to the *NPT*II gene.

Transformation of *A. thaliana trp*1-100

Agrobacterium carrying pHS107 (Fig. 3) was used to transform *A. thaliana trp*1-100. After cocultivation of the root explants with *Agrobacteria*, remaining bacteria were killed by adding timentin (100 mg/L) on MS medium supplemented with IAA (0.05 mg/L),

Fig. 4. Southern blot analysis for the genomic DNA isolated from transgenic and non-transgenic plants. 1.1 kb *Eco*RI fragment of *PAT*1 gene was used as probe. A-B, transgenic plants carrying exogenous *PAT*1 gene; C, non-transgenic plant; M, BRL's 1 kb DNA ladder.

IPAR (7 mg/L) and Kanamycin (50 mg/L). Calli formed on the root explants after 2 weeks and finally shoots were induced on the same medium. Rooting

of the transformants was done on MS medium containing IBA (1 mg/L) and Kanamycin (50 mg/L). The integration of *PAT*1 gene in the transgenic plants were confirmed by southern hybridization of the plant genomic DNA. While there was a single band observed from the nontransformed control plant, three band appeared at about 13.8 kb, 7.1 kb and 3.3 kb from the transgenic plants (Fig. 4). This result showed that a single copy of *PAT*1 gene was introduced into transformed plants.

A

B

C

Fig. 5. Photographs of non-fluorescenced cotyledons of the transformant due to the genetic complementation. A, *Arabidopsis trp*1-100 (*pat*1/*pat*1) as control; B, wild type as control; C, *Arabidopsis trp*1-100 carrying exogenous *PAT*1 gene (*PAT*1/pat1).

Complementation of Blue Fluorescence in *A. thaliana trp*1-100

Germinated seedlings of T_2 were examined under UV light. Fig. 5 shows the results of the complementation assay for the blue fluorescence in the T_2 plants. About 30% of transgenic plants were still showing blue fluorescence that revealed the segregation of introduced *PAT*1 gene by Mendelian ratio. As control, *trp*1-100 show blue fluorescence while wild type plant has non-fluorescence.

DISSCUSSION

We studied gene targeting as a strategy to induce gene disruption by homologous recombination in *A. thaliana*. The *PAT*1 gene was selected as a target locus because the disruption leads to a change in blue fluorescence which is easily distinguishable from the wild phenotype as shown in the results of complementation experiment.

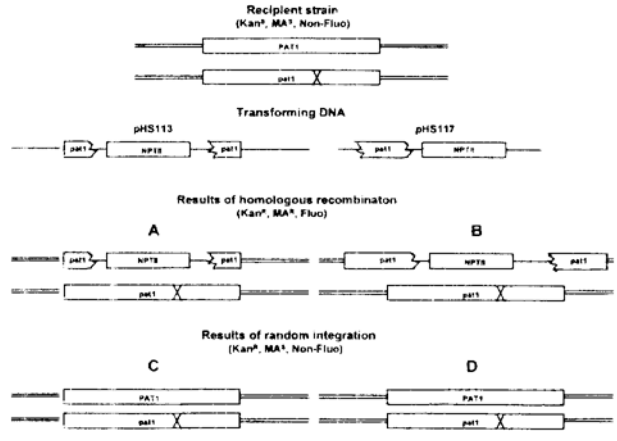

Fig. 6. Schematic diagram of gene targeting to knock out *PAT*1 gene in *A. thaliana*. The *pat*1 and *NPT*II gene can be introduced into plant cells by either homologous recombination (A, B) or random integration (C, D). The recipient plant is a heterozygous tryptophan mutants carrying one functional *PAT*1 gene. The plants show non-fluorescence and sensitivity to both 5-MA and Kanamycin. A, The functional *PAT*1 gene is replaced with *NPT*II gene through two recombination events on each side of the *PAT*1 locus; B, The functional *PAT*1 gene is splitted by insertion of the homologous sequences carried on the vector so that two unfunctional *pat*1 genes are arranged with *NPT*II gene; C-D, The homologous sequences can be incorporated into plant genome randomly so that *PAT*1 gene is remained functionally. As the results, all transformed cells (A,B,C and D) can resist to the Kanamycin but only homologous recombinants can (A and B) survive in the presence of 5-MA.

In a first experiment, we constructed two targeting vectors containing different segments of the *PAT*1 gene. Using pHS113, the disruption of *PAT*1 is expected to result from a double recombination event between the DNA sequences flanking *PAT*1 carried on the vector and the corresponding homologous regions of the *Arabidopsis* genome. Two recombination event at each end of the *PAT*1 locous should result in replacement of endogenous *PAT*1 gene with the gene for Kanamycin resistance (Fig. 6A). Gene disruption of *PAT*1 using pHS117 is expected to result from a single recombination event between the *PAT*1 locus in the genome and the homologous DNA carried on the vector. The integration by the single recombinational event will result in a direct repeat of *PAT*1 gene sequences in which only that portion of the gene carried by the plasmid is duplicated. Consequently, each of the repeated copies of the *PAT*1 gene will be now incomplete (Fig. 6B). Most current work involves the replacement type of genomic alternation as shown in pHS113 (Bertling, 1995).

Although disruption of a gene results in a recessive mutation on one of the pair of the gene in a diploid state, the phenotype of the resulting transformant is complemented by wild type of the corresponding gene. For this reason, the heterozygous *Arabidopsis* strain (*PAT*1/*pat*1) should be used to transfer reconstituted DNA into endogenous *PAT*1. It was intended that homologous sequences target functional *PAT*1 gene between two endogenous *PAT*1 loci in the heterozygous plant. The exact targeting will result in homozygous recessive mutation (*pat*1/*pat*1) so that the color phenotype of the regenerated transformant will be easily detected with the blue fluorescence.

Based on the analogy to selections for microbial mutants, mutants in the key regulatory enzyme, PAT, can be obtained by selection for plants that are able to grow on anthranilate analog 5-MA (Last and Fink, 1988). The strategy for such selections can be applied to the micro-colonies during the protoplast culture. When protoplasts from heterozygous *Arabidopsis* strain (*PAT*1/*pat*1) are directly transformed, the resulting microcolonies will be screened for the homologous recombination by selecting on the media containing both 5-MA and Kanamycin. Finally, plants regenerated with double resistances will be checked for the blue fluorescence.

Gene transfer by transformations is a crucial step in the development of a gene targeting system. The transformation schemes in plants are based on the integration of donor DNA into a random locus of genomic DNA (Deroles and Garder, 1988; Gheysen *et al.*, 1991). Among them, a direct transformation method to protoplasts could be applied most efficiently because a batch of transformants are screened for the homologous recombination. Although *A. thaliana* has been probed to be a useful model system to study molecular genetics in plants, a routine method for protoplast isolation and regeneration has not been established for various ecotypes or mutants since the optimum condition is variable among different genotypes (Wench and Marton, 1995). As a first step in gene targeting, it is one of the main limitations for the further use of *Arabidopsis* in plant molecular biology.

The gene targeting strategies we have developed are simple and can be used easily in *Arabidopsis* mutant *trp*1-100. Furthermore, any interesting genes in *trp*1-100 (*pat*1/*pat*1) can be targeted by using a universal vector containing PAT1 expression cassette. We are working on the construction of the vector system. These systems will certainly contribute to the elucidation of the genetic regulation of *PAT*1 gene and other genes. It will be expanded the range of genetic manipulations in plants.

ACKNOWLEDGEMENT

This work was supported by the Research Fund for the Genetic Engineering Program from the Ministry of Education (1995).

LITERATURE CITED

Baur, M., I. Portrykus and J. Paszkowski. 1990. intermolecular homologous recombination in plants. *Mol. Cell. Biol.* **10**: 492-500.

Bollag, R.J., A.S. Waldman and R.M. Liskay. 1989. Homologous recombination in mammalian cells. *Ann. Rev. Genet.* **23**: 199-225.

Bertling, W.M. 1995. Gene targeting. *In* Gene targeting. M.A. Vega (ed.). CRC Press. Boca Raton, pp. 1-44.

Deroles, E.S. and R. C. Gardner. 1988. Analysis of the T-DNA structure in a large number of transgenic petunias generated by *Agrobacterium* mediated transformation. *Plant Mol. Biol.* **11**: 365-377.

Engels, P. and P. Meyer. 1992. Comparison of homologous recombination frequences in somatic cells of petunia and tobacco suggest two distinct recombination pathways. *Plant J.* **2**: 59-67.

Gheysen, G., R. Billarroel and M. van Montagu. 1991. Illegitimate recombination in plant: a model for T-DNA integration. *Genes Dev.* **5**: 287-297.

Klee, H.J., M.F. Yanofsky and E.W. Nester. 1985. Vectors for transformation of higher plants. *Biotechnology.* **3**: 637-642.

Last, R.L. and G.R. Fink. 1988. Tryptophan-requiring

mutants of the plant *Arabidopsis thaliana*. *Science*. **240**: 257-364.

Murray, M.G. and W.F. Thompson. 1980. Rapid isolation of high molecular weight plant DNA. *Nucl. Acids Res.* **8**: 4321-4325.

Offringa, R., M. Groot, H.J. Hagsman, M.P. Does, P. Elzen and P.J. Hookaas. 1990. Extrachromosomal homologous recombination and gene targeting in plant cells after *Agrobacterium* mediated transformation. *EMBO J.* **9**: 3077-3084.

Peterhans, A., J. Schlupmann, C. Basse and J. Paszkowski. 1990. Intrachromosomal recombination in plants. *EMBO J.* **9**: 3437-3445.

Rose, A.B., A.L. Casselman and R.L. Last. 1992. A phosphoribosylanthranilate transferase gene is defective in blue fluorescent *Arabidopsis thaliana* tryptophan mutants. *Plant Physiol.* **100**: 582-592.

Shortle, D., J.E. Haber and D. Botstein. 1982. Lethal disruption of the yeast actin gene by integrative DNA transformation. *Science*. **217**: 371-373.

Wench, A.R. and L. Marton. 1995. Large-scale protoplast isolation and regeneration of *Arabidopsis thaliana*. *BioTechniques*. **18**: 640-643.

Zimmer, A. 1992. Manipulating the genome by homologous recombination on embryonic stem cells. *Ann. Rev. Neurosci.* **15**: 115-137.

Received March 8, 1997
Accepted July 21, 1997