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Random Insertional Mutagenesis with Subtracted cDNA Fragments in *Arabidopsis thaliana*

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We have evaluated a new mutagenesis strategy called random insertional mutagenesis with subtracted cDNA fragments. The cDNAs from long day *Arabidopsis* plants were subtracted by cDNAs from short day plants using PCR based cDNA subtraction. The subtracted cDNAs were inserted between 35S promoter and 3′-NOS terminator regardless of orientation. When the cDNA library was used for the random insertion into Arabidopsis genome by *Agrobacterium*-mediated transformation, approximately 15% of transformants showed abnormal development in leaf, floral organ, shoot apex. When 20 mutants were further analyzed, 12 mutants showed single cDNA fragment insertion and 8 mutants showed more than 2 transgene insertions. Only two mutants among 12 mutants that have single cDNA insert showed consistent phenotype at T2 generation, suggesting the genetic instability of the mutants.

key words: Random insertional mutagenesis, cDNA subtraction, antisense, posttranscriptional gene silencing

INTRODUCTION

The function of a gene can be revealed by genetic defect when the gene is mutated. The primary tool of mutagenesis is screening loss-of-function mutants using ethyl-methane sulfonate (EMS), fast-neutron bombardment, X-ray or γ-ray radiation. However, loss-of-function mutant screening has a limitation due to redundancy of genes and embryo-lethality. When a gene involved in specific genetic pathway is duplicated during evolutionary process, loss-of-function mutant of the gene cannot reveal any phenotype due to functional redundancy [1]. Likewise, when a gene is involved in both embryogenesis and later developmental process, the loss-of-function mutant cannot reveal phenotype at later developmental stage. To overcome such problems, activation tagging mutagenesis and random antisense mutagenesis have been recently developed [2,3].

The activation tagging mutagenesis is a random insertion by *Agrobacterium* transformation of four repeats of 35S enhancer derived from cauliflower mosaic virus into the plant genome [3,4]. The insertion of 35S enhancer by *Agrobacterium*-mediated transformation usually causes the overexpression of nearby gene. Thus, the activation tagging mutagenesis is an approach of gain-of-function mutant screening, an opposite approach of loss-of-function mutagenesis. Using activation tagging, the problems with gene redundancy and embryo lethality can be overcome. An alternative approach is a random antisense mutagenesis in which antisense cDNAs are randomly introduced into plant genome to block the function of individual gene [2,5]. Since antisense effect is variable according to the expression level of antisense in individual transgenic line and the homologous gene can be silenced based on sequence similarity, the problems with gene redundancy and embryo-lethality can be overcome. However, random antisense mutagenesis has also a limitation. Firstly, the copy number of mRNA for each gene is extremely variable and it is not easy to equalize the amount of antisense cDNAs in the library. Thus, most of the transgenic lines from random antisense mutagenesis would have antisense transgenes of which the gene is highly expressed. Secondly, the key regulatory genes are very often expressed less abundantly, thus the chance to get antisense transgenics of such genes is very low. To overcome such problems, we have developed random cDNA fragment insertional mutagenesis using equalized cDNA fragments.

In this study, we made equalized cDNA fragments by PCR based cDNA subtraction and performed random insertional mutagenesis with the equalized cDNA fragments that are under the regulation of 35S promoter. This approach is different from random antisense mutagenesis such that the orientation of cDNA fragments can be both sense and antisense. If cDNA fragments are inserted in antisense orientation, the result would be the same with random antisense mutagenesis [6]. If cDNA fragments are inserted in sense orientation, we expected two different effects. One is dominant negative effect, that is, part of the gene expression causes malfunction of the endogenous gene. Such effect can be usually found in transcription factors and protein kinases [7,8]. The other is posttranscriptional gene silencing (PTGS);

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when aberrant mRNA of certain genes are overexpressed, PTGS mechanism degrades normal mRNA of endogenous genes as well as aberrant mRNA [9]. Here, we report the results of random cDNA fragment insertional mutagenesis in *Arabidopsis*.

MATERIALS AND METHODS

Plant Materials and Growth. *Arabidopsis thaliana*, ecotype Columbia seeds were sterilized with 75% ethanol/0.05% Triton X-100 and sown on 0.5 ×Murashige-Skoog(MS) media for cold treatment at 4°C for 3 days, then transferred into the growth room. The 7 days old seedlings were transferred to soil and grown at 23±2°C, 60±10% relative humidity in long days (16 hours light/8 hours dark) under cool white fluorescent light. To isolate poly(A)$^+$ RNA for cDNA subtraction, plants were grown in long days and short days (8 hours light/16 hours dark) for 3 weeks. Long day grown plants produced flowers but short day grown plants were still in vegetative phase, thus producing only rosette leaves.

DNA and RNA Extraction. Genomic DNA from Arabidopsis plants was extracted by cetyl trimethylammonium bromide (CTAB) method as described [10]. Total RNA was extracted as described before [11].

cDNA Subtraction. Poly(A)$^+$ RNAs were extracted from the whole plants grown under long days and short days for 3 weeks using magnetic oligo d(T) dynabeads (Dynal AS) as described [12]. Double stranded cDNAs synthesis and cDNA subtraction were performed according to manufacture's manual (CLONTECH PCR-Select cDNA Subtraction Kit, Clontech). Briefly, double stranded cDNAs were synthesized using 2 μg poly(A)$^+$ RNAs with MMLV reverse transcriptase (200 units) and second-strand enzyme cocktail (DNA polymerase I, RNase H and DNA ligase mixture). The synthesized double stranded cDNAs were phenol extracted, precipitated with ethanol and resuspended with distilled water. The resuspended cDNAs from long day and short day grown plants were digested with *RsaI*. The cDNA fragments from long day plants were used as tester cDNA and the cDNA fragments from short day plants were used for driver cDNA. The tester cDNA fragments were divided into two and ligated with two different adaptors, IL38 and IL40 (both have *BamHI* recognition sequences as shown in Table 1) using T4 DNA ligase. The IL38 and IL40 adaptor ligated tester cDNAs were separately denatured at 98°C for 1.5 minutes and hybridized with 50 times excess amount of the *RsaI* digested driver cDNA fragments in hybridization buffer (5 mM HEPES, pH7.5, 50 mM NaCl and 20μM EDTA) at 68°C for 8 hours. The two samples from first hybridization were mixed together and freshly denatured driver cDNA fragments (30 times excess) were added to further enrich for differentially expressed sequences. The mixtures were hybridized in the same hybridization buffer at 68°C overnight. The hybridization mixtures were amplified by polymerase chain reaction (PCR) with IL42 primer (specific primer for IL38 adaptor) and IL44 primer (specific primer for IL40 adaptor) in 25μl reaction mixture (the sequence of primers in Table 1). PCR conditions were as follows: 75°C (5 min); 27 cycles of 94°C (30 sec), 68°C (30 sec), 72°C (1.5 min). After first PCR reaction, 0.1μl aliquots were used for second PCR reaction using nested primers IL43 (nested primer for IL38 adaptor) and IL45 (nested primer for IL40 adaptor) with the same PCR condition. In principle, the cDNA fragments that were ligated with both IL38 and IL40 adaptors would be amplified exponentially and the final PCR products would represent equalized cDNA fragments.

Construction of Binary Vector Library and Arabidopsis Transformation. The subtracted cDNA fragments were digested with BamHI and introduced into the BamHI site of pCGN18 binary vector [13] that is located between 35S promoter and 3′-NOS terminator (Fig. 1). The ligation products were used for the transformation of *E. coli* and plasmid DNAs were extracted using Wizard plus SV miniprep Kit (Promega). The plasmid DNAs

Table 1. The Sequence of adaptors and primers used in cDNA subtraction.

cDNA synthesis primer	5′TTGTACAAGCTT173′
IL38(adaptor)	5′CTAATACGACTCACTATAGGGCTCG***GGATCC***GGGCGCCGCAGGT3′ 3′CGCGGCGTCCA5′
IL40(adaptor)	5′TGTAGCGTGAAGACGACAGAAAGG***GGATCC***TGCGGAGGGCGGT3′ 3′GCCTCCCGCCA5′
IL42(PCR primer)	5′CTAATACGACTCACTATAGGGC3′
IL44(PCR primer)	5′TGTAGCGTGAAGACGACAGAAAG3′
IL43(nested PCR primer)	5′TCGGGATCCGGGCGCGGCAG3′
IL45(nested PCR primer)	5′AGGGGATCCTGCGGAGGGCGGT3′

* *BamHI* sites in IL38, IL40 primers are marked as italic, bold and underlined

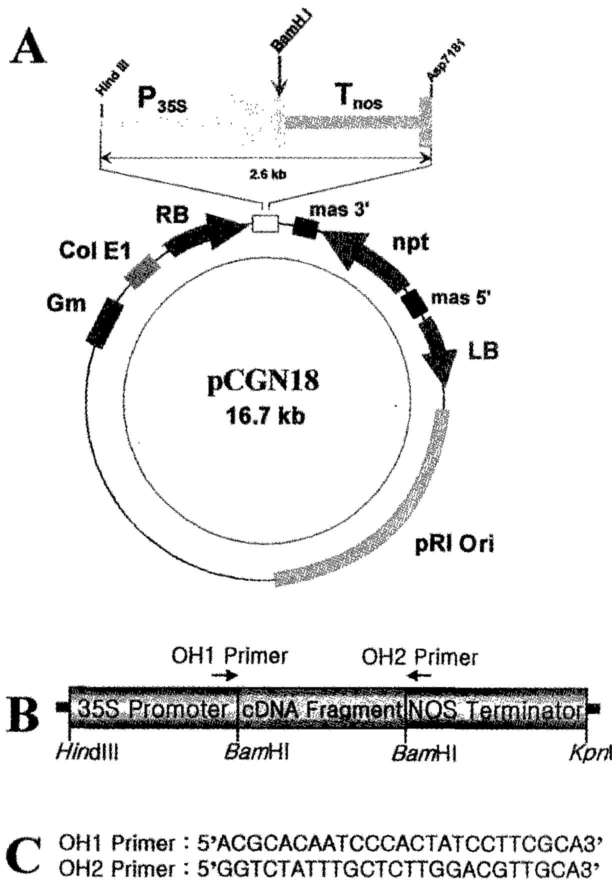

Figure 1. Map of binary vector pCGN18 and a schematic representation of cDNA construct. A. pCGN18 vector map. Gm, gentamycin resistant marker; npt, kanamycin resistant marker; RB, right border; P_{35S}, 35S promoter; T_{nos}, nos terminater; LB, left border. B. Schematic representation of cDNA fragment insertion between 35 promoter and nos terminator. C. The sequence of 35S and nos specific primers, OH1 and OH2.

were used for the transformation of Agrobacteria strain ASE. The plasmid constructs were introduced into Arabidopsis, ecotype Columbia, by vacuum infiltration [14,15].

RESULTS

Evaluation of Library Constructs. After plasmid constructs were introduced into *E. coli*, 30 independent plasmids were isolated from *E. coli* strains and digested with *BamHI* to check the insertion efficiency. Because the *BamHI* digested pCGN18 vectors were dephosphorylated with calf intestinal alkaline phosphatase (Promega) before ligation with subtracted cDNAs, most of the plasmid constructs contained inserts. When 30 plasmids were checked, 28 plasmids contained one insert and 1 plasmid contained two inserts and 1 plasmid had no insert (Fig. 2). The size of inserts were from 400 bp to 100 bp. To evaluate the subtraction efficiency, colony hybridization was performed using 200 independent *E. coli*

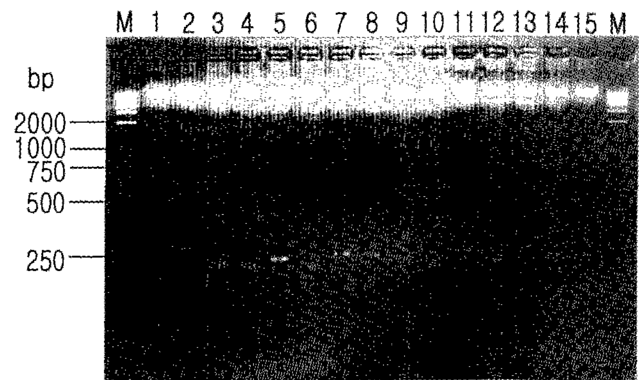

Figure 2. Representative clones from cDNA library. After *BamHI* digestion, the DNAs were electrophoresed in 2% agarose gel. First and last lanes are molecular markers (M) and the size of each fragment is presented as base pair. All the clones except clones 4, 14, 15 show single insert. Clones 4 and 14 show two inserts and clone 15 shows no insert.

strains. After 200 *E. coli* strains were grown on nitrocellulose membrane, hybridizations were performed using 20 independent inserts as probes. Thirteen inserts showed no cross hybridization except its own clone, three inserts showed cross hybridization with one other clone, two inserts showed cross hybridization with two other clones, two inserts showed cross hybridization with three other clones (data not shown). Thus, these results show that at least two-thirds of the binary vectors used for plant transformation contained a single cDNA fragment and the library was relatively well equalized.

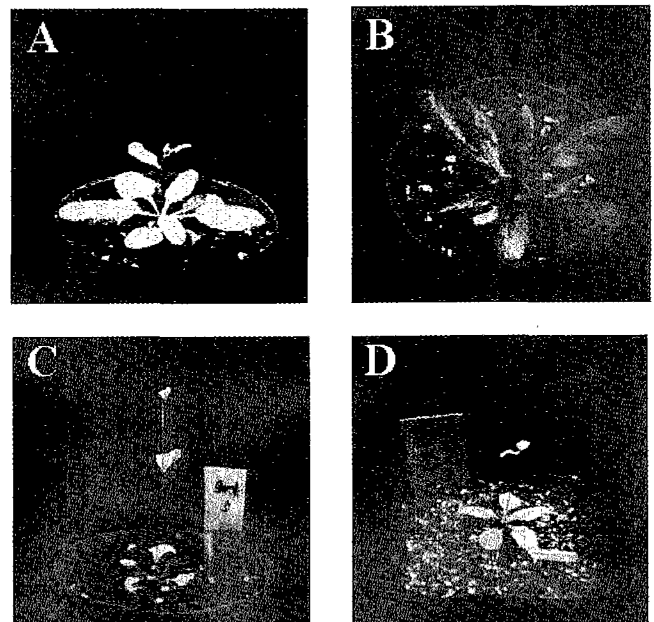

Figure 3. Morphology of mutant phenotype. A. wild type. B. late-flowering mutant C. early-flowering mutant D. pale and early-flowering mutant.

Table 2. Summary of mutant phenotypes in T1 transgenic plants.

Abnormal organs	Abnormal phenotype	Number of mutants
Cotyledon	Abnormal number of cotyledon	5
	Heart-shaped cotyledon	1
Leaf	Disordered phyllotaxy	18
	Small leaves	2
	Narrow leaves	8
	Irregular shaped leaves	20
	Curling leaves	3
Flowering-time	Early-flowering	7
	Late-flowering	1
	Abnormal shoot apex	34
Floral organ	Defects in seed production	20
	Abnormal petal development	9
	Elongated stamen	2
	Abnormal carpel	1
	Dwarfism	1
	Retardation of senescence	3
Pleiotropic phenotypes	Small leaves and abnormal shoot apex	8
	Irregular shaped leaves and defects in floral organs	9
	Irregluar shaped leaves and early-flowering	4
	Dwarfism and defects in seed production	4
	Early-flowering and pale color	1
	Sum	161

Screening of Mutants. We screened total of 1,091 T1 transgenic plants out of 1.8×10^5 primary transformed seed pools. The efficiency of transformation were about 0.6% that is relatively lower than previous report [15]. Among 1,091 T1 transgenics, 161 T1 plants showed abnormal phenotypes as shown in Table 2 and Fig. 3. Thus, approximatly 15% of transgenic plants showed abnormal phenotype. The commonest abnormal phenotypes were found in leaf morphology. Among them, 17 mutants showed disordered phyllotaxy of leaf formation, 2 mutants showed small leaves, 8 mutants showed narrow leaves, 20 mutants showed irregular shaped leaves and 3 mutants showed curling leaves. Total of 6 mutants showed abnormal cotyledons; 2 mutants showed a single cotyledon and 3 mutants showed three cotyledons instead of two. Total of 34 mutants showed abnormal shoot apex; some showed no shoot apex and some showed fasciation of shoot apices, resulting in two or three primary shoot apices. Flowering-time mutants were also identified as 7 early-flowering mutants and 1 late-flowering mutant. Defects in floral organ development were also found in the mutants. In addition, one mutant showed dwarfism and one mutant showed delayed leaf senescence. As well as the mutants showing simple phenotype, total of 26 mutants showed pleiotropic phenotypes.

Among 161 T1 mutants that showed abnormality, 20 mutants were further analyzed for the copy number of transgenes. The copy number of transgenes were determined by the segregation ratio between kanamycin resistant and sensitive progenies among T2 population derived from self-pollination. When the ratio is in the range of 3:1 (resistant:sensitive), it is indicative of single transgene but the ratio is higher than 15:1, it is indicative of more than two transgene insertions. Twelve mutants among 20 showed single insertion and 8 mutants showed more than two insertions. We have isolated the cDNA inserts from 12 mutants that showed single insertion by genomic PCR using 35S promoter and 3' -NOS terminator specific primers (Fig. 1). All of 12 mutants showed single cDNA fragment insertion (data not shown). When the mutant phenotype was confirmed in T2 generation, only 2 mutants among 12 showed consistent phenotype; one was early-flowering mutant, the other was small size silique producing mutant.

DISCUSSION

With the multinational efforts, the complete genome sequence of *Arabidopsis* has been reported [16]. However, the functions of most of the genes are largely unknown. To

reveal the function of each gene, the most valuable resource would be mutants of the genes. Currently, two different mutant strategies are commonly used; one is loss-of-function mutagenesis and the other is activation tagging mutagenesis [3]. Random antisense mutagenesis is also recently adopted [2]. However, all of three mutageneses have their own limitations. Thus, we report here alternative strategy to get more valuable mutants.

In this study, we have evaluated the strategy of random cDNA fragment insertional mutagenesis after cDNA subtraction. The cDNAs were made from the tissues of long day grown plants that were already producing flowers and the tissues of short day grown plants that were still in vegetative phase. When the cDNAs from long day grown plants were subtracted by cDNAs from short day grown plants, most of the highly abundant cDNAs were subtracted out. Thus, the PCR based cDNA subtraction had been very successful and resulted in equally normalized cDNA library.

The most popular mutant phenotype from this mutagenesis was found in leaf morphology. Approximately 30% of mutants obtained showed aberrant leaf morphology. It may suggest that the cDNA library have the genes involved in leaf development abundantly. Because a large portion of the tissues of long day grown plants is leaf tissue (approxiamtely 50% of tissues are from leaves), it also indicates that our cDNA library is well equalized. The other popular phenotypes were found in shoot apex and floral organ development. Because the tissues contain lots of floral organs, the abnormal phenotype in floral organ development is expected but the shoot apex abnormality could not be explained currently. The pleiotropic phenotype mutants suggested that many genes are involved in several different developmental processes.

Unfortunately, most of the mutants had lost mutant phenotype at next generation. Among 12 mutants that showed single cDNA inserts, 10 mutants lost the phenotype at T2 generation. It suggests that the mutants are genetically unstable. Such instability may be due to the instability of post-transcriptional gene silencing as reported [17]. For the analysis of gene function, genetic stability is important. Therefore, phenotypes of the mutants obtained with our strategy must be confirmed at the next generation.

The library we constructed could have both sense and antisense orientation. Such a strategy may have its own merit because we could expect dominant negative mutants from sense cDNA inserted transgenics. Such dominant negative mutants could be rarely expected from common loss-of-function mutants or random antisense mutants. Thus, this strategy would give us a new window to get novel mutants. In addition, the antisense cDNA inserted transgenics would give us variable mutant phenotypes depending on the positional effect. Such variation would be very helpful to overcome the problem with embryo-lethality or functional redundancy. Further analyses of the mutants obtained will provide us the feasibility to get such mutants.

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