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SCREENING OF *ARABIDOPSIS* MUTANTS FOR FUNCTIONAL GENOMIC STUDIES

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Abstract

Eight photosynthetic *Arabidopsis* mutants were screened for co-segregation of a photosynthetic phenotype with the T-DNA insertion. These mutants were selected from 80 photosynthetic mutants with genetic background of Columbia-0. Two different screening approaches were used to study the T-DNA insertion in the genome of mutant *Arabidopsis* lines. The sulphonamide sulfadiazine was found to be an effective selective agent and a single copy of sulfonamide resistant gene was found to be completely resistant to the optimal concentration i.e., 5mg mL^{-1}. The maximum number of *Arabidopsis* mutant plants had confirmed insertions. Some of the plants did not show any amplification with gene specific primer combination, and it was assumed that either they were wild type plants or they had random T-DNA insertion and the insertion was not found in the gene under study but it could be found in any where in the genome. Some mutant plants were morphologically different from the wild type plants e.g., ALP105. These plants grew as small in size and dark green in color. After PCR screening with gene specific and T-DNA border primers all such mutant plants were confirmed as heterozygous T-DNA insertion plants.

Introduction

The *Arabidopsis thaliana* is described as a model genetic organism by the Arabidopsis research community. *Arabidopsis* genome is organized into five chromosomes and contains an estimated 20,000 genes. However, due to the small size of meiotic chromosomes and absence of polytene chromosomes, cytogenetic studies of its chromosome structure are limited but visualization has been improved with the aid of *in-situ* hybridization methods. (Fransz, 1998). A number of approaches are being used to clone and gather information about the function(s) of gene(s). Among them, insertional mutagenesis has been effectively exploited for cloning genes, characterization and identification of genes and promoter elements in *Arabidopsis*. Positional cloning is being employed to cause and find out the mutation in the genomes. This strategy is facilitated in model species such as *Arabidopsis,* for which dense genetic maps with many visible and molecular genetic markers exist, and for which complete physical map consisting of a collection of overlapping cloned DNA fragments and total genome sequences are already available. The limiting factors for this approach include the time and efforts required for creating the mapping population and fine mapping of the mutant locus. Gene suppression (deletion / mutation knock out) or over-expression permits the gene sequence to be linked to a phenotype from which the function of the gene can be deduced (Matzke & Matzke, 1995). TILLING (Targeting Induced Local Lesions In Genomes), another modern

strategy for high throughput screening for point mutations (McCallum *et al*., 2000a; McCallum *et al*., 2000b; Perry *et al*., 2003; Till *et al*., 2003; Henikoff *et al*., 2004), which allows identification of allelic series of induced point mutations in genes of interest. Conversely map based cloning is another tool to identify a gene known only by its phenotype (Komori *et al*., 2004; Ohno *et al*., 2004; Sun *et al*., 2004). The chemical agents and radiation are used for induction of mutations. Among the chemical mutagens, Ethyl methane sulfonate (EMS) is easy to use and typically causes single base pair exchanges, and is more likely to create mutations with special properties such as weak, dominant, or conditional alleles (Lukowitz, 2000),

Insertional mutagenesis is an alternative means of disrupting gene function and is based on the insertion of foreign DNA into the gene of interest. Identification of insertion and point mutations in all *Arabidopsis* genes is a major goal of the Multinational Coordinated *Arabidopsis* 2010 project on functional genomics. On the subject of insertional mutagenesis, the identification of a T-DNA or transposon insertion in a gene of interest is exceptionally uncomplicated. Gene-specific primers and T-DNA specific or transposon-specific primers are used in PCR amplification from a DNA template that is derived from pools of mutant plants. The presence of specific PCR products in a particular pool indicates that a T-DNA or transposon has inserted in or near the gene of interest. In addition to the T-DNA insertion mutant collections (Azpiroz-Leehan & Feldmann, 1997; Feldmann, 1991), new mutant populations are available (with gene and promoter trap inserts) that drive specific expression of b-glucuronidase (GUS) and green fluorescent reporter proteins (Devic, 1995; Kiegle, 2000) and with activator T-DNA tags that facilitate screening for dominant mutations (Weigel *et al*., 2000). Saturation T-DNA mutagenesis is now performed using in plant transformation (Bechtold, 1993) and exploited to identify gene mutations by direct sequencing of transposon and T-DNA insert junctions (Balzergue, 2001; Liu, 1995; Tissier, 1999). Despite the advantages, this technique has some draw backs like after PCR amplification of complex pool samples, non specific amplification products often appears. This problem can be overcome by introducing a hybridization step (Southern blotting with a gene specific label primers) to identify the true-positive pools. This is by far the most widely used reverse genetic approach in *A. thaliana* (McKinney, 1995). The limitations of insertional mutagenesis includes the predominance of loss-of-function alleles, the biased distribution of insertions in the genome, the inability to characterize lethal mutations, and the difficulty of generating populations that are large enough to reach complete saturation of the genome (Krysan, 2002). The identification of mutations in a specific gene requires the screening of large numbers of mutagenized lines, necessitating the construction and assaying of large number of plant pools. This requirement has prompted the development of more sophisticated pooling strategies that minimize the number of assays required, but still allow the identification of an individual mutant line in one-step or two-step screens (Winkler & Feldman, 1998). The objective of the present studies was to formulate the screening strategies for the mutant *Arabidopsis* plants for the functional genomics.

Materials and Methods

Plant material: Eight GABI-KAT mutants (PAM-60, ALP-105, PAM-74, PAM-66, ALP-100, GABI- 080C08, GABI-134E03 and GABI-161D06), with genetics background of Columbia-0 were analyzed in this study. The sterilized seeds were grown on the MS medium plates with and without sulfadiazine (2.5mg/L). Fifty seeds of each line were grown on each plate. Plates were kept at 4^{o}C for one day and then transferred to a growth chamber with constant temperature of 22^{o}C till the required growth stage of the plants (3-

4 weeks). Mutants were sown in separate small pots kept at 4°C for 24 hours and then transferred to a greenhouse under normal growth conditions at 22°C and 16 hours day length period. After two weeks of germination, 54 plants of each mutant were transplanted separately to 54 well trays.

Sterilization of seeds: A beaker was filled with 100ml Sodium Hypochloride and 3 ml of HCl concentrated and placed with small microcentrifuge tubes (1.5mL) stand into a desiccator. Seeds were placed in tubes 1.5ml micro centrifuge tubes and the tubes were kept open in desiccator for 6-7 hours. The whole process was done under a fume hood then tubes were immediately closed while under the hood after 6 hours sterilization.

Genomic DNA extraction: Seedlings (Leaves) were harvested with a sharp pincet and transferred to eppendorf tubes and immediately frozen in liquid nitrogen and stored at -80°C. Frozen dry leaves from each plant were grounded to a fine powder in a 2-mL micro centrifuge tube with one 3-mm glass bead to a fine powder. 500µl of extraction buffer was added and shaken with horizontal shaker for complete homogenization. After adding 400µl of Phenol/Chloroform, the centrifugation was done at 13000rpm for 15 minutes at 20°C. The supernatant was transferred to fresh eppendorf tubes. DNA was precipitated with 0.8 volumes Isopropanol by centrifugation at 13000rpm for 15 minutes at 20°C. The pellet was washed with 70% ethanol and dissolved in 40-80 µl of 0.1XTE pH 8.0 containing 10 µg/ml of RNase A.

Data analysis: All the information relevant to T-DNA insertional *Arabidopsis* mutants are available on the web site of SALK institute Genomic analysis Laboratory (SIGNAL) http://signal.salk.edu/cgi-bin/tdnaexpress.By analyzing the sequence of the inserted gene by BLAST search http://www.ncbi.nlm.nih.gov/BLAST the orientation of the T-DNA in the genome of *Arabidopsis* was determined. The forward and reverse gene specific primers were designed for each mutant by flanking the regions of inserted T-DNA segment. The right border and left border T-DNA primers were also designed (Table 1).

Results and Discussion

Selection of mutants on sulfadiazine: *Arabidopsis* mutant plants were grown on the MS media with sulfadiazine and on MS media without sulfadiazine to check the germination of the mutants. It was observed that the germination rate of mutant line ALP-100 and a mutant line PAM-66 was low as compared to other mutant plants. It was also observed that nearly all plants of mutant line PAM-60 and mutant line ALP-105 were sulfadiazine resistant (Fig. 1).

Due to a reason that in any transformation experiments only a small portion of the target region is transformed and most of the region remained untransformed therefore a precise system is necessary to identify the transformed cells. Normally the selection of the transformed cells involves the germination of the putative transformants on a selective medium containing specific chemical that will inhibit the growth of the non-transformed individuals. The early attempts indicated that the most common selective marker gene in plants is the neomycin phosphotransferase II gene (*npt II*) (Beck *et al*., 1982; Bevan *et al*., 1983; Herera-Estrella *et al*., 1983), which confers resistance against the common selective agents like aminoglycoside antibiotics kanamycin. In addition, hygromycin, BASTA herbicide, methotrexate and sulfadiazine have also been used in *Arabidopsis*. The sulphonamide sulfadiazine was found to be an effective selective agent (Guerineau *et al*., 1990). The binary transformation vectors designed for activation

Table 1. Gene specific forward and reverse primers along with GK-LB and GK-RB primers.

S.No.	Primer name	Left primer	Right primer
1.	165B07	GAAACAGATCAACAAACCAAACAG	GTTCGAAAGAGAGAATCCAAATGT
2	060B06	GAGGAAGCTGGTTGATAAAGTTGT	AATTTTACAACGGGATGATGAAGT
3..	072A01	TAACTATCATGCTTTGGAATCACC	TTCGTCATTTTGATAACCTCTGAA
4.	120D02	ATGAGAATTCAGACGAATTTGTGA	GCATATGAAGAAAAGGACCACTCT
5.	120C01	TGTGTTATCACCAAACACAAACTG	TCATGTTTTTCCCCTTCTTACAAT
6.	080C08	TTTTTCAAGGTCAATTTCACTGAG	AACGGAAGGTAATCTTGGTTTATG
7.	134E03	TTGGATGGTAAACCATTGAAGTAA	ATACCTGGGAACACTAGTTGCATT
8.	161D06	ATGGCAATGGATGAGTCTGTACTA	TGCGTTCAGACAAAACAAAAGTAT
9.	GK-LB	ATATTGACCATCATACTCATTGC	
10.	GK-RB	CCAAAGATGGACCCCCACCCAC	

Fig. 1. Resistant plants of Arabidopsis T-DNA mutants germinated on MS having Sulfadiazine.

Table 2. Detail of GABI mutant plants germinated on MS media with sulfadiazine and as well as on simple MS media.

	Name	Total seeds	Germinated seedlings on sulf plates	Germinated seedlings on simple MS	Resistant	Non resistant
1.	060B06 (2)	50	42	45	42	0
2.	060B06 (4)	50	25	32	9	16
3.	060B06 (5)	50	14	19	7	7
4.	ALP-105 (2)	50	17	6	9	8
5.	ALP-105 (4)	50	50	32	50	0
6.	ALP-105 (5)	50	50	45	50	0
7.	PAM-74 (3)	50	12	11	9	3
8.	PAM-74 (4)	50	25	38	20	5
9.	PAM-74 (5)	50	3	2	3	0
10.	PAM-66 (10)	50	11	10	9	2
11.	PAM-66 (11)	50	4	11	4	0
12.	PAM-66 (12)	50	5	13	3	2
13.	ALP-100 (1)	50	9	4	0	9
14.	ALP-100 (2)	50	14	6	4	10
15.	ALP-100 (6)	50	13	6	2	11

tagging to generate the T-DNA population contain the *SUL*^{r} *ORF* gene for resistance against the herbicide sulfadiazine (4-amino-*N*-2-pyrimidinyl benzene sulfonamide, Sigma-Aldrich, Germany) (Rosso *et al., 2003*). Hadi *et al., 2003* described a versatile selection procedure for *Arabidopsis* transformants in the green house conditions. They used the inert growth substrate Grodan and proved that the sterilized transgenic seeds could be screened with kanamycine, hygromycine, BASTA, sulfadiazine and methotrexate (Table 2).

In this study the seeds were germinated on the MS media in tissue culture under sterile conditions (Grevelding *et al*., 1992). It was observed that the minimal concentration of the sulfadiazine for the optimal growth of *Arabidopsis* ecotypes was 5mg mL^{-1}. This supports the findings of Hadi *et al*., (2002). They checked the sensitivity of the two *Arabidopsis* ecotypes with different concentrations and found the best germination of the mutants with a minimal concentration of 5mg mL^{-1} sulfadiazine. A single copy of the T-DNA having P^{S001} sulfonamide resistant gene is present in the mutant *Arabidopsis* plants (Reiss *et al*., 1996). Since all the sensitive mutant plants die after emergence, it was confirmed that a single copy of sulfonamide resistant gene was completely resistant to the optimal concentration i.e., 5mg mL^{-1}. The survived plants were either in a homozygous or heterozygous T-DNA state. This was also explained by the Hadi *et al*., (2002) that a mutant T-DNA plant segregated with a clear 3:1 Mandelian ratio, which was acceptable for a monogenic Mandelian trait. Some of the mutant plants did not grow on the MS Media containing sulfadiazine, the germination capacity was then further tested on the simple MS media and it was assumed that the seeds of such mutant plants were not capable to germinate on the MS media although good germination was observed of these mutants under green house conditions. It was observed that the plants of the mutant line PAM 74 turned pale green and died on normal MS media after one week of germination. Fumiyoshi *et al*., (2006) identified 38 albino pale green mutants in a study for identifying nuclear genes responsible for chloroplast development and pigment synthesis. Noutoshi *et al*., (2005) isolated 10 pale green mutants which exhibited pale green cotyledons and true leaves at the juvenile stage.

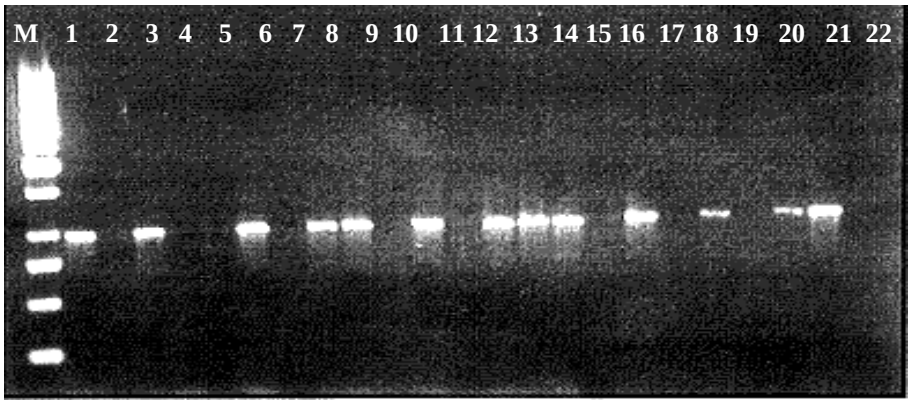

Fig. 2. PCR amplification of the PAM-74 mutant with gene specific primer.

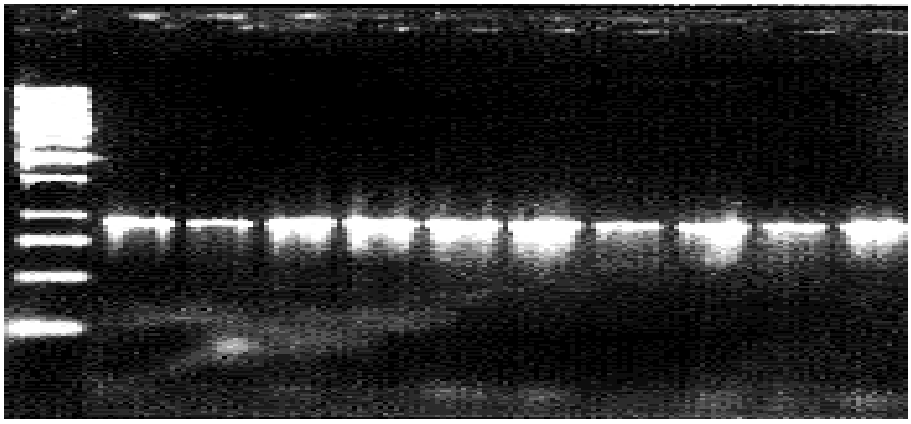

Fig. 3. PCR amplification of mutant PAM-66 with gene specific primers.

Fig. 4. PCR amplification of TDNA heterozygous mutant (PAM-66) with gene specific forward and GK-LB primer.

PCR screening of the mutants: One of the important features of GABI-KAT lines is that it has a Flanking Sequence Tags (FST) and a wide FST based database consisting more than 108000 mapped FSTs from approximately 64000 *Arabidopsis* lines covering almost 64% annotated *Arabidopsis* protein-coding genes. Along with other relevant informations the graphical display of the gene and the FST provides the gene-specific primers, insertion sequences and segregation data. According to Alonso *et al.,* (2003) the T-DNA express at SIGnAL http://signal.salk.edu/cgi-bin/T-DNAexpress) is the most comprehensive database for the mapped FSTs. It also includes the FST data from the Wisconsin T-DNA population (Sussman, 2000) and the RIKEN transposon population (Kuromori, 2004). In the present research the respective gene specific and T-DNA right border or T-DNA left border primers (Table 1) of 8 GABI KAT *Arabidopsis* mutant lines were synthesized by exploiting the informations from the FST database and BLAST. Different labs described the methodologies and the results out comes in the identification of *Arabidopsis* insertions mutants using PCR based strategies. Bouchez & Höfte, (1998) and Tissier *et al*., (1999) devised a pooling strategy by combining 20-100 insertion lines together and then gene specific and insert specific primers were used for PCR screening. They also suggested that the DNA from pools can also be combined into super pools and PCR could be performed in different stages. Sessions *et al.*, (2002) described a thermal asymmetric interlaced PCR for amplification of the unknown flanking sequences of the T-DNA insertions. Regarding the TAIL-PCR methodology, it requires the specific conditions to obtain the specific results, two series of primers AD and DL, are used. The major flaw of the TAIL-PCR is that this system does not have stability for *Arabidopsis* system. Young *et al*., (2001) used the degenerate primers on a large DNA pool and screen *Arabidopsis* H^+ -proton ATP*ase* gene family. The Gabino Rios *et al.*, (2002) devised another detection system for the T-DNA tagged genes in *Arabidopsis*. They amplified the T-DNA insert junctions from the pooled T-DNA samples and identified the insertions with direct sequencing. Yan *et al*., (2003) used another efficient PCR strategy to amplify the T-DNA flanking region known as T-linker PCR. They amplified the template molecules in three steps. First genomic DNA was digested with $3^{/}$ overhang enzymes. Secondly, a single A-tail was generated on the 3' unknown end of the target molecule, and then a $3^{/}$ overhang-T linker was ligated on to the target. Thirdly, the target was amplified by nested PCR with specific primers and T-linker primers. The screening of the T-DNA insertions in the eight GABI mutants under study was done by the gene specific primers and T-DNA border primers as shown in Figures 2, 3 & 4.

It was found that out of all the *Arabidopsis* mutants maximum number of plants had confirmed insertions. Some of the plants did not show any amplification with gene specific primer combination, and it was assumed that either they were of the wild type plants or they have random T-DNA insertion and the insertion was not found in the gene under study but it could be found in any where in the genome. Some mutant plants were morphologically different from the wild type plants e.g. ALP105. These plants grew as small in size and dark green in color. After PCR screening with gene specific and T-DNA border primers all such mutant plants were confirmed heterozygous T-DNA insertion plants.

In the functional genomic studies T-DNA insertion lines play a vital role and number of insertion lines of different crop plants will be available in the near future. The present study describes the efficient and more reliable procedures for screening the insertional mutagenized T-DNA lines, which would provide an efficient base for the analysis of novel gene function relationship.

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