High-throughput generation of sequence indexes from T-DNA mutagenized *Arabidopsis thaliana* lines

Nicolai Strizhov, Yong Li, Mario G. Rosso, Prisca Viehoveer, Koen A. Dekker, and Bernd Weisshaar

Max Planck Institute for Plant Breeding Research, Cologne, Germany

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A pipeline has been created for the characterization of Arabidopsis thaliana mutants by generating flanking sequence tags (FSTs) and optimized for economic, high-throughput production. The GABI-Kat collection of T-DNA mutagenized A. thaliana plants was used as a source of independent transgenic lines. The pipeline included robotized extraction of genomic DNA in a 96-well format, an adapter-ligation PCR method for amplification of plant sequences adjacent to T-DNA borders, automated purification and sequencing of PCR products, and computational trimming of the resulting sequence files. Data quality was significantly improved by (i) restriction digestion of the adaptor-ligation products to reduce trivial sequences caused by co-amplification of fragments derived from the free plasmid, and (ii) the design of the adaptor primers for the second amplification step to enhance selective generation of single PCR fragments, even from lines with multiple T-DNA insertions. Gel-purification was avoided by including these steps, the number of amplification reactions per line was reduced from four to three, and the percentage of lines that yielded at least one FST was increased from 66% to 86%. More than 58,000 FSTs have been submitted to GenBank® and are available at <http://www.mpiz-koeln.mpg.de/GABI-Kat/>.

INTRODUCTION

Random insertional mutagenesis, in which the foreign DNA not only disrupts a gene but also provides a tag for the characterization of the mutation, is used for the study of gene function relationships in a variety of organisms (1–4). In the model plant *Arabidopsis thaliana*, large collections of insertion-tagged mutants have been established by *Agrobacterium*-based transformation (so called T-DNA mutants) (5–7). The publication of the complete *A. thaliana* genome sequence (8) stimulated the systematic identification of the T-DNA insertion sites by sequencing (9–11). However, there are several factors that have a negative effect on efforts to characterize such insertion-tagged populations by sequencing. T1 seeds (see Materials and Methods), and plants generated from these seeds are inhabited by the *agrobacteria* used in transformation. Thus, DNA extracted from T1 leaves can contain the T-DNA plasmid. Plasmid-derived sequences may amplify preferentially during recovery of DNA fragments from the

T-DNA insertion sites. Another reason for poor quality sequences are truncated, tandem, or multiple insertions that often occur in complex patterns, resulting in complicated amplification products (12). Separation of unwanted fragments by gel electrophoresis is tedious, while a low success rate in flanking sequence tag (FST) production is expensive.

We optimized the characterization process of *A. thaliana* T-DNA mutants by FST generation through the introduction of simple steps to deal with the above-mentioned problems in an economic way.

MATERIALS AND METHODS

Plant Material and Growth Conditions

A. thaliana ecotype Columbia (Col-0) was used for plant transformation by *Agrobacterium*-mediated transfer with the flower-dip method (7). Plants containing the binary vector pAC161 (GenBank® accession no. AJ537514), conferring sulfadiazine resistance, were

randomly selected from the GABI-Kat (<http://www.mpiz-koeln.mpg.de/GABI-Kat/>) population. Plants were grown under natural greenhouse conditions with additional light to cover 16 h.

Isolation of Plant Genomic DNA

Preparation of genomic DNA from 300–500 mg leaf material of T1 plants was adapted to 96-well polypropylene 2.2-mL deep well blocks (HJ-Bioanalytik, Moenchengladbach, Germany) and an automated Biomek FX 96-channel pipettor (Beckman, Fullerton, CA, USA). We use the term T1 for the seeds and the plants that grow out of the embryos in these seeds, which are set by the infiltrated plants (T0 plants). T1 plants are hemizygous for the T-DNA insertion and contain the selectable marker. Blocks stored at -80°C were cooled with liquid nitrogen, and leaf material was ground with two stainless steel ball bearings (outer diameter 3.5 mm) per well by shaking for 30–45 s at 30 Hz in a Retsch 300 Matrix Mill homogenizer (Qiagen, Hilden, Germany).

Genomic DNA was prepared from ground leaf material according to a modified cetyltrimethylammonium bromide (CTAB)-DNA preparation protocol (13), replacing chloroform with dichloromethane (CH₂Cl₂). Finally, 0.8–1.6 µg DNA was dissolved in 80 µL TE (10 mM Tris-buffer, 0.1 mM EDTA, pH 8.0) and stored at -20°C.

Amplification of T-DNA Tagged Plant Sequences

DNA amplifications were carried out in 96-well Thermo-Fast plates (ABgene, Epsom, Surrey, UK) (14). About 100 ng of genomic DNA per line was digested with 4 to 5 U *Bfa*I (New England Biolabs, Frankfurt, Germany) in 20 µL total volume for approximately 5 h at 37°C. The reaction mixture contained 5 µg RNase A (Roche, Mannheim, Germany). An aliquot of 5–10 µL from the restriction reaction was used for ligation [20 µL total volume, approximately 3 Weiss U of T4 DNA ligase (New England Biolabs), for 11 h at 14°C] with 12.5 pmol of the adaptor T732/APL16OH. This asymmetrical adaptor was generated by the annealing of two oligonucleotides, T732 and APL16OH, at an equimolar

Table 1. Sequences of the Oligonucleotides Used in this Study

Name	Sequence	Purpose and Step Used
APL16OH	5'-TATGGGATCACATTAA-ddC-3'	Adaptor oligonucleotide, strand rendering 5'-TA overhang
T732	5'-GAGTAATACGACTCACTATAGGGAGGATCCCA-3'	Adaptor oligonucleotide, strand ligated to genomic DNA
8603	5'-CCCATTTGGACGTGAATGTAGACAC-3'	LB primer, 1st amplification
8474	5'-ATAATAACGCTGCGGACATCTACATTTT-3'	LB primer, 2nd amplification
3087	5'-CCAAAGATGGACCCCCACCCAC-3'	RB primer, 1st amplification
2895	5'-CAGTCTCAGAAGACCAAAGGGC-3'	RB primer, 2nd amplification
T726	5'-GAGTAATACGACTCACTATAGGGAGG-3'	Adaptor primer, 2nd amplification
adpA	5'-CTCACTATAGGGAGGATCCCATAGA-3'	Adaptor primer, 2nd amplification
adpC	5'-CTCACTATAGGGAGGATCCCATAGC-3'	Adaptor primer, 2nd amplification
adpG	5'-CTCACTATAGGGAGGATCCCATAGG-3'	Adaptor primer, 2nd amplification
adpT	5'-CTCACTATAGGGAGGATCCCATAGT-3'	Adaptor primer, 2nd amplification
8409	5'-ATATTGACCATCATACTATTGC-3'	LB sequencing primer
3144	5'-GTGGATTGATGTGATATCTCC-3'	RB sequencing primer

LB, left border; RB, right border.

concentration of 25 μ M (see Table 1 for all oligonucleotide sequences). Subsequently, the ligase was inactivated by incubation at 66°C for 17 min. Five microliters of the ligation mixture was digested with either *Xho*I (for the left border, LB) or *Pae*I (for the right border, RB) for 5 h in 15 μ L at 37°C. The first linear amplification was performed in 55 μ L with the whole *Xho*I or *Pae*I digest using primer 8603 (for LB) or primer 3087 (for RB) at a concentration of 200 nM, 250 μ M of each dNTP, and 1.5 U *Taq* DNA polymerase (Invitrogen, Karlsruhe, Germany). Amplification was performed in a PTC-225[®] Thermal Cycler (MJ Research, Waltham, MA, USA) using the following parameters: 94°C for 2 min, 30 cycles of 94°C for 35 s, 64°C for 1 min, and 73°C for 1.5 min, and then elongation at 73°C for 3 min. When postligation digestions were not performed, a 5- μ L aliquot of the ligation reaction was directly subjected to the first amplification. One microliter from the first amplification reaction was used as a template for the second PCR amplification in 50 μ L with nested T-DNA primers (8474 for LB and 2895 for RB) and adaptor primers adpA, adpC, adpG, adpT, or T726 at 200 nM each. The PCR reagents and cycling parameters were identical to the first amplification step.

Automated Sequencing and Computational Sequence Analysis

The PCR products were purified over a Sephadex[™] G50 superfine col-

umn (Amersham Biosciences, Freiburg, Germany). Dry Sephadex was filled into 96-well Multiscreen HV-plates (Millipore, Schwalbach, Germany),

and 300 μ L water was added per well for swelling. After 3 h, the plates were centrifuged at 910 $\times$ g for 5 min, washed with 150 μ L water, and again centri-

Table 2. Effect of Various Pipeline Modifications on Final Flanking Sequence Tag Yield

Methods	Amplification Reactions per Line	Sequencing Reactions per Line	Genome Hits ^a per Line	Lines with Hits (%)
I. Standard: no digestion; second amplification with adaptor primer T726	4	2	0.72	66
II. Digestion (<i>Xho</i> I, LB; <i>Pae</i> I, RB); second amplification with adaptor primer T726	4	2	0.80	74
III. No digestion; second amplification with adpA, adpC, adpG for LB (no adpT) adpA, adpG, adpT for RB (no adpC)	4	2	0.55	50
IV. Digestion (<i>Xho</i> I, LB only); second amplification with adpA and adpC or adpG and adpT	3	2	1.05	86
V. Digestion (<i>Pae</i> I, RB only); second amplification with adpA and adpC or adpG and adpT	3	2	0.26	25
VI. Digestion (<i>Xho</i> I, LB; <i>Pae</i> I, RB); second amplification with adpA and adpC or adpG and adpT	6	4	1.22	90

^aOnly unique hits are counted.
LB, left border; RB, right border.

fused for 5 min at 910× g. An aliquot of 16 µL of the second amplification reaction was applied to the minicolumns and centrifuged, and 2.5 µL of the eluate was used for cycle sequencing with the ABI PRISM® Big Dye Terminator Cycle Sequencing Ready Reaction kit v3.0 (Applied Biosystems, Weiterstadt, Germany) using primer 8409 for LB and primer 3144 for RB.

Unincorporated fluorescent nucleotides were removed by another centrifugation through a separate Sephadex minicolumn. The trace files generated by the ABI PRISM 96-capillary sequencer were fed into the computational data-processing pipeline (15). Information on the source plant of the PCR fragment analyzed was written into the respective trace file via the sample description

file of the sequencer. Each resulting sequence was assayed for T-DNA vector sequence before alignment against the *A. thaliana* nuclear genome sequence obtained from MATDB version 110103 (16) using BLASTN. A “genome hit” was defined as a FST of at least 30 bp in length with expectation values smaller than 5×10^{-4} . Resulting data were stored in the SimpleSearch database (15) that can be accessed at <http://www.mpiz-koeln.mpg.de/GABI-Kat/>.

RESULTS AND DISCUSSION

We developed and tested several approaches to improve the quality of FSTs, resolve amplification of multiple fragments, and reduce consequences of plasmid-vector contamination of plant genomic DNA to establish a pipeline for efficient and economical high-throughput generation of T-DNA FSTs. The evaluation experiments were carried out on a sample of 363 lines transgenic for pAC161. Based on the pAC161 sequence, *Bfa*I (CTAG) was selected as the restriction enzyme of choice for generation of DNA fragments spanning the T-DNA to genomic DNA junctions (Figure 1).

The common procedure for adaptor ligation-based FST production required, for each border, two nested amplifications and one cycle-sequencing reaction. When applied to both the left and right borders (four amplifica-

Figure 1. Overview of the final pipeline for the sequencing of genomic fragments flanking the known T-DNA insertion. The separate steps can be summarized as: (1) digestion of genomic DNA with *Bfa*I and ligation of the asymmetrical adaptor T732/APL16OH; (2) removal of plasmid-derived fragments by digestion with *Xho*I; (3) linear amplification with T-DNA primer 8603; (4) PCR amplification with T-DNA primer 8474 and adaptor primers with A plus C or G plus T; (5) cycle sequencing with T-DNA primer 8409. A. th., *Arabidopsis thaliana*.

tions plus two sequencing reactions), about 66% of the lines yielded at least one genome hit, with an average of 0.72 genome hits per plant (Table 2, Method I). To prevent amplification of plasmid-borne *Bfa*I fragments, we tested the inclusion of a restriction enzyme digest that splits such fragments. Two appropriate enzymes were used in the postligation digestion, *Xho*I for LB and *Pae*I (equals *Sph*I) for RB amplifications. Both enzymes are 6-bp cutters, and the losses of FSTs containing the sites for these enzymes were estimated to be moderate. Moreover, cleavage of the plant genomic DNA with *Xho*I (CTCGAG) is impaired by the CG methylation found in plants, and thus, the sites are protected, in contrast to the DNA derived from the free-living agrobacteria. Inspection of the resulting data (Table 2, Method II) showed a more than 10% increase in the number of LB-originated FSTs after *Xho*I digestion. No difference was found when RB-specific amplifications were performed either with or without *Pae*I treatment. There is no definite explanation for the latter case, because agarose gel analyses demonstrated that plasmid-borne PCR fragments (512 bp) disappeared after *Pae*I digestion. It could be that gains in the number of FSTs yielded were compensated by losses resulting from the use of unprotected *Pae*I (GCATGC) sites.

Another approach to avoid amplification of free plasmid-borne fragments involved a special design of adaptor-specific primers for the second amplification reaction (Table 2, Method III). The primers adpA, adpC, adpG, and adpT are homologous to the 3' part of the adaptor sequence and encompass a *Bfa*I site and extend by the last 3' end nucleotide into the adjacent plant sequence (Table 1). The last 3' end nucleotide is different in all four adaptor primers. In order to prevent amplification of free plasmid-borne fragments with T-DNA borders, only three adaptor primers were used in the PCR step for each border, and one primer containing the 3' end nucleotide corresponding to the plasmid fragment was omitted (adpT for LB and adpC for RB, respectively). It was anticipated that template fragments with bases that are noncomplementary to the 3' end of the primer

would not be amplified. However, one-fourth of all FSTs was expected to be lost, as not only free plasmid-borne fragments were incapable of amplifying, but also those FSTs that contain the corresponding nucleotide adjacent to the *Bfa*I site in the plant DNA. Indeed, with this method, we observed more losses than gains. The number of FSTs obtained for both the LB (41%) and the RB (18%) were significantly lower compared to the ordinary procedure with T726 adaptor primer.

The use of the 3' end specific adaptor primers was also attempted to resolve multiband patterns to single-band ones. Preliminary experiments that used only one of the 3' end specific adaptor primers (adpA, adpC, adpG, or adpT) demonstrated that this was possible (data not shown). However, it was not considered economical (due to high sequencing costs) to perform four different sequencing reactions per border. Therefore, two second-amplification reactions were performed for each line followed by sequencing. Each of the two reactions combined two adaptor primers, either adpA and adpC or adpG and adpT.

Combining the *Xho*I digestion with a specific primer set resulted in 66% (adpA and adpC) and 74% (adpG and adpT) genome hits at LB. After subtracting the 33% common hits produced by both reactions, an average 1.05 hits/line was obtained from the LB amplifications and 86% of all lines contributing an FST (Table 2, Method IV). Including RB reactions increased the yield further (Table 2, Method V). However, the RB yielded only 14% (adpA and adpC) and 20% (adpG and adpT) FSTs with 25% common hits (Table 2, Method V). The difference in amplification efficiency between LB and RB has been noticed before (17), but no quantitative data was provided. Part of an explanation for the difference could be that many of the RB sequences are present in inverted, head-to-head T-DNA arrangements (LB-RB:RB-LB) and are therefore not productive in FST generation. In fact, many of the 33% common genome hits at LB are from two LB-derived FSTs in opposite orientation.

With application of *Xho*I digestion only, 67% of the analyzed lines yielded FSTs from LB. For LB, the use of the

specific primer sets increased FST yield to 86%. If the contribution from RB amplifications is considered, 90% of the lines produced at least one FST with a yield of 1.22 hits/line. These values are in agreement with genetic analyses of the GABI-Kat population (segregation for sulfadiazine resistance) where we found approximately 1.3 insertions per line. We note that not all inserted T-DNAs are detected by genetic analysis. However, in terms of productivity, performing the two additional RB amplifications was not economical. Therefore, FST generation at RB was omitted from the pipeline.

Agarose gel separation is commonly used either for purification of PCR fragments before sequencing (18,19) or for fragment "picking" followed by reamplification (17). These tedious and DNA-damaging procedures greatly hamper automation of the FSTs' production process. Using additional restriction digestion and selective primers, we avoided agarose gel electrophoresis, which considerably accelerated the pipeline. The 96-well format was conveniently kept throughout the whole procedure.

With the assumption of a 50% GC content, every second line carrying two T-DNA insertions and/or generating a double PCR band gel pattern should be resolved by our method and produce two different FSTs. The remaining lines could still produce two overlapping sequences in one sequence file. For three or more fragments, the situation is more complicated. Some of the multiple-band patterns were resolved by computational trimming of the sequence files. In the case of two overlapping sequences, the 5' part of the sequence trace was of poor quality. However, 3' parts of the trace, corresponding to the longest PCR fragment, yielded unambiguous reads and produced valuable FSTs.

In summary, the final FST production pipeline included an additional restriction digestion and no gel purification. The number of amplification reactions per line was reduced from four to three (LB only), and yet the percentage of lines that yielded at least one FST was increased. We found an average of 1.05 genome hits per analyzed plant. We have currently generated

more than 58,000 FSTs from GABI-Kat using the pipeline described. These FSTs are available to assist the scientific community with the study of gene function relationships in *A. thaliana*.

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Address correspondence to Bernd Weisshaar, Bielefeld University, Department of Biology, D-33594 Bielefeld, Germany. e-mail: genomforschung@uni-bielefeld.de