fectly analyzed on both strands within only one sequencing reaction. Instead of using ethanol precipitation after the first PCR, one can use filtration methods in a 96-well format with QIAquick™ 96 (Qiagen GmbH, Hilden, Germany) or NucleoSpin® Multi-96 (Macherey-Nagel GmbH, Düren, Germany). These methods remove the PCR primers more efficiently and resulted in a remarkable reduction of amplification peaks in the intron sequences. However, the quality of exon sequences was the same compared to the purification by ethanol precipitation. The filtration methods are more comfortable in handling but increase the costs per sample. Since both methods revealed good quality of exon sequences, one can choose between the two cleanup methods.

Several attempts were made to adapt the EARL strategy to three exons. The reverse primer for exon 9 and the forward primer for exon 15 carried an *Xba*I restriction site, whereas the reverse primer of exon 15 and the forward primer of exon 16 carried a *Cla*I site. Multiplex PCR obtained the three amplification products, and ligation was performed after simultaneous *Xba*I and *Cla*I restriction. Re-amplification revealed products of expected size in addition to nonspecific smaller fragments, which caused problems in the correct evaluation of the complete sequences of the three exons. Smaller re-amplification products presumably result from false ligation. We suggest applying the EARL strategy for only two exons to avoid problems in the evaluation of sequencing data.

In summary, by applying the EARL method and a DNA sequencing system for intermediate throughput, we were able to analyze the sequence of four exons on both strands in 96 individuals within one week. This strategy increases the efficiency of the screening of gene polymorphisms and can also be adapted to other genes.

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Improved PCR-Walking for Large-Scale Isolation of Plant T-DNA Borders

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Nowadays, plant transformation is a standard method in plant genetics. This tool has been used to generate large collections of T-DNA or transposon insertion mutants for gene-tagging approaches. Several PCR-based methods have been developed for the recovery of genomic fragments flanking a known sequence [e.g., inverse PCR (6), TAIL PCR (5), and PCR-walking (7)]. These methods have been adapted for the cloning of plant genomic DNA flanking T-DNA (2,8) or transposon (4) insertions. However, these techniques were not optimal for the analysis of a large number of lines and needed further improvements for high-throughput processes. A PCR-walking technique (2,8) has been adapted for the cloning of the flanking sequence tags (FSTs) of a large number of T-DNA insertions from a collection of transgenic Arabidopsis lines (1).

The PCR-walking method consists of three main steps: (*i*) restriction digestion of the genomic DNA, (*ii*) ligation of an asymmetrical adaptor, and (*iii*) PCR amplification between two primers specific for the T-DNA and the adaptor, respectively. Both the asymmetry of the adaptor and its internal 3′ end blocked with an NH2 group prevent PCR amplification that could result from two adaptors (2,7). Two successive PCRs nested for both adaptor and T-DNA primers are subsequently carried out to avoid nonspecific PCR amplifications.

To optimize throughput, adaptations to the original technique have been made in all steps (extraction, restriction, ligation, and PCR), and reactions were performed in 96-well plates. DNA restriction and ligation were carried out in a single step. Finally, a protocol to isolate a single PCR product from each sample (i.e., individual line) was set up.

Seeds from T-DNA insertion lines of the Versailles's T-DNA collection (1) were sown in a Petri dish on 2 mL water. This growing condition limited the de-

velopment of microorganisms and thus allowed the rescue of plant material with no need for seed surface sterilization. Six-day-old seedlings were harvested, and 30–50 mg (fresh weight) were placed into 96-well plates (Corning Costar, Acton, MA, USA). Manual grinding of plant tissues is time consuming and not suitable for high-throughput experiments. However, grinding is required for the efficient extraction of good-quality DNA required for these experiments. Therefore, plant material was lyophilized overnight in a freeze dryer (FTS Systems, New York, NY, USA) and ground to dry powder with glass beads (4 mm diameter; Prolabo, Fontenay sous bois, France) for 2 min. with a modified RETSCH® MM200 oscillogrinder (Retsch, Haan, Germany), which allowed two 96-well plates to be processed simultaneously. Genomic DNA was extracted by a cetyltrimethylammonium bromide (CTAB) method adapted from Doyle and Doyle (3). The powder resulting from grinding was homogenized in 400 µL extraction buffer preheated to 65°C. Proteins were removed with 400 µL chloroform, and nucleic acids were then precipitated with one volume isopropanol in a 96-well Ultra-Clear EVERGREEN® plate (V bottom; Polylabo, Strasbourg, France). The pellets were dried at 65°C for 15 min and dissolved in 30 µL water. Ten microliters of the DNA sample were separated by electrophoresis through a 1% TAE agarose gel (Life Technologies, Burlington, Canada) to control both DNA quantity and quality.

DNA restriction and ligation steps were conducted simultaneously with no decrease of efficiency in the subsequent PCR amplifications. This protocol reduced the number of DNA manipulations and increased the reproducibility of the experiment (Figure 1). Also, the precipitation of the digested DNA before the ligation step was avoided. Several different restriction enzymes yielding blunt-ended DNA fragments may be used for PCR-walking from T-DNA insertion genomic borders (2). A computer analysis using Arabidopsis genomic sequences available in the public databases indicated that *Dra*I and *Eco*RV restriction enzymes should generate FSTs with an average length of 500 bp (data not shown). This length was compatible with PCR amplification, and the sequence data obtained were more than adequate for assigning the location of the amplified sequences when compared with Arabidopsis genome databases. Restriction of genomic DNA by a mixture of the two enzymes increased the number of borders recovered. By combining both of these restriction enzymes, correct amplification products were obtained for 80% of the lines tested (n = 7000). In practice, 500 ng genomic DNA were digested and ligated with 1 U both *Dra*I and *Eco*RV restriction enzymes (Life Technologies) and 1 U T4 DNA ligase (Life Technologies) in a 40-µL reaction volume. The reaction mixture contained T4 DNA ligase ligation buffer at the appropriate concentration and 25 µM of the adaptors. The reaction was conducted overnight at room temperature.

Primers

Since the right and left T-DNA borders do not have the same structure and function, the efficiency of plant genomic DNA rescue may differ depending on the border considered. This hypothesis was tested on a sample of 864 lines. Amplifications were more efficient for the left than for the right border (data not shown). Therefore, the T-DNA left border was chosen for the following PCR amplifications. Our preliminary sequence analyses suggested that the difference in amplification yield was most probably due to the accuracy of border integration during T-DNA insertion (data not shown). Primers used in this work were purchased from Genset SA (Evry, France). The specific primers for the adaptor were AP1 (5′-GGATCCTAATACGACTCACTATAGGGC-3′), and AP2 (5′-CTATAGGGCTCGAGCGGC-3′). Specific primers matching the T-DNA sequence were designed for both borders: RB1 (5′-CTGATACCAGACGTTGCCCGCATAA-3′), RB2 (5′-TCGTTAAAACTGCCTGGCACAG-3′), and RB3 (5′-CCAGACTGAATGCCCACAGGCCGTC-3′) for the right border and LB1 (5′-CGGCTATTGGTAATAGGACACTGG-3′), LB2 (5′-CAACCCTCAACTGAAACGGGCCGGA-3′), and LB3 (5′-TCCAGGGCGTGTGCCAGGTGC-3′) for the left border. Nested sequencing primers were designed for both borders, which were RB4 (5′-TCACGGGTTGGGGTTTCTACAGGAC-3′) for the right border and LB4 (5′-CGTGTGCCAGGTGCCCACGGAATAGT-3′) for the left border.

The adaptor consisted of two oligonucleotides (ADA1: 5′-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGAGGT-3′ and ADA2: 5′P-ACCTCCCC-N3′) at equimolar concentration. The adaptor was generated by heating the ADA1 and ADA2 to 80°C and then allowing it to cool to room temperature for annealing. The adaptor stock solution was diluted to the right concentration before use.

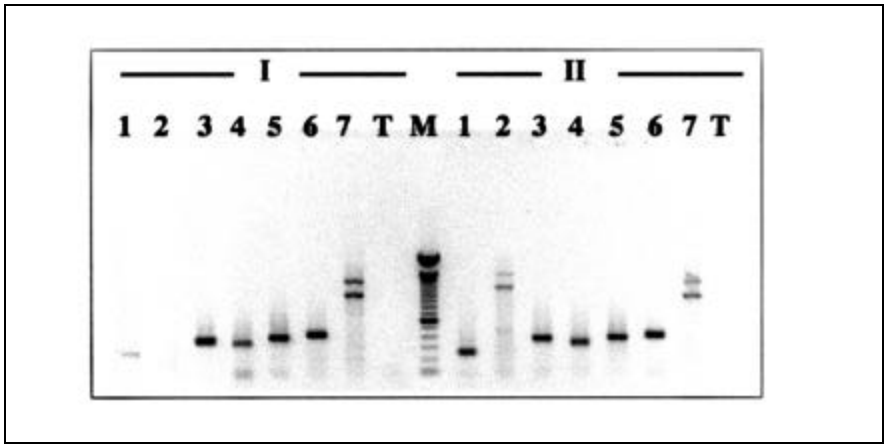

Figure 1. Efficiency of the digestion/ligation protocol. Panel I, lanes 1–7 show PCR II products obtained from different transgenic lines. DNA matrix was digested by both *Dra*I/*Eco*RV, precipitated and ligated with the adaptor, before PCR amplification. T, negative control with water; M, 100-bp molecular size marker (Life Technologies). Panel II, lane identification is similar to panel I. The genomic DNA is digested with both *Dra*I/*Eco*RV and simultaneously ligated with adaptor before PCR amplification.

PCR Amplifications

The first PCR was conducted in a 20-µL reaction containing 1× PCR buffer (Life Technologies) (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM each dNTP, 1 µL digestion/ligation product, 1 U *Taq* DNA polymerase (Life Technologies), and 10 µM each primer (i.e., AP1 and LB1 or RB1). PCR amplifications were performed in a PTC-100™ thermal cycler (MJ Research, Waltham, MA, USA) and sealed with thermo-well sealers (Corning, Costar®, New York, NY, USA). PCRs were conducted as follows: 30 cycles of 2 min at 94°C, 30 s at 94°C, 45 s at 67°C, 2.5 min at 72°C, followed by a final elongation step at 72°C for 5 min, performed in a 96 Thermowell plate (Model P; Corning Costar). The first PCR products were diluted 200-fold before being used as the template for the second amplification, which was conducted with the second set of nested primers (AP2 and LB2 or RB2) under the same conditions as PCR I.

The *Arabidopsis thaliana* lines used for FST isolation carry an average of 1.5 insertion loci, each of them possibly containing more than a single T-DNA copy (1). Thus, several FSTs might be co-amplified for each line. Amplified products were loaded on a 1% agarose gel and submitted to electrophoresis. Amplification products were picked from the agarose gel with a 10-µL tip and diluted, according to band intensity, to a concentration of 10 pg/µL. A third primer, AP3 (5′-GGCCGCCCGGGGAGGT-3′), was designed based on the adaptor 3′-end sequence, for use in a third PCR with AP3 and LB3 or RB3. In this reaction, the diluted PCR II fragment was used as the template to obtain a large quantity of high-quality single PCR products suitable for direct sequencing. Several bands were often visible on the gel after PCR II for any sample. After picking and dilution, amplification of the upper band always yielded several bands in the third PCR, whereas amplification of the lowest band yielded a clean single PCR product (Figure 2, lines 2–3 and 8–9). Therefore, for each sample, only the smallest band was used for the third PCR. The PCR products were then directly sequenced using a T-DNA specific primer, RB4 or LB4 for right and left borders, respectively.

To date, about 7000 T-DNA insertion lines have been processed using this protocol. A PCR product has been obtained for 80% of these lines. The absence of a PCR product in a fifth of the lines may be due to complex insertion loci or partial deletions of the T-DNA borders, thereby preventing PCR amplification. The sequence obtained from the PCR products revealed that 85% of the fragments can be considered as useful FSTs. Indeed, some sequences were discarded because of their poor quality or because they did not contain the expected residual T-DNA border. This latter FST class contains nonspecific amplifications or specific amplifications of truncated T-DNA borders, long enough for annealing of the specific primer but insufficient for the identification of the T-DNA by sequencing. The overall yield of this procedure is thus about 70 useful FSTs obtained per 100 lines processed.

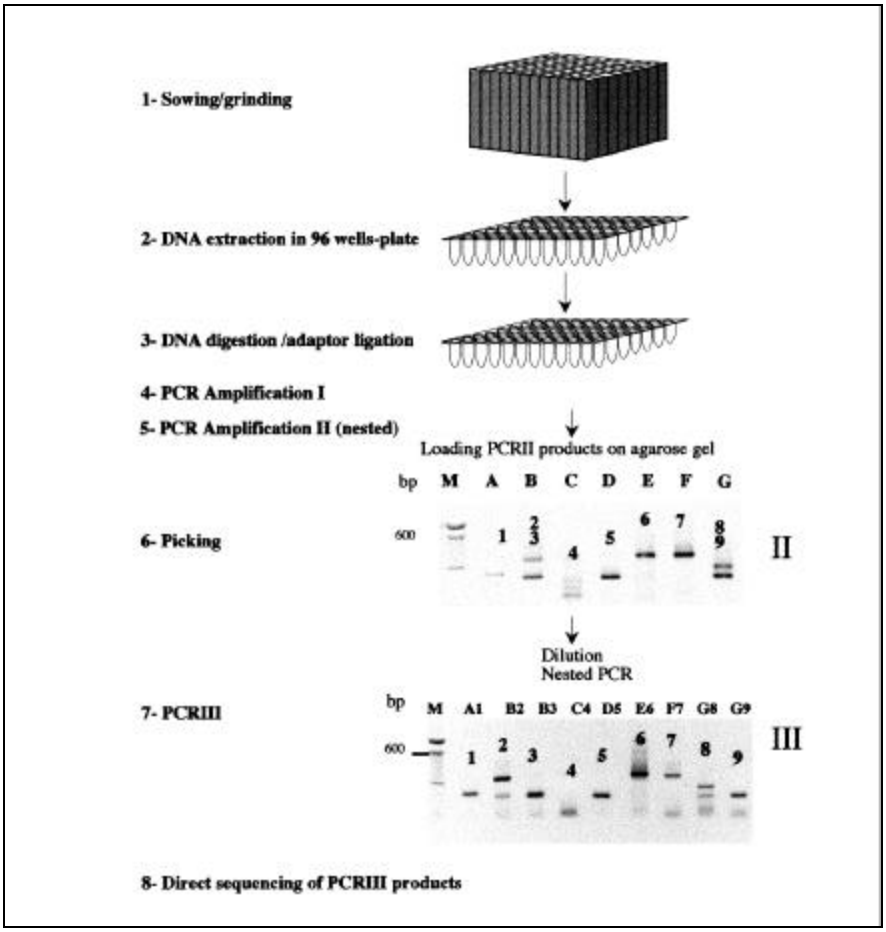

Figure 2. Scheme of the steps used in high-throughput PCR-walking. Step 1: seeds were sown in Petri dishes and germinated in temperature-controlled growth chambers for eight days. Each plate was filled with 96 individual lines. Samples were lyophilized, and DNA was extracted. Step 2: DNA was transferred into 96-well microplates, restriction digested (*Eco*RV and *Dra*I), and ligated to the adaptor (Step 3). The digestion/ligation product was then PCR-amplified (Step 4), and the diluted PCR was amplified with two nested primers (Step 5). PCR II products were electrophoresed onto a 1% agarose gel containing ethidium bromide, and the lowest bands were picked (Step 6). Lanes A–G show results from PCR II obtained from different transgenic lines. T, negative control with water; M, 100-bp molecular size marker (Life Technologies). The band was picked and diluted (Step 7), and a third PCR amplification was performed. Lanes A–G show results obtained after picking and PCR amplification of band from PCR II (numbered 1–9). Products from PCR III were loaded onto an agarose gel to check for purification and directly used for sequencing.

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Use of Fluorescently Labeled DNA and a Scanner for Electrophoretic Mobility Shift Assays

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The electrophoretic mobility shift assay (EMSA) is commonly used to determine the presence of specific transcription factors within the nucleus. In this assay, nuclear extracts from cells are mixed with a radioactively labeled oligonucleotide or DNA fragment containing a sequence known to bind to the transcription factor. This mixture is then electrophoresed through a non-denaturing acrylamide gel, and the gel is subjected to autoradiography. Binding of the oligonucleotide, and hence the presence of the transcription factor, is evidenced by a change or shift in the migration of the oligonucleotide. This method is quite sensitive but has the drawback that it relies on the labeling of DNA with a radioisotope. These probes are short lived, require special precautions for handling and disposal, and pose the health risk associated with the use of radioisotopes. To overcome these problems, we wanted to determine if a fluorescently labeled oligonucleotide could be used in an EMSA.

To determine if non-isotopic methods could be used to detect the binding of NF-κB, several oligonucleotides were obtained from commercial sources (Research Genetics, Huntsville AL, USA) (Table 1). Oligo 1 contained the fluorescent dye, carboxyfluorescein (FAM) at the 5′ end, while Oligo 1 unlabeled, which had the same sequence and therefore serves as a competitor for Oligo 1, was not fluorescently tagged. Oligo 1 complement binds to Oligo 1 and Oligo 1 unlabeled. To create an oligonucleotide that would not serve as a competitive inhibitor for NF-κB binding to Oligo 1, we synthesized a mutated version (and its complement) of the unlabeled oligonucleotide. Mutated Oligo 1 contained two point mutations that have been previously shown to abolish NF-κB binding (1).

Nuclear extracts were prepared as described previously (2). U-87 MG cells were seeded onto 150-mm culture plates and allowed to become approximately 80% confluent. Cells were removed from the plates by trypsinization and recovered by centrifugation at 2000× *g* for 5 min. The supernatant was discarded, and cells were washed in 1 mL buffer A [10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% Nonidet™ P-40 (NP-40), and 0.5 mM dithiothreitol (DTT)] and pelleted at 2000× *g* for 5 min. The supernatant was again discarded, and cells were resuspended by gentle pipetting in 80 µL buffer A containing 0.1% Triton® X-100. After incubation at 4°C for 10 min, the homogenate was centrifuged at 2000× *g* for 5 min, and the nuclear pellet was washed in 60 µL 20 mM HEPES, pH 7.9, 0.42 M NaCl, 25% (v/v) glycerol, 1.5 mM $MgCl_2$, and 0.2 mM EDTA. This suspension was incubated on ice for 30 min and then centrifuged at 16 000× *g* for 20 min at 4°C. The supernatant, which represented the nuclear extract, was stored at -80°C until use. Total protein concentrations of the extracts were determined using the bicinchoninic acid (BCA) assay (Pierce Chemical, Rockford, IL, USA) using bovine serum albumin (BSA) as the standard.

Assays were performed in 40-µL volumes containing 20 µg nuclear protein incubated with 1 pmol Oligo 1 and its complement in binding buffer (10 mM HEPES, 50 mM KCl, 2.5 mM DTT, 0.2mM, 0.05% NP-40, 10% glycerol, pH 7.9) for 30 min at room temperature. Competition experiments were performed by mixing Oligo 1 and its complement and 50 pmol of the oligonucleotides used for competition. The supershift assays were performed by adding 0.5 pmol antibody to the p65 subunit of NF-κB (Upstate Biotechnology, Lake Placid, NY, USA) to reactions that contained Oligo 1 and its complement as well as nuclear extract. Samples were loaded on a 7% native polyacrylamide gel, and electrophoresis was performed in 50 mM Tris, pH 8.3, 380 mM glycine. Following electrophoresis, the gel was analyzed by blue-excited fluorescence scanning with an 860 Storm™ FluorImager (Amersham Pharmacia Biotech, Piscataway, NJ, USA).