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Fast and reliable genotype validation using microsatellite markers in *Arabidopsis thaliana*

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Abstract In this paper we show how rogue genotypes in the parental stocks or contaminants among the crossed progeny of *Arabidopsis thaliana* can be readily identified and excluded from the breeding process using microsatellite markers derived from a small quantity of intact leaf tissue which has been alkali-treated. This method is fast and cost effective as it does not require DNA extraction, is highly reliable, and is less damaging to small plants where only limited quantities of plant tissue are available. Furthermore, a large number of samples can be processed in 1 day, facilitating the identification process prior to selfing or crossing the plants. In addition, the procedure could potentially be automated since no centrifugation is required.

Key words *Arabidopsis* · Microsatellites · DNA markers · PCR

Introduction

The accuracy and reliability of breeding programmes are clearly of prime concern and methods involving molecular-marker techniques have long been advocated as a means of quality control. To be effective, however, these techniques need to be simple, robust, reliable and suitable for a large-throughput system. They also need to be non-destructive, applicable when the plant material available for analysis is limited in quantity, and sufficiently quick to allow early decisions to be made prior to further breeding.

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Our current research programme, in which we are trying to produce overlapping contiguous chromosomal lines (OCLs) for the whole genome of *Arabidopsis thaliana*, provides a good example of the need for marker-aided quality control. We are using the recombinant inbred lines (RILs) extracted from the Columbia (Col) × Landsberg erecta (Ler) cross (Lister and Dean 1993), together with the Col and Ler parents, in a complex backcrossing and selfing programme in order to construct lines with defined chromosomal tracts from the Col and Ler parents. These OCLs will eventually be used to identify quantitative trait loci (QTLs), particularly those of small effect, to study their action, and to locate them sufficiently accurately to initiate map-based gene cloning (Kearsey and Pooni 1996). Accuracy of each step of the program is, therefore, essential.

In this paper, we describe a simple molecular technique to authenticate the pedigree of each and every individual prior to its crossing or selfing, and to identify those progeny among the segregating generations, such as the F_2 or the backcrosses, which need to be advanced to the next generation. The technique applied is quick, cost effective and reliable, and has already proved invaluable in identifying errors in the parental lines and in the crossing programme.

Materials and methods

The material

The programme involved crossing the Ler and Col parental lines to several RILs which had been chosen for their suitability for generating the required genotypes with overlapping segments of each chromosome. These RILs were selected according to their RFLP genotypes out of the 96 that were available from the Col × Ler cross (Lister and Dean 1993). Seeds of the parents and RILs were obtained initially from the Nottingham *Arabidopsis* Stock Centre in 1993 and have been maintained by controlled selfing at Birmingham since then. Currently, we are concentrating on the

Table 1 Band sizes (bp) of various PCR products and their putative chromosomal locations for the Ler and Col parents, and the five RILs (adapted from Bell and Ecker 1994 and the internet site)

Line	Locus			
	ATEAT1	*ca72*[a]	*nga76*	*nga139*
Col	172	124	231	174
Ler	162	110	> 250	132
RIL32	172	110	> 250	132
RIL160	162	110	> 250	132
RIL161	172	110	Not available	132
RIL190	162	110	231	174
RIL257	Not available	110	231	Not available
Location	Chromosome I	Chromosome V	Chromosome V	Chromosome V

[a] Observed fragment sizes were different (see text)

crosses of RIL32, RIL160 and RIL257 with Col, and of RIL190 and RIL161 with Ler, to generate the selfed or backcross progenies which will lead to the production of OCLs for chromosomes 1 and 2**.** Prior to performing these crosses, we have attempted to establish the true identity of the individuals to be crossed and rogue out any contaminants. In other words, we have sought to determine with a high degree of confidence if each individual belongs to its designated family (parent or RIL). For this purpose, individuals from each male and female parent were tested using four microsatellite loci (namely *ATEAT1*, *ca72*, *nga76* and *nga139*; Bell and Ecker 1994), chosen because they were polymorphic in the parents and their amplification products could be easily scored on a multipurpose agarose gel. The primer sequences, and their expected product sizes for RILs and the parents, were taken from Bell and Ecker (1994) and the internet site *http://cbil.humgen.upenn.edu/~atgc/*. Further information on the genotypes of the parents and RILs together with band sizes is provided in Table 1.

The method

We have opted to use small leaf cuttings as a source of the genomic target for PCR (Klimyuk et al. 1993; Clancy et al. 1996) in order to avoid causing serious damage to the plants. Leaf tissue (1–2 cm long) was finely chopped and transferred into a sterile 1.5-ml microfuge tube containing 200 µl of 0.25 M NaOH. The microfuge tube was then put on a floating rack and immersed in boiling water for 30 s. After the hot-water treatment, the tube was removed from the water bath and 200 µl of 0.25 M HCl and 100 µl 0.5 M Tris-HCl pH 8.0, [0.25% v/v Nonidet P40 (Sigma)] added into it. The microfuge tube was once again incubated in the boiling water for 2 min and a small piece ($\cong 2\,mm^2$) of alkali-pre-treated leaf tissue used as a source of genomic target for running the PCR reactions.

The amplification reactions were performed in a volume of 20 µl containing 200 µM each of dNTP, 10 pmole of each of the primers (synthesized by Operon), 2 U of *Taq* polymerase and 1 × buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.1% Tween20]. A small piece of alkali-treated leaf tissue served as a source of genomic DNA, which was scooped out of the microfuge tube (see above) with the help of a sterile pipette tip and dropped into the PCR mix. The mixture was overlaid with 2 drops of light mineral oil. Amplification was achieved in a Hybaid-Omnigene thermal cycler programmed as follows: one cycle at 95°C for 1 min followed by 40 cycles at 94°C for 30 s, 55°C (61°C for *ca72*) for 30 s and 72°C for 1 min. Aliquots of 5 µl of amplification products mixed with 1 × loading buffer were loaded on a 4% multipurpose agarose gel and electrophoresed in 1 × TBE buffer at 200 V for 2 h. The electronic image of the ethidium bromide-stained gels was captured using a Flowgen IS500 imaging system.

To check the efficiency and reliability of using pre-treated leaf pieces as a template, we also extracted DNA from leaf tissue using the CTAB miniprep method of Virk et al. (1995).

Results and discussion

We obtained the same band intensity using DNA extracted from leaf tissue and the alkali-pre-treated intact leaf tissue as a genomic target (see Fig. 1 a, lanes 7–10 for example). Similar results were also obtained for other loci, which confirmed that intact leaf tissue could be used effectively for amplifying microsatellites in *Arabidopsis*. Miniprep DNA extraction requires 50 mg of leaf tissue from each plant, costs more than 114 pounds sterling for 50 samples (DNeasy Plant Mini Kit; QIAGEN), and takes almost 1 day to complete the protocol. Using the alkali pre-treatment method, the same number of samples can be prepared for PCR in about 2 hours and they cost less than one pound sterling, i.e. less than 1% of the cost of DNA extraction. As we have to genotype several thousand samples in this project, we shall be making considerable savings of time, money and storage space by using the alkali pre-treatment method.

To test the authenticity of the RILs which we have used as female parents in all the crosses, three individuals from each of the five RILs were subjected to PCR for four microsatellite loci, giving us 16 possible genotypic fingerprints and a $>93\%$ $[=1-(1/2)^4]$ probability of ascertaining the expected genotypes (assuming that all microsatellite loci are independently segregating). However, this probability is reduced to 84% when linkage between the three markers located on chromosome V is taken into account. All the RILs and the Ler and Col individuals amplified the correct fragments for all four microsatellite loci, with the exception of *ca72*. The expected fragment size for *ca72* locus is 124 bp for Col and 110 bp for Ler (Bell and Ecker 1994) whereas we obtained band sizes of $\cong 250$ bp and $\cong 220$ bp respectively (Fig. 1 a). Also, there was no within-genotype variation for four of the RILs, but

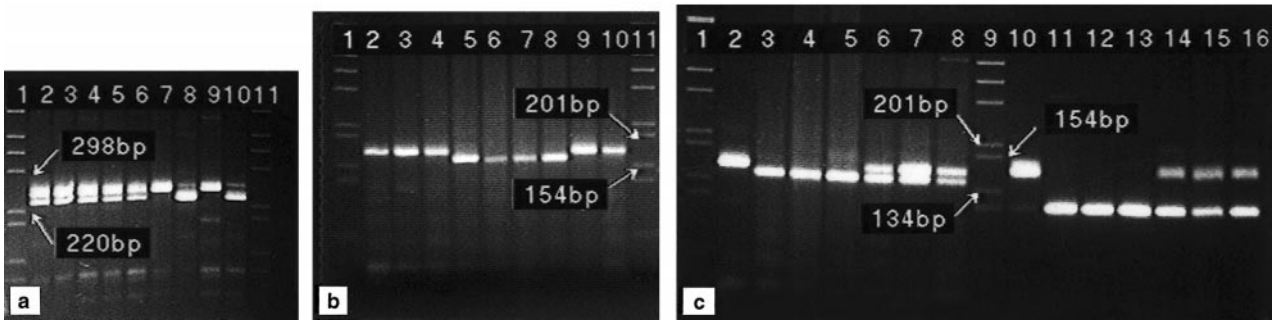

Fig. 1 a Amplification of locus *ca72* using genomic DNA and alkali-treated leaf tissue [*lanes 2–6*, alkali-treated leaf tissue of five F_1 (RIL257 × Col) individuals; *lanes 7–8*, genomic DNA of Col and Ler; *lanes* 9–*10*, alkali-treated leaf tissue of Col and Ler; *lanes 1 and 11*, 1-kb ladder]. **b** Products of locus *ATEAT1* from the alkali-treated leaf tissue of three individuals each from RIL32 (*lanes 2–4*), RIL 190 (*lanes 5–7*) and RIL 161 (*lanes 8–10*). *Lanes 1 and 11*, 1-kb ladder. **c** Amplification of locus *ATEAT1* (*lane 2*, Col; *lanes 3–5*, three individuals of female parent RIL160; *lanes 6–8*, three hybrid plants of RIL160 × Col cross; *lanes 1 and 9*, 1-kb ladder) and locus *nga139* (*lane 10*, Col; *lanes 11–13*, three individuals of female parent RIL32; *lanes 14–16*, three hybrid plants of RIL32 × Col cross) for the various genotypes

there was for RIL161 where the amplification products of the *ATEAT1* locus showed marked variation among the three individuals (see Fig. 1 b, lanes 8–10). This RIL was expected to have the Col allele (= 172-bp fragment) and while two out of three individuals had the expected allele, the third had the Ler allele (162-bp fragment). Another sample from RIL161 also gave similar results indicating that the seed of this line is not pure, and we are currently verifying the original seed sample prior to attempting fresh crosses.

One polymorphic locus is all that we need to validate each cross. Crosses RIL32 × Col and RIL190 × Ler were validated using the *nga139* locus, while *ATEAT1* and *ca72* were used to confirm the purity of the RIL160 × Col and RIL257 × Col crosses. All the F_1 individuals from each of these crosses possessed alleles of both parents, confirming that they were true hybrids (see Fig. 1). This shows that the crossing procedure employed was adequate and reliable.

The present study has demonstrated that accurate microsatellite profiles can be produced quickly from the intact leaf tissue of *Arabidopsis*, and has been found to work satisfactorily in barley (freeze-dried or fresh leaf tissue) and rice (fresh tissue) and there is every reason to believe that the same approach would apply in other plant species. Because no DNA extraction and hence centrifugation is required, it would be a simple matter to automate the whole procedure for large-scale genetic fingerprinting.

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