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Ty1-*copia* retrotransposon-based SSAP marker development in cashew (*Anacardium occidentale* L.)

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Abstract The most popular retrotransposon-based molecular marker system in use at the present time is the sequence-specific amplification polymorphism (SSAP) system. This system exploits the insertional polymorphism of long terminal repeat (LTR) retrotransposons around the genome. Because the LTR sequence is used to design primers for this method, its successful application requires sequence information from the terminal region of the mobile elements . In this study, two LTR sequences were isolated from the cashew genome and used successfully to develop SSAP marker systems. These were shown to have higher levels of polymorphism than amplified fragment length polymorphic markers for this species.

Introduction

The development and subsequent application of molecular marker systems have greatly increased the numbers of markers that can be identified between two parents and, consequently, significantly enhanced the power of genetic analysis in both plant and animal species. Molecular markers have revolutionized the genetic analysis of crop plants, where they play a vital role in linkage analysis, physical mapping, quantitative trait locus (QTL) analysis, marker-assisted selection and map-based cloning. However, the usefulness of any given marker system depends on the species under study and the chosen application. Microsatellite markers are powerful tools but require a significant input for their discovery. Therefore, for crops where there is no or little DNA sequence information, anonymous marker systems such as the amplified fragment length polymorphism (AFLP) system have been favoured.

Long terminal repeat (LTR) retrotransposons are a class of mobile genetic elements that have been harnessed for the development of molecular markers in plants (Schulman et al. 2004). LTR retrotransposons move and replicate around their host genomes via RNA intermediates. The two major groups of LTR retrotransposons are Ty1-*copia* and Ty3-*gypsy*. LTR retrotransposons are present as large heterogeneous populations in all plant genomes (Konieczny et al. 1991; Flavell et al. 1992; Pearce et al. 1997; Suoniemi et al. 1998), and they show great variations in copy number and genome localization, even between closely related species (Pearce et al. 1996a, b; Kumar et al. 1997).

The sequence-specific amplification polymorphism (SSAP) approach is the most popular transposon-based molecular marker method at the present time (Waugh et al. 1997; Schulman et al. 2004). This method exploits the variation generated by retrotransposon movement and reveals higher levels of polymorphism between individuals than the AFLP marker system in barley, pea, wheat and alfalfa (Waugh et al. 1997; Ellis et al. 1998; Flavell et al. 1998; Gribbon et al. 1999; Kalendar et al. 1999; Porceddu et al. 2002; Queen et al. 2004). The transposon display approach, which uses another transposon type called miniature inverted repeat transposable

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elements (MITEs) is virtually identical to SSAP and has been deployed in maize (Casa et al. 2000).

SSAPs require sequence information from the terminal region of the mobile element in order to design the primers required for the method (Waugh et al. 1997). The SSAP marker approach is similar to that of AFLPs in that genomic DNA is digested with restriction enzymes, enzyme-specific adapters are ligated to the restriction products and the resulting fragments are PCR-amplified. The final step differs between the two approaches, with AFLP using two different adapter-specific primers carrying selective bases to reduce the number of amplified marker bands to a manageable level, whereas SSAP uses a single adapter-specific primer, together with a transposon-specific, labelled primer with selective base(s) on the 3′-end.

Cashew (*Anacardium occidentale* L.) (Anacardiaceae family) is a tree which is indigenous to northeastern Brazil. The plant produces one of the most economically important and widely relished nuts and has become an important crop world wide, especially in Vietnam, India, Brazil and Africa (FAOSTAT 2002). Although, cashew is mainly cultivated as nut crop, its apples (hypocarp) are also used both as fresh fruits and for making a popular brew in India, Brazil and several other countries (Archak et al. 2003). Cashew is an outbreeding species, with entomophilous pollination (pollen dispersal by insects; Nambiar and Pillai 1985). It has a haploid chromosome number of 21 but the ploidy level is not very clear (Purseglove 1968; Archak et al. 2003).

Genetic studies in cashew have been hampered by its long generation time (approx. 5 years) and difficulty in crossing. Controlled crosses between cashew trees are both time-consuming and prone to failure with less than 9% of the manual crosses typically generating a single seed. Consequently, a sizeable mapping population is still unavailable for this species. Furthermore, little attention has been given to the development of molecular tools to aid the breeding effort. Recently, Archak et al. (2003) described various fingerprinting techniques, including random amplified polymorphic DNAs (RAPDs), inter-simple sequence repeats (ISSRs) and AFLPs, with varying degrees of polymorphisms and efficiency in cashew. We report here the development of a SSAP marker system for cashew which offers a significant advantage over the other methods used to date.

Materials and methods

Plant material

Parental clones were selected from a primary cashew (*Anacardium occidentale* L.) population maintained in the breeders‘ germplasm collection of the Brazil Embrapa Agroindústria Tropical at the Pacajus Experimental Station, Pacajus, State of Ceará, Brazil. Since the efficiency of cashew crossing is very low, only a small segregating F_1 progeny of 28 individuals was created between two parental clones that had been selected on the basis of the following contrasting features:

1. CP 1001—commercial dwarf clone, exhibiting high yields of small nuts, susceptible to anthracnose and highly susceptible to black mould.
2. CP 96—tall, high yields of large nuts, moderate resistance/tolerance to anthracnose and a high resistance to black mould.

All of these traits segregate within the F_1 population. The ultimate aim is to extend the population to allow the creation of the linkage map of the crop to aid QTLs identification and markers linked to useful traits.

Isolation and characterization of retrotransposon LTR sequences from cashew

DNA was extracted using a modified CTAB extraction method (Saghai-Maroof et al. 1984; Virk et al. 1999). For isolating cashew LTR sequences, we used the protocol of Pearce et al. (1999), with the following modifications. The original approach used affinity purification of the PCR products, which had been primed from a biotinylated RNAseH motif primer, to eliminate a background of AFLP products lacking a retrotransposon-specific sequence. Instead of this, we used hot start *Taq* DNA polymerase to amplify the RNaseH-LTR junction region.

Briefly, genomic DNA (0.5–1 μg) was digested with *Mse*I [New England Biolabs (NEB), Beverly, Mass.]. Aliquots of 500 ng of *Mse*I adapters (Vos et al. 1995) were ligated to the digested DNA in NEB ligation buffer to prepare the template DNA for pre-amplification. Template DNA (50 ng) was used to amplify the RNAseH motif-LTR junction regions using the motif 1 primer (Pearce et al. 1999: 5′-MGNACNAAS-CAYATHGA-3′) and *Mse*I adapter primer. When non-phosphorylated adapters are used, one of the adapter strands is unligated from the PCR product and dissociates during the hot start step (Vos et al. 1995). These sequences are the priming sites for PCR amplification and, therefore, the AFLP products, which contain adaptors at both ends of the amplicon, cannot be amplified. Amplification can only take place after the RNAseH motif primer copies the ligated adaptor strand to re-create the complete adapter. Only then can the adapter-specific primer bind to the adapter region and be amplified by PCR.

Using this method, a smear of bands was usually amplified using motif 1 primer (data not shown). These PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, Calif.) and re-amplified using a nested primer corresponding to the conserved RNAseH motif 2 (GCNGAYATNYTNAC-NAA; Pearce et al. 1999) and the *Mse*I adapter-specific primer. This second amplification resulted in much

stronger DNA smears, corresponding to a variety of putative Ty1-*copia* retrotransposons in the cashew genome. The amplified fragments were purified as described above and cloned using pGEM-T easy cloning kit (Promega, Madison, Wis.). Fifty colonies having potential LTR-containing inserts were initially used directly as templates in PCR reactions to identify clones of the desired size. The PCR conditions consisted of 1.5 m *M* $MgCl_2$, 200 μM dNTPs, 10 pmol of each of the primers T7 (GTAATACGACTCACTATAGGGC) and SP6 (TATTTAGGTGACACTATAG) and 0.5 U Hotstar *Taq* DNA polymerase (Qiagen) in a 20-µl final volume. Cycling included a denaturation step of 15 min at 95°C followed by 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, with a final extension of 7 min. Amplified fragments were resolved on 1.5% agarose gels, and clones having inserts larger than 300 nucleotides but representing various size fractions were sequenced.

SSAP analysis

The SSAP procedure was essentially performed as described by Waugh et al. (1997) with the following modifications. All restriction digestions and ligations were performed using NEB buffers and enzymes. Between 300 ng and 500 ng genomic DNA was digested overnight with the *Pst* I and *Mse*I restriction enzyme in a final volume of 25 µl. The digested DNA samples were incubated at 80°C for 20 min to inactivate the restriction enzymes. This was followed by the ligation of adapters (*Mse*I: 5′-GACGATGAGTCCTGAG and TACTCAGGACTCAT; *Pst*I: 5′CTCGTAGACTGCGTACATGCA and TGTACGCAGTCTAC) to the restriction fragments. Ligations were performed for 3 h at room temperature in a final volume of 32 µl with 1× NEB T4 ligation buffer, 25 µl digested DNA, 500 ng *Mse*I and 250 ng *Pst*I adapters and 200 U T4 DNA ligase (NEB). The ligation mixture was again incubated at 65°C for 10 min this time to inactivate the enzyme. The adapter-ligated DNA was used for pre-amplification with a primer pair (*Mse*I: GATGAGTCCTGAGTAA; *Pst*I: GACTGCGTACATGCAG) based on the sequences of the *Pst*I and *Mse*I adapters with no selective nucleotides, in a final volume of 25 µl containing 100 ng of each primer, 200 μM dNTPs, 1 U *Taq* DNA polymerase (Promega) and 2.5 µl pre-amplified DNA in a PTC-225 DNA Engine Tetrad (MJ Research, Waltham, Mass.) at 95°C for 1 min followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C and with a final extension of 7 min at 72°C.

Selective amplification was performed with primer pairs containing two or three selective nucleotides on the adapter primer (*Mse*I or *Pst*I) and one on the [γ^{33}] P-labelled retrotransposon-based primer using the touch-down PCR conditions of Vos et al (1995). The amplified fragments were separated on 6% polyacrylamide sequencing gels and visualized by autoradiography. Retrotransposon-specific primers were *Tao1* (GTCCTGAGTAAACTAGTCAACA plus the T, A or G selective base) and *Tao2* (AGTAATGGAATAAAATCTCAACA plus the T, A or G selective base).

Results

Isolation of LTR sequence from cashew

The development of SSAP requires sequence information for the terminal regions of the retrotransposon (Pearce et al. 1999). The LTR sequences of Ty1-*copia* retrotransposons are present at the ends of the transposon and consist of identical direct repeats that are typically several hundred base pairs in length (Fig. 1). Unfortunately, the LTRs do not contain any conserved motifs. Therefore, to isolate LTR sequence information, it is necessary to perform an amplification between a conserved region in the RNAseH gene and a restriction site in the adjacent LTR sequence or flanking genomic DNA (Pearce et al. 1999). The LTR sequence thus

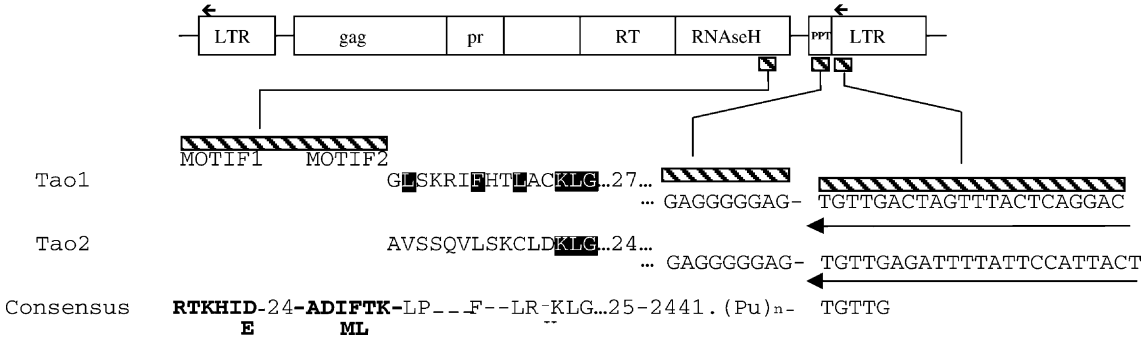

Fig. 1 Structural features of RNAseH, polypurine tract (*PPT*) and LTR terminal sequences for Ty1-*copia* retrotransposons. The sequences of *Tao1* and *Tao2* clones obtained in this study are compared with the consensus region of Ty1-*copia* sequence (not to scale; adapted from Pearce et al. 1999). The complimentary sequences and polarities of the LTR primers for the SSAP experiments is shown with *arrows* on the diagram and *below* the sequences themselves. The SSAP marker method amplifies fragments extending *leftwards from the leftmost arrow* in the figure

obtained from one end of the retrotransposon can then be used to design primers facing outwards from the LTR at the other end.

To obtain a set of RNAseH-LTR junction clones, we used a modification of the method of Pearce et al. (1999), which involves the replacement of biotinylated RNAseH primers, together with affinity purification of PCR products, with the use of normal primers and hot-start PCR (see Materials and methods). The population of amplified putative RNAseH-LTR junction fragments were subcloned and sequenced. The derived sequences were individually compared with the consensus RNAseH-LTR region (Pearce et al. 1999). Two convincing retrotransposon LTR sequences were obtained (Fig. 1), which we have termed *Tao1* and *Tao2* (transposon, *Anacardium occidentale*). Following motif two, the RNAseH open reading frame, which contains conserved amino acid residues, continues for 15 codons before a stop codon. This is followed by a spacer region (27 bp and 24 bp for *Tao1* and *Tao2*), respectively, then an identical polypurine tract (PPT) follows and a TGTTG motif, characteristic of the end of an LTR.

SSAP marker development in cashew

The SSAP primers specific for *Tao1* and *Tao2*, were designed to amplify DNA in a leftwards direction as shown in Fig. 1. Both primers contain a single selective base (T, A or G) at their 3′-ends, which are mismatched with the PPT sequence to prevent internal SSAP priming into the RNAseH gene from the right-hand LTR.

The two SSAP primers were used to optimize the SSAP protocol for cashew, initially by using a few samples of an F_1 mapping population and its two parents. Firstly, a combination of three selective bases with the adapter primer and one with the LTR primer were used for the selective amplification step. This combination did not produce enough bands for a good marker system (data not shown), indicating a comparatively low-copy number of these retroelements in the cashew genome. We next tested for two selective bases attached to the *Mse*I adapter primer and one T base added to the *Tao1* LTR primer. This combination gave a good SSAP marker profile (Fig. 2a), with an excellent reproducibility of bands between duplicates. In total, 54 bands were observed generating six (11%) markers polymorphic within the F_1 population (markers are indicated with an arrow in Fig. 2a). All SSAP profiles in Fig. 2a were carried out in duplicate, derived from separate pre-amplification PCR products, in order to check the consistency and reproducibility of the SSAP markers.

Figure 2b and 2c show the SSAP profiles of *Tao1* and *Tao2* LTR primers amplified with another selective adapter primer (CT). *Tao1* LTR and *Tao2* LTR primers generated a total of 75 and 68 bands with six (8%) and five (7%) polymorphic bands (band present or absent in one of the two parents), respectively, in the F_1 population for these primer combinations. Given the dominant inheritance of SSAP markers, there should be two patterns of segregation within the F_1 population. First, in instances where both parents are heterozygous for a given marker, the offspring should segregate for the presence of that marker in a 3:1 (presence: absence) ratio. Second, where a marker is present in the heterozygous state in one parent only, the offspring should segregate in a 1:1 (presence: absence) ratio. Careful analysis of the markers described above revealed that 10/11 markers (Fig. 2b,c) segregated according to expectations, with five markers segregating 3:1 and five markers segregating 1:1. The remaining one marker exhibited skewed segregation (Chi-squared, $P < 0.05$). Table 1 summarizes information on each SSAP marker, its scores (presence:absence), segregation ratio and χ^2 values. This table also shows that most of the markers are segregating according to the expected segregation ratios (1:1 or 1:3), with only one marker deviating from the normal segregation ratio.

SSAP versus AFLP for cashew

In principle, SSAP should generate more polymorphic markers than AFLP due to the higher mutation rate of retrotransposition relative to base mutation. In order to directly compare the efficiencies of the SSAP and AFLP marker systems, the same pre-amplification PCR products with *Mse*I and *Pst*I were used in an AFLP experiment using the *Pst*I primer with AAT selective bases and the *Mse*I primer with CT selective bases at their 3′-end. In total, 80 scoreable AFLP markers were generated with only three (4%) being polymorphic between the two parents (shown with arrows in Fig. 2d) for this combination of selective bases. Table 1 presents information on each AFLP marker, its scores (presence:absence), segregation ratio and $\chi 2$ values. All three markers segregated according to a 1:1 ratio. In comparison, six and five SSAP markers were obtained for *Tao1* and *Tao2*, respectively. Thus, in this limited comparison, SSAP appears to give very approximately twice the polymorphism level of AFLP. In particular, the proportion of SSAP bands which are polymorphic is much higher than that seen for AFLP (we obtained almost twofold more bands from the SSAP data in Fig. 2a–c than from the AFLP data in Fig. 2d).

Discussion

In this study, we have isolated two cashew LTR sequences, *Tao1* and *Tao2*, by means of unidirectional PCR from conserved regions in the RNAseH gene of cashew Ty1-*copia* LTR retrotransposons (Pearce et al. 1999). Both LTRs were successfully used to develop SSAP marker systems using several individuals of an F_1 population. The efficiencies of SSAP and AFLP technologies were compared using the same selective bases in

SSAP and AFLP experiments. In our limited analysis, SSAP appears to give a comparable number but higher proportion of polymorphic markers compared to AFLP.

In addition to the amount of polymorphism, there is very little technical difference between SSAP and AFLP marker technology. In fact, SSAP should be considered to be an alternative to AFLP technology (Schulman et al. 2004). The only disadvantage of the SSAP markers is the need to acquire a retrotransposon LTR sequence from a given species. The availability of efficient and fast LTR isolation methods (Pearce et al. 1999) and the increase in available genome sequences have facilitated the development of SSAP marker systems. The higher levels of polymorphisms for SSAP markers make it a less labour-intensive protocol so that more markers can be developed relatively easily and at a reduced cost.

There is another advantage of the SSAP marker system over AFLP. When the fluorescent labelling of

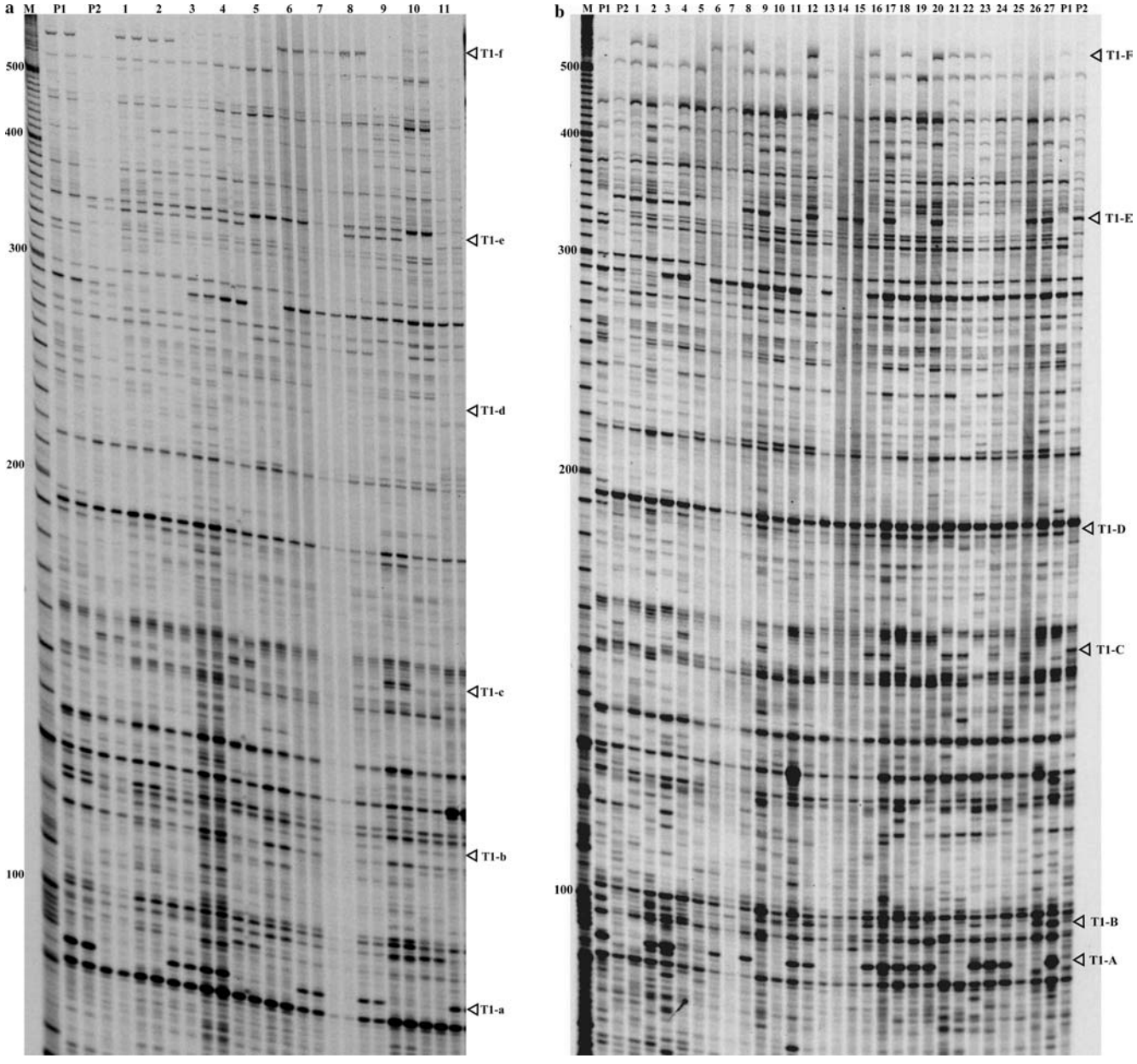

Fig. 2 **a** SSAP marker profiles of the two parents, CP 96 (*P1*) and CP 1001 (*P2*), and 11 (*1–11*) duplicate individuals (from different pre-amplification products) of their F_1 mapping population using the *Tao1* LTR primer with a T selective base and the *Mse*I primer with AC selective bases. **b** SSAP marker profiles of the two parents and of individuals of their F_1 mapping population (*1–27*) using the *Tao1* LTR primer with a T selective base and the *Mse*I primer with CT selective bases. **c** SSAP for the *Tao2* LTR with a T selective base and the *Mse*I primer with CT selective bases. **d** AFLP profiles of the two parents along with the individuals of their F_1 mapping population (*1–28*) using *the Pst*I-AAT and *Mse*I-CT primer combinations. *Arrows* indicate polymorphic markers. *M* Marker lane. Each band interval represents ten bases

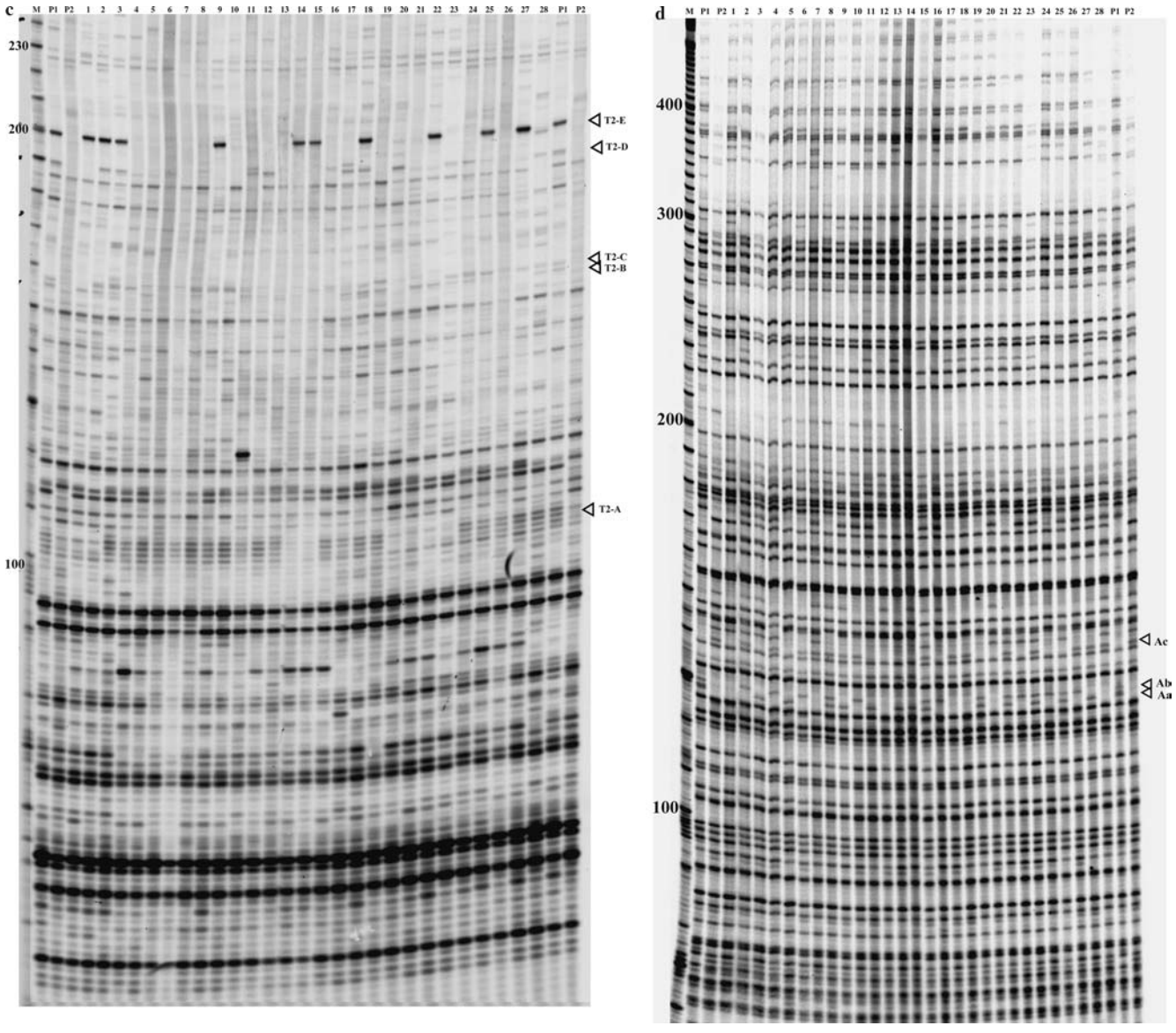

Fig. 2 (Contd.)

AFLP primers is used for automated detection bands, the cost of running the SSAP marker system is dramatically lower, as only one LTR primer is needed to be fluorescently labelled in combination with standard adapter-based primers. In contrast, a large number of primers need to be fluorescently labelled to generate corresponding AFLP marker profiles.

Unfortunately, some SSAP marker bands can be weaker and difficult to score than those of AFLP. Also, in some cases, SSAP markers are present among the parents but are missing from the progeny, and vice versa. This could merely be because of the small sample size of the mapping population (28 individuals in the present investigation), segregation distortion or the methylation status of the parents and F_1 progeny (*Pst*I is susceptible to DNA methylation, which might differ between parents and offspring). This problem needs further attention and may be solved by employing larger F_1 populations as well as by studying the genetic basis of segregation distortion and linkage disequilibrium in cashew. The presence or absence of bands in the parents and progeny could be addressed by using methylation sensitive-enzymes (*Msp*I and *Hpa*II) in AFLP experiments. Nevertheless, SSAP remains an attractive alternative to AFLP, and its benefits to cashew genetic analysis should still outweigh its disadvantages.

The provision of primers for SSAP in cashew greatly enhances the capacity to generate large numbers of polymorphic markers for marker-assisted breeding in this species. This capacity is particularly useful for tree crops such as cashew, where an extended period of juvenility (typically 3—5 years) delays both the phenotypic evaluation of segregating progenies and also the production of subsequent generations. More specifically,

Table 1 Quantitative information for all SSAP and AFLP markers is presented along with their observed numbers (presence or absence), segregation ratios, and χ^2 values

Markers	Allele	Present	Absent	Ratio	χ^2 (1*df*)
AFLP markers (Fig. 2d)					
A-a-P-AAT:M-CT-128	CP96	13	15	1:1	0.1428
A-b-P-AAT:M-CT-130	CP96	14	14	1:1	0.0000
A-c-P-AAT:M-CT-140	CP1001	14	14	1:1	0.0000
SSAP markers (*Tao1*, Fig. 2b)					
T1-a-T:M-CT-93	CP96	14	13	1:1	0.0370
T1-b-T:M-CT-100	CP96	17	10	3:1	2.0864
T1-c-T:M-CT-157	CP1001	9	18	3:1	1.0000
T1-d-T:M-CT-194	CP96	26	1	3:1	6.5308*
T1-e:T-M-CT-341	CP1001	13	14	1:1	0.1428
T1-f-T:M-CT-560	CP96	12	15	1:1	0.3333
SSAP markers (*Tao2*, Fig. 2c)					
T2-a-T:M-CT-104	CP96	24	4	3:1	1.7142
T2-b-T:M-CT-156	CP96	12	16	1:1	0.5714
T2-c-T:M-CT-157	CP96	14	14	1:1	0.0000
T2-d:T-M-CT-190	CP96	9	19	3:1	0.7919
T2-e-T:M-CT-200	CP96	10	18	3:1	1.7142

the availability of these primers enables the rapid accumulation of polymorphic markers to create a linkage map of the crop and the eventual identification of association between these markers and segregating traits of interest (Cardoso et al. 1999; Cavalcanti et al. 2000, 2003). This preliminary analysis of the patterns of segregation shown by SSAP markers in cashew indicates that a useful number of markers are generated that are relatively easy to score and behave according to Mendelian expectations. This finding is consistent with results from previous studies that have used SSAP for mapping purposes in temperate crops (Ellis et al. 1998; Nagy and Lelley 2003; Queen et al. 2004). Now that effective marker systems are available for cashew, the next stage will be the creation of a sizeable mapping population for this species. This will allow QTL mapping and marker-assisted selection strategies for the useful traits that are segregating in the lines described here. Such investigations will require a concerted effort over several years, but the rewards will most certainly justify the efforts.

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