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Intron targeted amplified polymorphism (ITAP), a new sequence related amplified polymorphism-based technique for generating molecular markers in higher plant species

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Abstract

Based on the principles of sequence related amplified polymorphism (SRAP), target region amplification polymorphism (TRAP) and conserved region amplification polymorphism (CoRAP), we developed a new molecular marker technique called intron targeted amplified polymorphism (ITAP). For ITAP, primer pairs were exploited to conduct the polymerase chain reaction (PCR). One primer targeting intron was retrieved from the SRAP technique. The other was designed from the 3' widely distributed conserved intron-exon splice junction sequences. Amplification was carried out according to the procedure of the SRAP technique. The PCR products were resolved through standard agarose gel electrophoresis. To test this technique, we have applied it to fingerprint three plant species including banana (*Musa spp.*), longan (*Dimocarpus longan Lour.*), and cultivated peanut (*Arachis hypogaea L.*). Depending upon the different primer pairs, 3-11 bands were detected among these three plant species. Of these bands, 80%, 79.25%, and 45.30% were polymorphic in banana, longan, and cultivated peanut, respectively. Cluster analysis of ITAP markers was largely consistent with previous studies. Since primers were designed from the 3' widely distributed intron-exon splice junction sequences in higher plant species assuming these primers should be universal across other plant species. This technique provides an alternative way to produce molecular markers for plant genotyping and fingerprinting.

Keywords: Molecular markers; fingerprinting; genetic diversity; intron targeted amplified polymorphism (ITAP); sequence related amplified polymorphism (SRAP); intron-exon splice junction (ISJ).
Abbreviations: ITAP_ Intron Targeted Amplified Polymorphism; RAPD_Random Amplified Polymorphic DNA; ISSR_Inter-Simple Sequence Repeat; AFLP_Amplified Fragment Length Polymorphism; SRAP_Sequence Related Amplified Polymorphism; TRAP_Target Region Amplification Polymorphism; CoRAP_Conserved Region Amplification Polymorphism; SCoT_Start Codon Targeted Polymorphism; CDDP_Conserved DNA-Derived Polymorphism; EST_Expressed Sequence Tag; CTAB_Cetyl Trimethyl Ammonium Bromide; UPGMA_Unweighted Pair Group Method with Arithmetic Averages; ISJ_Intron-exon Splice Junctions; ISAP_Intron Sequence Amplified Polymorphism; IT-ISJ_Intron Targeted Intron-exon Splice Junction; BPS_Branch Point Signal.

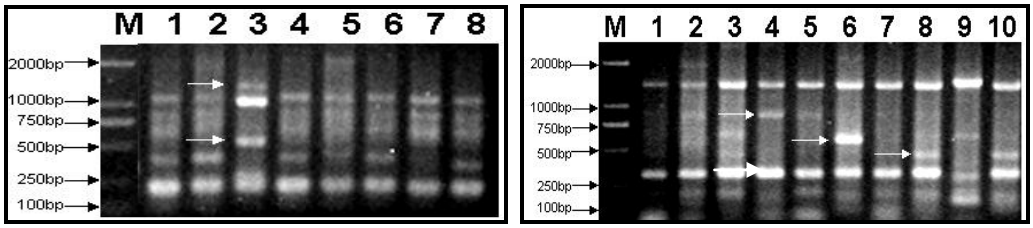
Introduction

Since 1980, molecular marker techniques including random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), and amplified fragment length polymorphism (AFLP) (Williams et al., 1990; Zietkiewicz et al., 1994; Vos et al., 1995) have been widely utilized because of their advantages of simplicity, informativeness, cost-effectiveness, efficiency, and no requirement for prior sequence information. However, they also have some certain disadvantages. For example, RAPD has low stability and reproducibility. AFLP has two major disadvantages of prohibitive expenses and tedious procedures. And the common disadvantage of RAPD, ISSR and AFLP techniques is that markers developed from them are probably distant from targeted genes. With the rapid growth of plant genomics and functional genomics research over the past two decades, many novel gene-targeted molecular marker techniques have been developed such as sequence related amplified polymorphism (SRAP), target region amplification polymorphism (TRAP), conserved region amplification polymorphism (CoRAP), start codon targeted polymorphism (SCoT), and conserved DNA-derived polymorphism (CDDP) (Li and Quiros, 2001; Hu and Vick, 2003; Wang et al., 2009; Collard and Mackill, 2009a; Collard and Mackill, 2009b). Among which the most widely used is SRAP. The SRAP method, developed by Li and Quiros (2001), is a novel molecular marker system that utilizes a pair of arbitrary primers, one expected to target exons and the other introns. The intervening fragments between the binding sites of forward and reverse primers were expected to be amplified.

Table 1. Polymorphism among eight banana varieties and ten longan cultivars revealed by ITAP primer combinations.

Primer combinations	TNB	NPB	PPB（%）	PI	PIC
Polymorphism among eight banana cultivars					
em4-ITPR1	10	7	70.00	2.11	0.563
em4-ITPR2	9	7	77.78	1.70	0.406
em8-ITPR3	7	6	85.71	1.82	0.781
em5-ITPR5	7	6	85.71	1.80	0.750
em6-ITPR5	7	6	85.71	2.01	0.813
Mean	8	6.40	80.98	1.89	0.663
Total	40	32	—	—	—
Polymorphism among ten longan cultivars					
em4-ITPR1	10	9	90.00	1.62	0.340
em4-ITPR2	9	4	44.44	0.72	0.180
em8-ITPR3	7	6	85.71	1.80	0.800
em10-ITPR3	8	8	100.00	2.68	0.860
em5-ITPR5	12	10	83.33	3.02	0.880
em6-ITPR5	7	5	71.43	0.90	0.180
Mean	8.83	7	79.15	1.79	0.540
Total	53	42	—	—	—

Note: Total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (PPB), and polymorphic index (PI).



A **B**

Fig 1. The ITAP amplification results from eight banana varieties by primer combinations em4-ITPR2 (A) and ten longan accessions by primer combinations em8-ITPR3 (B). The codes for eight banana varieties and ten longan accessions are shown in Fig .2. The white arrows indicate polymorphic bands.

On the basis of the SRAP strategy, TRAP was established by substituting one of the SRAP arbitrary primers with one fixed primer designed from the expressed sequence tag (EST) sequences (Hu and Vick, 2003). Recently, Lu et al (2008) developed a gene targeted molecular marker technique termed intron sequence amplified polymorphism (ISAP) in cotton. ISAP technique utilized a pair of primers designed from conservative consistent sequences of intron splicing sites of donor and acceptor sites to conduct the polymerase chain reaction (PCR). Nearly at the same time another intron related marker technique called intron targeted intron-exon splice junction (IT-ISJ) marker has been developed by Zheng et al. (2008). IT-ISJ technique also utilized a pair of primers targeting conserved consensus splice junction sequences of donor and acceptor sites to conduct PCR. More recently, a novel technique termed CoRAP was developed based on SRAP and TRAP techniques. The most striking feature of this technique was the use of an arbitrary primer targeting an extremely conservative sequence in most plant introns and the use of fixed primers targeting specific coding sequences (Wang et al., 2009). In the present study, the 3' widely distributed consensus intron-exon splice junction sequences were also used to design primers which can be utilized in combination with the other arbitrary primers retrieved from SRAP technique. This technique called intron targeted amplified polymorphism (ITAP) was described and used for fingerprinting banana, longan, and cultivated peanut.

Results

ITAP markers among banana and longan

ITAP technique was firstly validated by fingerprinting banana and longan varieties. For banana varieties, analysis of the 8 accessions with 5 ITAP primer combinations identified a total of 40 scorable fragments. Among them, 32 (80.00%) were polymorphic. The number of fragments detected by each primer combination ranged from 7 (em5-ITPR5, em6-ITPR5, and em8-ITPR3) to 10 (em4-ITPR1), with an average of 8.00. The number of polymorphic fragments for each primer combination ranged from 6 (em5-ITPR5, em6-ITPR5, and em8-ITPR3) to 7 (em4-ITPR1, em4-ITPR2), with an average of 6.40. The percentage of polymorphism for each primer combination varied from 70.00% (em4-ITPR1) to 85.71% (em5-ITPR5, em6-ITPR5, and em8-ITPR3) with an average of 80.98%. The polymorphic index (PI) for each primer combination ranged from 1.70 (em4-ITPR2) to 2.11 (em4-ITPR1) with an average of 1.89. And polymorphism information content (PIC) for each primer combination varied from 0.406 (em4-ITPR2) to 0.813 (em6-ITPR5) with an average of 0.663 (Table 1). The amplification result from em4-ITPR2 among the eight banana varieties is shown in Fig.1A. For longan accessions, six sets of primer combinations that revealed polymorphisms in all 10 longan genotypes were used for data analysis. A total of 53 bands with a mean of 8.83 and ranging from 7 (em6-ITPR5, em8-ITPR3) to 12 (em5-ITPR5) per primer combination were observed. Among these bands, 42 (79.25%) were polymorphic, ranging from four (em4-ITPR2) to ten (em5-ITPR5) per primer combination, with an average of 7.00 per primer combination. The percentage of polymorphisms for each primer combination varied from 44.44% (em4-ITPR2) to 100% (em10-ITPR3) with an average of 79.15%. The PI ranged from 0.72 (em4-ITPR2) to 3.02 (em5-ITPR5) with an average of 1.79. And PIC varied

Table 2. Polymorphisms among sixteen cultivated peanut cultivars revealed by ITAP primer combinations.

Primer combinations	TNB	NPB	PPB（%）	PI	PIC
em1-ITPR1	8	6	75.00	2.34	0.906
em3-ITPR1	6	3	50.00	0.71	0.539
em4-ITPR1	7	2	28.57	0.73	0.632
em5-ITPR1	4	1	25.00	0.43	0.430
em6-ITPR1	4	1	25.00	0.22	0.219
em7-ITPR1	7	2	28.57	0.73	0.477
em8-ITPR1	5	1	20.00	0.50	0.500
em11-ITPR1	6	2	33.33	0.80	0.617
em6-ITPR2	5	2	40.00	0.34	0.227
em7-ITPR2	7	2	28.57	0.34	0.227
em1-ITPR3	4	1	25.00	0.22	0.219
em7-ITPR3	5	1	20.00	0.38	0.375
em9-ITPR3	5	4	80.00	1.64	0.781
em10-ITPR3	9	7	77.78	2.75	0.891
em11-ITPR3	8	2	25.00	0.52	0.320
em7-ITPR4	11	8	72.73	1.94	0.672
em10-ITPR4	6	2	33.33	0.87	0.586
em7-ITPR5	6	3	50.00	1.10	0.711
em9-ITPR5	4	3	75.00	1.33	0.625
Mean	6.16	2.79	42.78	0.94	0.524
Total	117	53	—	—	—

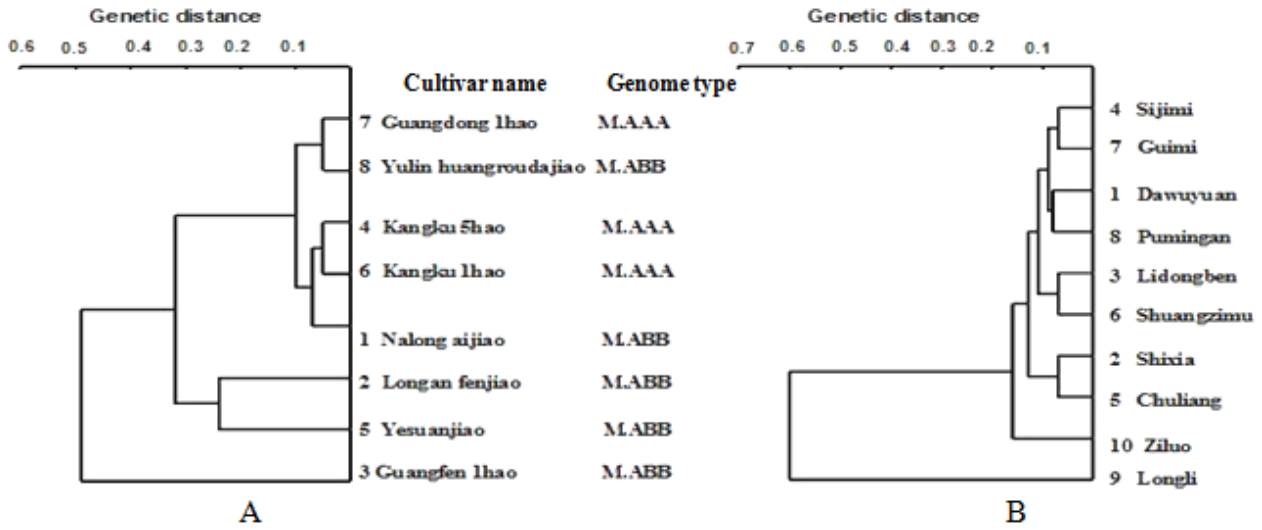


Fig 2. Dendrograms generated for eight banana varieties (A) and ten longan accessions (B) from ITAP markers by cluster analysis. The codes for banana and longan cultivars were shown in the dendrograms.

from 0.180 (em4-ITPR2, em6-ITPR5) to 0.880 (em5-ITPR5) with an average of 0.540 (Table 1). Fig .1B illustrates the amplification profile of em8-ITPR3.

Based on the total scored bands produced with ITAP technique among banana and longan varieties, 0-1 binary matrices were built and genetic distance analysis and cluster analysis were conducted. Fig .2A and Fig .2B illustrate the dendrograms of cluster analysis for banana and longan varieties.

Survey of polymorphism among cultivated peanut based on ITAP technique

We tested a total of 34 primer combinations. Nineteen successfully produced 117 reproducible and unambiguous bands of 100 to 2,000 bp from the tested peanut genotypes. The mean number of bands was 6.16, and ranged from 4 to 11 per primer combination. Of these, 53 (45.30%) were polymorphic. From one to eight polymorphic bands were amplified from each primer combination, with an average of 2.79. The primer combinations exhibited different levels of polymorphism, ranging from 20.00% to 80.00% with an average of 42.78%. The PI per primer combination varied from 0.22 to 2.75 with an average of 0.94. And PIC varied from 0.219 to 0.906 with an average of 0.524 (Table 2). Representative band pattern of ITAP markers is presented in Fig.3. These results indicated that certain DNA polymorphism could be detected among peanut botanical varieties using ITAP markers.

Discussion

Principle of ITAP technique

Introns, which are non-coding sequences that belong to parts of genes, are interspersed throughout the genome. Compared with exons, introns are much more variable and suitable for DNA marker development (Hawkins, 1998). Previous studies

Table 3. Summary of data generated by ITAP primer combinations for three higher plant species in this study.

Parameters	Species		
	Banana	Longan	Peanut
Number of accessions	8	10	16
No. of tested primer combinations	5	6	34
No. of primer combinations detecting polymorphism	5	6	19
Percentage of polymorphic primer combinations (%)	100	100	55.88
Total bands	40	53	117
Polymorphic bands	32	42	53
Average bands per primer combination	8.00	8.83	6.16
Average polymorphic bands per primer combination	6.40	7.00	2.79
Percentage of polymorphic bands (%)	80.00	79.25	45.30
Average polymorphic index	1.89	1.79	0.94
Average polymorphism information content	0.663	0.540	0.524

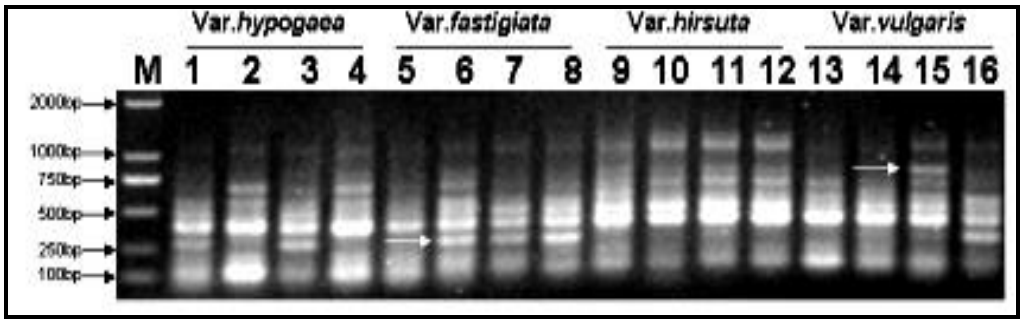


Fig 3. The ITAP amplification result from sixteen cultivated peanut accessions by primer combinations em1-ITPR1. Lanes 1 to 16 represent the sixteen cultivated peanut genotypes (1. 93-8116; 2. Lishan dali; 3. Baise longchuandou; 4. Linyi dali; 5. Huapi 1hao; 6. Huapi 2hao; 7. PI393518; 8. Mini; 9. Zhangmu dahuasheng; 10. Longtan dagouyao; 11. Weizhouhainan huasheng; 12. Rongxian niujiaodou; 13. Ganyu xiaohuasheng; 14. Namuhong; 15. Xiekangqing; 16. Guangxi4825). The white arrows indicate polymorphic bands.

have confirmed that intron-exon splice junction sequences are conserved across species. So intron-exon splice junction sequences are informative resources for primer design and marker development. Weining and Langridge (1991) proposed the idea of carrying out PCR using semi-specific primers targeting semi-conservative consensus intron-exon splice junctions (ISJ). In the present study, the 3' widely distributed consensus intron-exon splice junction sequences were also used to design primers which can be utilized in combination with the other arbitrary primers retrieved from SRAP technique. Due to the principle of primer design, the DNA polymorphisms revealed by ITAP technique could be attributed to intron length polymorphism and point mutations of ITAP primers binding sites.

Validity of the primer combinations of ITAP technique

In this study, validity of the primer combinations was confirmed by comparing the PCR amplification results of the same SRAP primer with the different intron targeted primer, the different SRAP primer with the same intron targeted primer, and the different SRAP primer with the different intron targeted primer. For example, differences in the intron-targeted primers (em4-ITPR1 and em4-ITPR2) generated different band patterns. Similarly, differences in the SRAP primers with different intron targeted primers (em5-ITPR5 and em8-ITPR3) produced different band profiles, and em1-ITPR1 and em11-ITPR1 differed in SRAP primers generated different band patterns (Data not shown). These results demonstrated the effectiveness and validity of the ITAP primer combinations. Therefore, ITAP is regarded as a new molecular marker technique.

Genetic diversity and fingerprinting analysis of three plant species as revealed by ITAP technique

To validate the adaptability and efficacy of the ITAP marker technique, we firstly applied it to fingerprint two plant species, banana and longan. Although only five (for banana) or six (for longan) primer combinations were used, abundant DNA polymorphisms and good DNA fingerprinting were achieved. And all banana and longan cultivars can be discriminated. Additionally, several accession-specific markers were generated by ITAP technique. As for banana, we compared the cluster result of this study with our previous study by branch point signal (BPS) analysis with the same banana accessions. The result showed that cluster result of banana varieties obtained by ITAP technique was similar to cluster result obtained by BPS technique (Xiong et al., 2011b). In addition to banana and longan, cultivated peanut was also used to validate the efficiency of ITAP technique. Genetic studies in peanut are far behind than other crops due to its narrow genetic base (Kochert et al., 1996), so it is difficult to yield molecular markers using existing molecular marker techniques (He et al., 1997; Halward et al., 1992; Subramanian et al., 2000; He and Prakash, 2001; Raina et al., 2001; Tang et al., 2007; Varshney et al., 2009; Xiong et al., 2011a). In our experiments, nineteen of the thirty four primer combinations produced a total of 117 bands in the tested peanut accessions, of which 53 were polymorphic. The result of cluster analysis was generally in accordance with our previous studies using SCoT markers (Xiong et al., 2011a). All these results indicated that ITAP technique can be used to produce molecular markers for the genetic diversity and fingerprinting analysis.

Table 4. The primer sequences used for ITAP analysis.

Primers targeting intron retrieved from SRAP technique			
Primer name	Primer sequence (5' to 3')	Primer name	Primer sequence (5' to 3')
em1	GACTGCGTACG**AATT**AAT	em7	GACTGCGTACG**AATT**ATG
em2	GACTGCGTACG**AATT**TGC	em8	GACTGCGTACG**AATT**AGC
em3	GACTGCGTACG**AATT**GAC	em9	GACTGCGTACG**AATT**ACG
em4	GACTGCGTACG**AATT**TGA	em10	GACTGCGTACG**AATT**TAG
em5	GACTGCGTACG**AATT**AAC	em11	GACTGCGTACG**AATT**TCC
em6	GACTGCGTACG**AATT**GCA		
Primers designed from the 3' intron-exon splice junction sequences			
Primer name	Primer sequence (5' to 3')	Primer name	Primer sequence (5' to 3')
ITPR1	GACTGCGT**ACCTGCA**AAT	ITPR4	GACTGCGT**ACCTGCA**ATG
ITPR2	GACTGCGT**ACCTGCA**TGC	ITPR5	GACTGCGT**ACCTGCA**GCT
ITPR3	GACTGCGT**ACCTGCA**GAC		

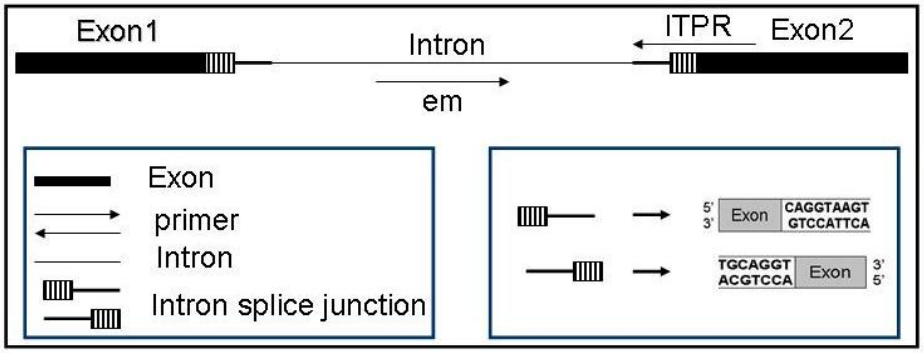


Fig 4. The schematic drawing describes the primer design of ITAP technique. The primer ITPR targeting the 3' intron-exon splice junction for acceptor sites can be used in combination with SRAP primer (em) freely. Abbreviation: ITPR: intron targeted primer. The drawing was not scaled.

To fullly appreciate the potential of ITAP technique for assaying DNA polymorphisms in diverse plant species, data were collected for banana, longan, and peanut (Table 3). ITAP technique can detect more obvious DNA polymorphisms in banana and longan than in cultivated peanut which could be due to its low genetic diversity.

Advantages of ITAP

ITAP technique has three advantages over many existing molecular marker techniques. Firstly, compared with RAPD, ISSR, and AFLP, ITAP is a novel gene-targeted molecular marker technique which can amplify targeted functional DNA region using primers designed from the 3' widely distributed consensus intron-exon splice junction sequences. Secondly, although a small set of primers were designed from the 3' intron-exon splice junction sequences and used in the present study, much more additional primers can be redesigned with minor alterations. Thirdly, ITAP technique can be applied to other higher plant species because primers designed from the 3' widely distributed consensus intron-exon splice junction sequences are conserved across the plant kingdom.

Materials and methods

Plant materials and DNA isolation

Three plant species including banana (*Musa spp.*), longan (*Dimocarpus longan Lour.*), and cultivated peanut (*Arachis hypogaea* L.) were used in the present study. For cultivated peanut, a collection of sixteen peanut accessions, four from each of the four botanical varieties, were selected. Of sixteen accessions, fourteen were from China and two were from America. Ten seeds from each accession were sown in a greenhouse. Young leaves from two week-old seedlings of the same accession were ground in liquid nitrogen for DNA extraction using the cetyl trimethyl ammonium bromide (CTAB) method (Tang et al., 2007; Xiong et al., 2011a). The DNA yield was examined on 1% agarose gels and DNA concentration was estimated by spectrophotometry at 260 and 280 nm. The final concentration of all DNA samples for PCR analysis was adjusted to 50 ng/ul. The detail information for plant materials used in this study can be seen from Fig .2 and Fig.3.

Primers design

Primer pairs were used for ITAP analysis. One primer targeting intron was retrieved from the SRAP technique. The other primer was designed from the 3' widely distributed consensus intron-exon splice junction sequences (Table 4, Fig.4). The primers designed from intron-exon splice junction sequences were 18-mer in length with a core of the reverse complementary sequence 'ACCTGCA' of the 3' conserved intron splice junction sequence 'TGCAGGT' (Brown, 1986; Weining and Langridge, 1991). These primers were supplemented with additional filler bases 'GACTGCGT' at the 5' end to extend the primers length and with three random selective bases at the 3' end (Table 4). There were no degeneracies. In theory, the primers retrieved from the SRAP technique can be used in combination with primers designed from the 3' widely distributed consensus intron-exon splice junction sequences for ITAP analysis. For ITAP technique, the schematic drawing for the primer design was described in Fig .4.

PCR amplification and electrophoresis

Primer sequences were synthesized by Shanghai Sangon

Biological Engineering Technology and Service Co. Ltd (Table 4). A total of 20 μl PCR reaction mixture contained 0.2 mM dNTPs, 0.5 μM each of forward and reverse primer, 1.5 mM $MgCl_2$, 1.25 U Taq DNA polymerase (TaKaRa Biotechnology, Dalian, China), 1 × PCR buffer, and 50 ng genomic DNA. Amplifications were conducted in a thermocycler using the following cycling parameters: 4 min denaturing at 94 ºC, 5 cycles of 1 min denaturing at 94 ºC, 1 min annealing at 35 ºC and 1 min elongation at 72 ºC. In the following 35 cycles, the annealing temperature was increased to 50 ºC, with a final elongation of 5 min at 72 ºC. All amplification products were separated on 1.5% agarose gels using 1× TAE buffer. Gels were visualized under UV light after staining with ethidium bromide (0.5 μg/ml). The consistency of PCR-amplified products was checked by performing all reactions at least twice. Bands that were unambiguous and reproducible in successive amplifications were selected for scoring.

Band scoring and data analysis

ITAP fragments are generally dominant. So each band on agarose gels was scored as present (1) or absent (0). Genetic similarity between accessions i and j was calculated using the similarity coefficient of Nei and Li, $S_{ij}=2N_{ij}/(N_i+N_j)$, where N_{ij} is the number of common bands in accessions i and j, and N_i and N_j are the total number of bands in accessions i and j (Nei and Li, 1979). Genetic distance (D_{ij}) between accessions i and j was calculated as $D_{ij}=1–S_{ij}$. Polymorphic index (PI) was calculated by a previous method (Raina et al., 2001). Polymorphism information content (PIC) was calculated as $PIC=1–\sum f_i^2$, where f_i^2 is the frequency of the ith allele. The computing for parameters as D_{ij}, PI, and PIC was conducted by our self-compiled software. And dendrograms were obtained using unweighted pair group method with arithmetic averages (UPGMA) under the use of our self-compiled software (Tang et al., 2007, 2008).

Conclusion

In summary, with the ITAP technique, many DNA polymorphisms could be detected among tested banana, longan, and peanut accessions. This technique is simple, rapid, and efficient for generating molecular markers in higher plant species. As an alternative to SRAP, TRAP, and CoRAP, this method could be employed in the fields of genetic diversity analysis, DNA fingerprinting, gene tagging, and QTL mapping.

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