Theor Appl Genet (1995) 91:465–470

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Large-scale, cost-effective screening of PCR products in marker-assisted selection applications

Received: 30 May 1994 / Accepted: 29 December 1994

Abstract A simple, PCR-based method has been developed for the rapid genotyping of large numbers of samples. The method involves a alkaline extraction of DNA from plant tissue using a slight modification of the procedure of Wang et al. (Nucleic Acids Res 21:4153–4154, 1993). Template DNA is amplified using allele-specific associated primers (ASAPs) which, at stringent annealing temperatures, generate only a single DNA fragment and only in those individuals possessing the appropriate allele. This approach eliminates the need to separate amplified DNA fragments by electrophoresis. Instead, samples processing the appropriate allele are identified by direct staining of DNA with ethidium bromide. Total technician time required for extraction, amplification and detection of 96 samples is about 4 h, and this time requirement can be reduced by automation. Excluding labor, cost per sample is less than $0.40. The method is tested using the codominant isozyme marker, alcohol dehydrogenase (*Adh-1*) gene in pea (*Pisum sativum*), and applied to the screening of photoperiod genes in common bean (*Phaseolus vulgaris* L.).

Key words RAPDs · Electrophoresis · Allele-specific associated primers · Common bean · Pea

Introduction

The use of arbitrary primers for the development of genetic markers (Williams et al. 1990; Welsh and McClelland 1990) has significantly increased the speed and ease by which DNA fragments tightly linked to genes of interest can be identified. However, one of the major problems encountered when attempting to apply either restriction length fragment polymorphisms (RFLPs) or polymerase chain reaction (PCR) markers in breeding or seed quality control has been the expense of performing such tests. The expenses include not only materials and supplies (Ragot and Hoisington 1993), but also less definable costs such as quality technical support, laboratory space, radioisotope permits, etc. A second problem, associated particularly with procedures involving PCR as modified for random amplified polymorphic DNA (RAPD), is the relatively low reliability (5–10% error rate) of the phenotypes (Weeden et al. 1992). This slight but critical lack in reproducibility of the RAPD phenotypes appears to be caused at least partly by imprecise matches between the oligonucleotide primer and the template DNA at the low (35 °C) annealing temperature usually employed in this procedure. A 5% error intrinsic to the procedure greatly compromises the value of the technique for marker-assisted selection and virtually precludes its use in seed quality control applications.

Michelmore and coworkers (Paran and Michelmore 1992; Kesseli et al. 1992) were able to increase the reliability of RAPD markers by sequencing the two ends of the RAPD fragment and synthesizing two longer primers (24 mers) homologous to each end. These two primers could be used in a more traditional PCR cycle (annealing temperatures of 50 °C–65 °C) to amplify the RAPD fragment specifically and reliably. Paran and Michelmore (1992) referred to these markers as sequence characterized amplified regions (SCARs).

We have used a similar approach to increase the reliability of RAPD markers. However, our goal has been to develop a procedure that is fast, economical and applicable to large numbers of samples. To achieve this goal, we needed not only to increase the reliability of the PCR reaction, but also to develop DNA extraction and detection methods compatible with large-scale screening.

To simplify the DNA extraction procedure, Chunwongse et al. (1993) extraction DNA from rice and

Communicated by J. S. Beckmann

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wheat by preincubating a half seed in an extraction buffer without grinding. Using an alkaline (NaOH) solution, two groups (Wang et al. 1993; Klimyuk et al. 1993) extracted DNA from leaf tissue with simple procedures. Both the preincubating and alkaline solution treatment methods represented significant improvements in DNA extraction technology, but also contained important limitations. The method developed by Chunwongse et al. (1993) did not work for a number of crop species, including small-seeded species such as tomato and cabbage or large-seeded legumes such as pea and bean (J. Chunwongse, personal communication; Gu unpublished results). In addition, this procedure included several steps (seed treatment with buffer, incubating at 50 °C, boiling at 100 °C and centrifugation) that made it difficult to automate. On the other hand, the use of leaf tissue in the alkaline extraction methods delays analysis for 5–10 days until the seeds have germinated and the first leaves developed. As seed is a convenient and often desirable tissue to use for marker-assisted selection and quality control purposes, we endeavored to develop a method compatible with this tissue.

DNA marker detection usually requires the electrophoretic separation of the PCR products or restriction fragments. However, if primers specific to a single sequence (allele) are used in the PCR reaction, only a single product will be amplified, and only in samples containing that allele. RAPDs are usually dominant, allele-specific markers. Unfortunately, the single short oligomer typically employed in RAPD procedures usually generates several to many fragments. By extending the length of the primer (using sequence data derived from the amplified RAPD) it would be theoretically possible to create a pair of primers, one complementary to each of the RAPD fragment, that would be long enough to make the PCR amplification fragment-specific (only a single fragment generated) and yet short enough to retain allele specificity. Indeed, several such cases have already been reported (Paran and Michelmore 1992; Timmerman et al. 1994).

DNA markers that can be amplified as a presence/absence polymorphism would not require electrophoresis before scoring because only one fragment is being generated and only in certain plants. All that would be required is a system that could distinguish double-stranded DNA from a mixture of individual nucleotides. Several options appear to be available. Direct measurement of absorbance at 260 nm should work because the molar absorbance of the nucleotides in native DNA is about 60–70% lower than that of an equivalent mixture of the free nucleotides, and about 40% lower than that for denatured DNA (Zubay 1988). Alternatively, compounds such as ethidium bromide (EB) bind to a DNA double helix and dramatically enhance its fluorescence but would not bind to the free nucleotides in the PCR mixture (Heller and Greenstock 1994). The method we present here is based on these considerations and provides a tool for rapidly screening large numbers of samples.

Materials and methods

Plant material

Beans

Two populations of recombinant inbred lines (RILs) of common bean (*Phaseolus vulgaris* L.) were used in this study. F_8 RILs from a cross of 'Redkloud' × 'Redkote' (Wallace et al. 1993) and F_7 RILs from a cross between 'Redkloud' and 'Rojo70' were developed by D. H. Wallace. The lines were generated by single-seed descent. 'Redkloud' is a photoperiod-insensitive, day-neutral variety. 'Redkote' is a photoperiod-sensitive variety, displaying a delay in time to flower when daylength is increased beyond 12 h. 'Rojo70' is a variety that is extremely photoperiod sensitive, and it will not flower if the daylength exceeds 12 h.

Peas

A F_2 population produced from a cross 'Almota' × 'ADH-fast' of pea (*Pisum sativum*) was used to further examine the allele-specific associated primer (ASAP) technique. 'Almota' is a variety homozygous *Adh-1b/Adh-1b*. 'ADH-fast' is a inbred line homozygous *Adh-1a/Adh-1a* developed at Cornell University (N. F. Weeden).

DNA extraction method

DNA for ASAP analysis was extracted from leaves following the method of Wang et al. (1993). For seeds, the procedure of Wang et al. (1993) was modified as following: seeds were placed in water and allowed to imbibe for 12–24 h. Approximately 5 mg was removed from the end of each seed analyzed. For each milligram of tissue, 20 µl of 0.5 *N* NaOH was added, and the tissue was manually crushed using a mortar and pestle. A 10-µl aliquot of this liquid suspension was diluted with 100–200 volumes 100 m*M* Tris-HCl buffer (pH 8.0).

Isozyme analysis

Alcohol dehydrogenase phenotypes of pea plants were determined on root tissue from each F_2 plant in the 'Almota' × 'ADH-fast' family as described by Weeden and Provvidenti (1988).

Cloning of the RAPD marker

RAPD markers linked to each of the photoperiod genes in common bean have been identified (Gu et al. 1993, 1994). A 1.6-kb fragment, P5b, generated by the primer 5'-TCTCTGTCCC, is about 3 cM from *ppd*, the gene conferring insensitivity to photoperiod. A 600-bp fragment, BC303, generated by the primer 5'-GCGGGAGACC, lies within 4 cM of *hr*. The dominant allele at *Hr* conditions extreme sensitivity to photoperiod in *Ppd* plants. A 1-kb RAPD marker within 2 cM of the *Adh-1* locus in pea (Yu et al. 1995) was generated using primer P256 (5'-GATCCACGGA). These three markers were cloned with a TA Cloning Kit (Invitrogen). The procedure was as described by the manufacturer except for certain modifications. For the cloning of P5b and P256, the ligation reaction contained 2 µl undiluted PCR reaction product and was incubated at 12 °C overnight. Recombinant clones were screened for appropriately sized inserts. For the other marker (BC303), the individual RAPD fragment was isolated and reamplified. One microliter of the amplified product was used for ligation.

End-sequencing cloned DNA fragments

Both ends of each DNA insert were sequenced on an ABI 373 Automated Sequencer (Applied Biosystems, Inc.) using a *Taq* DyeDeoxy™ Terminator Cycle Sequencing kit (Applied Biosystems).

Template DNA preparations were done with a modified mini-alkaline-lysis/PEG precipitation procedure as recommended by ABI. The sequence reaction mixture included 9.5 µl reaction premix, 6 µl DNA template solution and 4.5 µl M13 primer at a concentration of 10 ng/µl in a 0.5-µl microfuge tube. Amplification was performed on a PTC-100 Thermal Cycler (MJ Research) using cycle parameters of 96 °C for 30 s (60 s for the first cycle), 50 °C for 15 s. and 60 °C for 4 min for 25 cycles. The extension products were purified by phenol/chloroform extraction (ABI manual) and air-dried before running on the sequencer.

PCR amplification with ASAP primers

A pair of primers specific to sequences at each end of each cloned RAPD fragment were synthesized and used to amplify the particular DNA fragment linked to the respective gene. Each reaction tube contained 0.2 µ*M* of each of two specific primers, 0.13 m*M* of each of four dNTP, 20 ng of genomic sample DNA, 2.5 µl 10 × reaction buffer (Promega), 1.5 µl 25 m*M* $MgCl_2$, 0.5 unit of *Taq* polymerase (Promega) and 18 µl sterilized H_2O, making the total volume 25 µl. Initial PCR amplification was conducted on a PTC-100 Thermal Cycler. In order to make the PCR reaction conditions sufficiently stringent to avoid the non-specific amplifications, PCR cycle parameters were modified until only one allele generated a product. Amplification in micromtiter plates was performed on a Coy Tempcycler II (Coy Laboratory Products).

Comparison of various methods for marker detection

Two methods were used to directly analyze the PCR products. For UV absorption at 260 nm, 10 µl of reaction product in each of ten tubes (five with genomic DNA carrying the marker sequence and five lacking) was added to 2 ml distilled H_2O and A_{260} measurements were taken on a Gilford Spectrometer 250. The remainder of the PCR products were subjected to electrophoresis on a 2% agarose gel.

For direct staining of the PCR product with ethidium bromide, 1 µl ethodium bromide solution (50 µl/ml) was added to the PCR reaction after amplification. To compare electrophoretic analysis with the direct staining method, the directly stained products were removed from the thin-walled tubes or microtiter plate wells and loaded on an agarose gel for electrophoresis.

Analysis of accuracy of screening procedure

To assess the accuracy of the direct screening procedure developed, samples of known genotype were analyzed using the simplified DNA extraction procedure followed by amplification with the allele-specific associated primers. Both incorrect and questionable resuts were treated as erroneus data, and the sum of these was divided by the total number of samples analyzed to obtain a percent error for the analysis.

Results

Simplified DNA extraction procedure

The DNA isolated using the simplified extraction procedure was of sufficient quality for PCR amplification. With the longer primers and stringent annealing conditions (60°–65 °C), we obtained identical results in comparisons between DNA samples isolated from either leaves or imbibed seeds. DNA extracted from dry seeds was not as clean as that from leaves or imbibed seeds and often failed to amplify.

Cloning and end-sequencing the RAPD fragments

Direct ligation of all reaction products generated with P5b marker (four major bands) gave us plasmids with different sized inserts. Comparison of these inserts with the original size of the P5b fragment (1.6 kb) indicated that 3 of the 24 clones examined contained inserts of the correct size. A similar comparison indicated that 1 out of 18 clones from P256 ligation had the correct insertion. All 9 clones from the BC303 ligation were of the correct size. One P5b clone, the appropriately sized P256 clone, and 1 of the BC303 clones were selected for sequencing. About 300 bases were sequenced from each end of cloned fragments of P5b and BC 303 markers and 200 bases from each end of the P256 marker (Fig. 1).

Marker amplification with specific primers

The P5b marker was best amplified using the cycle parameters: 94 °C for 30 s followed by 64 °C for 4 min for 50 cycles with a 4-min-long final soak at 64 °C. The P5b fragment was clearly amplified with the template DNA from photoperiod-insensitive RILs (*ppd/ppd*) but not with the template DNA from photoperiod-sensitive

Fig. 1 DNA sequence of the P5b marker, the BC303 marker and the P256 marker. RAPD and ASAP primers are indicated by *underlined* and *bold* text, respectively. *A* Adenine, *G* guanine, *T* thymine, *C* cytosine, *N* not determined

P5b sequence from both ends

TCTCTGTCCCATGCACCAACTTAACACTCTCAGAAATTGAGCTTCTTCCAGCTCAAGGAGACATAGTGCAT

GACCCTTTCTGTTGGAATGCATATGGAGATTTGGAGACACTAACCATTCCACCAGTCTCATGCTTGCTCGAGGGC

ACTCCTCAATCCGTGTTAGACTTTGACATAACTCGTTGCTGACCCACTACATTATGTGTTGGGCAAAAATGTGA

GCATGTATATAATTATTTACTAGGGAAGTGATTAGGNCATTAGGGAATACATAGCATGATGTTGANTCTAATT

ATCT-------

TCTCTGTCCCCACCACTGTACTACCTTGGTAGTAATACAAATTGTTCTTCCTCACGCCTTTCAACATCACCA

GTGCTCCTGAAGTTGCTTTAAGAATTCCATCTCGCATCGNCACAACTAGGCCTTTAAGTTCTAAAGCCCCCAAT

GAGATGAGATTCTTCTTCAACTTTGGNACGTACCGTACATCCCGCAAAATTCTGGTGGATCCATCGTGATTCCGC

AACTTGATTGAACCTATCCCGACTGTCTTACATGGATTATCATTACCCATGTAAACAACCCCGCCAT-----

BC303 sequence from both ends

GCGGGAGACCATGACCAATGAATATTGGTAACTGATCCANTGAATATATTCAATGATGGCTATTCGAAAGT

TTTTTGGNATTGTNACTCTCTCTTGAAAATGTAATGAGCATTAATTTCTTTATGAAGAAGNCNNCCNCTCATTCT

CTGTATNAAGGAAACTTTGGGGAAGAGAAAAACAAGTAAGTGAGAAAAGCAACATAGAGAGAAAGGAGAGAA

TAAATATTCACCATNGTGGTGAGATTATAAATCCCNNCNGAGATNCTAGAGAGANTTNGNGTTCCANCCCTNCT

TGAGTGAGANNATAAATCCTANCGAGATNCTAGAGAG---

GCGGGAGACCTAGTCCAGAAAATGTCTTCAGTATGAAAGTAGGATTACAATAACTGTTTCTCTCACTCTTT

GAGGATAACACTCAATCTCACCCAACAAGGATGGAATACAAAGTCTCTCTAGCATCCCACTAGGATNTCTAAT

CTCACCCAAGAAGGATGGAATACAAAGTCTTTCTAGCATCTCACTAAGATTTCTNATCTCACCCAAGAAGGAT

GTTATATAAAGTCTCTCTAGCATCTCACTAGGATTTATNATCTCACTCAAGANGGATGGGAATACAAAGTCTCT

CTAGCATCTCAGGAGGATNTATAATCNCANCACTATGGGGAATATTTATTCTCTCCCNNCNCT—

P256 sequence from both ends

GATCCACGGACAAAAGGGAAAGCAAAATCAAAATCTTCCTTGAGGGAGAACTCAGTGGGGAAATTGAAAG

GACATACACTTTCTGCTTAAGGTTGACAACTCTNTTGGAGAAAAGNTTTTTTTCCNCCGGGGATAATCAT---

GATCCACGGAATCACACCATTTACTGATTATCATTCCTTCAATAAAATTTGAGACTTTATACATTTATTTG

NAATTCTTATTTTATTTTATTCTATCTTGCAAAATTTCATTTTTACTTTTGATAGCAAGCCT---

RILs (*Ppd/Ppd*) (Fig. 2). In the case of the BC303 marker, the initial two primers (24 mers), as well as the second pair (18 mers), generated a fragment of identical size in both *hr/hr* and *Hr/Hr* genotypes irrespective of annealing temperature. A pair of shorter primers (15 mers) gave intensity differences between the two genotypes but did not function as ASAPs. However, by combining one of the shorter primers with one of the longer primers (an 18 mer) we were able to amplify the appropriate fragment only from plants containing the *hr* gene (Fig. 3). The PCR conditions used were: 50 cycles of 94 °C for 30 s and 62 °C for 3 min.

The P256 marker was also amplified from the genotype of *Adh-1b/Adh-1b* using the cycle parameters: 94 °C for 35 s, 63 °C for 30 s, and 64 °C for 3 min for 50 cycles with a 4-min final soak at 64 °C.

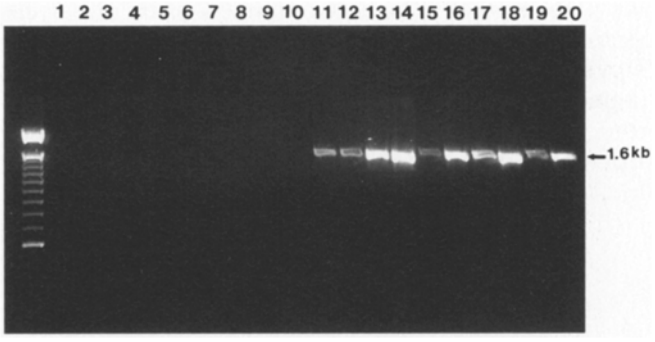
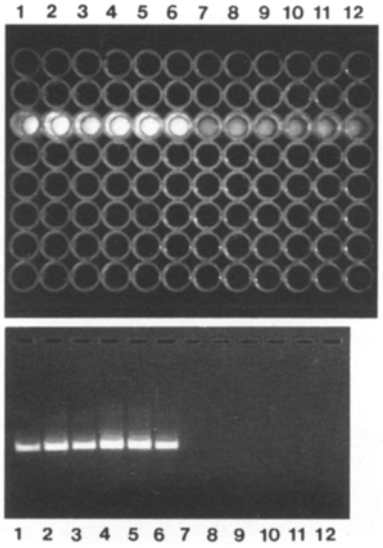


Fig. 2 Amplification of P5b by specific primers with different plant DNA templates. *Lanes 1–10* were photoperiod-sensitive and lanes *11–20* were photoperiod-insensitive varieties and RILs

Fig. 3 Amplification of BC303 by specific primers with different plant DNA templates. The *first 6 lanes* were *hr/hr* genotypes and the *following 6 lanes* were *Hr/Hr* genotypes. The PCR products were detected using both direct staining with ethidium bromide (*top*) and electrophoresis (*bottom*)



Direct detection of amplified DNA

The three markers were amplified on the Coy Tempcycler II using 96-well microtiter plates. Minor adjustments in the cycling parameters were necessary to obtain the same results on this instrument as we obtained on the PTC-100 Thermocycler with individual samples. The program used for P5b amplification was 49 cycles at 94 °C for 35 s and annealing and extension at 62 °C for 4 min. These PCR products were used for testing different detection methods.

Direct measurement of absorbance at A_{260}

We could not observe a consistent difference in the absorbance at 260 nm between samples in which a DNA fragment had been amplified and those in which no amplified product was observed (Table 1). As a further confirmation of these results we could not detect a difference in absorbance between amplified and unamplified RAPD reaction solutions (data not shown).

Ethidium bromide stain

The addition of 1 µl of a 50 µg/ml ethidium bromide staining gave a strong fluorescence in samples predicted to contain an amplified DNA fragment but only weak fluorescence in the samples in which no fragment was predicted. These direct staining results were completely verified by electrophoresis (Fig. 3). The time required for the direct staining procedure was 10–20 min, whereas electrophoresis required more than 4 h.

Directly staining PCR products of the F_2 population of pea

When the PCR products of the F_2 population of pea were stained with ethidium bromide, no difference was observed in the fluorescence of homozygous *Adh-1b/Adh-1b* and heterozygous genotypes (Fig. 4). Both of those genotypes could be clearly distinguished from the homozygous *Adh-1a/Adh-1a* genotype that gave a weak fluorescence. There was no difference between the *Adh-1a/Adh-1a* genotype and any of several negative controls (Fig. 4).

Table 1 Measurement of UV light absorption at 260 nm PCR products amplified with different DNA templetes

Template DNA in PCR product	UV absorption of PCR products for five different pairwise comparisons					Average
ppd/ppd[a]	0.036	0.031	0.036	0.033	0.027	0.0326
Ppd/Ppd[b]	0.032	0.036	0.025	0.035	0.032	0.0320

[a] Genomic DNA carrying marker sequence
[b] Genomic DNA lacking marker sequence

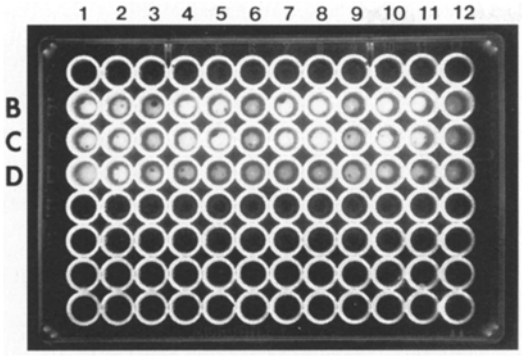


Fig. 4 Direct staining of PCR products of a F_2 population of pea. For the 12 wells of *rows B* and *C* the DNA template were arranged into four groups each with *Adh-1b/Adh-1b*, *Adh-1b/Adh-1a* and *Adh-1a/Adh-1a* genotypes. *Row D* included 'Almota' (*Adh-1b/Adh-1b*) (*wells 1 and 2*). ADH-fast (*Adh-1a/Adh-1a*) (*wells 3 and 4*), reaction mixture lacking polymerase (*wells 5 and 6*), reaction mixture lacking polymerase (*wells 7 and 8*), reaction mixture lacking template DNA (*wells 9 and 10*) and reaction mixture not run on PCR (*wells 11 and 12*)

Determination of accuracy of ASAP procedure

When leaves or seeds for which the genotype had been previously established were subjected to the entire procedure, the error rate was determined to be less than 1.5% (Table 2). In nearly all cases the error was due to the lack of amplification in a sample that contained the allele producing the fragment.

Discussion

We describe a procedure that greatly simplifies PCR product analysis for marker screening. This method has been applied to the screening for the presence of photoperiod genes in common bean (*Phaseolus vulgaris* L.) and has proven to be highly reliable, fast and convenient. Amplifiable DNA can be extracted from leaf or seed tissue using simple procedures. Elctrophoresis, usually associated with resolving DNA markers, has been eliminated by the direct staining of PCR products. The key to the technique is the availability of a pair of primers that will specifically amplify only a DNA fragment tightly linked to one allele at a locus of interest, and we refer to such pairs as allele-specific associated primers (ASAPs). A error rate of about 1.5% was determined for the procedure. Although this error rate is significant, for many applications in breeding and seed production it is acceptable, particularly considering the increase in convenience and reduction in costs. As most errors involved cases in which the fragment was not amplified, greater accuracy could be achieved by a second amplification of those samples lacking a product. We have found the DNA extraction procedure is very dependable, and a second amplification usually identifies positive samples that failed to generate products in the first amplification.

In their studies on the development of SCARs from RAPDs, Michelmore and coworkers found that the use of longer primers often amplifies fragments in both genotypes (Paran and Michelmore 1992; Kesseli et al. 1992). Occasionally, these allelic fragments were of distinct sizes and could be resolved on agarose gels. In other cases, fragments of identical size could be differentiated by endonuclease restriction patterns. However, allele specificity was not a primary concern of their investigations.

Our approach requires that the longer primers maintain the original presence/absence polymorphism observed for the RAPD. The optimal primer length for ASAPs appears to be shorter than the 24 mers typically used for SCARs, the longer primers being more likely to produce fragments in all genotypes. Although we report the development of only three ASAPs in this paper, with one of three (BC303) requiring experimentation with several primers, the approach appears to be applicable to most RAPDs. Timmerman et al. (1994) developed ASAPs for the gene *er-1*, which confers resistance to powdery mildew in pea, and we have two additional ASAPs for other regions of the pea genome (Yu, et al. 1995; Gu, unpublished). In each of these latter cases allele specificity was obtained with the first set of primers synthesized, and normal (60°–65 °C) annealing temperatures were employed.

In our experience, the modification of primer length appears to be the most effective approach to obtain allele specificity. However, Xu and Hall (1994) reported that the allele-specific DNA fragment can be easily obtained by two runs of PCR amplifications, with three primers in the first run and two specific primers in the second run. They referred to the technique as SASA (simplified allele-specific amplification). The application of this technique is somewhat complicated but offers an alternative to the approach we used.

Once the ASAP primers have been developed, the DNA product generated in a PCR reaction is easily visualized by ethidium bromide, which binds to the

Table 2 Reproducibility of DNA extraction and ASAP method tested with known genotypes

DNA source	DNA marker score[a]			Total error	Number of plants analyzed	Percent error
	Present	Absent	Uncertain			
Leaf						
Current score	285	186	0			
ASAP score	280	191	1	6	471	1.3
Seed						
Current score	60	12	0			
ASAP Score	59	13	0	1	72	1.4

DNA double helix to generate a highly fluorescent complex but not to the free nucleotides in the PCR mixture. Direct staining could not distinguish between plants heterozygous for the allele and homozygous plants. Thus ASAPs, like RAPDs, are dominant markers. In marker-assisted selection applications the ASAP marker should be developed to select the desired genotype or genotypes. Alternatively, two ASAP markers, one for each allele could be developed and two amplifications performed to identify both alleles.

Ragot and Hoisington (1993) compared the costs of three molecular marker protocols: chemiluminescent restriction fragment length polymorphisms, radioactivity-based restriction fragment length polymorphisms and RAPDs. Although their analysis focused on studies involving large numbers of probes/primers, and thus is not totally appropriate for marker-assisted selection applications, their breakdown of costs for RAPD analyses indicated that nearly half the costs could be attributed to DNA extraction and detection steps. For ASAPs, material costs associated with the extraction and detection steps are less than 5% of those involved with amplification (*Taq* polymerase, primers, deoxynucleotides, thin walled microcentrifuge tubes, etc.). Hence, per sample analysis costs for latter procedure are determined primarily by the price of *Taq* polymerase, currently about $0.20 per assay, plus about 3 min of personnel time.

The high success rate of this approach suggests that ASAPs also may be derived from RFLPs and other DNA sequence varaiation identified near or within a gene of interest. The method appears to be applicable to animal and microbial testing, and although designed for large-scale screening, ASAPs may be useful in small programs with minimal funds to support electrophoresis or additional personnel.

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