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Robust allele-specific polymerase chain reaction markers developed for single nucleotide polymorphisms in expressed barley sequences

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Abstract Many methods have been developed to assay for single nucleotide polymorphisms (SNPs), but generally these depend on access to specialised equipment. Allele-specific polymerase chain reaction (AS-PCR) is a method that does not require specialised equipment (other than a thermocycler), but there is a common perception that AS-PCR markers can be unreliable. We have utilised a three primer AS-PCR method comprising of two flanking-primers combined with an internal allele-specific primer. We show here that this method produces a high proportion of robust markers (from candidate allele specific primers). Forty-nine inter-varietal SNP sites in 31 barley (*Hordeum vulgare* L.) genes were targeted for the development of AS-PCR assays. The SNP sites were found by aligning barley expressed sequence tags from public databases. The targeted genes correspond to cDNAs that have been used as restriction fragment length polymorphic probes for linkage mapping in barley. Two approaches were adopted in developing the markers. In the first approach, designed to maximise the successful development of markers to a SNP site, markers were developed for 18 sites from 19 targeted (95% success rate). With the second approach, designed to maximise the number of markers developed per primer synthesised, markers were developed for 18 SNP sites from 30 that were targeted (a 60% success rate). The robustness of markers was assessed from the range of annealing temperatures over which the PCR assay was allele-specific. The results indicate that this form of AS-PCR is highly successful for the development of robust SNP markers.

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Introduction

Single nucleotide polymorphisms (SNPs), represent the most common type of sequence polymorphism found in plant and animal genomes, with well over a million SNPs detected and catalogued for the human genome alone (Sachidanandam et al. 2001). SNPs are likely to be the basis for many polymorphisms that are detected using systems such as restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLPs) (Schork et al. 2000). However with recent advances in DNA sequencing technology and hence output, it is now cost effective to detect SNPs directly and for species which have publicly available expressed sequence tag (EST) databases, putative SNPs located within genes can be identified *in silico*. There are a number of reasons why markers located within genes can be desirable and SNPs have been shown to occur at high frequency in expressed barley sequences (Bundock et al. 2002; Kanazin et al. 2002; Kota et al. 2001). This makes them much more ideal for mapping genes than simple sequence repeats (SSRs or microsatellites) which occur in only a small proportion of ESTs (Holton et al. 2002; Kota et al. 2001; Varshney et al. 2002).

A wide variety of different methods have been developed to carry out genotyping for detected SNPs, some using automated systems developed for high throughput (Gupta et al. 2001; Gut 2001). These methods are often reliant on expensive equipment and require high development costs and the marker assays generated are commonly not transferable between laboratories due to the diversity of assay technologies. A simple cost-effective method for SNP marker genotyping would

greatly improve marker transferability and application. The cleaved amplified polymorphic site (CAPS or PCR-RFLP) method has been adopted by a number of groups to enable mapping of SNP markers found in ESTs (e.g. in barley, Graner 2004; Sato 2004). However, only a proportion of SNPs are amenable to CAPS and the system can be unreliable and time consuming.

An alternative method is allele-specific PCR (AS-PCR). The original two primer, single product AS-PCR (Wu et al. 1989) has likely been used for many assays; however, it has a reputation for a low success rate for producing robust markers. A competitive PCR reaction, in which more than one product is or can potentially be amplified, is one way to increase the stability of AS-PCR assays (Newton et al. 1989). A common approach that also enables heterozygotes to be identified in the one assay, has been to include four primers in the one PCR mix, these include tetra primer PCR (Ye et al. 1992), tetra primer ARMS-PCR (Ye et al. 2001), Bi-PASA (Liu et al. 1997) and CTTP (Hamajima et al. 2000). A simplification of these approaches using three primers has been described by Soleimani et al. (2003). Rarely however, are any of these methods subjected to analyses to determine how successful they are for marker development. In the work described here we examine the success of undertaking SNP marker development based on the three primer nested PCR approach of Soleimani et al. (2003). We have targeted SNPs discovered using public domain EST sequences of barley.

Materials and methods

Mining barley ESTs for SNP sites

Expressed genes (cDNAs) from barley (*Hordeum vulgare*) developed for use as RFLP probes (ABC and BCD series cDNAs) were targeted for inter-varietal SNP discovery. Most of the cDNAs chosen had been mapped in barley and sequence and mapping information are available from the GrainGenes probe repository (http://wheat.pw.usda.gov/ggpages/probes/index.shtml) or from a search of the Entrez Nucleotides database at the NCBI website (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) using the probe clone name. EST clusters for in silico SNP discovery were obtained from Plant GDB website (http://www.plantgdb.org) and Sputnik (now known as openSputnik http://sputnik.btk.fi/). Sputnik also has results from running the SNiPper algorithm to search for SNP sites in these clusters (Rudd et al. 2003; Kota et al. 2003). The score given in Sputnik for each mismatch between ESTs in a cluster was used as a basis for choosing EST clusters for the development of three-primer AS-PCR assays. EST accession numbers were used to find the respective matching cluster (where available) in the *Hordeum vulgare* section of the Sputnik website. A suitable accession number (with a two letter code) could be found from an NCBI Entrez search using the probe cDNA name or from a matching EST from a blastn of the cDNA sequence on the barley TUG (or EST) section of the PlantGDB blast site (http://www.plantgdb.org/cgi-bin/PlantGDBblast). Member sequences of matching clusters were downloaded from the plantGDB website and imported into the program Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA) for auto-aligning and identification of SNP sites. For larger clusters, a subset of sequences with representatives from each of the barley varieties present was chosen for alignment in Sequencher against the Sputnik cluster consensus sequence. The varietal origin of each sequence was identified from either the FASTA annotation or from the Genbank entry of each sequence. Contigs were assessed in Sequencher with the aim of identifying SNP sites polymorphic between barley varieties but monomorphic within each variety. Likely SNP sites flanked by reliable sequence became the focus of primer design for three primer AS-PCR.

Primer design

A three-primer nested system was used for allele-specific amplifications (Fig. 1). At potential SNP sites, two generic primers (forward and reverse orientations) were designed that flanked the SNP containing region. Allele-specific primers (ASPs) were then designed for each SNP site. All primers were designed using the program MacVector™ 6.5 (Accelrys, San Diego, CA, USA). Two separate approaches were taken with regard to the number of ASPs designed for each barley gene. In the first approach, a single SNP site within each SNP containing EST cluster (contig), one that was considered highly reliable, was targeted. For this SNP site, eight ASPs were synthesised in two sets of four. In the first set there are two primers (one for each SNP allele at the last base position) in each orientation (forward and reverse) i.e. two orientations for each allele. The second set of primers are identical to the first except with a mismatch three base pairs from the 3′ end, designed to increase primer specificity (Kwok et al. 1990; Zhang et al. 2003). The mismatch was designed to pair the base on the template with a base of the same identity (i.e. A-A, T-T, C-C and G-G) at this position on the primer. The only parameter considered in the design of primers using MacVector™ for this approach was the melting temperature (T_m), which was as close as possible to the T_m of the competing flanking-primer. In the second approach, designed to reduce primer synthesis costs, only a subset of ASPs were synthesised. Candidate ASPs were removed from consideration for synthesis based on warnings displayed in MacVector™ that are considered to impact on the likelihood of primer success/failure. Remaining candidate primers were chosen based on closeness of T_m to the competing flanking-primer. All AS-PCR amplifications were run as nested PCRs with the two flanking-primers and the internal ASP present in the reaction mix.

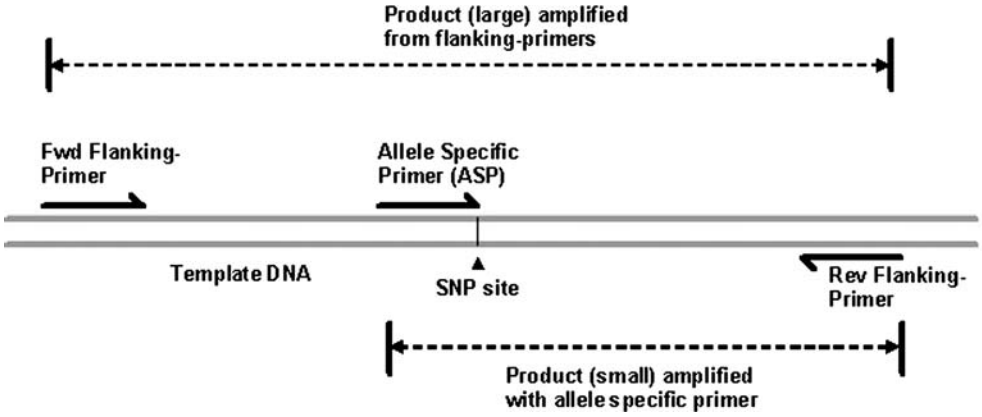

Fig. 1 Diagram illustrating the position and orientation of PCR primers for the three primer allele-specific PCR relative to the matching template DNA

PCR amplifications

Initial PCR amplifications were carried out in a gradient thermal cycler (Palm-Cycler™, Corbett Research, Sydney, Australia) to determine the robustness and optimum annealing temperature for each allele-specific reaction. For the annealing temperature gradient there was a 1° difference in temperature between adjacent wells across the 12-well block for annealing (an 11° difference between the highest and lowest annealing temperatures) from 59 to 70°C. The cycling parameters were as follows: 95°C 2 min, [94°C for 30 s, 59–70°C for 30 s, 72°C for 30 s] for 35 cycles, 72°C for 5 min, ambient hold. The following components at the indicated concentrations were included in a 10 μL volume for each PCR reaction: each primer 0.2 µM, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.02 U/μL Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and approximately 10 ng of template DNA. It should be noted that Platinum Taq is a 'hot start' Taq polymerase, and like other Taq polymerases has no 3′–5′ proof reading ability which could interfere with allele specific amplifications.

Plant material

Barley (*H. vulgare* and *Hordeum spontaneum*) genotypes were obtained from the Australian winter cereals collection at Tamworth (NSW, Australia). DNA was extracted from fresh leaf material approximately 1 week post germination, with a pool of two to four individuals represented (not equally) in each extraction. The following varieties were used to test amplification and verify SNP position: Akashinriki, Barke, Golden Promise, Haruna Nijo, Morex, Optic, Saana, Tokak. Publicly available EST sequences from barley originate almost entirely from these varieties. The following barley varieties and accessions were used to determine SNP frequency and are parents of mapping populations included in the Australian National Barley Molecular Marker Program: Alexis, Arapiles, Barque, Chebec, Clipper, Dash, Franklin, Galleon, Halcyon, Harrington, Haruna Nijo, *Hordeum spontaneum* 7128448, Kaputar, ND 11231-12, Patty, Sahara 3771, Sloop, Tallon, TR232, VB9104, VB9524, WABAR2080.

Gene identities

For each cDNA containing a SNP verified using AS-PCR, identification of the closest matching gene has been carried out using a BLASTX of the NCBI nucleotide (nt) database of sequences from all species.

Results

We attempted to find in silico SNPs in EST sequence clusters corresponding to 100 barley cDNAs used as RFLP probes (Table 1). Six cDNAs from these 100, did not have sequence information available in the public domain, for nine cDNAs there was no matching EST cluster to the cDNA sequence and for ten cDNAs there was no evidence for a SNP site in the cDNA cluster (Table 1). There was evidence for one or more SNP sites for the remaining 75 probes. However, some cDNA probes appeared to originate from the same gene, ABC154 matching ABC305 and ABC255 matching ABC465. There was thus evidence for in silico SNP sites for 73 out of 92 cDNA probes for which there was unique sequence (79%). Thirty-one of these 73 were targeted for the development of allele-specific SNP markers at one or more SNP sites (Table 2).

Primers were designed to interrogate 49 SNPs in 31 sequence clusters, using the nested three-primer system. Of these 49 SNP sites, robust AS-PCR markers were developed for 36 located in 28 EST clusters. The cDNAs corresponding to each of these clusters, their RFLP map positions and the identity of the best matching gene to each cDNA (as revealed by a BLAST search) is presented in Table 3. There were a number of different banding patterns produced across the temperature gradient and between markers. The most common banding pattern is characterised by amplification from the two flanking-primers to give a large band in each amplifi-

Table 1 Result of mining ESTs for in silico SNPs using 100 barley cDNA probes

cDNA probe sequence not found (6)	Matching EST cluster not found (9)	SNP sites not present in cluster (10)	Likely SNP site found in EST cluster (75)
ABC157, ABC314, ABC460, ABC482, BCD002, BCD221	ABC162, ABC166, ABC171, ABC257, ABC321, ABC706, BCD269, BCD339, BCD758	ABC160, ABC168, ABC174, ABC308, BCD001, BCD111, BCD129, BCD340, BCD351, BCD402	ABC151, ABC152, ABC153, ABC154, ABC155, ABC156, ABC158, ABC161, ABC163, ABC164, ABC165, ABC172, ABC175, ABC176, ABC252, ABC254, ABC255, ABC261, ABC302, ABC303, ABC304, ABC305, ABC306, ABC309, ABC310, ABC451, ABC454, ABC455, ABC465, ABC466, ABC468, ABC483, ABC717, BCD008, BCD015, BCD021, BCD022, BCD098, BCD102, BCD127, BCD131, BCD134, BCD135, BCD147, BCD175, BCD205, BCD207, BCD249, BCD263, BCD265, BCD266, BCD276, BCD292, BCD298, BCD304, BCD310, BCD334, BCD342, BCD355, BCD372, BCD386, BCD410, BCD442, BCD451, BCD453, BCD454, BCD508, BCD512, BCD589, BCD808, BCD809, BCD828, BCD1072, BCD1130, BCD1434

cation with a second smaller band present in amplifications where the corresponding allele matching the AS primer is present in the template DNA (Fig. 2). The second most common banding pattern is characterised by amplification of the large band (from the flanking-primers) only when the allele of the corresponding AS primer is absent, being replaced (or almost so) when that allele is present, by the smaller band amplified from the internal primer (Fig. 3). The sequence of the most robust primers giving allele-specific amplifications for each SNP site, the corresponding annealing temperature range for successful amplification and the resulting banding patterns and band sizes for alternative genotypes are presented as supplementary data (Supplementary Table).

From the 19 SNPs targeted using the first approach of primer saturation (of designing eight ASPs to each SNP site), robust AS amplification was observed for at least one of the two alternative alleles at 18 SNP sites—a 95% success rate for marker development to a site. From the 30 SNPs targeted using the second approach of designing only the 'best' primers, amplifications for 18 SNPs were found to give robust AS-PCR assays, which is a 60% success rate for marker development to a site. Using the first approach, the development of markers for both SNP alleles at a site was achieved for 16 sites out of 19 or an 84% success rate. For the second approach the aim was for efficient marker development and the number of primers synthesised per SNP site with marker developed was smaller for this approach (if one flanking-primer pair per SNP site is assumed there are 6.2 (111/18) primers per SNP site marker for the second approach compared with 10.6 (190/18) for the first approach). Thus, although no assay was developed for 40% of the SNP sites targeted using the second method; it was more cost effective in producing markers being 60% of the cost of the first method for primer synthesis per SNP site marker.

The third base back mismatch primers (comprising half the primers used in the first approach) provided successful allele-specific amplifications for 16 out of 19 SNP sites, with a total of 38 (out of 76) of these primers giving allele-specific amplifications. This compared to successful allele-specific amplifications for 41 (out of 76) of the corresponding primers without this mismatch. The average temperature range for allele-specific amplification for the third base back mismatch primers

Table 2 Barley cDNA probes homologous to EST clusters that were targeted for the development of AS-PCR markers

cDNA Probe Name	Number SNP sites targeted	Number AS primers tested	Number SNP sites with marker(s) developed
ABC151	1	8	1
ABC153	1	8	1
ABC154/305	1	8	1
ABC155	2	4	2
ABC156	3	5	2
ABC158	1	8	0
ABC161	1	8	1
ABC163	1	8	1
ABC165	2	4	2
ABC172	3	4	0
ABC176	1	8	1
ABC252	2	4	2
ABC254	1	8	1
ABC255/465	5	13	3
ABC261	3	4	1
ABC310	1	2	1
ABC451	2	4	1
BCD008	1	8	1
BCD021	1	8	1
BCD127	1	2	1
BCD134	2	4	1
BCD175	1	8	1
BCD207	1	8	1
BCD265	1	2	0
BCD266	1	8	1
BCD276	1	8	1
BCD304	4	7	3
BCD410	1	8	1
BCD442	1	8	1
BCD453	1	8	1
BCD589	1	8	1
Total	49	203	36

Table 3 Map location and details of closest matching gene for barley cDNA probe sequences developed into AS-PCR markers

cDNA name	No. SNP sites with marker(s) developed	Map location (chromosome/arm) [A]	Sputnik Cluster	Closest matching gene	Database ID number	Species of origin	Expected value
ABC151	1	7H +	C_BQ464039	Multifunctional aminoacyl-tRNA ligase-like protein	gi|22136084|	*Arabidopsis thaliana*	1.00E-128
ABC153	1	2HL	C_BG299704	Predicted protein of unknown function	gi|38344803|	Rice	8.00E-25
ABC154	1	6H	C_BG366112	Leucyl aminopeptidase (EC 3.4.11.1)	pir||T14912	Barley	9.00E-80
ABC155	2	5HL	C_BM442818	Sucrose synthase 1 (sucrose–UDP glucosyltransferase 1)	sp|P31922|SUS1_HORVU	Barley	0
ABC156	2	7H +	C_BE427780	pZE40 protein	pir||T06174	Barley	2.00E-13
ABC161	1	3HL	C_BQ466763	50S Ribosomal protein L27, chloroplast precursor	gi|20139372	Rice	1.00E-74
ABC163	1	6HL	C_AL506101	F1-ATP synthase delta subunit	|BAA77508.1|	*Ipomoea batatas*	1.00E-53
ABC165	2	2HL	C_BG367758	Hydroxymethyltransferase	gi|7268097	*A. thaliana*	1.00E-177
ABC176	1	3H	C_BI949259	Expressed protein supported by cDNA	|NP_568653.1|	*A. thaliana*	9.00E-38
ABC252	2	2HL	C_BF261131	Adenosylhomocysteinase (*S*-adenosyl-L-homocysteine hydrolase)	Sp|P32112|SAHH_WHEAT	Wheat	0
ABC254	1	7H	C_AV909243	Probable glucan-1,3-β-glucosidase (EC 3.2.1.58) ExoII	pir||T04414	Barley	0
ABC255	1	7H	C_BG417785	Sucrose synthase (EC 2.4.1.13)	pir||S24966	Barley	0
ABC261	1	1HL	C_AV917375	Putative ribosomal protein L18a, cytosolic	ref|NP_916142.1|	Rice	1.00E-92
ABC310	1	7HL	C_BF264462	Elongation factor 2	emb|CAC12817.1|	*Nicotiana tabacum*	1.00E-17
ABC451	1	2HL	C_AL510682	*S*-Adenosylmethionine decarboxylase precursor	gb|AAD17232.1|	Wheat	3.00E-75
ABC465	2	7HS	HVtuc02-11-10.237	Sucrose synthase (EC 2.4.1.13)	pir||S24966	Barley	0
BCD008	1	4A (wheat)	C_BI950124	Omnipotent suppressor protein SUP1 homolog (clone G18)	pir||S31328	*A. thaliana*	2.00E-40
BCD021	1	6HS +	C_BI948013	Putative ubiquitin-conjugating enzyme	gb|AAN04972.1|	rice	1.00E-85
BCD127	1	3HS	C_AV915904	Pyruvate kinase, cytosolic isozyme (PK)	sp|Q42954	*Nicotiana tabacum*	1.00E-160
BCD134	1	3HL	C_BE060523	Predicted protein of unknown function	gi|52076355|	Rice	5.00E-75
BCD175	1	2HS	C_BG343178	Unknown	gb|AAO72677.1|	Rice	7.00E-78
BCD207	1	1HL	C_BE438982	Putative protein	ref|NP_567969.1|	*A. thaliana*	1.00E-143
BCD266	1	2HL	C_BG365646	Cysteine protease component of protease-inhibitor complex	dbj|BAA88898.1|	Maize	1.00E-106
BCD276	1	6HL	C_AL507626	Betaine aldehyde dehydrogenase	dbj|BAB62846.1|	Barley	0
BCD304	3	1HL	C_BM101162	AFG1-like ATPase family protein	gi|42567204	*A. thaliana*	1.00E-48
BCD410	1	2HL +	C_BQ463645	No match	NA	NA	NA
BCD442	1	1HL	C_BM817164	Unknown protein	gb|AAK50581.1	Rice	0
BCD453	1	2HL +	C_AV929639	Extracellular invertase; β-fructofuranosidase	gb|AAN80141.1|	*Triticum monococcum*	7.00E-51
BCD589	1	3HL +	C_BJ469856	Similar to phytochrome A supressor spa1	ref|NP_683567.1|	*A. thaliana*	5.00E-77

A The map location is the chromosome (arm) that is indicated in the Grain Genes database (http://www.graingenes.org). A plus symbol (+) indicates that more than one RFLP was mapped using that cDNA probe. In this case the chromosome indicated is that found most commonly to harbour a mapped RFLP for that probe
NA not applicable

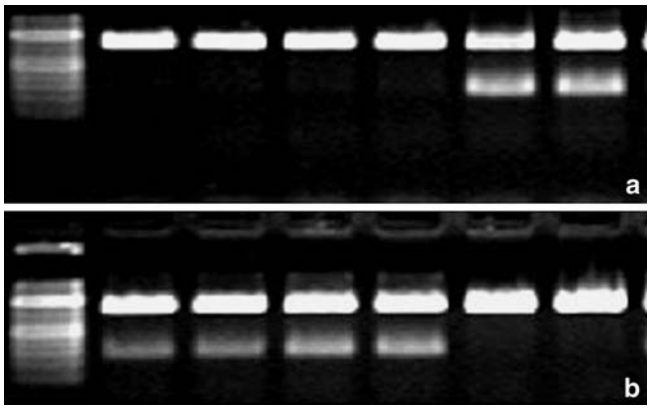

Fig. 2 Agarose gel photo of allele-specific (AS) amplifications showing the most commonly observed banding pattern. **a** *Lane 1* is a 50 bp ladder followed by AS-PCRs in *lanes 2–7* with template DNA derived from: *2* Arapiles, *3* Barque, *4* Chebec, *5* Clipper, *6* Dash, and *7* Franklin. The AS-PCRs include the AS primer ABC155s516CBO_R (C allele). **b** *Lane 1* is a 50 bp ladder the AS-PCRs are amplified from the same templates in the same order as in (**a**) above. However the AS-PCRs include the AS primer for the alternative allele ABC155s516H_R (T allele). In the banding pattern shown in this figure, the upper band amplifies independently of the targeted SNP genotype and the presence of the lower band indicates the presence of the SNP allele being tested.

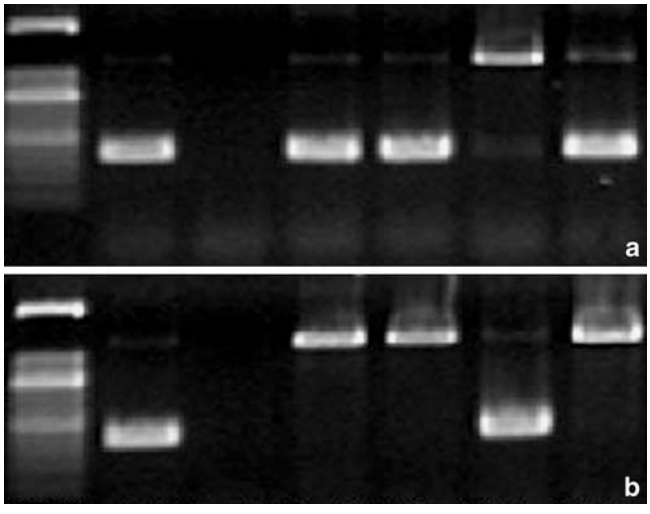

Fig. 3 Agarose gel photo of AS amplifications showing an alternative banding pattern. **a** *Lane 1* is a 50 bp ladder followed by AS-PCRs in *lanes 2–7* with template DNA derived from: *2* Barke, *3* no DNA, *4 Hordeum spontaneum* 7128448, *5* Sahara 3771, *6* Haruna Nijo and *7* TR232. The AS primer is ABC255s572C-BO_R (A allele). **b** *Lane 1* is a 50 bp ladder followed by AS-PCRs in *lanes 2–7* with template DNA derived from: *2* Haruna Nijo, *3* no DNA, *4 H. spontaneum* 7128448, *5* Sahara 3771, *6* Haruna Nijo (a separate extraction), *7* TR232. The AS primer is ABC255s572H_R (G allele). In the banding pattern shown in this figure, amplification of the upper band is dependent on the genotype being tested. The presence of the lower band indicates the presence of the allele being tested and the presence of the upper band indicates the absence of this allele

was approximately 4°C compared with 6°C for the primers without this mismatch, a difference in means that is statistically highly significant (*t* test, $P < 0.01$).

SNP allele frequencies

The AS-PCR assays developed here were used to determine the presence of SNP alleles in a group of 22 genotypes. This was carried out to give some idea of how commonly each SNP allele occurs, although this was assessed in terms of the number of genotypes with the SNP allele not as an estimate of allele frequency (Supplementary data). For some SNP sites, some varieties appear not to be pure for one SNP allele and are assumed to be mixtures of the two alleles. In these cases there were generally weak band intensities for one or both of the alleles for a genotype.

Failure to develop allele-specific assays

Since the aim of this work has been to develop AS-PCR assays we have attempted to determine the main reasons for assay failure. The result of amplification with each AS primer was classified into one of six outcomes which were then tallied (Table 4). From this it can be seen that the main cause of assay failure was due to non amplification of a product from the AS (internal) primers (39 instances). A total of 20 primers did not display allele-specific amplification. From these there were 15 targeting nine SNP sites that were verified as SNPs by other primers. A maximum of only five primers that targeted four SNP sites, could be considered as possible failures due to SNP misidentification from sequencing errors (although any or all of these sites might be genuine SNPs). It was expected that assays would fail due to the presence of introns. Based on the sizing of PCR products on agarose gels, introns were detectable in 17 amplification products and absent in 14. However, there were very few failures of amplification from flanking-primers with only two products failing to amplify. The failure rate of the flanking primers was thus as low as 0.03 (2/66) if we assume that failure to amplify a product was caused by only one of each pair of primers. By contrast the failure rate of amplification from the AS primers was 0.2 (39/191).

Discussion

We have shown here that AS-PCR using the three primer nested approach, is a highly successful method for the development of SNP markers. A high proportion of the ASPs led to the development of a robust SNP assay. Other papers have described various technical modifications that may make AS-PCR more useful but generally the number of assays developed and published has been very limited (Ye et al. 2001; Liu et al. 1997; Imyanitov et al. 2002; Chiapparino et al. 2004). This work describes the successful development of, to our knowledge, the largest number of AS-PCR markers published in one article (36 SNP sites in 29 genes).

The two approaches to the development of SNP markers taken in this paper reflect two potentially

Table 4 Outcomes from allele specific (AS) PCR assays

	Number of AS primers	Number of SNP sites
Both small (AS) PCR product and large product (from flanking-primers) failed to amplify	12	2
Large PCR product amplified—AS product did not	39	15
Amplification not AS—evidence for SNP from other assays	15	9
Amplification not AS—only in silico evidence for SNP	5	4
Assay AS but not robust	11	8
Successful three primer assay	113	36

different motives for developing markers. The first approach is a 'saturation' approach designed to provide a high probability of developing an assay for each SNP polymorphism. This approach involved designing a primer for both alleles in both orientations and also a corresponding set of primers with third base back mismatches. The second approach involves selecting a minimum number of the 'best' AS primers to enable a greater coverage of SNP sites for a given outlay on primers. As expected the saturation approach produced a higher success rate for development of one or more markers to each SNP site, (18 SNP sites out of 19 targeted or 95%) compared with the second approach (18 from 30, or 60%). However, the cost of oligonucleotide primers for the first approach per SNP site developed was on average nearly twice the cost of the second approach and in addition there were a larger number of tests to determine useful primer combinations. Thus, although no assay was developed for many individual SNP sites, this second method of selecting a small number of the 'best' primers was more cost effective in producing markers. For a marker development project a combination of the two approaches carried out here could perhaps be used to efficiently develop a large number of markers i.e. an initial round of primer design using the more efficient 'best ' primer strategy followed by a second round using the saturation approach where marker development failed in the first round.

The results indicate that the SNP identification process has been extremely reliable in this study and at most only a very minor cause of assay failure. The main cause of assay failure was absence of an amplification product from the AS primer. Amplification failure is a problem with PCR in general but there are several reasons why amplification failure from internal primers might be higher for the type of assay designed here. Firstly, the flanking primers were designed as a pair, whereas the AS primers were designed separately but were required to pair with an independently designed flanking-primer for amplification. The result of this has probably been to reduce the likelihood of success for the AS primers relative to the flanking primers. The alternative approach to primer design, designing flanking primers to pair with the respective AS primer, would probably increase the frequency of AS primers amplifying but reduce the likelihood of flanking primers amplifying a competing product. This strategy is thus likely to perform no better than the strategy used here since amplification from the flanking primers is essential to provide a competitive product for allele absent templates (genotypes). Another reason why AS primers failed to amplify is that the sequence of internal AS primers is entirely dictated by the position of the SNP site, whereas for the flanking-primers (and PCR primers in general) the best choice is made from many possibilities. This has meant that it is likely that many of the internal primers designed for the AS-PCR amplifications had suboptimal qualities for PCR, likely resulting in amplification failure. An indirect cause of failure of AS primers may be introns. In some cases it appears that introns may have prevented the development of AS assays where the product amplified from flanking-primers was large (due to the presence of intronic sequences) and the AS product was much smaller. In these cases the large product may not have acted as a sufficiently effective competitor to prevent non AS amplification.

The data indicate that the method utilized here should be broadly applicable to the development of SNP markers where there is sufficient knowledge of DNA sequence around a SNP site. The nested three-primer AS-PCR should enable the development of markers that can be used to map the transcriptome, for general linkage mapping for QTL detection, as perfect markers for the candidate gene approach, for varietal identification and to perform the function of DNA markers in general. These markers could act as an adjunct to CAPS where sites are not sensitive to restriction enzyme assay or as a more reliable replacement. In particular, they will be useful for projects that are on a modest budget or where sophisticated equipment is not available.

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References

Bundock PC, Christopher JT, Eggler P, Ablett G, Henry RJ, Holton TA (2003) Single nucleotide polymorphisms in cytochrome P450 genes from barley. Theor Appl Genetics 106:676–682

Chiapparino E, Lee D, Donini P (2004) Genotyping single nucleotide polymorphisms in barley by tetra-primer ARMS-PCR. Genome 47:414–420

Graner A, Kota R, Perovic D, Potokina E, Prasad M, Scholz U, Stein N, Thiel T, Varshney RK, Zhang H (2004) Molecular

mapping: shifting from the structural to the functional level. In: Oral presentations. Proceedings 9th International Barley Genetics Symposium, Brno, Czech Republic, 20–26 June 2004, p 49

Gupta PK, Roy JK, Prasad M (2001) Single nucleotide polymorphisms: a new paradigm for molecular marker technology and DNA polymorphism detection with emphasis on their use in plants. Curr Sci 80:524–535

Gut IG (2001) Automation in genotyping of single nucleotide polymorphisms. Hum Mutat 17:475–492

Hamajima N, Saito T, Matsuo K, Kozaki K, Takahashi T, Tajima K (2000) Polymerase chain reaction with confronting two-pair primers for polymorphism genotyping. Jpn J Cancer Res 91:865–868

Holton TA, Christopher JT, McClure L, Harker N, Henry RJ (2002) Identification and mapping of polymorphic SSR markers from expressed gene sequences of barley and wheat. Mol Breeding 9:63–71

Imyanitov EN, Buslov KG, Suspitsin EN, Kuligina ES, Belogubova EV, Grigoriev MY, Togo AV, Hanson KP (2002) Improved reliability of allele-specific PCR. Biotechniques 33:484–490

Kanazin V, Talbert H, See D, DeCamp P, Nevo E, Blake T (2002) Discovery and assay of single-nucleotide polymorphisms in barley (*Hordeum vulgare*). Plant Mol Biol 48:529–537

Kota R, Varshney RK, Thiel T, Dehmer KJ, Graner A (2001) Generation and comparison of EST-derived SSRs and SNPs in barley (*Hordeum vulgare* L.). Hereditas 135:145–151

Kota R, Rudd S, Facius A, Kolesov G, Thiel T, Zhang H, Stein N, Mayer K, Graner A (2003) Snipping polymorphisms from large EST collections in barley (*Hordeum vulgare* L.). Mol Gen Genomics 270:24–33

Kwok S, Kellogg DE, McKinney N, Spasic D, Goda L, Levenson C, Sninsky JJ (1990) Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res 18:999–1005

Liu Q, Thorland EC, Heit JA, Sommer SS (1997) Overlapping PCR for bidirectional PCR amplification of specific alleles: a rapid one-tube method for simultaneously differentiating homozygotes and heterozygotes. Genome Res 7:389–398

Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC, Markham AF (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res 17:2503–2516

Rudd S, Mewes H-W, Mayer KFX (2003) Sputnik: a database platform for comparative plant genomics. Nucleic Acids Res 31:128–132

Sachidanandam R, Weissman D, Schmidt SC, Kakol JM, Stein LD, Mullikin JC, Mortimore BJ, Willey DL, Hunt SE, Cole CG et al (2001) A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. Nature 409:928–933

Sato K, Nankaku N, Motoi Y and Takeda K (2004) A large scale mapping of ESTs on the barley genome. In: Oral presentations. Proceedings 9th International Barley Genetics Symposium, Brno, Czech Republic, 20–26 June 2004, p 79

Schork NJ, Fallin D, Lanchbury JS (2000) Single nucleotide polymorphisms and the future of genetic epidemiology. Clin Genetics 58:250–264

Soleimani VD, Baum BR, Johnson DA (2003) Efficient validation of single nucleotide polymorphisms in plants by allele-specific PCR, with an example from barley. Plant Mol Biol Rep 21:281–288

Varshney RK, Thiel T, Stein N, Langridge P, Graner A (2002) *In silico* analysis on frequency and distribution of microsatellites in ESTs of some cereal species. Cell Mol Biol Lett 7:537–546

Wu DY, Ugozzoli L, Pal BK, Wallace RB (1989) Allele-specific enzymatic amplification of β-globin genomic DNA for diagnosis of sickle cell anemia. Proc Natl Acad Sci USA 86:2757–2760

Ye S, Dhillon S, Ke XY, Collins AR, Day INM (2001) An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Res 29:e88

Ye S, Humphries S, Green F (1992) Allele specific amplification by tetra-primer PCR. Nucleic Acids Res 20:1152

Zhang W, Gianibelli MC, Ma W, Rampling L, Gale KR (2003) Identification of SNPs and development of allele-specific PCR markers for γ-gliadin alleles in *Triticum aestivum*. Theor Appl Genet 107:130–138