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Conversion of an AFLP fragment linked to the carrot Y_2 locus to a simple, codominant, PCR-based marker form

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Abstract Recent advances have expanded the potential usefulness of molecular techniques for plant genetic research. AFLP is a powerful technique, allowing rapid and reliable analysis of multiple, potentially polymorphic sites in a single experiment. Because AFLP technology requires no a priori knowledge of genome structure or preparation of molecular probes, it is immediately useful for a wide variety of plant species. However, because AFLP markers are dominant, costly, and technologically demanding, the technique has limited application for large-scale, locus-specific uses. In carrot, the Y_2 locus controls carotene accumulation in the root xylem core. Although carrot is an important source of dietary carotene, little is known about the regulation and biosynthesis of carotenes in carrot. We identified six AFLP fragments linked to the Y_2 locus through a combination of F_2 mapping and bulked segregant analysis. We have developed a procedure for generating simple, codominant, PCR-based markers from dominant AFLP fragments using a Y_2-linked AFLP fragment as a model. Our converted marker requires only a simple PCR followed by standard agarose gel electrophoresis. It is rapid, simple, reliable, comparatively inexpensive, codominant, and non-radioactive. Conversion of AFLP fragments to forms better adapted to large-scale, locus-specific applications greatly expands the usefulness of this molecular technique.

Key words AFLP · Bulked segregant analysis · *Daucus carota* · Inverse PCR · Marker conversion

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Introduction

With recent improvements in marker technologies, molecular maps have been developed for a wide variety of important agronomic and horticultural crops (Davis and Yu 1997; Halldén et al. 1996; Jiang et al. 1997; King et al. 1998; Mudge et al. 1996; Paillard et al. 1996) and genes conditioning disease and insect resistance, yield, plant architecture, and other important traits have been mapped and/or cloned (Cai et al. 1997; Lu et al. 1996; Sourdille et al. 1996; Veldboom and Lee 1996; Warburton et al. 1996). Yet despite increasing marker availability for many species, molecular biology remains largely inaccessible for locus-specific applications requiring the characterization of large plant populations, and marker-aided selection is frequently little more than theory. This is due in part to the fact that markers used to generate linkage or genetic maps are frequently poorly adapted to other, larger scale uses. Markers that are useful must allow rapid characterization of hundreds or thousands of individuals with a minimal input of resources, including money and skilled labor. They must also be reliable with low error rates and typically should be codominant. Finally, in most instances, the markers must be non-radioactive.

The Amplified Fragment Length Polymorphism™ (AFLP; Vos et al. 1995; Zabeau and Vos 1993) is growing in popularity. Combining the specificity of restriction enzyme analysis with the ease and specificity of the polymerase chain reaction (PCR), this technique requires no genomic or cDNA library construction and no specific a priori knowledge of the plant genome. The technique is rapid and reliable, allowing the researcher to simultaneously evaluate 50 or more potential polymorphisms on a single polyacrylamide gel. For plant researchers the technique offers quick linkage map construction and rapid identification of markers linked to genes of interest. Limitations to the large-scale, locus-specific application of AFLP include the requirement

of fairly sophisticated equipment and training, relatively high costs, and a general requirement of radioactivity. Additionally, AFLPs are a dominant marker class. Schondelmaier et al. (1996) discuss some of the potential limitations of using dominant AFLP markers for plant improvement.

The Y_2 locus in carrot controls carotene accumulation in the root xylem core (Buishand and Gabelman 1979; Simon 1996). The recessive allele conditions high carotene accumulation and a bright-orange color. The exact role of Y_2 in carotene accumulation is not clear, but it is likely that it is a regulatory gene rather than in the carotene synthesis pathway itself (Buishand and Gabelman 1979). Growing interest in the health benefits of carotenes necessitates an improved understanding of carotene biosynthesis and regulation, particularly in carrot, an important source of dietary carotene.

In this paper, we report the mapping of the Y_2 carotenoid locus in carrot and the identification of six linked AFLP markers. Using one linked AFLP fragment as a model, we demonstrate a procedure to convert dominant AFLP fragments to simple, codominant PCR-based marker forms. Our procedure relies upon inverse PCR (iPCR) to characterize genomic regions adjacent to the AFLP fragment, sequence comparison of regions associated with the various alleles of interest, and the development of PCR strategies to capitalize upon genomic differences. For large-scale, locus-specific applications such conversion techniques are necessary and will become increasingly important given the popularity of the AFLP procedure, its immediate applicability to a very broad range of plant species, and its ability to rapidly identify linkages to genes of interest. Our converted marker is well-adapted to large-scale, locus-specific applications as it is reliable, rapid, simple to generate and evaluate in terms of equipment and skilled labor requirements, comparatively inexpensive, non-radioactive, and codominant.

Materials and methods

Plant materials and DNA samples

An F_2 carrot mapping population of 103 individuals was constructed from the cross B9304 × YC7262 (Vivek 1997). Morphological traits segregating in this population include high versus low carotene accumulation in the root xylem core, conditioned by the Y_2 locus (Buishand and Gabelman 1979; Simon 1996). Root xylem core color is a reliable indicator of plant genotype at the Y_2 locus and carotene levels: yellow = Y_2Y_2 or Y_2y_2 = relatively low carotene content; orange = y_2y_2 = relatively high carotene content. F_2 individuals were visually scored for root core color. F_2 leaf material was used for DNA extractions (Murray and Thompson 1980). For bulked segregant analysis (BSA; Michelmore et al. 1991), DNAs were combined based on phenotypic evaluations to create a "yellow-cored bulk" (Y_2-; 64 individual DNAs) and an "orange-cored bulk" (y_2y_2; 25 individual DNAs) such that each individual F_2 provided an equal amount of DNA to the bulks. Fourteen F_2s were not phenotypically classified and were omitted from the bulks.

Mapping and marker characterization

AFLP markers linked to Y_2 were identified by both F_2 mapping and by a combination of BSA (Michelmore et al. 1991) to identify likely candidate primer combinations and subsequent F_2 mapping. AFLP procedures were performed according to the manufacturer's (Gibco-BRL Life Technologies, Gaithersburg, Md.) instructions except that all reaction volumes were reduced to 25% of the recommended volumes. Linkage analyses were performed using MAPMAKER MacIntosh version 2.0 (Lander et al. 1987) as described by Vivek (1997). AFLP bands linked to Y_2 were excised from the dried polyacrylamide gel with a sterile razor blade and incubated in 350 µl elution buffer (0.5 *M* NH_4Ac, 10 m*M* $MgAc_2$, 1 m*M* EDTA pH 8.0, 0.1% SDS) overnight at 37°C with agitation (150 rpm). The fragments were reamplified in a reaction volume of 50 µl [5 µl eluate, 2.5 U AmpliTaq DNA Polymerase (Perkin Elmer, Branchburg, N.J., 1 × reaction buffer with 1.5 m*M* $MgCl_2$ (supplied by manufacturer), 40 µl preamp primer mix or 150 ng each of the appropriate selective primers; 94°C (30 s), 47°C (30 s), 72°C (60 s), 40 cycles total]. Twenty microliters of each of the resulting PCR products were electrophoresed through a 1% agarose gel in TAE. Following surface staining of the gel in a weak ethidium bromide solution, the appropriately sized band for each reaction was excised and placed in a sterile microfuge tube. DNA was extracted from the gel fragment using GeneClean II Kit or MERmaid Kit (Bio101, Vista, Calif.), depending on fragment size. The resulting DNA was quantified by fluorometry. To verify that the PCR products were the correct size, we end-labeled each sample with [^{33}P] (10 ng DNA, 0.2 U T4 DNA kinase, 110 kBq γ[^{33}P]-dATP, 1 × reaction buffer; 37°C for 1 h) and ran it on a polyacrylamide gel adjacent to AFLP reactions bearing the original Y_2-linked fragment.

The purified DNA fragments were cloned into bacterial plasmid pGEM-T, transformed into bacterial strain JM109, and selected according to the manufacturer's (Promega, Madison, Wis.) instructions. Plasmid DNA was extracted using Wizard Plus SV Minipreps DNA Purification System (Promega) and quantified by fluorometry. To free the insert from the plasmid, we subjected 5 µl of each sample to double restriction enzyme digestion with *Apa*I and *Sac*I (10 U each) in a total volume of 20 µl at 37°C for at least 1 h. Digested samples were electrophoresed through a 1% agarose gel in TBE, which was subsequently surface-stained and photographed. The digested DNA was transferred to Zeta-Probe nylon membrane (Bio-Rad Laboratories, Richmond, Calif.) overnight by Southern blotting (Southern 1975) in 0.4 *N* NaOH + 0.6 *M* NaCl. Southern hybridizations were performed as previously described (Vivek 1997) using reserved purified, reamplified AFLP fragments as probes. Plasmid preparations containing the appropriate insert DNA were sequenced using pUC/M13 forward and reverse primers by the University of Wisconsin Biotechnology Center (Madison, Wis.).

Inverse PCR

When possible, iPCR primers were designed from the resulting AFLP fragment sequence using DNAStar Primer Select (DNAStar, Madison, Wis.). Primers were commercially synthesized (Gibco-BRL). One microgram of genomic DNA from yellow-cored F_2 individuals was singly digested with *Hsp*92II, *Msp*I, *Sac*I, and *Taq*I according to the manufacturer's instructions. Digested DNA was desalted by spin column (Bio-Gel P-G DG, Bio-Rad Laboratories) and quantified by fluorometry. Ligation reactions (0.5 m*M* dATP, 1 × ligation buffer, and 5 U T4 DNA ligase) containing DNA concentrations of 500 ng/ml, 100 ng/ml, and 20 ng/ml were prepared for each sample and incubated for 16–18 h at 14°C. Reactions were

desalted by spin column, dried to a powder under vacuum, and resuspended in TE to a final concentration of 1 ng/µl. Inverse PCRs [0.2 m*M* each dNTP, 1.25 U TaKaRa Ex Taq (PanVera Corp, Madison, Wis.), 1 × manufacturer-supplied reaction buffer, 50 pmol each iPCR primer, 1.25 ng template DNA; 95°C for 10 m (1 cycle only) followed by the addition of Ex Taq and 35 cycles at 95°C for 40 s, 44°C or 52°C for 2 min, 72°C for 4 min] were performed in a total volume of 50 µl. Inverse PCR products were electrophoresed through a 1% agarose gel in TBE which was subsequently surface-stained, photographed, and blotted to nylon membrane overnight. Blots were prehybridized, hybridized, washed, and autoradiographed as described previously (Vivek 1997). Probes used were purified, reamplified AFLP fragments. Inverse PCR products homologous to the original AFLP fragment were purified from an agarose gel in TAE, cloned into pGEM-T, and sequenced, as described above. The resulting sequence information was used to design a series of four standard PCR primers (DNAStar Primer Select) that were subsequently used to amplify fragments from orange- and yellow-cored F_2 individuals. The resulting PCR products were gel-purified, cloned, and sequenced as described above. PCR and/or iPCR DNA sequences from both Y_2- and y_2y_2 individuals were aligned using UW-GCG (Wisconsin Package Version 9.0, Genetics Computer Group, Madison, Wis.), visually edited, and compared.

Marker conversion

DNA sequence differences between the Y_2 alleles allowed development of a codominant, PCR-based marker from an AFLP fragment generated with primer pair E-ACT × M-CAA. PCR primers flanking a 138-bp indel (insertion/deletion), the presence of which is associated with the recessive allele, were designed and synthesized. Primer sequences are 5′-TAAAGTCGTATAGGAAGAACAT-3′ (22-mer) and 5′-TGGATCATCAGAACTCAACT-3′ (20-mer). With this primer pair, bands characteristic of both Y_2 and y_2 were amplified from genomic DNA samples [50 ng genomic DNA, 50 pmol each primer, 1 U AmpliTaq DNA polymerase, 1 × manufacturer supplied reaction buffer containing 1.5 m*M* $MgCl_2$, 0.2 m*M* each dNTP; 95°C (30 s), 42°C (30 s), 72°C (60 s), 35 cycles] and visualized by standard gel electrophoresis. All F_2s evaluated were scored as Y_2Y_2, Y_2y_2, or y_2y_2 based on this marker. Representative DNAs from each genotype were Southern-blotted as described above and the resulting blots probed with the appropriate iPCR fragment. PCR fragments characteristic of both alleles were cloned and sequenced, as was an unexpected third fragment characteristic of the heterozygote. To further examine the nature of the heterozygote-specific fragment, we subjected 1:1 artificial mixtures of PCR fragments characteristic of both alleles to one PCR cycle [95°C (2 min), 42°C (30 s), 72°C (30 s)] or kept them on ice. The resulting reactions were visualized by standard gel electrophoresis, blotted to nylon membrane and subjected to Southern hybridization, as described above.

Results

Mapping of seven AFLP primer pairs (E-AAC × M-CAA, E-AAC × M-CAT, E-AAC × M-CTA, E-AAG × M-CAA, E-AAG × M-CTT, E-ACA × M-CAG, and E-ACT × M-CTG) on the F_2 mapping population revealed a single linkage of 7.6 cM between the Y_2 locus and a 73-bp fragment generated with primer pair E-AAC × M-CAA. BSA followed by mapping on individual F_2s identified an additional five AFLP fragments linked to Y_2 (generated by primer pairs E-AAG × M-CAG, E-ACT × M-CAA, E-ACT × M-CAG, E-AGC × M-CTA, and E-AGG × M-CTC) from among the remaining 57 commercially available primer pairs (Fig. 1). Linkages ranged from 3.8 cM to 15.8 cM and fragment lengths from 67 bp to 360 bp (Table 1). AFLP fragments linked to the Y_2 locus and identified with BSA were all associated with the dominant allele, as might be expected since the yellow-cored bulk contained both Y_2Y_2 and Y_2y_2 individuals (and therefore

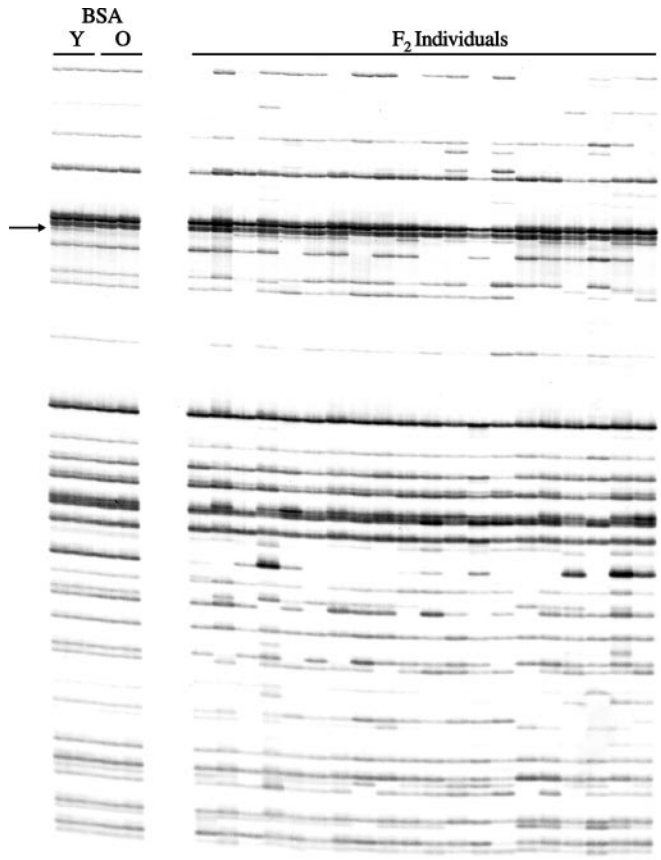

Fig. 1 Autoradiograph demonstrating carrot AFLP patterns generated with primer pair E-ACT × M-CAA for BSA bulks (*Y* yellow-cored bulk = Y_2-, *O* orange-cored bulk = y_2y_2) and 20 representative F_2 individuals. Notice the AFLP fragment linked to the Y_2 locus (*arrow*). This band is present in the Y bulk, absent in the O bulk, and segregating in the F_2s

Table 1 AFLP fragments linked to the carrot Y_2 locus

AFLP primer pair[a]	Fragment size (bp)	Distance from Y_2 (cM)[b]
E-AGC × M-CTA	102	15.8d
E-AGG × M-CTC	67	7.6d
E-AAC × M-CAA	73	7.6d
E-AAG × M-CAG	225	3.8d
E-ACT × M-CAA	264	6.6p
E-ACT × M-CAG	360	9.5p

[a] Y_2-linked fragment generated by primer pair E-ACT × M-CAA selected for marker conversion
[b] d, Distal; p, proximal

both alleles), whereas the orange-cored bulk contained only y_2y_2 individuals.

Inverse PCR amplifies DNA sequences outside of a characterized region. From a known sequence, iPCR primers are designed such that primers point away from each other into the unknown DNA sequence. Genomic DNA is then digested and ligated under conditions that favor the formation of circular DNA molecules (Collins and Weissman 1984). Consequently, the iPCR primers, which once pointed away from each other, now point towards each other around the circular molecule, and the intervening DNA is largely formerly uncharacterized sequence. DNA sequence obtained from a cloned 264-bp AFLP fragment (generated with primer pair E-ACT × M-CAA) associated with the Y_2 locus was used to generate iPCR primers. DNA regions appropriate for iPCR primer design were not identified for three of the six AFLP fragments, each of which contained less than 100 bp of genomic sequence. Likewise, a 360-bp AFLP fragment generated with primer pair E-AAG × M-CAG, with a G : C content of only 35.4%, could not be used for iPCR primer design. Our observations suggest that for most effective iPCR primer design one should begin with AFLP fragments greater than 100 bp in length with a minimum G : C content of 50%.

Template DNA preparation is a limiting step in the iPCR procedure (Ochman et al. 1993), and preparation conditions must be optimized. Restriction enzymes selected for the generation of the template must not cut within the characterized fragment. Furthermore, it is desirable that the overall iPCR product length be less than approximately 3 kb. It is possible to characterize restriction site patterns around the AFLP fragment of interest by Southern blotting using the cloned AFLP fragment as a probe (not shown), but it is time-consuming and costly to do so. Hartl and Ochman (1996) suggest that the use of a single, random restriction enzyme with a recognition sequence of 4 bp will result in successful iPCR amplification 50% of the time. We arbitrarily chose one 6-bp-cutting (*Sac*I) and three 4-bp-cutting (*Hsp*92II, *Msp*I, and *Taq*I) restriction enzymes for iPCR template generation. Amplification products were generated from both *Hsp*92II and *Msp*I templates but not from *Sac*I or *Taq*I templates. Products of the former reactions were shown to be the desired products by Southern hybridization to the original AFLP fragment (Fig. 2). Successful iPCR also requires optimization of template DNA ligation conditions. Ochman et al. (1993) suggest overnight ligations at 14°C and three different genomic DNA concentrations: 500 ng/ml, 100 ng/ml, and 20 ng/ml. We found that all three of these concentrations yielded the desired template molecules.

From a 264-bp fragment generated with AFLP primer pair E-ACT × M-CAA, nearly 2.0 kb of additional DNA sequence was obtained via iPCR (Fig. 3). Using that iPCR sequence, which was obtained from

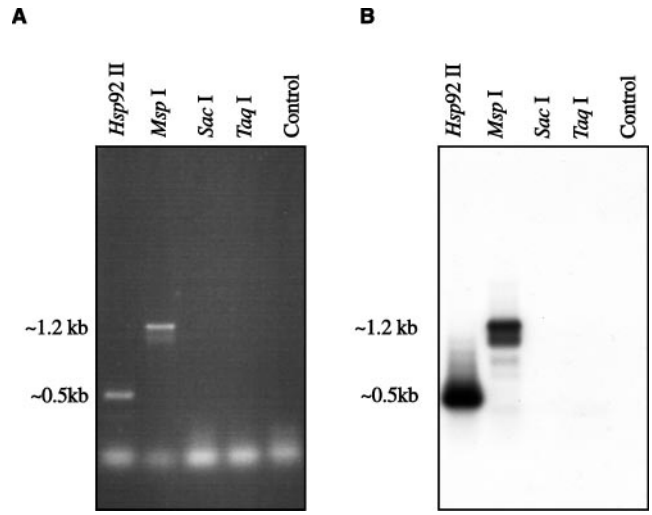

Fig. 2A, B Photograph of an agarose gel (**A**) and the corresponding autoradiograph (**B**) illustrating results of iPCR using primers generated from the Y_2-linked AFLP fragment in Fig. 1. Template DNA is carrot total genomic DNA from a yellow-cored (Y_2-) F_2 individual. Different lanes indicate restriction enzymes used to generate template DNA: *Hsp*92II, *Msp*I, *Sac*I, and *Taq*I. Digested DNA was ligated into circular form prior to iPCR. "Control" lane is a negative control iPCR reaction lacking template DNA. Fragments generated were shown to be homologous to the AFLP fragment by Southern hybridization

regions associated with Y_2, we designed a series of four standard PCR primers and amplified 1.2 kb of DNA associated with y_2 (Fig. 3). The amplification products were cloned and sequenced.

The nature of genomic polymorphisms responsible for AFLPs will differ from fragment to fragment and can alter the way in which one approaches marker conversion. For the Y_2-linked AFLP fragment generated with primer pair E-ACT × M-CAA, opportunity existed to develop a simple PCR-based converted marker form. Only point mutations were observed between the dominant and recessive Y_2 alleles in the region of the AFLP fragment itself. However, adjacent to that region was a 138-bp indel, the presence of which was associated with the recessive (y_2) allele. Primers flanking this indel amplified a 172-bp fragment characteristic of the Y_2 allele and a 310-bp fragment characteristic of the y_2 allele (Fig. 3).

The converted marker was used to characterize 93 F_2 individuals from the carrot mapping population (Fig. 4). All individuals could be confidently classified as Y_2Y_2, Y_2y_2, or y_2y_2 using this assay. Although observed data failed to fit the expected 1 : 2 : 1 segregation for these genotypes ($P = 0.125$), skewed marker segregation is likely a result of skewed gene segregation. Vivek (1997) noted that approximately 25% of all marker loci tested in this same carrot mapping population failed to fit expected segregation ratios. Furthermore, the observed root core phenotypes failed to fit the expected 3 : 1 segregation ($P = 0.645$). Until F_3 material is available for study, we cannot confirm that

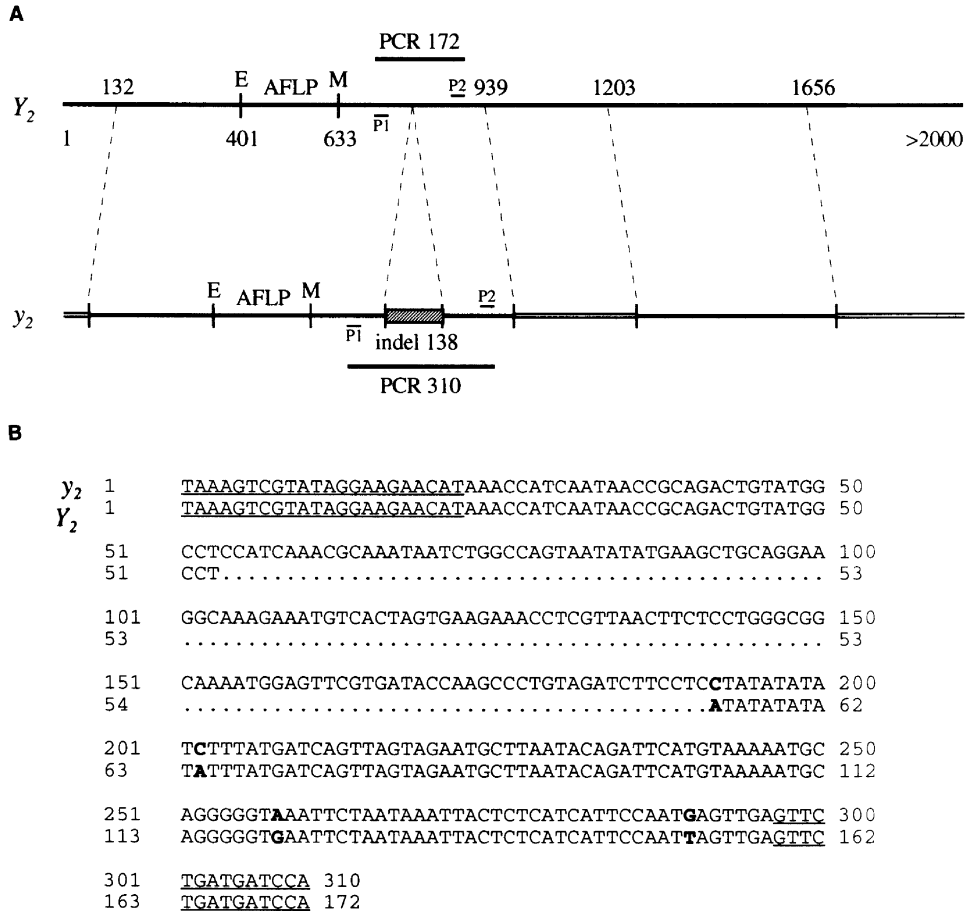

Fig. 3A, B **A** Schematic of more than 2.0 kb of genomic sequence generated from the regions adjacent to the Y_2 allele (*top*). *AFLP* indicates the location of the genomic portion of the original AFLP fragment shown in Fig. 1. The region is flanked by *Eco*RI (*E*) and *Mse*I (*M*) restriction enzyme sites. The DNA sequence generated via iPCR from the Y_2 allele was used to design a series of standard PCR primers which amplified 1.2 kb from regions adjacent to the y_2 allele (*solid lines*, *bottom*). *Open lines* indicate regions not characterized for the y_2 allele. *Hatched box* indicates a 138-bp indel linked to the Y_2 locus. Its presence is characteristic of the y_2 allele. Standard PCR primers (*P1* 20-mer, *P2* 22-mer) flanking the indel generated a 172-bp fragment from regions linked to the Y_2 allele and a 310-bp fragment from regions linked to the y_2 allele. The result is a simple PCR-based marker linked to the Y_2 locus. **B** PCR fragment sequences of the converted marker

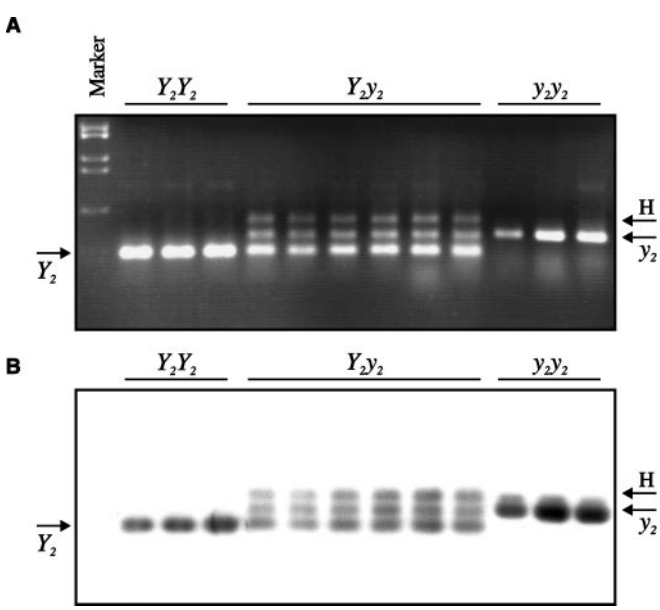

Fig. 4A, B **A** Photograph of an agarose gel illustrating PCR products generated from genomic DNA of 12 representative F_2 individuals using the described PCR assay. Bands labeled are Y_2-specific, y_2-specific, and heterozygote-specific (*H*) fragments. **B** Corresponding autoradiograph using iPCR product as a probe for Southern hybridization. It demonstrates that each of the three PCR products are homologous to each other and to the genome region in question

our genotypic classifications based on the converted marker are correct. However, as might be expected, predictions of plant phenotypes are identical for both the converted marker and its parent AFLP marker, and both markers map to the same location (6.6 cM away from the Y_2 locus).

Interestingly, in addition to Y_2- and y_2-associated fragments, the newly developed assay generates a third, seemingly larger PCR band that is specific to the heterozygote (Fig. 4). This slower moving band is a heteroduplex resulting from one Y_2-associated DNA strand pairing with one y_2-associated DNA strand. Although the strands from the different alleles are homologous for a large portion of their length, the presence of the indel on the y_2-associated strand results in conformational change in the heteroduplex. This change results in retarded migration through an agarose gel, giving the appearance of a larger DNA fragment. Artificial 1:1 mixtures of the Y_2- and y_2-associated fragments resulted in the generation of the heteroduplex band if the mixture is first heat-denatured and then allowed to reanneal. Unheated samples, in contrast, showed no or very little heteroduplex band formation (Fig. 5). Southern hybridization using the appropriate iPCR fragment as a probe revealed that the three bands observed in this renaturation experiment are homologous (not

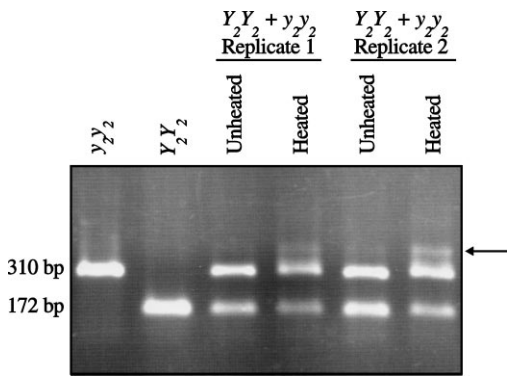

Fig. 5 Photograph of an agarose gel showing results of the reannealing experiment (see Materials and methods). The *first two lanes* are PCR products generated from genomic DNA using the described PCR assay. Predicted genotypes are y_2y_2 and Y_2Y_2 as shown by the presence of a single, allele-specific band in each of these lanes. *Arrow* shows the location of the heterozygote-specific PCR fragment. Artificial 1 : 1 mixtures of y_2y_2 and Y_2Y_2 PCR products result in little if any of the heterozygote-specific product if the mixtures are kept on ice (*lanes 3 and 5*). If the same samples are subjected to a single round of denaturation and reannealing, the heterozygote-specific band is visible (*lanes 4 and 6*), suggesting that the seemingly larger heterozygote-specific band is actually a heteroduplex resulting from one strand of the Y_2-specific product and one strand of the y_2-specific product

shown). Additionally, cloned heteroduplex bands yielded plasmid inserts of only two sizes: 172 bp (identical in size to the Y_2-associated insert) and 310 bp (identical in size to the y_2-associated insert). As expected, the DNA sequence of the smaller and larger heteroduplex inserts matched exactly those of the Y_2- and y_2-associated inserts, respectively. In no case did the heteroduplex yield an insert size larger than the y_2-associated insert. The presence of this band in a PCR sample serves as an added confirmation of heterozygosity.

Discussion

Popular DNA marker technologies include restriction fragment length polymorphism (RFLP; Botstein et al. 1980), random amplified polymorphic DNA (RAPD; Williams et al. 1990), and simple sequence repeat (SSR; Tautz 1989; Weber and May 1989). While each of these marker classes has important applications for plant genetic research, limitations exist for large-scale, locus-specific use. The RFLP is perhaps among the most reliable of DNA markers, and the molecular basis of polymorphisms is well understood. RFLP markers are generally codominant. However, the RFLP procedure is time-consuming, costly, and requires fairly sophisticated equipment and training. The need for genomic or cDNA library construction or other methods for obtaining RFLP probes adds substantially to the cost and complexity of the procedure and limits its usefulness for less well-characterized plant species. Additionally, RFLPs are often detected through the use of radioactive material, although non-radioactive alternatives do exist. In contrast to RFLPs, RAPDs are simple, requiring only a single PCR and standard agarose gel electrophoresis. No probe sources are necessary, and there is no need for a priori knowledge of genome structure, meaning RAPDs can be used immediately for any plant species. Furthermore, RAPDs are comparatively inexpensive and are non-radioactive. Limitations for large-scale, locus-specific use of RAPD markers arise from their generally dominant nature and frequent difficulties with repeatability and reliability (Weeden et al. 1992; Bradeen and Havey 1995; Staub et al. 1996). Like RAPDs, SSRs require only a simple PCR followed by agarose gel electrophoresis, are less expensive than RFLPs, and require no radioactivity. SSRs offer the added advantages of a generally codominant nature and improved reliability. Once identified, SSRs are well-adapted to large-scale, locus-specific applications. However, the identification of SSRs requires some knowledge of the plant genome or, more specifically, regions containing runs of oligonucleotide repeats. Modifications of procedures to identify SSRs may make the marker class available to a broad range of plant species (Wu et al. 1994; Zietkiewicz et al. 1994; Brown et al. 1996).

Attempts have been made to convert poorly adapted molecular markers to forms useful for large-scale, locus-specific applications. For example, RAPD markers have been successfully converted to sequence characterized amplified region (SCAR) markers (Garcia et al. 1996; Naqvi and Chattoo 1996). These markers are generated by sequencing the ends of RAPD bands shown to be linked to genes of interest. The resulting DNA sequence information is used to generate longer, more specific PCR primers that amplify only the product of interest. In some cases codominant SCARs have been developed from dominant RAPD markers (Adam-Blondon et al. 1994; Paran and Michelmore 1993; Williamson et al. 1994).

In attempting to convert AFLP markers to codominant, simple PCR forms, the SCAR approach (generating PCR primer pairs from AFLP fragments) is intuitive. However, there are reasons why this approach will not be generally successful. Chiefly, while RAPD products may be 500–1500 bp in length, the average size of AFLP fragments of interest identified in this study was less than 182 bp, including 32 bp contributable to the *Eco*RI and *Mse*I adapters. That means our average AFLP fragment consisted of less than 150 bp of genomic sequence. Even if differences existing between regions linked to Y_2 and y_2 allowed codominant PCR-based marker development, it is unlikely that appropriate PCR primers could be designed from such short DNA sequences or that the resulting PCR products could be distinguished by standard agarose gel electrophoresis. Consequently, it was necessary to consider a different approach, beginning with the

generation of larger tracks of DNA sequence. Inverse PCR was the key to our success in converting the Y_2 marker. Further refinement of the iPCR approach should allow a more standardized and routine generation of sequence information outside of the AFLP fragment.

The health benefits of carotenes are of increasing interest. β-carotene is an important dietary precursor of vitamin A, and carrots account for 30% of the vitamin A in the average American diet (Simon 1992). Carotenes have also been implicated in the prevention of cancer and heart disease and as enhancers of immune function (reviewed in Stavric 1994). Ironically, while genes involved in carotene synthesis have been characterized from several plant species including tomato, pepper, and corn (eg. Buckner et al. 1990; Bartley et al. 1992; Kuntz et al. 1992; Römer et al. 1993; Buckner et al. 1996), little is known about carotene synthesis and regulation in carrot, despite its importance.

High-carotene carrots arose in cultivation. Wild and feral carrots are frequently white or pale-yellow in color, lacking discernible amounts of carotene. Banga (1957) studied changes in carrot color throughout cultivation by examining European art depicting contemporary carrots. He concluded that orange carrots were selected from yellow types and first arose in the Netherlands in the 17th and 18th Centuries. Changes in carrot pigmentation under domestication probably were the result of human selection for more "attractive" phenotypes. The Y_2 locus may have played an important role in the origin of high-carotene carrots and is of great importance to modern day, high-carotene cultivars. The converted PCR marker we developed for Y_2 will be useful for characterizing wild and feral carrot populations and historic carrot cultivars. By an examination of the allelic diversity and frequency at the Y_2 locus in these populations it may be possible to better understand what role Y_2 played in the origin of the orange carrot.

Other important uses of our converted marker include mapping and genetic analyses, including aiding the development of populations for these applications. Finally, although carrot core color is easily visually scored, suggesting a molecular marker for Y_2 may be of limited use for plant breeding, our converted marker could prove useful for selection of Y_2Y_2 homozygotes from heterozygous backcross progeny in introgression programs.

Molecular markers continue to play an important role in plant genetic research. While the AFLP is well-adapted to linkage mapping and diversity assessment, it is poorly adapted to large-scale, locus-specific uses. The conversion procedure we have developed resulted in an AFLP-derived, PCR-based marker for the Y_2 locus that is simple, rapid, reliable, comparatively inexpensive, non-radioactive, and codominant. As AFLP technology continues to grow in popularity, conversion procedures will expand the usefulness of this marker class. Our converted marker for Y_2 serves as an important model for conversion procedures.

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