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ORIGINAL PAPER

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Development of a codominant PCR-based marker for allelic selection of the pink trait in onions (*Allium cepa*), based on the insertion mutation in the promoter of the anthocyanidin synthase gene

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Abstract Bulb color in onions (*Allium cepa*) is an important trait and is inherited in a complex manner. However, the mechanism of color inheritance is poorly understood at the molecular level. A previous study showed that pink bulb color in onions is inherited as a single recessive trait. This trait is attributable to a significantly reduced transcription of the anthocyanidin synthase (*ANS*) gene. In this study, we developed a PCR-based marker for an allelic selection of the *ANS* gene to avoid the laborious progeny tests traditionally employed. To identify polymorphisms between pink and red alleles of the *ANS* gene, promoter sequences of both alleles were isolated. There was 97% nucleotide sequence identity between the promoter sequences of the two alleles. A 390-bp insertion was identified 632 bp upstream from the putative transcription start site in the pink allele. A pair of primers was designed on the flanking sequences of the inserted region and utilized as a PCR-based marker for allelic selection of the *ANS* gene. The reliability of the marker was tested using parents, F_1 hybrids, and F_3 lines whose genotypes had been identified by progeny tests. The marker was also used to evaluate the distribution of the pink allele in white and yellow breeding lines. The results indicated that a majority of the breeding lines tested were homozygous recessive.

Introduction

Bulb color in onions (*Allium cepa*) is a significant trait and has been used as a major category for classifying onion cultivars as white, yellow, or red onions. Bulb colors are inherited in a complex manner involving epistatic interaction among several loci. Five major loci, determining qualitative color differences, have been reported (Clarke et al. 1944; Davis and El-Shafie 1967; Reiman 1931).

The *I* locus, a color inhibitor, is a dominant factor. Colors can develop only when the genotype of the *I* locus is homozygous recessive (*ii*). Otherwise, bulb color remains white. Onions developing from these bulbs are known as dominant white onions. The *C* locus is a basic color factor, of which at least one dominant allele is required for any color development. When the *C* locus is in a homozygous recessive state (*cc*), bulb color is white regardless of the genotype of other loci. The white onions that result from these bulbs are called recessive white onions. The third locus, *G*, yields chartreuse bulb color when its genotype is homozygous recessive. The last two loci, *L* and *R*, are complementary factors, both of which are required for red color development (Davis and El-Shafie 1967).

Compounds responsible for bulb color in onions are known as flavonoids. Red color is attributed to cyanidin derivatives, particularly anthocyanidins (Fuleki 1971; Fossen et al. 1996). Flavonoids are a secondary metabolite in plants and are involved in UV protection, plant–microbe interaction, and fertility (Zeback et al. 1989; Li et al. 1993; Shirley 1996). The role of flavonoids in pigmentation is most conspicuous and results in a variety of colors in flowers and fruits. In addition, flavonoids have become an attractive research area as their health-promoting roles such as antioxidant agents have been revealed (Cook and Samman 1996; Keli et al. 1996; Knekt et al. 1996; Braca et al. 2002; Bastianetto and Quirion 2002; Kobayashi et al. 2002)

The anthocyanin biosynthesis pathway has been extensively studied, and most genes encoding enzymes in the pathway have been isolated in many species (Fig. 1). Genes involved in the anthocyanin synthesis pathway are conveniently classified into two groups. The first contains the structural genes encoding enzymes in the

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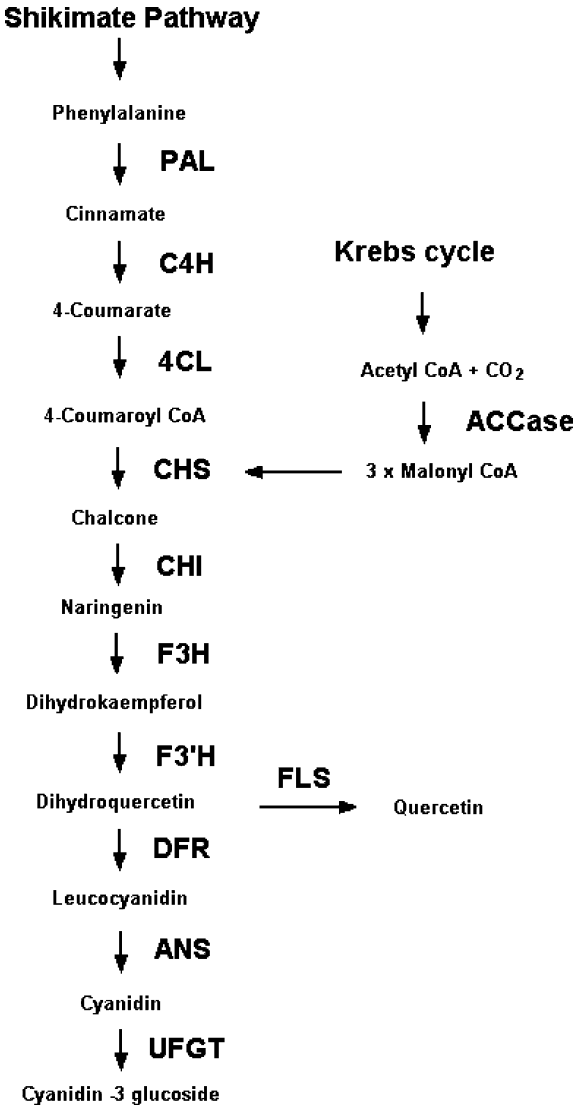

Fig. 1 Scheme of the anthocyanin biosynthesis pathway in onions. This pathway was based on reports that quercetin derivatives are the most abundant flavonoid (Rhodes and Price 1996), and cyanidin derivatives are the major anthocyanin in red onions (Fossen et al. 1996). *PAL* Phenylalanine ammonia lyase, *C4H* cinammate 4-hydroxylase, *4CL* 4-coumaroyl-coenzyme A ligase, *ACCase* acetyl CoA carboxylase, *CHS* chalcone synthase, *CHI* chalcone isomerase, *F3H* flavanone 3-hydroxylase, *F3′H* flavonoid 3′-hydroxylase, *DFR* dihydroflavonol 4-reductase, *ANS* anthocyanidin synthase, *FLS* flavonol synthase, *UFGT* UDP glucose-flavonoid 3-*o*-glucosyl transferase

pathway. The second contains regulatory genes that control the expression of the structural genes in response to environmental stimuli, such as light and temperature. Mutations in either structural or regulatory genes can produce mutants with missing or reduced pigmentation (Goodrich et al. 1992; Quattrocchio et al. 1993; Holton and Cornish 1995; Spelt et al. 2000; Endt et al. 2002; Bharti and Khurana 2003; Yamazaki et al. 2003).

Few studies on bulb color inheritance at the molecular level have been carried out in onions. Epistatic interaction among loci suggests that these loci are sequentially involved in a pigment synthesis pathway. Since pigments of bulb colors are known to be flavonoid compounds, different bulb colors may result from mutations in the genes involved in the anthocyanin biosynthesis pathway. In fact, a previous study showed that inactivation of the dihydroflavonol 4-reductase (*DFR*) gene in the yellow onion results in a lack of anthocyanin production (Kim et al. 2004b).

A new pink bulb color trait that is conditioned by a single recessive gene, the *Pink* (*P*) locus, was reported in the previous study. A significantly reduced transcription of the anthocyanidin synthase (*ANS*) gene was responsible for the pink trait (Kim et al. 2004a). Pink is considered an unwanted trait in red onion breeding programs. Unfortunately, a pink trait might persist in populations when a very low gene frequency of the recessive *P* locus exists, since the trait is inherited as a recessive trait. In addition, the phenotype of the *P* locus is visually undetectable in yellow and white onions in which earlier steps in the anthocyanin pathway are disabled. The presence of a homozygous recessive *P* locus would be a barrier to crossing among differently colored onions. Therefore, a molecular marker for the *P* locus would be a very useful breeding tool. Onion is a biennial crop and, at this time, time-consuming progeny tests are required for genotyping the *P* locus without molecular markers.

In this study, promoter sequences of both pink and red alleles of the *ANS* gene were isolated from onions. Significant mutations in a pink allele causing a reduction in *ANS* gene transcription were identified. A co-dominant PCR-based marker was developed based on a 390-bp indel mutation in the promoter sequence.

Materials and methods

Plant materials

A cross was made between a yellow male-sterile breeding line (‘506L’) and a red doubled haploid line (‘H6’). An F_2 population was produced from F_1 hybrids. The color of the F_2 population segregated with a ratio of 3 red to 1 yellow. Two hundred nine fertile F_2 plants were self-pollinated to produce F_3 families for the progeny test and to identify the gene determining the color difference. There were three groups of F_3 families: all yellow, segregating with yellow and red, and all-red F_3 plants. Ten specimens of all-red F_3 lines were planted in the field to evaluate a variation of red intensity between lines. One of ten lines was homogeneous pink, and four lines showed homogenous dark-red bulb color. Five lines yielded a mixture of pink and red onions. A detailed description of the pedigree was described by Kim et al. (2004a). A pink F_3 line and a homogeneous dark-red F_3 line were used as plant materials for promoter cloning. A PCR-based marker was developed and tested with parents, F_1 hybrids, and F_3 families.

Sixteen white and yellow breeding lines and two commercial cultivars ('Texas Legend' and 'Texas Early White') were used to evaluate the distribution of the pink allele in our breeding lines, using the PCR-based marker developed in this study.

DNA extraction and PCR amplification

Total genomic DNA was extracted from a bulk sample of approximately 20 two-leaf stage seedlings of F_3 lines, using a commercial DNA extraction kit (DNeasy Plant Mini Kit, QIAGEN, Valencia, Calif., USA). The primers for the amplification of the onion *ANS* gene were designed based on the genomic sequence of onion *ANS* alleles (GenBank accession numbers: red *ANS* allele, AY585677 and pink allele, AY585678). All primers for the PCR amplification of genomic DNA were mostly 27-mers with T_m values of higher than 70°C. PCR reactions were performed in a 50-µl reaction mixture containing 0.05 µg template, 5 µl 10× PCR buffer, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 1 µl dNTP (10 mM), and 1 µl polymerase mix (Advantage 2 polymerase mix, Clontech, Palo Alto, Calif., USA). PCR amplification was carried out with an initial denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min, and then a final 10-min extension at 72°C.

Isolation of *ANS* gene promoter sequences and promoter analysis

The isolation of a promoter region of ANS genes was carried out using a commercial genome walker kit (Universal GenomeWalker Kit, Clontech, Palo Alto, Calif., USA) according to the manufacturer's instructions. Both homogeneous pink and dark-red F_3 lines were used as plant materials for constructing genome-walking libraries. The PCR-amplified promoter regions were directly sequenced via primer walking.

Sequencing of PCR products

The PCR products were purified with a PCR-product purification kit (QIAquick PCR Purification kit, QIAGEN) after confirming that a clear, single band was amplified when run on a 1% agarose gel. The purified PCR products were directly used as templates for sequencing reactions and sequenced by automated Big Dye DNA cycle sequencing (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits, Applied Biosystems, Foster City, Calif., USA) by the LPGT (Lab for Plant Genome Technologies) core sequencing facility at Texas A&M University, with a capillary sequencer (ABI 3100 Genetic Analyzer, Applied Biosystems).

Results

Isolation of *ANS* promoter from onion genomic DNA

From the putative transcription start site of the promoter region of the onion *ANS* gene, 1,193 bp were isolated using a genome walker kit. There was 97% nucleotide sequence identity between pink and red allele promoter sequences. However, a 390-bp insertion was identified 632 bp upstream from the putative transcription start site in the pink allele promoter (Fig. 2). Except for this insertion, only point mutations were distributed throughout the promoter. Therefore, this insertion is likely to have a significant effect on the transcription of the *ANS* gene in pink onions.

Development of a PCR-based marker for an allelic selection of the *ANS* gene

Two primers binding to the flanking sequences of the inserted region were designed to be used as a PCR-based marker for allelic selection of the *ANS* gene at the *P* locus (Fig. 3a). The reliability of the marker was tested using parents, F_1 hybrids, and F_3 lines whose genotypes of the *P* locus were identified by progeny tests in the previous study (Kim et al. 2004a). Bulk DNA of approximately 20 seedlings of each F_3 line was used as a template. The result demonstrated the perfect identity of the marker with the genotypes of the *P* locus in the pedigree (Fig. 3b). A faint band between large and small bands was detected in all heterozygous genotypes. This band turned out to be a heteroduplex. Equal amounts of the large and small bands amplified from each parent were mixed, denatured at 94°C for 3 min, and reannealed, as described by Bradeen and Simon (1998). As a result, the heteroduplex displayed the same band pattern

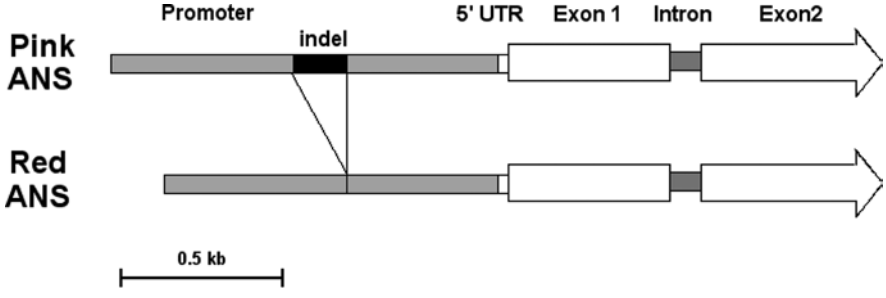

Fig. 2 Onion anthocyanidin synthase (*ANS*) gene structure of the pink and red alleles. The insertion in the pink allele promoter region is shown as a *black box*. The *vertical line* on the red allele promoter indicates the position of the indel region. *Arrow-shaped boxes* indicate a 5′-to-3′ direction

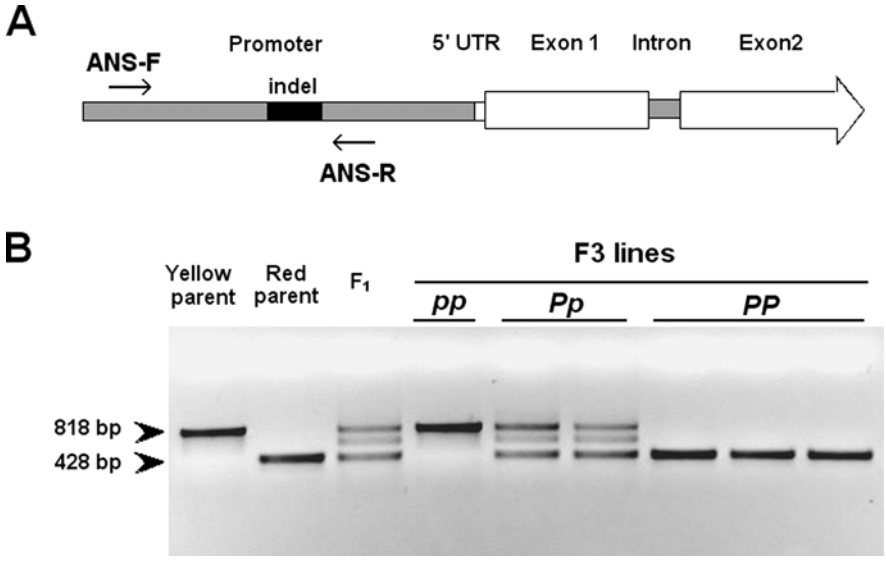

Fig. 3 Design of the PCR-based marker and its reliability test, using populations whose genotypes were already identified by progeny tests. **a** The position of the primer pair that was used as a PCR-based marker: ANS-F (TTTGCTCGATCGTTTAGCRGAAGAAGA), ANS-R (TGAGGATGATGACAAAGTTAGCGGAGCA). **b** PCR products run on 1% agarose gel. *P* Locus responsible for the pink trait in onions

as heterozygous types. On the contrary, the mixture of the two bands without denaturation resulted in two distinct bands with no heteroduplex band (data not shown).

Evaluation of a distribution of the pink allele in white and yellow breeding lines and commercial cultivars

The PCR-based marker developed in this study was used to evaluate the distribution of the pink allele in our white and yellow breeding lines and commercial cultivars. DNA extracts of 20–30 10-day-old seedlings of each line were used as templates. Clear bands of expected sizes were amplified in all samples. The result showed that the majority of white or yellow breeding lines were homozygous recessive for the *P* locus. Only one line (34133) out of 16 was heterogeneous. In this line, a high frequency of the pink allele seemed to be present, since the intensity of the bigger band was higher than that of the smaller one. A white commercial cultivar, 'Texas Early White', was also heterogeneous for the *P* locus. Only one breeding line (33048) was homozygous dominant for the *P* locus (Fig. 4). This result also suggests that this PCR-based marker could be applied to the screening of a broad range of breeding lines.

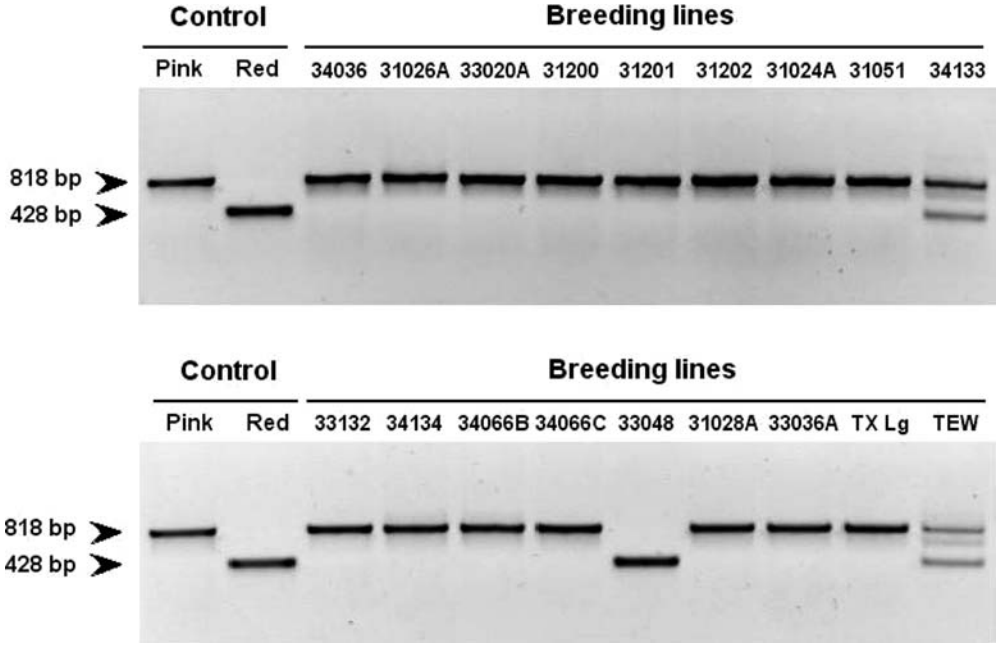

Fig. 4 Genotyping of white and yellow breeding lines and two commercial cultivars, using the developed PCR-based marker. *Control* PCR products amplified from the both parents in Fig. 3, *Tx Lg* Texas Legend, *TEW* Texas Early White

Discussion

Isolation of promoter sequences of the *ANS* gene from onions

The promoter sequences of the onion *ANS* gene were efficiently isolated using a commercial genome walker kit. The huge genome size of the onion [17.9 pg or 15,290 mega base pairs per 1C, which is 107 times bigger than that of *Arabidopsis* (King et al. 1998)] renders a genome walker kit more efficient than construction of a genomic library for isolating promoter regions.

The major purpose of this study was the development of a reliable PCR-based marker for allelic selection. A 390-bp insertion was identified approximately 600 bp upstream from the putative transcription start site of the pink allele of the *ANS* gene, which distinguished it from the red onion *ANS* allele. Elucidation of the exact role of this insertion is beyond the scope of this study. However, this insertion may affect protein–protein interactions among transcription factors or disrupt a specific promoter region, for example, a binding site for transcription factors.

We did not confirm that this insertion is the exact cause of reduced transcription of the *ANS* gene in the pink allele. In the case of insertion mutations, transposable elements are frequently inserted and disrupt a function of the gene. In this case, no sequence showed homology with the inserted sequence. In addition, this sequence did not have any characteristic terminal repeat sequences of transposable elements. For example, an insertion of an 876-bp transposable element (*dTdic1*) with 7-bp terminal inverted repeats within anthocyanin biosynthesis genes encoding DFR and chalcone isomerase resulted in disruption of those genes in carnations (Itoh et al. 2002). Alternatively, it is possible that the 390-bp sequence was deleted in the red allele promoter, causing over-expression of the *ANS* gene in red alleles.

Development of a co-dominant PCR-based marker for an allelic selection of the *ANS* gene

Molecular markers for important traits are especially useful for onion breeding, since the onion is a biennial crop. Thus, utilizing molecular markers makes it possible to reduce substantially the breeding period. A molecular marker for an allelic selection of the *P* locus was developed in this study. Pink color is considered an undesirable trait in red onion breeding but, because of its recessive inheritance, cannot be eradicated by simply discarding the pink onions. In the case of open-pollinated varieties, most pink alleles exist in heterozygous plants, as indicated by Falconer and Mackay (1996), and very few homozygous individuals appear in a population. Therefore, a molecular marker for the pink allele would be a very useful tool for removing the pink allele from red onion breeding lines.

The developed marker is equally useful for selecting white or yellow parents when crosses are made with red onions to transfer important traits such as disease resistance. If the white or yellow onion had a pink allele, a pink trait would segregate in the segregating populations. Enzymatic steps before ANS in the anthocyanin synthesis pathway are disabled in white or yellow onions. Therefore, the genotypic composition of the *P* locus cannot be detected until crosses are made with red onions and their F_2 progenies are analyzed. By utilizing our PCR-based marker, such laborious crossing and progeny tests could be avoided for genotyping the *P* locus. In fact, most of the white or yellow breeding lines tested in this study were shown to contain homozygous recessive genotypes for the *P* locus (Fig. 4).

For breeding purposes, molecular markers should allow analysis of a large number of samples with minimal cost and basic skills. For reliable results, the molecular marker should be PCR-based and co-dominant. The molecular marker developed in this study meets these requirements. In addition, the developed marker is a direct marker, not a linked marker, for the gene responsible for the targeted phenotype. Few direct PCR-based markers have been developed. Most PCR-based markers were developed by conversion of AFLP markers (Bradeen and Simon 1998; Mienie et al. 2002) or RFLP markers (Arru et al. 2003) linked to a locus responsible for a specific trait. Since the marker developed in this study is a direct marker, recombination between a locus and the marker is unlikely to occur. Therefore, the marker can be applied to diverse plant materials. The unique marker developed in this study for the allelic selection of the *P* locus would be a potent tool for onion breeding programs.

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