Theor Appl Genet (2005) 111: 120–127
DOI 10.1007/s00122-005-2000-1

ORIGINAL PAPER

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The *L* locus, one of complementary genes required for anthocyanin production in onions (*Allium cepa*), encodes anthocyanidin synthase

Received: 29 November 2004 / Accepted: 8 March 2005 / Published online: 26 April 2005

Abstract Bulb color in onions (*Allium cepa*) is an important trait, but its complex, unclear mechanism of inheritance has been a limiting factor in onion cultivar improvement. The identity of the *L* locus, which is involved in the color difference between Brazilian yellow and red onions, is revealed in this study. A cross was made between a US-type yellow breeding line and a Brazilian yellow cultivar. The segregation ratio of nine red to seven yellow onions in the F_2 population supports the involvement of two complementary genes in anthocyanin production in the F_1 hybrids. The high-performance liquid chromatography (HPLC) and reverse-transcriptase (RT)-PCR analysis of the Brazilian yellow onions indicated that the genes are involved late in the anthocyanin synthesis pathway. The genomic sequence of the anthocyanidin synthase (ANS) gene in Brazilian yellow onions showed a point mutation, which results in an amino acid change of a glycine to an arginine at residue 229. Because this residue is located adjacent to a highly conserved iron-binding active site, this mutation is likely responsible for the inactivation of the ANS gene in Brazilian yellow onions. Following the isolation of the promoter sequence of the mutant allele, a PCR-based marker for allelic selection of the ANS gene was designed. This assay is based on an insertion (larger than 3 kb) mutation. The marker perfectly co-segregated with the color phenotypes in the F_2 populations, thereby indicating that the *L* locus encodes ANS.

Communicated by I. Paran

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Introduction

Onion (*Allium cepa*) bulb color is a major trait in breeding and a basic criterion for cultivar classification. Homogeneous bulb coloration is as important as uniform bulb shape. However, because of its complex genetic segregation, bulb color has become a barrier to crossing differently colored onion cultivars. This results in a limitation of transferring important traits, such as mildness and disease resistance, among differently colored onion cultivars. Therefore, an understanding the molecular mechanism(s) governing onion bulb color inheritance is of great importance.

Flavonoids are very common plant secondary metabolites that have been reported in various forms and modifications in numerous plant species. Thousands of different flavonoids are known to exist, and it has been proposed that they perform several functions, such as UV-protection, plant-microbe interaction, fertility, and pigmentation (Shirley 1996). Flavonoids have recently been shown to function as antioxidant agents, thereby inducing health-promoting effects (Cook and Samman 1996; Keli et al. 1996; Knekt et al. 1996; Braca et al. 2002). The best-characterized function of the flavonoids is pigmentation. The pigments of a variety of flowers, fruits, and vegetables are due to specific flavonoids known as the anthocyanin compounds. One example of this is the red color of certain onion bulbs, which is attributable anthocyanin (Fossen et al. 1996; Rhodes and Price 1996).

The anthocyanin synthesis pathway is a well-characterized biochemical pathway (Fig. 1). Nearly all of the structural genes that encode the enzymes involved in this pathway have been identified in many different species (Holton and Cornish 1995). Other regulatory genes controlling the expression of the structural genes in response to external or internal stimuli, such as light, temperature, or wound stress, have also been identified in some species; for example, petunia, Arabidopsis, and maize (Goodrich et al. 1992; Quattrocchio et al. 1993;

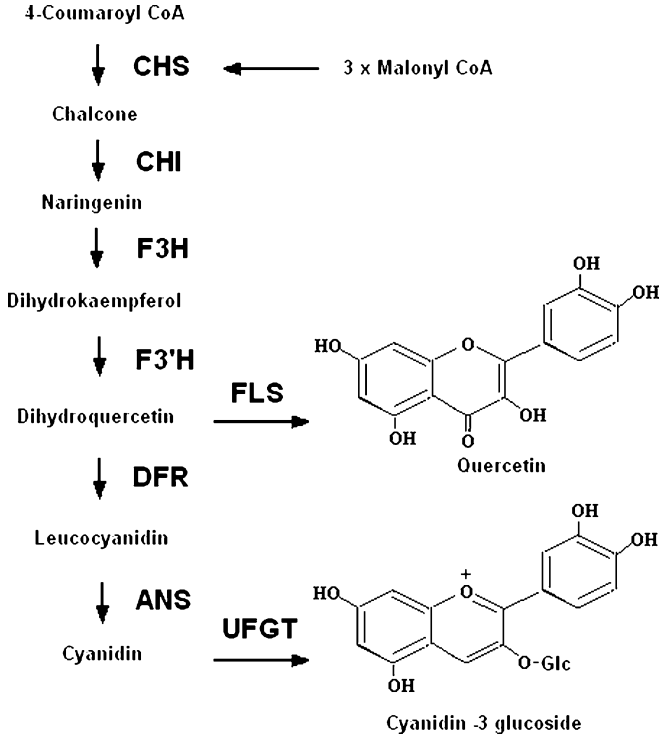

Fig. 1 Anthocyanin biosynthesis pathway in onions. This pathway was inferred on the basis of reports that quercetin derivatives are the most abundant flavonoid (Rhodes and Price 1996) and that cyanidin derivatives are the major anthocyanin in red onions (Fossen et al. 1996). *CHS* Chalcone synthase, *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

Spelt et al. 2000; Endt et al. 2002; Bharti and Khurana 2003; Yamazaki et al. 2003). The regulatory genes typically encode proteins with a basic helix-loop-helix (bHLH), MYB, or WD40 repeat domains (Yamazaki et al. 2003). Mutations in either the structural or regulatory genes result in changes in the color of the bulb or a lack of pigmentation. In fact, many natural color variants of flowers and fruits are due to such mutations (Holton and Cornish 1995).

There are varieties of onion in which the bulb colors range from white to yellow and red. The intensity and tone of the yellow and red colors also vary in different cultivars and in response to environmental effects. The inheritance of bulb color is complex with epistatic interaction among several major loci. To date, five loci involved in qualitative color differences have been reported (Clarke et al. 1944; Davis and El-Shafie 1967; Reiman 1931). The *I* locus, which encodes the color-inhibiting factor, is incompletely dominant over the recessive allele so that in the homozygous dominant state (*II*), the bulb color is white regardless of the other loci, while in the heterozygous state (*Ii*), bulb color is cream or buff. The *C* locus, which encodes the basic color factor, is required for the development of any color which means that homozygous recessive bulbs (*cc*) are white in color. The *G* locus is involved in chartreuse color development, such that homozygous recessive (*gg*) bulbs are chartreuse in color. The dominant allele of the *G* locus produces yellow onions. Gold-colored onions, which are similar to chartreuse onions but are devoid of the greenish tone, were identified in a previous study which demonstrated that a premature stop codon in the chalcone isomerase (CHI) gene is responsible for this gold color (Kim et al. 2004c). The *R* locus is a color-determinant between US-type yellow and red onions (Clarke et al. 1944) and encodes dihydroflavonol 4-reductase (DFR). A 3′-end deletion in the DFR gene in US-type yellow onions results in a lack of anthocyanin production (Kim et al. 2004b, 2005b). The *L* locus was described by Davis and El-Shafie (1967) to explain a complementation between US-type yellow and Brazilian yellow cultivars. The color of F_1 hybrids between the US-type yellow and Brazilian yellow cultivars is light red. This led to the recent identification of another locus, *Pink* (*P*), which controls a pink trait in onions (Kim et al. 2004a). The *P* locus encodes anthocyanidin synthase (ANS), and the significantly reduced transcription of the *p* allele of the ANS gene is likely the cause of the pink bulb color.

In the present study, we demonstrate that the *L* locus is the ANS gene. The molecular mechanism responsible for the inactivation of the ANS gene in this mutant allele of Brazilian yellow onions was examined. Based on promoter sequence polymorphisms of different alleles, we developed a PCR-based marker for allelic selection of the onion ANS gene.

Materials and methods

Plant materials

A US-type yellow onion (*Allium cepa*) breeding line, 1627B, was crossed with the Brazilian yellow onion cultivar Serrana. Since both parental lines were fertile, the F_1 hybrids were selected by the presence of a light-pink bulb, which was the result of complementation between the *R* and *L* loci (Davis and El-Shafie 1967). The F_1 hybrids were self-pollinated to produce a F_2 segregating population that was grown to bulbils in the greenhouse and subsequently classified into yellow and red onions for a segregation ratio analysis. Any F_2 onion developing a red color was classified as a red onion.

A recessive white onion cultivar, Texas Early White, a yellow doubled-haploid (DH)line, H2, and a red DH line, (H6), were used for high-performance liquid chromatography (HPLC) analysis and differential expression study.

HPLC analysis

Three-month-old seedlings in which the colors had fully developed were used for HPLC analysis. One or two

layers of leaf sheath of 3-month-old seedlings were peeled and ground in liquid nitrogen. The ground tissue (0.5 g) was extracted with absolute ethanol and filter-purified (13-mm HPLC Syringe filter, 0.45-μm Nylon; Alltech Assoc, Deerfield, Ill.). Quercetin content of the extract was measured using HPLC (PE series 200; Perkin-Elmer, Shelton, Conn.) with a Bondapak C-18 column at 374 nm (Patil et al. 1995).

RNA isolation, cDNA synthesis, and reverse transcriptase-PCR (RT-PCR)

One layer of the outermost fresh leaf sheath of 3-month-old seedlings was used for RNA extraction. The detailed procedures of RNA extraction and cDNA synthesis are described in Kim et al. (2004a). Differential expression of the anthocyanin synthesis gene was examined using RT-PCR as described in Kim et al. (2004a). For this analysis, onion-specific primers were designed based on the cDNA sequences of the structural genes found from CHS to ANS in the anthocyanin synthesis pathway (Fig. 1). Onion anthocyanin synthesis genes had been isolated from a red DH line (H6) previously by Kim et al. (2004a, b).

DNA extraction and PCR amplification

Total genomic DNA was extracted from the leaf tissue of seedlings at the five-leaf stage using a commercial DNA extraction kit (DNeasy Plant Mini kit; Qiagen, Valencia, Calif.). The T_m values of all primers used in this experiment were at least 70°C for the PCR amplification of genomic DNA. The PCRs were performed in a 50-μl reaction mixture containing 0.05 μg template, 5 μl 10× PCR buffer, 1 μl forward primer (10 μ*M*), 1 μl reverse primer (10 μ*M*), 1 μl dNTP (10 m *M*), and 1 μl polymerase mix (Advantage 2 Polymerase Mix; Clontech, Palo Alto, Calif.). The 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, 65°C for 30 s, and 72°C for 3 min, and a final 10-min extension at 72°C.

Isolation of promoter sequences and sequencing

The promoter regions of DFR genes were isolated using a commercial genome walker kit (Universal GenomeWalker kit, Clontech) according to the manufacturer's instructions. After verifying the successful amplification of a single, clear band on a 1% agarose gel, PCR products were purified with a PCR product purification kit (QIAquick PCR Purification kit; Qiagen). The purified PCR products were sequenced by automated Big Dye DNA cycle sequencing (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kits; Applied Biosystems, Foster City, Calif.) by the LPGT (Laboratory for Plant Genome Technologies) core sequencing facility at Texas A&M University with a capillary sequencer (ABI 3100 Genetic Analyzer; Applied Biosystems).

Results

Analysis of the color segregation ratio in the F_2 population

The colors of F_2 onions segregated as yellow or red, with the intensity of the red color varying from light pink to red. Any F_2 onion that showed a tiny red spot was classified as a red onion. The observed segregation ratio was consistent with the expected ratio of nine red to seven yellow onions (Table 1) and supports the previous finding that two complementary genes are involved in red pigment development in onion bulbs (Davis and El-Shafie 1967).

HPLC analysis of the quercetin content of differently colored onions

To more accurately identify the gene responsible for yellow bulb color in Brazilian yellow onions, the quercetin content of differently colored onions was analyzed (Fig. 2). Quercetin normally constitutes more than 80% of the total flavonoids in onions (Rhodes and Price 1996), however the quercetin content of the white onions analyzed in this experiment was below the detection level of the assay, indicating that the earlier steps of the anthocyanin synthesis pathway might be blocked. In fact, the transcription of chalcone synthase (CHS) was significantly reduced in the white onions (Fig. 2). In contrast, the quercetin level of Brazilian yellow onions was almost equivalent to that observed in US-type yellow and red onions, suggesting that the enzymatic steps from CHS to flavonol synthase (FLS) are active in Brazilian yellow onions (Fig. 1). Based on previous re-

Table 1 Chi-square tests of the segregation ratios of colors in the F_2 population originating from the cross between a US-type yellow breeding line (1627B) and a Brazilian yellow cultivar ('Serrana'). (R: red, Y: yellow)

Populations	Genotypes	Expected (R:Y)	Observed (R:Y)	χ^2	*P*
'Serrana'	*RR ll*	0:1	0:12	0	1
1627B	*rr LL*	0:1	0:34	0	1
F_1	*Rr Ll*	1:0	N/A [a]		
F_2	9*R-L-*:7 *(R-ll, rrL-, rrll)*	9:7	189:139	0.25	0.62

[a] No F_1 seeds were available in this experiment, but in a previous experiment, all of the F_1 hybrids were all light red when they grew to bulbs previously

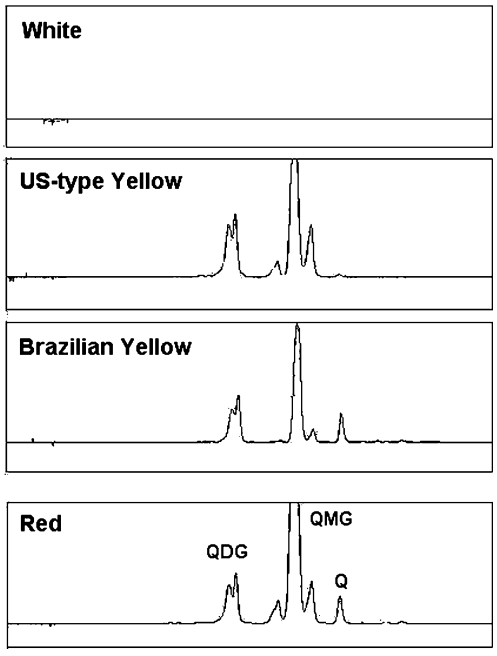

Fig. 2 HPLC analysis of the quercetin contents of differently colored onions. *White* White onion cultivar Texas Early White, *US-type yellow* yellow DH line H2, *Brazilian yellow* Brazilian yellow cultivar Serrana, *Red* red DH line H6. The *y-axis* is normalized to show the largest peak in the yellow onion as maximum. *QDG* Quercetin diglucosides, *QMG* quercetin monoglucosides, *Q* quercetin aglycone

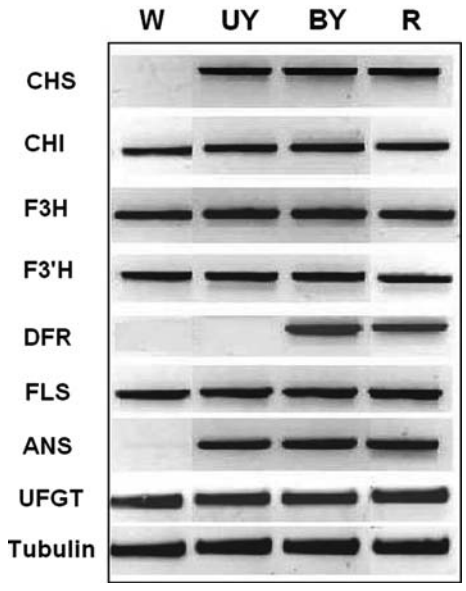

Fig. 3 RT-PCR analysis of the differential expression of onion structural genes in the anthocyanin synthesis pathway among differently colored onions. *W* White onion cultivar Texas Early White, *UY* US-type yellow DH line H2, *BY* Brazilian yellow cultivar Serrana, *R* red DH line H6. The onion tubulin gene (*AA451549*) was used as a positive control for cDNA synthesis. For the abbreviations of the enzymes see Fig. 1

sults showing that DFR is inactive in US-type yellow onions (Kim et al. 2004b, 2005b) , the dihydroflavonol 4-reductase (DFR) enzyme should be active in Brazilian yellow onions. An active DFR gene in Brazilian yellow onions could then complement an inactive DFR gene in US-type yellow onions to produce anthocyanin in the F_1 hybrids of US-type yellow and Brazilian yellow onions. Therefore, the genes encoding enzymes downstream of the DFR are potential candidate genes responsible for the lack of anthocyanin production in Brazilian yellow onions.

Differential expression of structural genes in the anthocyanin synthesis pathway among differently colored onions

The transcription of the structural genes in the anthocyanin synthesis pathway was compared among differently colored onions and found to be similar in Brazilian yellow and red onions for all of the genes in the pathway (Fig. 3). This suggests that there is a critical mutation(s) affecting the activities of ANS or UFGT UDP (glucose-flavonoid 3-*o*-glucosyl transferase) at the post-transcriptional level. Alternatively, other structural or regulatory genes that affect the late steps of the pathway and were not tested in this experiment may be inactive in Brazilian yellow onions.

Isolation of the onion ANS gene from Brazilian yellow onions

To identify any potential critical mutation(s) within the coding region of the onion ANS gene of Brazilian yellow onions, we isolated genomic sequences of the ANS gene from Brazilian yellow parental cultivars. There was greater than 99% nucleotide sequence identity between the red ANS allele and the Brazilian ANS allele, including an intron. Among the nine nucleotide differences, three resulted in amino acid changes (Pro-2 → Thr; Arg-64 → Ser; Gly-229 → Arg) (Fig. 4a). It is unlikely that substitutions at Pro-2 and Arg-64 affect the activity of onion ANS since these residues are not in the known active sites of the enzyme (Wilmouth et al. 2002), and they are not conserved among ANSs from other species. However, Gly-229 is located adjacent to a known active site, which forms the iron-binding motif and is conserved among ANSs from ten other species (data not shown). Therefore, this single nucleotide substitution at residue 229 is likely to affect the activity of the ANS in Brazilian yellow onions.

Design of a PCR-based marker for allelic selection of the onion ANS gene

If the ANS gene is responsible for the lack of anthocyanin synthesis in Brazilian yellow onions, this allele should co-segregate with color phenotypes in the F_2 segregating population originating from the cross between US-type yellow and Brazilian yellow onions. To

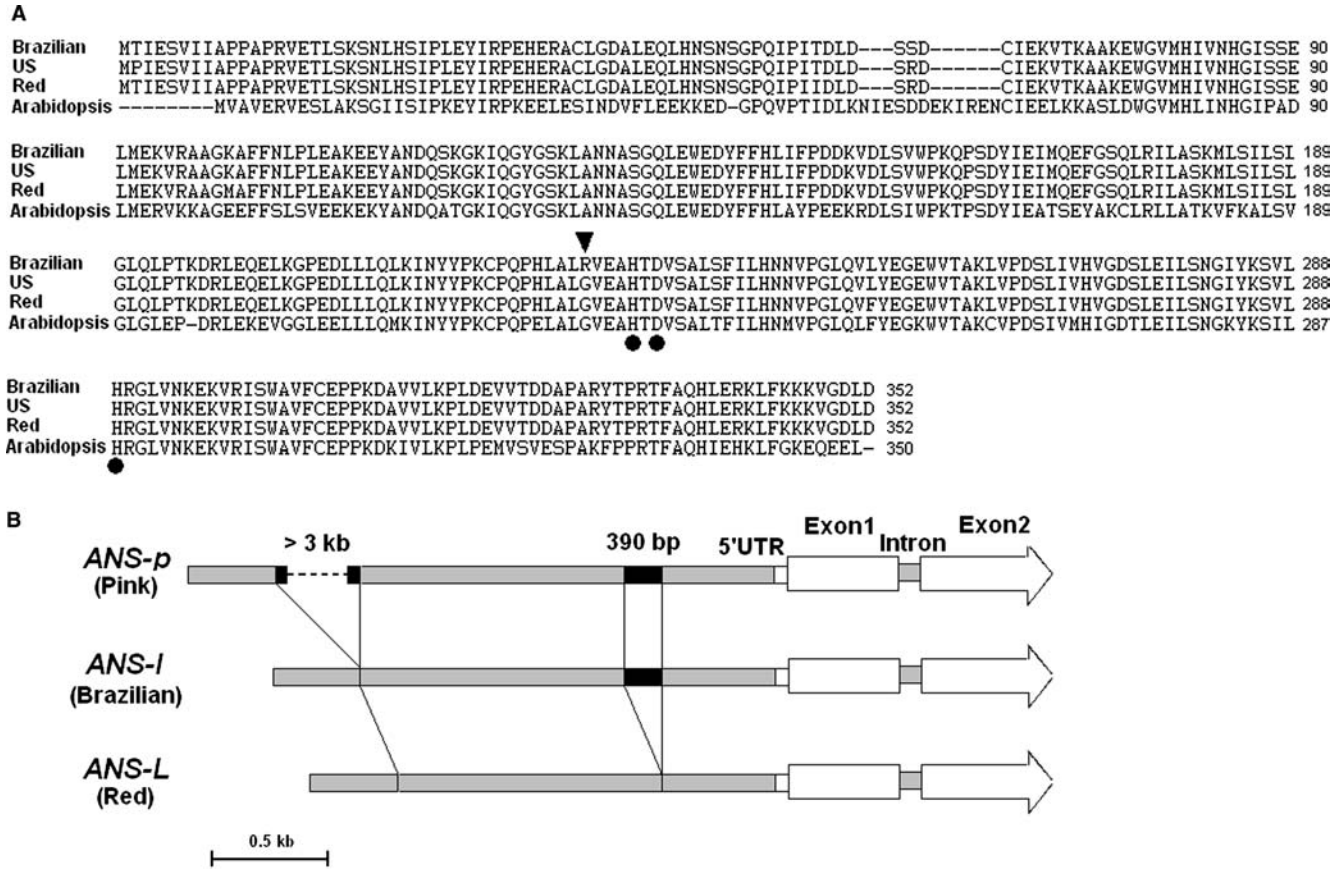

Fig. 4 **a** Alignment of the deduced amino acid sequences of anthocyanidin synthases from Brazilian yellow, US-type yellow, and red onions with that of Arabidopsis. **b** The gene structure of the *ANS-L*, *ANS-l*, and *ANS-p* alleles, including the polymorphic promoter sequences. *Filled black arrowhead* indicates the position of the amino acid change likely responsible for inactivation of the ANS gene in Brazilian yellow cultivars, *black circles* indicate residues in the iron-binding enzymatic active site, *filled black boxes* indicate the insertions, *dotted line* indicates the non-sequenced region within the insertion, *large arrow* denotes 5′-to-3′ orientation. *UTR* Untranslated region

examine this, a PCR-based marker was developed to genotype the F_2 segregating population for the ANS gene.

A PCR-based marker developed by Kim et al. (2005a) was used for the selection of a 390-bp insertion within the *p* allele of the ANS gene. If the *L* locus is the gene encoding anthocyanidin synthase, the *p* and *l* alleles should be multiple alleles of the same locus. Therefore, the three alleles of this locus are referred to here as *ANS-L* for the red, wild-type allele, *ANS-l* for the Brazilian yellow allele, and *ANS-p* for the pink allele.

The previously developed PCR-based marker was used to test whether the ANS alleles in both US-type yellow and Brazilian parental lines possessed *ANS-p* alleles. Both parents were found to have a 390-bp insertion in the promoter sequences (Fig. 4b). In addition, the US-type yellow parent was homozygous recessive for the *P* locus—i.e., it completely lacked the wild-type red allele. Interestingly, the 390-bp insertion was also present in the Brazilian ANS allele, however the transcription of the Brazilian ANS allele was normal compared to the significantly reduced transcription of the *ANS-p* allele. To identify additional polymorphisms between the *ANS-l* and *ANS-p* alleles, we isolated more distant upstream regions of the promoter sequences by genome walking. Another insertion was identified in the *ANS-p* allele promoter 2,177 bp upstream of the putative transcription start site (Fig. 4b). When primers annealing to the flanking regions of this insertion were used, no PCR product could be generated from the homozygous-recessive pink onions. This potentially means that the insertion size is greater than 3 kb, considering the fidelity of the polymerase mix used in this assay. The 3′-end sequence of the inserted region was isolated by genome walking and a primer annealing to the 3′-end of the insertion was used together with two primers annealing to the flanking regions of the insertion to generate a PCR-based marker for genotyping the *ANS-p* and *ANS-l* alleles (Fig. 5a).

Co-segregation of Brazilian ANS allele and the color phenotype in the F_2 population

When the co-segregation of the ANS alleles and color phenotypes was analyzed in the F_2 population, the

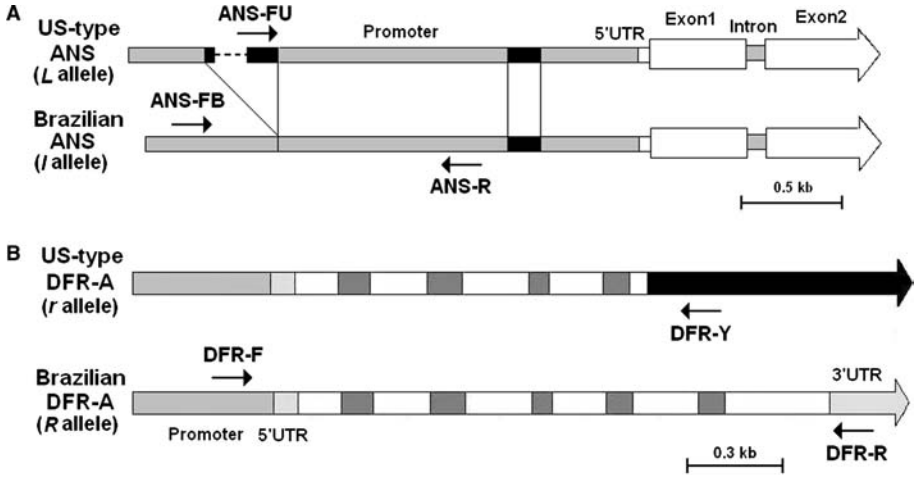

Fig. 5 Gene structures of the onion ANS (**a**) and DFR (**b**) alleles from US-type yellow and Brazilian yellow parents and the primer binding positions used as markers for genotyping F_2 onions. The *filled* and *open boxes* in the DFR gene represent introns and exons, respectively. *DFR-F* Forward primer (GGCCGCCATTAATGCATGTTCAACTTT), *DFR-Y* reverse primer1 (GAGTCGCAACAACGTTAAACGGGTCGT), *DFR-R* reverse primer2 (CACAGGGAATAGGGAAATTGGGGACA), *ANS-FU* forward primer1 (TCTTCCTTTTGTGCTTGGAGCTGATGC), *ANS-FB* forward primer2 (ATTCTGGGATGTTACACCTTGCATGCTTC), *ANS-R* reverse primer (GCCACCATCTCACATCATCCACAACCT)

genotypes of the DFR gene, the *R* locus, should be considered simultaneously. The genotypes of the onion *DFR-A* gene in the F_2 population was identified using another PCR-based marker developed by Kim et al. (2005b) (Fig. 5b). Twenty yellow and 20 red onions were randomly selected from the F_2 populations, and the genotypes of the individual onions were identified using the markers for both the DFR and ANS genes. The results show a perfect co-segregation of the marker for each allele and the phenotypes in the F_2 populations (Fig. 6). One or both of the two genes were homozygous-recessive in all of the yellow F_2 onions tested. In contrast, all of the red F_2 onions contained at least one dominant allele of either gene, thereby explaining the complementation between the two genes for anthocyanin production. This result suggests that the identity of the *L* locus in the Brazilian yellow onions is the onion ANS gene and supports the previous proposal that the *R* locus in the US-type yellow onions is the onion DFR gene.

Discussion

Identification of the *L* locus controlling anthocyanin synthesis in Brazilian yellow onions

Although the underlying mechanism of anthocyanin synthesis complementation between US-type and Brazilian yellow onions is unknown, Davis and El-Shafie (1967) identified two loci, the *R* and *L* loci, which are involved in anthocyanin synthesis. The *R* locus, first described by Clarke et al. (1944), encodes for the DFR enzyme involved in the anthocyanin synthesis pathway (Kim et al. 2004b, 2005b). In the present study, we demonstrated that the *L* locus encodes for the ANS enzyme. This finding is based on the observed perfect co-segregation of the ANS alleles and the color phenotypes in the F_2 segregating population.

A single nucleotide change is responsible for the inactivation of the ANS gene in Brazilian yellow onions. This point mutation changes a glycine residue to an arginine residue. This glycine residue is adjacent to the enzymatic active site and is conserved among ANS enzymes from other species. Therefore, this single change is likely to be the critical mutation involved in ANS inactivation, but comparative functional assays of the allele are necessary to verify this hypothesis.

A similar mutation has been described for the *le* allele of the gene encoding gibberellin 3β-hydroxylase in the pea (Lester et al. 1997). The *Le* locus, which controls stem length, was first identified by Gregor Mendel. Gibberellin 3β-hydroxylase is an iron-dependent oxygenases similar to ANS. A single point mutation, which changes an alanine residue to a threonine residue in the *le* mutant allele, results in significantly reduced enzymatic activity. The alanine involved is positioned two residues away from the conserved HXD iron-binding motif. This position is similar to Gly-229 of the onion ANS, which is only four residues from the same motif. Additionally, Gly-229 is highly conserved among ANS enzymes from other species and even among iron-dependent oxygenase families (Wilmouth et al. 2002). Therefore, a conformational change near the iron-binding motif induced by a single amino acid change of a small glycine residue to a positively charged arginine residue could result in the inactivation of the ANS enzyme in Brazilian yellow onions.

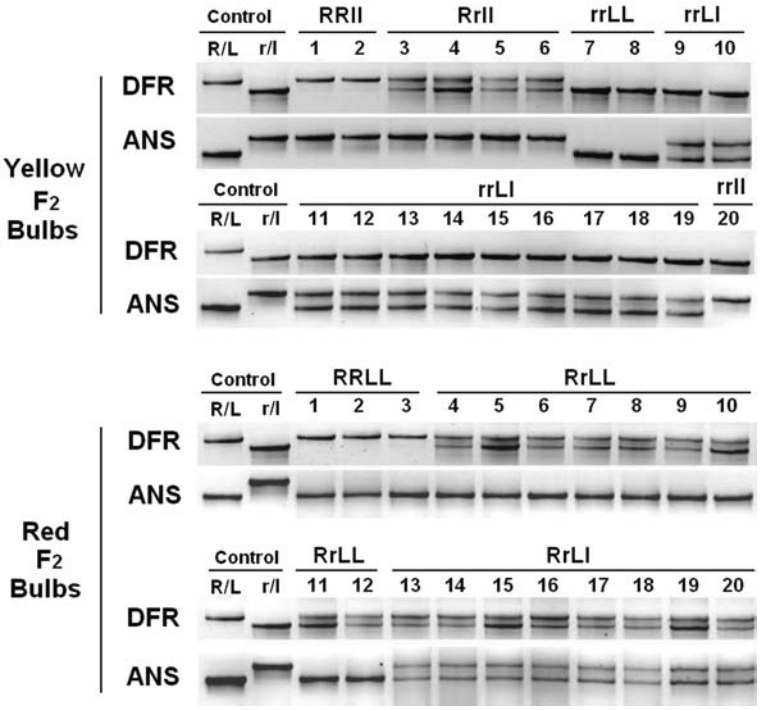

Fig. 6 Genotyping of 20 randomly selected yellow and red F_2 onion using PCR-based markers for allelic selection of the onion DFR and ANS gene. *Control* Homozygous-dominant and -recessive bands amplified from both parental *lines*

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

In addition to the *ANS-p* allele, which controls the pink trait in onions (Kim et al. 2004a), another mutant allele, *ANS-l*, was identified in this study. It has been proposed that a 390-bp insertion in the promoter sequence of the *ANS-p* allele is responsible for the significantly reduced transcription of this ANS allele. However, we were able to demonstrate that the *ANS-l* allele, which contains a similar insertion, exhibits a level of transcription similar to that observed with the red wild-type allele. Therefore, the larger insertion we identified further upstream of this site is probably responsible for the reduced transcription of the *ANS-p* allele.

The two insertions of the *l* and *p* alleles provide valuable polymorphisms for the generation of a PCR-based marker assay for ANS gene allelic identification. Development of the light-pink color of the F_1 hybrids between US-type yellow and Brazilian yellow onions is considered undesirable and a barrier to the transfer of useful traits, such as Fusarium basal rot resistance, between these cultivars. The complex segregation of color in the segregation population made it impossible to select desirable types of yellow onions without time-consuming and laborious progeny tests. Furthermore, both types of yellow onions are phenotypically indistinguishable. However, the cost-effective PCR-based marker assay developed in this study will enable onion breeders to perform genotypic selection of the desirable onions from the segregating populations at early generation and growth stages. The marker for multi-allelic selection of the ANS gene will facilitate gene transfer among differently colored onion cultivars and ultimately contribute to the development of improved onion cultivars.

Acknowledgements The authors thank members of the Vegetable & Fruit Improvement Center for their dedicated support of this research. This work was supported by produce industry member contributions to the Vegetable & Fruit Improvement Center and U.S. Department of Agriculture grant (CSREES 2001-34402-10543, "Designing Foods for Health").

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