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## ***Pink (P)*, a new locus responsible for a pink trait in onions (*Allium cepa*) resulting from natural mutations of anthocyanidin synthase**

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**Abstract** A new locus conditioning a pink trait in onions was identified. Unusual pink onions were found in haploid populations induced from an F<sub>1</sub> hybrid between yellow and dark red parents and in F<sub>3</sub> populations originating from the same cross. Segregation ratios of red to pink in F<sub>2</sub>, backcross, and F<sub>3</sub> populations indicated that this pink trait is determined by a single recessive locus. RT-PCR was carried out to look for any differential expression of anthocyanin synthesis genes between dark red and pink F<sub>3</sub> lines. The transcript level of anthocyanidin synthase (ANS) was significantly reduced in the pink line. To determine whether this reduced transcription is caused by other regulatory factors or by mutations in the ANS gene itself, ANS gene sequences from both dark red and pink F<sub>3</sub> lines were compared to detect any polymorphisms. Polymorphisms were identified, and subsequently utilized as molecular markers for the selection of ANS alleles. Absolute co-segregation of the pink allele and the ANS allele from the pink line was observed in parents, F<sub>1</sub> and F<sub>3</sub> populations. These results indicate that reduced transcription of the ANS gene caused by mutations in a *cis*-acting element is likely to result in the pink trait in onions.

**Keywords** Onion · Anthocyanidin synthase (ANS) · Bulb color inheritance · Molecular marker

### **Introduction**

Bulb color in onions (*Allium cepa*) is an important trait, and homogeneous coloration of commercial red onion cultivars is among the major goals of onion breeding programs. However, heterogeneous red onions are often found in commercial, open-pollinated varieties. There are four conventional classifications of bulb colors: white, yellow, brown and red, but intensity and shades of yellow and red colors can differ significantly depending on genetic and environmental factors. Flavonoids, one of the major classes of plant secondary metabolites, are thought to be the major pigments determining bulb color, and red color is attributable to the flavonoid anthocyanin (Fossen et al. 1996). Flavonoids have been recently attracting attention as their health-promoting roles have been discovered: in humans they act as antioxidants, free radical scavengers, and chelators of divalent cations (Cook and Samman 1996; Keli et al. 1996; Knekt et al. 1996; Hollman and Katan 1997; Braca et al. 2002; Bastianetto and Quirion 2002; Kobayashi et al. 2002). In plants, flavonoids also have a variety of functions; apart from pigmentation, they are involved in protection against UV radiation, in plant-microbe interactions, and fertility (Zeback et al. 1989; Li et al. 1993; Shirley 1996). Of these, pigmentation is the best known function, since the majority of flower and fruit colors are produced by anthocyanins. Anthocyanins absorb parts of the visible spectrum to produce red, orange, or blue colors depending on their specific structural modifications. The actual colors are further modified by pH, the presence of metals, and by interactions with other colorless flavonoids (Dooner et al. 1991; Holton and Cornish 1995; Mol et al. 1996; Shirley 1996).

The pathway of anthocyanin synthesis has been well studied and almost all of the genes encoding the enzymes comprising the pathway have been characterized in many species (Holton and Cornish 1995; Bharti and Khurana 2003). Genes affecting anthocyanin production

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are conveniently classified into two groups. The first group represents structural genes encoding enzymes in the biosynthetic pathway. Many genetic loci affecting color changes have been identified as alleles of structural genes (Holton and Cornish 1995; Bharti and Khurana 2003). The second group is comprised of regulatory genes which control the expression of the structural genes, and this regulation can be affected by many internal and external stimuli. Mutations in these regulatory genes that result in color changes have also been characterized in many species (Yamazaki et al. 2003).

Studies on the inheritance of bulb color in onions have revealed a complex pattern resulting from the interaction of five loci (Reiman 1931; Clarke et al. 1944; Davis and El-Shafie 1967). The first locus, *I*, which is incompletely dominant over *i*, is responsible for a color-inhibiting factor. Onions carrying the homozygous dominant *I* allele are completely white, irrespective of the genotypes at the other four loci. A second factor controlled by the locus *C*, which exhibits complete dominance over *c*, is also required for coloration of onion bulbs. Because both *cc* and *Ii* genotypes result in white bulbs, the term 'recessive white' is used to refer to bulbs lacking color due to the *cc* genotype. A third factor, *G*, produces golden yellow bulbs when present in either the homozygous or heterozygous state, while homozygous recessive *gg* bulbs are chartreuse in color. The final two loci, *L* and *R*, reportedly act in a complementary manner and result in the production of a red pigment (Davis and El-Shafie 1967). The early inheritance studies on bulb colors suggest that these five loci are closely related to the genes involved in anthocyanin biosynthesis, because the pigments of the different colored onions are considered to be flavonoid compounds (Fig. 1).

A new locus (*P*) conditioning a pink color in red onions was identified in this study. Unlike the five loci determining qualitative color changes, this locus is the first reported gene whose mutant phenotype results in reduced intensity of a color in onions. Similar 'pale' color phenotypes have been investigated in maize, petunia and carnation. Thus, a pale-pink color is conditioned by anthocyanin mutants, such as *bz2* of maize (Marrs et al. 1995), *an9* of petunia (Alfenito et al. 1998), and *F13* of carnation (Larsen et al. 2003). These genes were found to encode glutathione S-transferases that mediate vacuolar sequestration of anthocyanin. However, the *P* locus investigated in this present study is likely to be the gene encoding anthocyanidin synthase (*ANS*), one of the late genes in the anthocyanin pathway. Several natural mutants in the *ANS* gene have been reported, such as *A2* in maize (Menssen et al. 1990) and *Candi* in snapdragon (Martin et al. 1991). Their mutant phenotypes are colorless, since the anthocyanin pathway is completely blocked due to mutations in the *ANS* genes. In contrast, the phenotype of the *P* allele in onion is a pink color, which suggests that the mutation in the onion *ANS* gene does not cause a complete loss-of-function, but is instead a leaky mutation. Characterization of the *P* allele would be useful for both academic and practical applications. Since the

recessive *P* allele was detected in commercial yellow cultivars, a survey of the frequency of this recessive *P* allele in onion germplasm using molecular markers would provide valuable information on how these undesirable pink bulbs have arisen in breeding programs. In addition, this information could be used to design breeding strategies to efficiently remove the recessive allele or to deliberately utilize this for the development of novel pink onion cultivars.

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## Materials and methods

### Production of doubled haploid lines

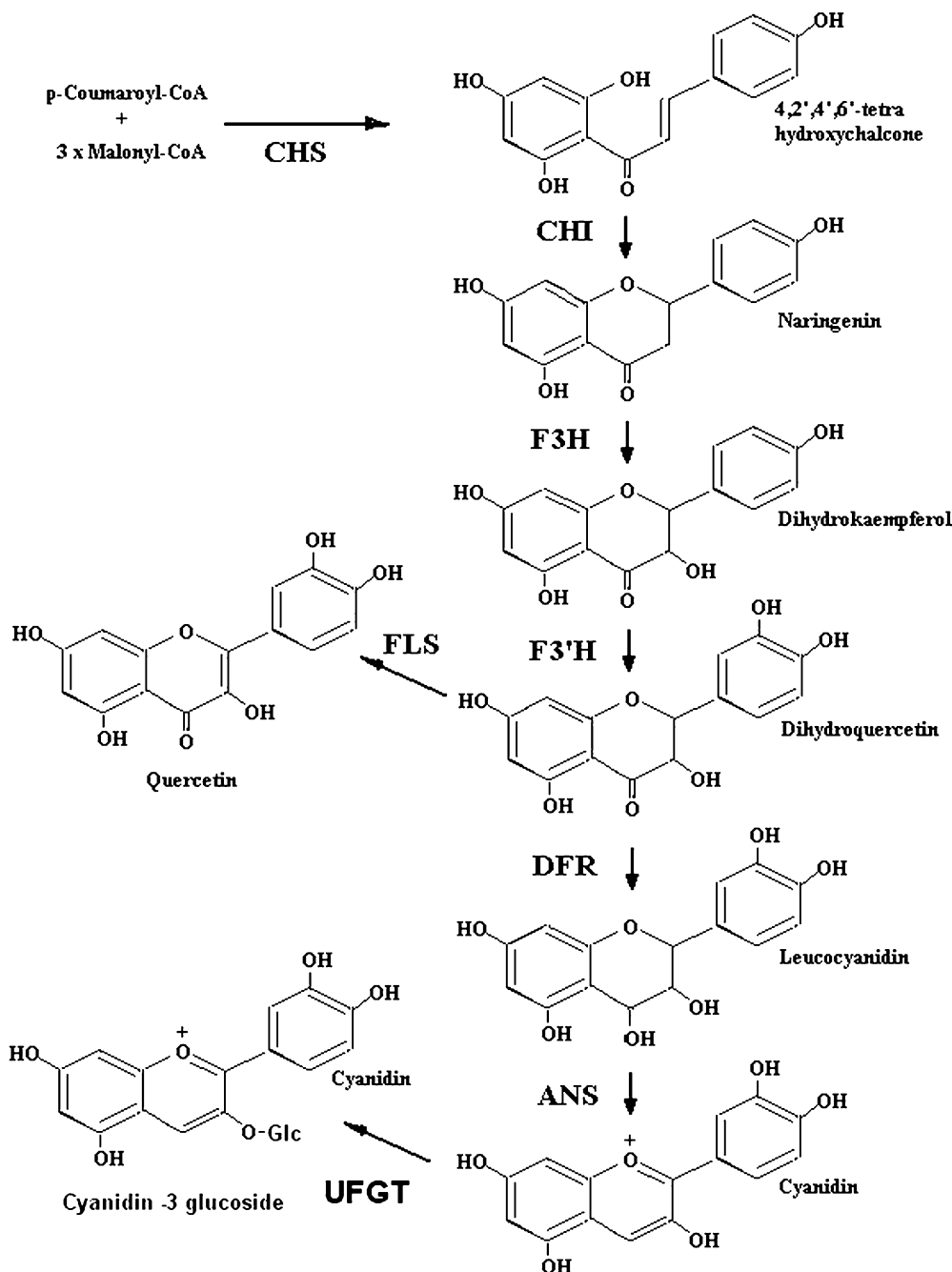
In vitro gynogenesis can be induced from unpollinated ovules, ovaries and whole flowers to produce haploid onions. Occasionally, doubled haploids may occur spontaneously. Alternatively, they may be induced by chemicals such as colchicines. Because doubled haploids are homozygous at every locus, they are particularly valuable for identifying recessive alleles in genetic studies and breeding programs (Muren 1989; Campion and Alloni 1990; Campion et al. 1992; Bohanec et al. 1995).

When the flowers of selected onions were about to open, they were excised with small scissors, and sterilized in a 5% bleach solution containing approximately three drops of Tween 20 for 1 h. The flowers were then washed with running water for at least 1 min, dried on sterile paper towels, and placed on culture medium (20–30 flowers per 90-mm Petri dish). The medium consisted of 100 g/l sucrose, MS salts (*PhytoTechnology Laboratories*, Shawnee Mission, Kan.), 0.1 g/l *myo*-inositol, 0.5 mg/l thiamine, 5 mg/l nicotinic acid, 1 mg/l pyridoxine, 2 mg/l benzyladenosine (BA), 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 7 g/l agar (*PhytoTechnology Laboratories*), with a pH of 5.8 (Bohanec et al. 1995). Haploid plants typically grow out of the ruptured ovary once the ovary color becomes brownish. It took about 60–150 days for the haploid shoots to emerge. The regenerated haploid plants were transferred to culture tubes containing MS basal medium with 0.5 mg/l naphthaleneacetic acid (NAA). After hardening, the regenerated plants were transplanted and placed in a growth chamber set at 25°C with a 16-h photoperiod. When the haploid plants had grown to the 4- to 5-leaf stage, they were transferred into a greenhouse to allow bulbing. Their ploidy level was determined by counting chromosomes under a microscope at 500× magnification (Bohanec et al. 1995). Only fertile plants were self-pollinated in individual cages with flies to produce doubled haploid lines (Pike 1986).

### Plant materials

Red doubled haploid lines (H1, H6, H8) and yellow (60708, 506L, 129) or white ('Texas Early White') male-sterile lines were crossed to produce F<sub>1</sub> populations. The

**Fig. 1** Proposed pathway of anthocyanin and quercetin biosynthesis in onions. This pathway is based on the observation that quercetin and cyanidin derivatives are the major flavonoids in 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



F<sub>1</sub> onions were then self-pollinated to produce F<sub>2</sub> populations. To identify the genotypes of F<sub>2</sub> plants, 209 fertile F<sub>2</sub> individuals of the cross 506L × H6 were self-pollinated to produce F<sub>3</sub> populations. The genotypes of individuals from the F<sub>2</sub> population were determined based on the results of the F<sub>3</sub> progeny test. To identify other modifying genes controlling the intensity of red color, ten homozygous red F<sub>3</sub> lines were transplanted to the field. Three replications of 10 entries were planted following a randomized complete block design in two locations: Weslaco (Texas) and Las Cruces (New Mexico). In Weslaco, three replications were placed in four rows with a distance of 87 cm between the rows. In Las

Cruces, three replications were placed in three rows with the same spacing between the rows. In each replication the space between the plants was 10 cm, and the interval between the entries was 174 cm. Approximately 100 seedlings were transplanted in each entry and all bulbs were used to study color inheritance.

#### RNA isolation and cDNA production

Yellow and red colors appear in only one or two layers of leaf sheath tissue at the seedling stage. Therefore, only a single layer of leaf sheath tissue was used for RNA

isolation. The peeled leaf sheath tissue was ground in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Total RNA extracted from 90-mg samples of ground tissue using an RNA extraction kit (RNeasy Plant Mini Kit; Qiagen, Valencia, Calif.) was treated with RNase-free DNase (Qiagen) to remove any DNA remaining after RNA purification. The concentrations of RNA were measured in a spectrophotometer, and 1- $\mu\text{g}$  of RNA was used (in a 20- $\mu\text{l}$  reaction volume) for reverse transcription with a commercial cDNA synthesis kit (Advantage RT-for-PCR kit; Clontech, Palo Alto, Calif.) according to the manufacturer's instructions.

### Design of degenerate primers

To design degenerate primers we searched homologous genes from other species in the Genbank database, and amino acid sequences were used to identify highly conserved blocks using Blockmaker software (Henikoff et al. 1995). Based on these conserved blocks several possible degenerate primers were created by CODEHOP software (Rose et al. 1998). The optimal degenerate primer sets were selected according to the following criteria: a PCR product size of approximately 400–600 bp, a GC content of  $\sim 50\%$ , and a minimum  $T_m$  value of  $60^{\circ}\text{C}$ . For the isolation of CHI and F3'H genes, one forward degenerate primer (CHI: 5'-GCCAA-GACCTTGTTCCCTCGGHGGYGCNGG-3'; F3'H: 5'-GACACGTCATCWAGYACSGTNGAMTGGGC-3') and Universal primer A mix (Clontech) were used for PCR amplification.

### Degenerate PCR and RACE

Degenerate PCRs were performed in 50- $\mu\text{l}$  reaction mixtures containing 3  $\mu\text{l}$  of template, 5  $\mu\text{l}$  of 10 $\times$  PCR buffer, 1  $\mu\text{l}$  of forward primer (10  $\mu\text{M}$ ), 1  $\mu\text{l}$  of reverse primer (10  $\mu\text{M}$ ), 1  $\mu\text{l}$  of dNTPs (10 mM), and 1  $\mu\text{l}$  of Taq polymerase mix (Advantage 2 polymerase mix, Clontech). PCR amplification was carried out with an initial denaturation at  $94^{\circ}\text{C}$  for 5 min, followed by 40

cycles of  $94^{\circ}\text{C}$  for 75 s,  $55^{\circ}\text{C}$  for 2 min, and  $72^{\circ}\text{C}$  for 2 min, and then a final 15-min extension at  $72^{\circ}\text{C}$  when two degenerate primers were used. When one degenerate primer was used with UPM (Universal primer A mix), the conventional degenerate PCR conditions were modified to reduce the number of background bands. RACE-ready cDNA was used as template and the amplification conditions for RACE PCR were applied, using a higher annealing temperature ( $68^{\circ}\text{C}$ ). RACE was carried out using a RACE kit (SMART RACE cDNA Amplification Kit; Clontech) according to the manufacturer's instructions.

### RT-PCR conditions

The onion-specific primers were designed after predicting possible splicing sites so that each PCR product included at least one intron to distinguish any unintentional amplification from DNA which might still be present in the RNA preparation (Table 1). The  $T_m$  values of all primers were above  $70^{\circ}\text{C}$  to ensure amplification of a single clear band. For the gene encoding UDP glucose-flavonoid 3-O-glucosyl transferase (UFGT), ten homologous sequences isolated from onions were selected from Genbank accessions based on the annotated function. One sequence (CF445052) was finally selected from among these because the deduced amino acid sequence showed the highest homology with UFGTs from grape, petunia, and maize, where the gene functions have been well studied (Holton and Cornish 1995; Boss et al. 1996). A primer set designed to amplify an onion tubulin gene (GenBank Accession No. AA451549) was used as a control to check the integrity of cDNAs. PCRs were performed in a 50- $\mu\text{l}$  reaction mixture containing 3  $\mu\text{l}$  of cDNA template, 5  $\mu\text{l}$  of 10 $\times$ PCR buffer, 1  $\mu\text{l}$  of forward primer (10  $\mu\text{M}$ ), 1  $\mu\text{l}$  of reverse primer (10  $\mu\text{M}$ ), 1  $\mu\text{l}$  of dNTPs (10 mM), and 1  $\mu\text{l}$  of polymerase mix (Advantage 2 polymerase mix; Clontech). PCR amplification was carried out with an initial denaturation at  $94^{\circ}\text{C}$  for 1 min, followed by 35 cycles of  $94^{\circ}\text{C}$  for 30 s,  $68^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 3 min, and then a final 10-min extension at  $72^{\circ}\text{C}$ .

**Table 1** Onion-specific primers used in RT-PCR amplification of genes encoding enzymes in the anthocyanin biosynthesis pathway

Gene	Primer sequences (5' to 3')	
	Forward	Reverse
CHS	GAGGGTCCAGCCACGGTGTAGCTG	ATCAATGGCCACACTCCTAAGCACC
CHI	CCCGGTTTCATCCAAAACGCACCTTC	CCAATACGGCTTGTGTCAGCGCCTTA
F3H	GAAGACGAGCGTCCCAAGATTGCAT	TCCACCATCTTTAGTCGCTTGCAGACC
F3'H	CCGGCCTCCTAACTCTGGTGCTGAG	CCGCCCAAACGGTATCAACTCAA
DFR	CTGCTGGAAGTGTAAATGTCGAGGAGC	GACCATCATCCAAATGAACCGGAACG
FLS	ACCACGGACCCGTACCCGAATTACC	GTTGGGGCAATGGACCTATCATCGTGT
ANS	CAACTCCGGCCACAAATTCCGATA	TGTGCAAACGTGCGAGGAGTGTACCTT
3GT	GATCACCGACGCGTTCCTATCCTTCAG	AGTTTCATGCCGTGGAGAAGCTTGAGG
Tub. <sup>a</sup>	CGTGACACCACAATTATCGCAAACACA	TGTGAAATCAACGGTTTTCGACATTCC

<sup>a</sup>This primer pair amplifies an onion tubulin sequence (AA451549)

**Table 2** Chi-square test of the segregation ratio of yellow to red in F<sub>2</sub>, F<sub>3</sub> and backcross populations from the crosses between yellow and red parents

Combinations (Yellow × Red) <sup>a</sup>	Populations	Observed (Red:Yellow)	Expected ratio (Red:Yellow)	χ <sup>2</sup>	P
60708 × H6	F <sub>2</sub>	212:62	3:1	0.82	0.37
506L × H8	F <sub>2</sub>	94:33	3:1	0.066	0.80
129 × H6	F <sub>2</sub>	144:68	3:1	5.66	0.017
60708 × H8	F <sub>2</sub>	209:71	3:1	0.019	0.89
129 × H8	F <sub>2</sub>	146:35	3:1	3.10	0.079
60708 × H1	F <sub>2</sub>	192:56	3:1	0.77	0.38
60708 × H1	BC <sup>(60708)</sup>	63:65	1:1	0.031	0.86
506L × H6	F <sub>3</sub>	52:107:50 (red:seg:yel) <sup>b</sup>			

<sup>a</sup>The lines 60708, 506L and 129 are yellow male sterile lines, and H1, H6 and H8 are red doubled haploid lines

<sup>b</sup>Seg: segregating, Yel: yellow

### Design of molecular markers for allelic selection

For the development of SNP (single nucleotide polymorphism) markers, genomic regions including introns were sequenced from both parents. Sequence data for the red ANS allele and the pink allele have been deposited with the GenBank Data Libraries under the Accession Nos. AY585677 and AY585678, respectively. After SNPs had been identified, PCR was carried out with primers flanking the selected SNPs. The PCR products were purified and directly sequenced by automated Big Dye DNA cycle sequencing (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits; Applied Biosystems, Foster City, Calif.) on a capillary sequencer (ABI 3100 Genetic Analyzer; Applied Biosystems) by the LPGT (Lab for Plant Genome Technologies) core sequencing facility at Texas A&M University.

## Results

### Inheritance of red color in segregating populations

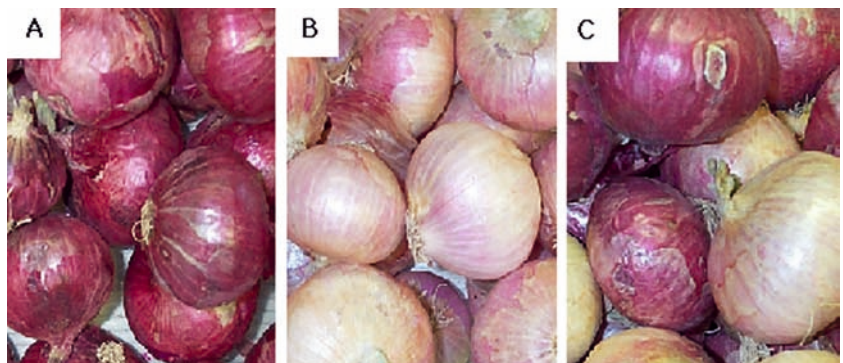
The colors of F<sub>1</sub> hybrids derived from crosses between yellow and red onions were always red, though the intensity of the red color was lighter than that seen in the red parents. This indicates that the red phenotype is partially dominant over the yellow phenotype. The results of color segregation in backcross and F<sub>2</sub> populations are shown in Table 2. The segregation ratios indicate that a single gene determines the expression of red color. To confirm the inheritance pattern and identify the genotype of F<sub>2</sub> individuals, F<sub>3</sub> populations were

produced from one of the F<sub>2</sub> populations (506 × H6) (Table 2). The segregation ratio is compatible with the single-gene inheritance hypothesis, and is consistent with previous studies of color inheritance (Reiman 1931; Clarke et al. 1944; Davis and El-Shafie 1967). The single red color-determinant was tentatively identified as a gene encoding dihydroflavonol 4-reductase (DFR) in the anthocyanin synthesis pathway. Our preliminary results indicated that the inactivation of DFR gene transcription in the yellow onions results in blockage of anthocyanin synthesis (Kim et al. 2004).

### Identification of the locus controlling pink color in red onions

An unusual pink color was detected in one of the F<sub>3</sub> lines originating from the cross between yellow and red parents (506L × H6) in the field trial. Because the DFR gene, the single red-color determinant, is fixed in the homozygous dominant state in every homogeneous red F<sub>3</sub> population, the difference in intensity of the red color between lines or within lines must result from the effects of other genes, environmental effects, or from an interaction between genetic and environmental effects. To distinguish between these possibilities, two sets of 10 red F<sub>3</sub> lines were planted in two different locations with three replications. The results revealed that four lines showed homogeneous red colors similar to that of the red parent, though the intensity and shade of red differed slightly between the lines, and five lines gave rise to a heterogeneous mixture with some pinks and others dark red (Fig. 2). Surprisingly, one of the homogeneous lines showed a uniform pink color that had not appeared

**Fig. 2A–C** Red F<sub>3</sub> populations derived from a cross between a red doubled haploid line and a yellow male-sterile line. A single red-color determining gene (DFR) is fixed as a homozygous dominant in all three F<sub>3</sub> lines, though the genotypes of the P locus are different. **A** Homogeneous red F<sub>3</sub> line. **B** Homogeneous pink F<sub>3</sub> line. **C** Segregating F<sub>3</sub> line with a mixture of red and pink onions



**Table 3** Chi-square test of the segregation ratio of pink to red in F<sub>2</sub>, F<sub>3</sub> and backcross populations

Combinations <sup>a</sup>	Populations	Observed (Red:Pink) <sup>b</sup>	Expected ratio (Red:Pink)	$\chi^2$	<i>P</i>
Yellow x Red					
60708 x H6	F <sub>2</sub>	171:41	3:1	3.62	0.057
506L x H8	F <sub>2</sub>	74:20	3:1	0.70	0.41
129 x H6	F <sub>2</sub>	115:29	3:1	1.82	0.18
60708 x H8	F <sub>2</sub>	172:37	3:1	5.94	0.015
129 x H8	F <sub>2</sub>	127:19	3:1	11.19	0.0008
60708 x H1	F <sub>2</sub>	176:16	3:1	28.44	<.0001
White x Red					
TEW x H6	BC <sub>(TEW)</sub>	15:16	1:1	0.032	0.86
Yellow x Red					
506L x H6	F <sub>3</sub> -005	64:18	3:1	0.41	0.52
	F <sub>3</sub> -112	45:11	3:1	0.86	0.36
	F <sub>3</sub> -230	46:8	3:1	2.99	0.084
	F <sub>3</sub> -290	62:18	3:1	0.27	0.61
	F <sub>3</sub> -308	75:20	3:1	0.79	0.37

<sup>a</sup>TEW is a white male-sterile line; 60708, 506L and 129 are yellow male-sterile lines; H1, H6 and H8 are red doubled haploid lines

<sup>b</sup>Red onions in segregating populations are further classified into red and pink color

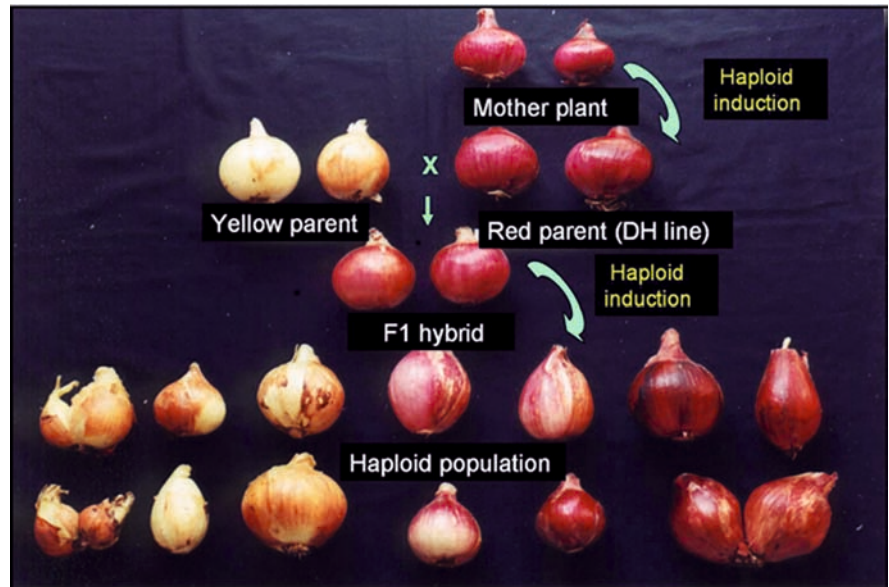
in either of the parental lines or in the F<sub>1</sub> (Fig. 2). The pink phenotype was also detected in heterogeneous F<sub>3</sub> lines and F<sub>2</sub> populations. After the phenotypes of the different lines had been scored in segregating F<sub>2</sub>, F<sub>3</sub>, and BC populations, it was found that the pink phenotype is conditioned by a single recessive gene (Table 3). A segregation ratio of 3 reds to 1 yellow was observed in F<sub>2</sub> populations originating from the crosses between yellow and red parents. Red F<sub>2</sub> individuals were further classified in the ratio of 3 red to 1 pink. However, the segregation ratio of red to pink did not fit the expected 3:1 ratio in half of the F<sub>2</sub> populations. This might be due either to misclassification of pink onions as red or to the small sample numbers. The subtle distinction between red and pink in some F<sub>2</sub> onions could result from an interaction between one major gene determining the pink trait, and DFR or other genes. In addition, environmental effects are also likely to have an impact on the degree of variation between pink and red coloration. Thus, we noted that the distinction between pink and red was more subtle in F<sub>2</sub> populations grown in Weslaco than in F<sub>2</sub> populations grown in Las Cruces (data not shown). In the case of F<sub>2</sub> populations derived from the cross between white and red, the segregation ratio of 9 red:3 yellow:4 white was observed, indicating that two-genes underlie the difference between white and red. Within red F<sub>2</sub> individuals, a segregation ratio of 3 red to 1 pink was observed as well. In a backcross population [BC<sub>(TEW)</sub>] originating from the cross between white and red onions, a segregation ratio of 2 white:1 yellow: 1 red was observed. Red BC individuals segregated in a ratio of 1 pink to 1 red (Table 3). This suggests that a single recessive gene determines the pink trait, and this gene is also independent from the genes that determine yellow and red colors. This single recessive gene was named *P* (*Pink*). Single-locus recessive inheritance was also observed in five heterogeneous red F<sub>3</sub> lines. Segregation ratios of 3 red to 1 pink were observed in all five heterogeneous F<sub>3</sub> lines (Table 3). Single-gene recessive inheritance was further supported by the haploid population produced from the F<sub>1</sub> hybrid (506L × H6), from which F<sub>3</sub> populations originated, by haploid culture of

unopened flowers. Haploid populations were initially produced to simplify the segregation pattern, because haploids exhibit no dominance effects and no heterozygosity. More importantly, recessive traits can be easily detected because of the lack of dominance. More than 15,000 flowers were cultured, but only 13 haploids formed bulbs. Although a total of 33 haploids were induced, many of them stopped growing by the two-leaf stage, and some seedlings withered during hardening. Although the size of the haploid population was too small for statistical analysis, the colors of haploids showed a striking variation (Fig. 3). This small haploid population showed two characteristics. First, almost half of them were yellow, in accordance with single-gene inheritance of a red-color determinant giving the ratio of 1 red to 1 yellow. Second, three out of seven red bulbs showed pink traits, not the parental-type red color (Fig. 3). Theoretically, half of the red haploids should be pink because of a single recessive gene conditioning the pink trait.

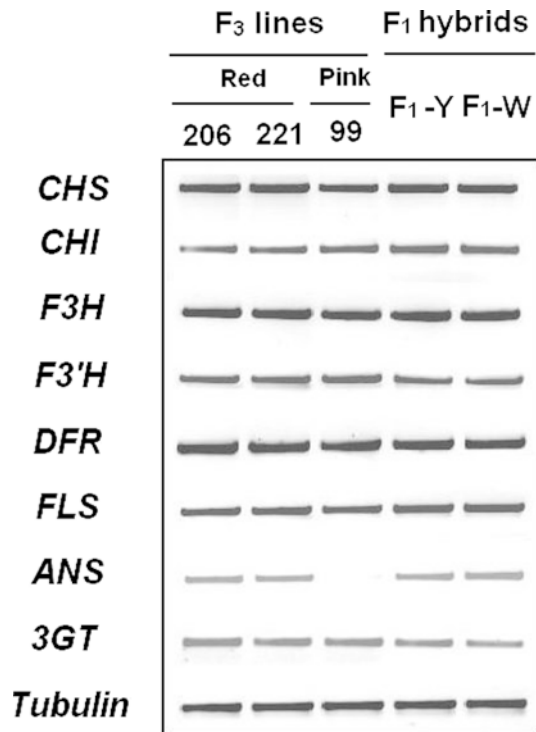
#### Differential expression of anthocyanin synthesis genes among F3 lines

In previous experiments, full-length cDNAs for the genes CHS, F3H, FLS, DFR and ANS were obtained by PCR using degenerate primers and RACE reactions (Kim et al. 2004). Isolation of full-length cDNAs for F3'H and CHI genes was also achieved using the same method, and sequences were deposited in the GenBank database. RT-PCR was carried out with onion-specific primers based on the cDNA sequences of anthocyanin synthesis genes in order to look for any differential expression of genes between red F<sub>3</sub> lines and pink F<sub>3</sub> lines. Two F<sub>1</sub> plants originating from the cross between red and yellow or white parents were included, because F<sub>1</sub> plants normally show a light-red phenotype, and the F<sub>1</sub> from the cross between white and red usually yields a lighter red than the F<sub>1</sub> with a yellow parent. RT-PCR results revealed that all genes were expressed normally in the dark red F<sub>3</sub> lines and F<sub>1</sub> plants, regardless of color

**Fig. 3** Pedigree of a haploid population originating from a cross between yellow and red onions (506L × H6). The red parent is a doubled haploid line induced from a dark red mother plant. Ploidy levels of haploids were determined from chromosome counts. Three out of seven red haploids showed a non-parental type pink color



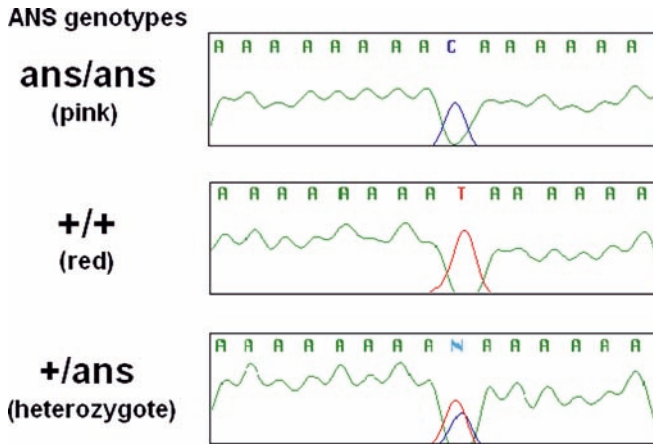
intensity (Fig. 4). However, the amount of anthocyanidin synthase (ANS) transcript was significantly reduced in the pink F<sub>3</sub> line. It is likely that the reduced transcription of the ANS gene in the pink line is caused by mutation(s) in *cis*-acting sequences of the ANS gene or by mutations in other genes for *trans*-acting factors.



**Fig. 4** RT-PCR analysis reveals differential expression of anthocyanin biosynthesis genes in onions with different intensities of red color. F<sub>3</sub>-206 and F<sub>3</sub>-221 are homogeneous dark red F<sub>3</sub> lines; F<sub>3</sub>-99 is a homogeneous pink F<sub>3</sub> line. F<sub>1</sub>-Y and F<sub>1</sub>-W are F<sub>1</sub> hybrids originating from crosses between yellow and red parents, and between red and white parents, respectively

Co-segregation of ANS alleles with pink phenotypes in segregating populations

To examine whether the reduced transcription of the ANS gene in the pink line is caused by mutations in the ANS gene itself or in other regulatory genes, we searched for polymorphisms between ANS genes from pink and dark red lines by direct sequencing. The onion ANS gene consists of two exons and one 107-bp intron. After aligning two ANS sequences from pink and dark red F<sub>3</sub> lines, four point mutations in the coding region, four point mutations in the intron, and a 10-bp deletion in the intron were identified. Although three point mutations in the exons resulted in amino acid changes, these mutations may not affect enzyme activity since the corresponding amino acid changes reside in non-conserved regions of anthocyanidin synthase, and none of them is in the known active sites of the enzyme (Wilmouth et al. 2002). To check for co-segregation of these two different alleles of the ANS gene with the pink phenotypes in segregating populations, SNPs were used as molecular markers for genotyping of the ANS alleles (Fig. 5). The ANS genes from parents, F<sub>1</sub>, and F<sub>3</sub> populations were directly sequenced after PCR amplification. DNA extracted from 10 plants of each F<sub>3</sub> line were bulked and used as a template for PCR amplification to distinguish homozygous from heterozygous F<sub>3</sub> lines using a single reaction per line. The pink allele was found to be homozygous in the yellow parent and the pink F<sub>3</sub> line, while the F<sub>1</sub> and segregating F<sub>3</sub> lines had heterozygous genotypes. On the other hand, the dark red allele was homozygous only in the red parent and homogeneous dark red F<sub>3</sub> lines (Table 4). Therefore, this result indicates that the pink allele of the ANS gene co-segregates with the pink phenotype in segregating populations, and it is therefore likely that the pink phenotype in onions is determined by mutations in the ANS gene itself. The critical mutations might reside in the promoter region or



**Fig. 5** An example of the SNP markers utilized for genotyping ANS alleles. PCR products amplified with primers flanking SNPs were sequenced directly. Heterozygous individuals and heterogeneous F<sub>3</sub> lines can be identified if both chromatographic peaks are present at the polymorphic sequences

other *cis*-acting sequences of the ANS gene, and may affect the transcription of the ANS gene, resulting in a significantly reduced transcription rate. The reduced transcription of the ANS gene might eventually lead to the pink phenotype in onions.

## Discussion

Utilizing doubled haploid onions, in which all genes are homozygous, as parents significantly simplified the interpretation of color gene inheritance, and made it possible to identify a new locus involved in determining the intensity of red color in bulbs. Furthermore, haploid populations induced from a F<sub>1</sub> hybrid enabled us to identify a recessive allele relatively easily. This study thus exemplifies how valuable doubled haploid lines are in the study of the genetic basis of complex phenotypes.

Davis and El-Shafie (1967) reported that the *L* and *R* loci were complementarily involved in red color production, based on their findings that light red F<sub>1</sub> hybrids were produced from crosses between yellow varieties from Brazil and American yellow varieties, and F<sub>2</sub> plants showed segregation ratios of 9 red:7

yellow. Thus, either *L* or *R* might be fixed as a homozygous dominant in both parents used in this study, since inheritance patterns resulting from the yellow × red crosses were consistent with a single-gene inheritance model. The single gene which determined the qualitative difference between yellow and red onions was recently proposed to be the gene encoding the DFR enzyme in the anthocyanin synthesis pathway (Kim et al. 2004). Unlike *L*, *R*, or other previously reported loci involved in bulb color determination, the *P* locus is the first gene reported to control a quantitative difference in color in onions. Our findings in the present study suggest that the *P* locus is identical to the gene encoding anthocyanidin synthase, which catalyzes the very next step after DFR in the anthocyanin pathway (Fig. 1). Because of this relationship between DFR and ANS genes, the *P* locus should affect red intensity only when at least one active copy of the DFR gene is present, so that leucocyanidin, the immediate precursor to anthocyanidin, is produced (Fig. 1). At least one dominant allele of both DFR and ANS genes would be required to produce the dark red phenotype. In other words, the *P* locus has no effect on yellow or white onions, and thus, this locus is undetectable in yellow or white onions. The existence of the recessive *P* locus in yellow or white onions is, however, relevant when they are utilized in breeding programs to develop red cultivars. When breeders use yellow or white breeding materials to transfer good traits from yellow or white breeding materials to red cultivars by the backcross method, they might happen to recover some unexpected pink onions if the yellow or white starting materials had the recessive *P* allele. If red cultivars were open-pollinated varieties, it would be difficult to remove the recessive *P* allele, since the heterozygous phenotype is visually indistinguishable from the normal dark red phenotype. In addition, it is known that when the frequency of an allele is low in a population, the rare allele exists mostly in heterozygotes and few homozygotes appear in the population. Thus, the complete removal of the recessive allele by discarding homozygotes is not as efficient as expected (Falconer and Mackay 1996). In fact, it may be speculated that a significant number of yellow or white onions in our breeding material collections may have

**Table 4** Co-segregation of ANS and *P* alleles in F<sub>3</sub> populations originating from the cross between yellow and red parents

Parameter	Parent		F <sub>1</sub>	F <sub>3</sub> lines								
	Yellow	Red		Pink	Red				Mixed <sup>a</sup>			
					1	2	3	4	1	2	3	4
Phenotype	Yellow	Red	Red	Pink	Red	Red	Red	Red	Mix	Mix	Mix	Mix
<i>P</i> alleles	<i>p/p</i> <sup>b</sup>	+/+	+/ <i>p</i>	<i>p/p</i>	+/+	+/+	+/+	+/+	+/ <i>p</i>	+/ <i>p</i>	+/ <i>p</i>	+/ <i>p</i>
ANS alleles	<i>ans/ans</i>	+/+	+/ <i>ans</i>	<i>ans/ans</i>	+/+	+/+	+/+	+/+	+/ <i>ans</i>	+/ <i>ans</i>	+/ <i>ans</i>	+/ <i>ans</i>

<sup>a</sup>Mixed means that both dark red and pink onions are mixed in F<sub>3</sub> lines

<sup>b</sup>The *P* genotype of the yellow parent is inferred to be homozygous recessive since no anthocyanin is produced in yellow onions



the recessive *P* locus, since the *P* genotype of yellow and white male-sterile lines used in this experiment and some yellow cultivars such as '1015 onion' and 'Texas Legend' were all homozygous recessive. Therefore, molecular markers which can be used to screen for the recessive *P* allele, especially in yellow or white onions, would be very useful for the efficient selection of starting materials for a breeding program.

Co-segregation of the ANS allele with the pink phenotype in segregating populations strongly suggests that the *P* locus is identical to the ANS gene. However, complementation tests examining whether the pink phenotype can be reverted to a dark red color by transforming pink onions with the dark red ANS gene would be necessary to confirm this definitively. It is possible that another regulatory gene controlling ANS gene transcription is very closely linked to the ANS gene, so that they segregate together 100% of the time in segregating populations. Additional supporting data might be obtained by sequencing promoter regions of ANS genes and identifying critical mutations affecting transcription levels. Sequencing of promoter regions would be also helpful in facilitating the design of more reliable PCR-based markers for the allelic selection of the ANS gene. The uniformity of red color is among the most important traits in red cultivars. The results of this work will contribute to the more efficient development of red cultivars with high color uniformity, and the recessive *P* allele may be a valuable resource for the development of novel cultivars with homogeneous pink color.

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