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ORIGINAL PAPER

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Development of a PCR-based marker utilizing a deletion mutation in the dihydroflavonol 4-reductase (DFR) gene responsible for the lack of anthocyanin production in yellow onions (*Allium cepa*)

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Abstract Bulb color in onions (*Allium cepa*) is an important trait, but the mechanism of color inheritance is poorly understood at the molecular level. A previous study showed that inactivation of the dihydroflavonol 4-reductase (DFR) gene at the transcriptional level resulted in a lack of anthocyanin production in yellow onions. The objectives of the present study were the identification of the critical mutations in the DFR gene (DFR-A) and the development of a PCR-based marker for allelic selection. We report the isolation of two additional DFR homologs (DFR-B and DFR-C). No unique sequences were identified in either DFR homolog, even in the untranslated region (UTR). Both genes shared more than 95% nucleotide sequence identity with the DFR-A gene. To obtain a unique sequence from each gene, we isolated the promoter regions. Sequences of the DFR-A and DFR-B promoters differed completely from one another, except for an approximately 100-bp sequence adjacent to the 5′UTR. It was possible to specifically amplify only the DFR-A gene using primers designed to anneal to the unique promoter region. The sequences of yellow and red DFR-A alleles were the same except for a single base-pair change in the promoter and an approximately 800-bp deletion within the 3′ region of the yellow DFR-A allele. This deletion was used to develop a co-dominant PCR-based marker that segregated perfectly with color phenotypes in the F_2 population. These results indicate that a deletion mutation in the yellow DFR-A gene results in the lack of anthocyanin production in yellow onions.

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Introduction

Bulb color is one of the most salient traits and has been used as a major criterion for classifying cultivars in onions. Despite the importance of this character, however, relatively few studies were carried out on bulb color inheritance in the mid-1900s (Reiman 1931; Clarke et al. 1944; Davis and El-Shafie 1967). The results of these early studies indicated that five major loci are involved in the determination of qualitative color differences in bulbs. The *I* locus is known as a color inhibitor, and its *I* allele is incompletely dominant over the *I* allele since heterozygous onions produce cream- or buff-colored bulbs. When the genotype of the *I* locus is in the homozygous-dominant state (*II*), the color of the bulb becomes white regardless of the genotypes of the other four loci. The *C* locus is a basic color factor, and at least one dominant allele is required to produce any color at all. The homozygous recessive (*cc*) genotype results again in the bulb having a white color regardless of the genotypes of the other loci. The *G* locus is involved in the production of a chartreuse-colored bulb, and the homozygous (*gg*) genotype results in a chartreuse-colored bulb. The *L* and *R* loci have been reported to be complementary factors in the production of red bulbs (Davis and El-Shafie 1967).

The epistatic interaction of these five loci implies that these loci are closely related to a pigment biosynthesis pathway, and the red bulb color in onions has been attributed to anthocyanin derivatives (Fuleki 1971; Fossen et al. 1996). Anthocyanin is a flavonoid compound and the primary pigment in a variety of flowers and fruits (Dooner and Robbins 1991; Mol et al. 1996). Flavonoids form one of the major categories of plant secondary metabolites, and in addition to playing a role in pigmentation they have been reported to be involved in UV protection, plant-microbe interaction, and fertility (Zeback et al. 1989; Li et al. 1993; Shirley 1996). Flavonoids have also been shown to have a variety of health-promoting effects, such as as antioxidants, and

these have been studied intensively (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). Such potential health benefits have recently made flavonoids an attractive research area.

The anthocyanin biosynthesis pathway has been well-defined on the basis of the results from extensive studies in maize, snapdragon, and petunia (Fig. 1) (Holton and Cornish 1995). Most of the genes encoding enzymes in the pathway have been isolated from many species, and an increasing number of regulatory genes controlling the anthocyanin synthesis genes have also been characterized in some species (Goodrich et al. 1992; Quattrocchio et al. 1993; Spelt et al. 2000; Endt et al. 2002; Bharti and Khurana 2003; Yamazaki et al. 2003).

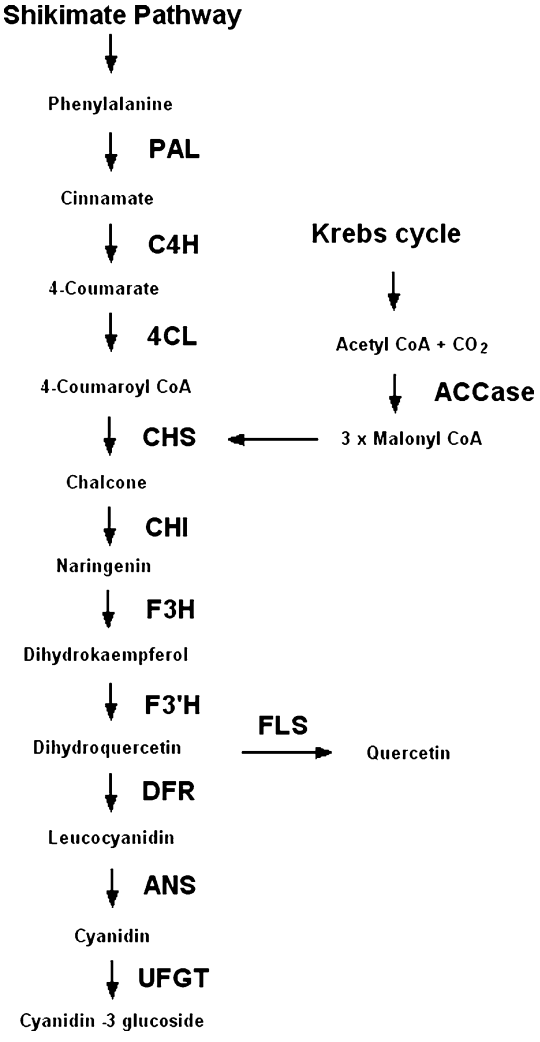

Fig. 1 Schematic anthocyanin biosynthesis pathway in onions. This pathway was inferred on the basis of reports that quercetin derivatives are the most abundant flavonoid (Rhodes and Price 1996) and that cyanidin derivatives are the major anthocyanin in red onions (Fossen et al. 1996). *PAL* Phenylalanine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

Color variety in flowers and fruits is mainly the result of mutations in the genes involved in the anthocyanin biosynthesis pathway. Many genetic loci are known to affect color change or colorless traits, and some have been revealed to be structural genes encoding enzymes in the pathway (Holton and Cornish 1995; Bharti and Khurana 2003) and others to be regulatory genes controlling whole or subsets of structural genes (Yamazaki et al. 2003).

In a previous study (Kim et al. 2004a), we showed that the lack of dihydroflavonol 4-reductase (DFR) transcription in yellow onions is responsible for the color difference between yellow and red onions. We also demonstrated co-segregation of the absence of DFR transcription and the color phenotypes in segregating F_3 families. However, we were unable to develop reliable molecular markers for the selection of yellow and red DFR alleles, possibly due to the existence of multiple genes with a very high degree of sequence homology.

In the investigation reported here, we examined three homologous onion DFR genes and the promoter regions from two of these genes in an attempt to find a unique sequence that could be used to detect the DFR gene of interest. Based on the unique sequences of the promoter regions, we were able to develop a reliable co-dominant PCR-based molecular marker and subsequently used this marker to show perfect co-segregation of the marker and color phenotypes in the F_2 population originating from the cross between yellow and red onions.

Materials and methods

Plant materials

A cross between a yellow male-sterile line (506L) and a red doubled-haploid (DH) line (H6) was made to study the inheritance of onion bulb colors. To avoid the complexity resulting from the heterogeneous genetic background in onion, which is a cross-pollinated crop, we used the DH line that had been produced and used as a red parent in our previous study (Kim et al. 2004a). From the F_1 hybrid, a F_2 population was produced, and 209 fertile F_2 plants were self-pollinated to produce F_3 families. The genotyping of the F_2 plants was carried out by a F_3 progeny test. For the reliability test of the developed marker, we selected 18 breeding lines or commercial cultivars. The original parental lines are presented in Table 1. More than ten seedlings of each line were bulk-sampled for DNA extraction and marker analysis.

RNA isolation and cDNA production

One or two layers of leaf sheath tissue of 3-month-old seedlings were used for RNA isolation. Total RNA extracted from 90 mg of ground tissue using an RNA extraction kit (RNeasy Plant Mini kit; QIAGEN,

Table 1 Original parental lines of the selected breeding lines used for the reliability test of the developed marker for allelic selection of the onion DFR gene

Breeding lines	Parental lines
34003	Ori × Ringold
33080	Ringer × Burgundy
31051	Buffalo × (Red Bone × 90977)
34086	1015 × Burgundy
43108	Grano 502 × Cardinal
31200	1015
31202	502
31051	Ori × Buffalo
34036	Ori
33048	Bison
33036	Bison × TG1015Y
31026	El Toro × 1015
33020	EEWSS × 1015

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 μg of RNA was used in a 20-μl reverse transcription reaction using a commercial cDNA synthesis kit (Advantage RT-for-PCR kit; Clontech, Palo Alto, Calif.) according to the manufacturer's instructions.

DNA extraction and PCR amplification

Total genomic DNA was extracted from the leaf tissue of five-leaf stage seedlings using a commercial DNA extraction kit (DNeasy Plant Mini kit; QIAGEN). A full-length onion DFR gene (AY221250) had been isolated previously (Kim et al. 2004a). The annealing temperatures (T_m) of all primers used in this experiment were at least 70°C for the PCR amplification of genomic DNA. 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 m M), and 1 μl polymerase mix (Advantage 2 Polymerase Mix; Clontech). 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 a final 10-min extension at 72°C.

Isolation of homologous DFR genes and the sequencing of PCR products

For the isolation of homologous DFR genes, the PCR products containing a mixture of more than one DFR gene were run out on a 1% agarose gel, and bands were cut and purified with a purification kit (NucleoTrap Nucleic Acid Purification kits; Clontech). The purified PCR products were cloned into a TOPO TA cloning vector for sequencing (Invitrogen, Carlsbad, Calif.). Purified clones 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 using a capillary sequencer (ABI 3100 Genetic Analyzer; Applied Biosystems). For direct sequencing, the PCR products were purified with a PCR Product Purification kit (QIAquick PCR Purification kit; QIAGEN) after checking out whether a clear single band was amplified by running out on an agarose gel. The purified PCR products were directly used as templates for sequencing reactions.

Isolation of DFR gene promoter sequences and promoter analysis

Isolation of the promoter regions of the DFR genes was carried out using a commercial genome walker kit (Universal GenomeWalker kit; Clontech) according to the manufacturer's instructions. The putative TATA box and transcription factor binding sites were analyzed using promoter analysis software (MATINSPECTOR; Genomatix, Munich, Germany).

Results

Isolation of three homologous DFR genes in onions

Whenever onion genomic DNA was used as a template for PCR amplification of the DFR gene, multiple PCR products were amplified together. However, these products all ran together when run out on a 1% agarose gel. The existence of homologous genes was suggested by the detection of duplicated peaks in the chromatogram when the PCR products were sequenced directly. To isolate the homologous DFR genes, we amplified the whole genomic DFR gene in two separate fragments, with an overlapping region as a convenience for cloning into a sequencing vector. Following sequence analysis of the clones, we were able to identify two different isoforms of the DFR genes. The normally transcribed onion DFR gene was labeled DFR-A, and the other DFR gene as DFR-B. The full-length DFR-B gene was obtained by combining each sequence of the two fragments. It contained the same number of introns at the same positions as the DFR-A gene and had 95% nucleotide sequence identity with the latter, including introns and untranslated regions (UTRs) (Fig. 2). The presence of these very homologous DFR genes in the onion genome had presumably caused a problem during the PCR amplification of the specific DFR gene which was normally expressed in red onions. The most conspicuous difference between the DFR-A and DFR-B genes was the length of a poly-A stretch in the 5′UTR: there were 20 'A' repeats in the DFR-A gene and six in DFR-B. We believed the DFR-B gene to be a pseudogene since no transcript was detected in the red onion cDNA pool (data not shown). In addition, there were

two point mutations, which resulted in changes of GT to CT in intron 1 and AG to AA in intron 2, in the conserved exon-intron boundary dinucleotide (GT and AG). When primer pairs that amplify the 5′ region of the DFR gene were used for PCR amplification, two clear bands were obtained. One of these was the band of the expected size and the other was smaller. Following cloning of the smaller PCR product into a sequencing vector, the sequence revealed another very homologous DFR gene that was distinct from DFR-A or DFR-B. This DFR gene was named DFR-C gene, and it had a 499-bp deletion that included intron 1, exon 2, intron 2, and a part of exon 3. There was 96% nucleotide sequence identity between the DFR-A and DFR-C genes (Fig. 2). We considered the DFR-C gene to be a pseudogene also because of the relatively large deletion in the coding region and because no transcript was detected in the red onion cDNA pool

Isolation of the DFR-A and DFR-B promoter

In order to design PCR-based markers which would discriminate between the DFR-A and DFR-B genes, the promoter sequences of these two genes were isolated using a commercial genome walker kit. Upstream regions of 2,618 bp and 1,625 bp from the putative transcription start site were isolated from the DFR-A and DFR-B genes, respectively. The sequence of a 96-bp region adjacent to the 5′UTR was the same in both DFR-A and DFR-B except for two point mutations. More distant upstream promoter sequences were completely different between the DFR-A and DFR-B genes (Fig. 3). Utilizing these promoter sequences, we were able to specifically amplify the DFR-A gene by designing forward primers in the DFR-A-specific promoter region.

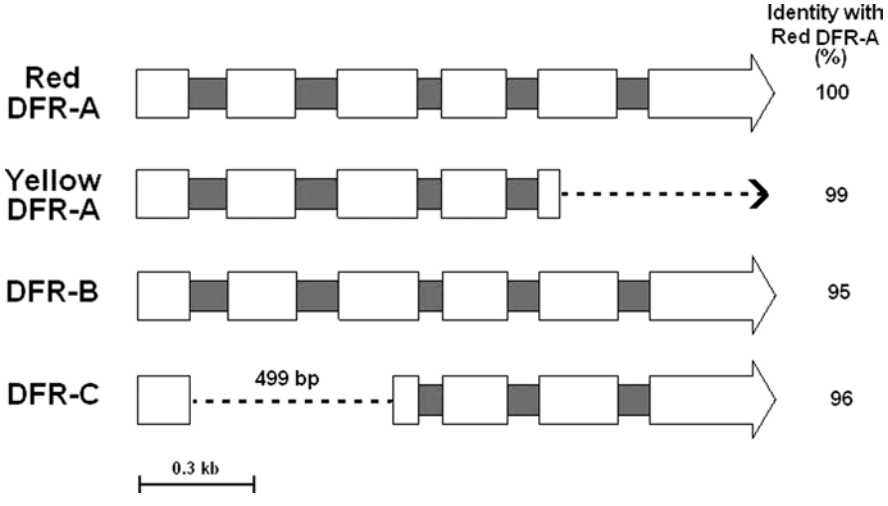

Fig. 2 Gene structures of three homologous onion DFR genes from the start to stop codon. *Shaded boxes* and *small empty boxes* Exons and introns, respectively, *arrow-shaped boxes* 5′-to-3′ direction, *dotted line* deleted region. The percentage identity indicates the nucleotide sequence identity of homologous regions of other DFR genes or alleles with the red DFR-A allele

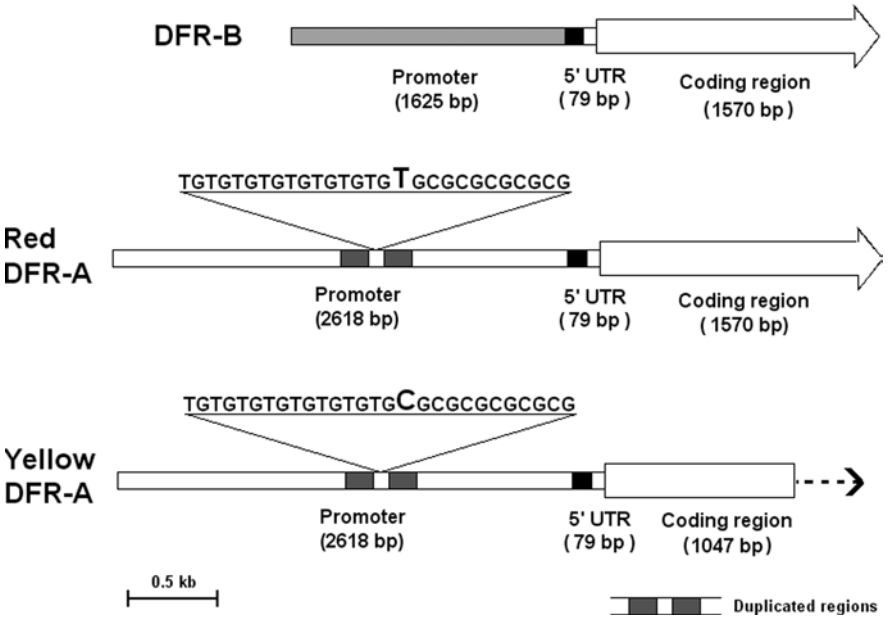

Fig. 3 Gene structure of the DFR-A and DFR-B genes including the promoter region. *Same-color boxes* Homologous sequences, *arrow-shaped boxes* 5′-to-3′ direction, *dotted line* deleted region. The position of the single nucleotide polymorphism between the red and yellow DFR-A gene promoter regions is represented as sequences with flanking simple sequence repeats

Analysis of promoter sequence of DFR-A gene

A putative TATA box and several MYB-like transcription factor binding sites were identified in the DFR-A promoter sequence using the promoter analysis software MATINSPECTOR (Quandt et al. 1995). We also identified a putative binding site for the maize P transcription factor, which regulates the expression of a subset of flavonoid synthesis genes in maize (Endt et al. 2002). One interesting feature of the promoter sequence was the existence of duplicated regions. A 150-bp block was duplicated with a 96-bp intervening sequence in the middle of the promoter sequence. There was 89% nucleotide sequence identity between these two duplicated regions (Fig. 3).

The promoter sequence of the yellow DFR-A gene, which was a non-functional allele, was also isolated. Surprisingly, there was only a single base-pair change between the functional red DFR-A and the non-functional yellow DFR-A alleles. The single base-pair change was located in the 95-bp intervening sequence between the duplicated regions. Specifically, this single nucleotide polymorphism was positioned between two simple sequence repeats (GT and GC). The last GT repeat in the red DFR-A allele was changed to GC in the yellow DFR-A allele, and as such, the result of this point mutation was simply a change in the number of repeats (Fig. 3).

Identification of a deletion in the yellow DFR-A gene and the development of a PCR-based marker

Repeated attempts to amplify the DFR-A gene from yellow onions using a forward primer binding to the DFR-A promoter region and reverse primers binding to the 3′end of the gene were unsuccessful. Since we used more than one reverse primer and since all of those tested had worked well in the red DFR-A allele amplifications, we speculated that the 3′end of the yellow DFR-A allele might be deleted.

To identify the approximate position of the deleted region in the 3′end of the yellow DFR-A allele, we used seven serial reverse primers annealing to all six exons and the 3′UTR for PCR amplifications. Our results indicated that the deletion was positioned after exon 4 (Fig. 2). The sequence following the deleted region in the 3′end of the gene was isolated using a genome walker library produced from the yellow onion genomic DNA. The deletion began at the 54th nucleotide in exon 5, and the sequence after that position was completely different from any of the sequences of the red DFR-A gene.

The deletion in the yellow DFR-A allele was utilized to develop a PCR-based marker to discriminate between the yellow and red DFR-A allele. A forward primer (5′-ATGCCAGTGGAGTGCATGTTGAATGGT-3′) binding to the promoter region of the DFR-A gene was used to ensure amplification of only the DFR-A gene. Two reverse primers were used: one binding to the genomic region beyond the deletion in the yellow DFR-A allele (5′-GAGTCGCAACAACGTTAAACG GGTCGT-3′) and the other binding to the 3′end of the red DFR-A allele (5′-TGGGTAGCGATTGGTTCA TTCTCT TCA-3′) (Fig. 4a). The PCR-based marker was tested using the F_2 segregating population, and our results showed perfect co-segregation of the marker and the color phenotypes (Fig. 4b).

The reliability of the developed marker for screening broader germplasm was tested using 18 selected breeding

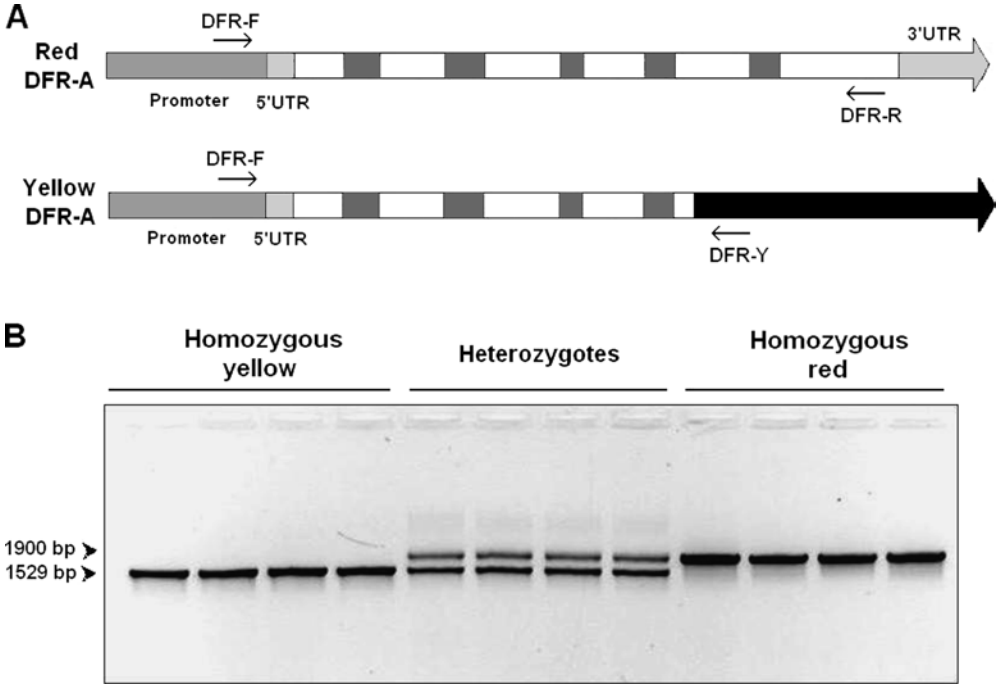

Fig. 4 Co-segregation of a PCR-based marker and the color phenotypes in the F_2 population. **a** The structure of red and yellow DFR-A alleles. *Empty boxes* and *shaded boxes* Exons and introns, respectively, *arrow-headed box* the 5′-to-3′ direction, *dark box in the 3′*end of the yellow DFR-A allele the sequence following the deleted region. *DFR-F*, *DFR-R*, *DFR-Y* primers used to develop a PCR-based marker. **b** PCR products of three different genotypes of F_2 onions using three primers in (**a**). The genotypes of F_2 onions were identified by means of a F_3 progeny test

lines or commercial cultivars. The marker was successfully utilized for genotyping all tested lines (Fig. 5). One of white lines was heterogeneous for the DFR gene, indicating both alleles existed in this line in either the homozygous or heterozygous states since the bulked DNA of more than ten seedlings of each line was used for the analysis. In the case of white onions, a bulb turned white irrespective of the genotypes of the DFR gene, since the basic color factor, the *C* locus, which is responsible for a white color, is dominant over the DFR gene

Discussion

Isolation of the promoter regions from DFR genes

Onions have a large nuclear genome (17.9 pg or 15,290 Mbp per 1C) which is 107-fold larger than that of Arabidopsis (King et al. 1998). Because of this huge genome size, the isolation of a promoter for specific genes from onion genomic DNA libraries would likely be laborious and inefficient. However, the isolation of DFR promoter regions using a genome walking kit was successful and efficient in onions.

The promoter regions of the DFR-A and DFR-B genes were found to be completely different except for an approximately 100-bp region upstream from the putative transcription start site. Even though the promoter of the DFR-C gene was not isolated, the sequence is likely to be different from that of DFR-A gene since only the DFR-A gene was amplified when the PCR amplifications were carried out with primers binding to the DFR-A promoter. Until the promoter sequences were isolated, no unique sequences had been available for reliable PCR amplification of the DFR-A gene in onions.

We hypothesized that transcription of the yellow DFR-A allele was likely inactivated because no transcript was detected in a cDNA pool from yellow onions. Thus, there might be a critical mutation in the promoter region that causes inactivation of the transcription initiation. However, there was only a single base-pair change between the red and yellow DFR-A promoter regions. This single base-pair change would not seem to be a critical mutation because the resulting change is simply a change in the number of simple sequence repeats and no known transcription factor would differentially bind based on this change. After we had found the deletion in the 3′end of the yellow DFR-A allele, we realized that no cDNA could be synthesized from the yellow DFR-A mRNA using the oligo-dT primer that had actually been used for cDNA synthesis. This conclusion was based on our observation that there seems to be no poly-A in the yellow DFR-A mRNA due to the 3′end deletion. A new pool of cDNA was then synthesized using a random hexamer primer instead of oligo-dT. Using this second cDNA pool and primers for DFR-A, we were able to amplify a smear band in yellow onions, indicating that transcription of the yellow DFR-A allele occurred but that the mRNA might be unstable due to the lack of poly-A in the 3′end of the DFR-A allele (data not shown). These results suggest that it is the deletion that is responsible for the inactivation of the DFR-A gene in yellow onions. In addition, this deletion may have occurred as a very recent event evolutionarily since only a single point mutation accumulated in the 3.6-kb promoter and coding regions between the yellow and red DFR-A alleles.

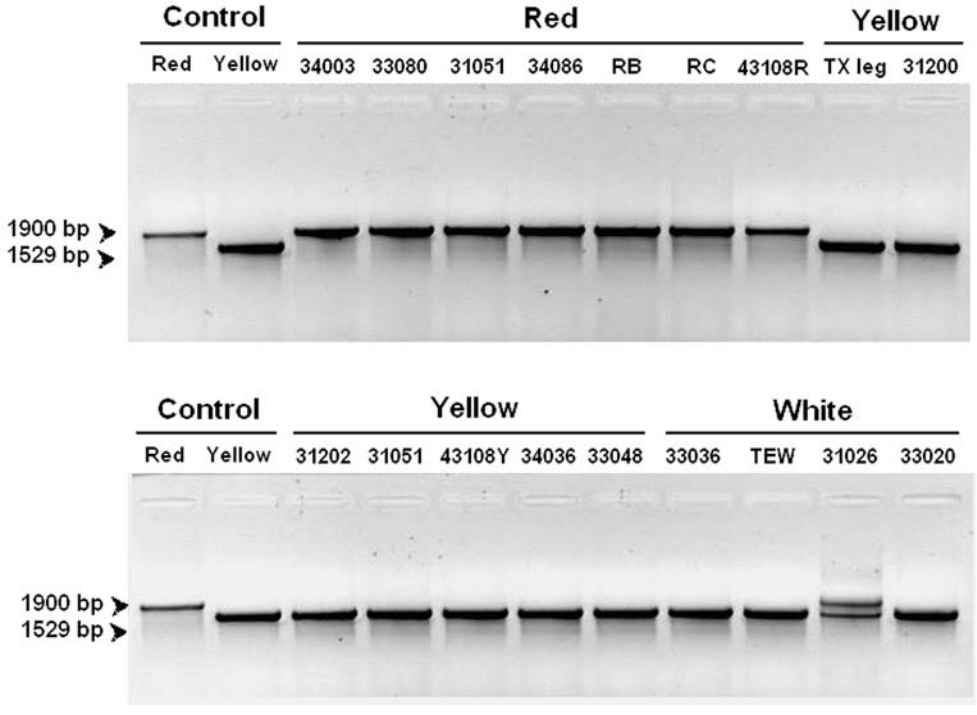

Fig. 5 Application of the newly developed PCR-based marker for allelic selection of the onion DFR-A gene during genotyping of the selected breeding lines. The original parental lines are presented in Table 1 (*numbers*). *Red* and *Yellow Control* plant materials are the parental lines of which the sequences were used to develop the marker. *RB* Red Bone, *RC* Red Comet, *TX leg* Texas Legend, *TEW* Texas Early White, *43108R and 43108Y* red and yellow selections from the same line, respectively

Development of a co-dominant PCR-based marker for allelic selection of the onion DFR-A gene

The PCR-based marker developed in this study is not a marker linked to a gene responsible for a specific trait but a direct marker for the gene causing the different phenotypes. This implies that no recombination can occur between the marker and the gene of interest and, consequently, that this marker could be used as a universal marker to screen diverse breeding materials. In addition, this marker is a co-dominant PCR-based maker, which from the viewpoint of breeding is a very important prerequisite because of its economical cost.

Molecular markers for important traits are especially useful in biennial crops such as onions in order to reduce the breeding period of 2 years per generation. The marker for allelic selection of the DFR gene would facilitate and expedite the screening of heterozygous red onions in segregating populations, thereby eliminating the need for a time-consuming progeny test. Another application of this marker is the classification of yellow onion germplasm into a DFR-mutant type or possibly into other types. Not all yellow onions have a DFR gene mutation: some Brazilian yellow onions are thought to have a different gene mutation because the bulbs of F_1 hybrids between Brazilian yellow and US-type yellow onions are light red due to complementation between two complementary factors (Davis and El-Shafie 1967). The identity of one of the two complementary factors is considered to be the DFR gene based on the results of this study. With visual examination, it is impossible to distinguish which yellow onions have a DFR gene mutation without a complementary test. The co-dominant PCR-based marker developed in this study can be utilized for this purpose to avoid numerous crosses and the growing out of their progenies.

Bulb colors have been barrier to transferring agronomically important traits among different colored onions because of the complex segregation of bulb colors in the segregating populations. In addition to the DFR gene, the *P* locus, which is responsible for a pink trait in onions (Kim et al. 2004b), has an epistatic interaction with the DFR gene and makes segregation more complex. For the efficient selection of desirable colors, the combined use of molecular markers for the major genes is required. A complete marker-assisted selection system for the major color-determining genes in onions would provide onion breeders with the freedom to select elite parental lines irregardless of bulb color. The PCR-based marker for allelic selection of the DFR-A gene developed in this study would be the first marker for the complete system and a valuable tool in onion breeding programs.

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