Theor Appl Genet (2009) 118:1391–1399
DOI 10.1007/s00122-009-0989-2

ORIGINAL PAPER

Identification of two novel inactive *DFR-A* alleles responsible for failure to produce anthocyanin and development of a simple PCR-based molecular marker for bulb color selection in onion (*Allium cepa* L.)

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Received: 17 December 2008 / Accepted: 4 February 2009 / Published online: 24 February 2009

Abstract Two novel inactive alleles of *Dihydroflavonol 4-reductase-A* (*DFR-A*) were identified in yellow onion (*Allium cepa* L.) cultivars and breeding lines from Korea and Japan. Unlike the previously reported inactive yellow *DFR-A* allele, designated as *DFR-A*^{TRN}, in which the 3′ portion of the coding sequences was deleted, an allele containing a premature stop codon, *DFR-A*^{PS}, was isolated from the majority of cultivars. Co-segregation of *DFR-A*^{PS} and color phenotypes in the F_2 population from a cross between yellow and red parents showed that inactivation of *DFR-A* was responsible for lack of anthocyanin in these yellow onions. In addition, RT-PCR analysis of F_2 population showed that the transcription level of the *DFR-A*^{PS} allele was significantly reduced owing to non-sense-mediated mRNA decay. A 20-bp deletion of a simple sequence repeat in the promoter region of the *DFR-A*^{PS} allele was used to develop a simple PCR-based molecular marker for selection of the *DFR-A*^{PS} allele. All genotypes of 138 F_2 individuals were clearly distinguished by this molecular marker. In addition to the *DFR-A*^{PS} allele, another *DFR-A* allele, *DFR-A*^{DEL}, was identified in some cultivars. In case of the *DFR-A*^{DEL} allele, no PCR products were amplified throughout *DFR-A* sequences including promoter regions, suggesting deletion of the entire *DFR-A* gene. Co-segregation of the absence of *DFR-A* and color phenotypes was confirmed in another F_2 population. Furthermore, RT-PCR results showed that no *DFR-A* transcript was detected in any yellow F_2 individuals.

Communicated by I. Paran.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-009-0989-2) contains supplementary material, which is available to authorized users.

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Introduction

Bulb color has become an important trait in onions (*Allium cepa* L.) as people pay more attention to health-promoting compounds in fresh vegetables. In addition to three major colors of white, yellow, and red, a variety of other bulb colors such as chartreuse (El-Shafie and Davis 1967) and gold (Kim et al. 2004c) exist in onion germplasm. The pigments responsible for bulb color are flavonoid compounds (Fossen et al. 1996; Rhodes and Price 1996). Flavonoids, plant secondary metabolites, are composed of more than 8,000 derivatives in plants, and 54 kinds of flavonoid have been reported in onions (Slimestad et al. 2007). Anthocyanin, which confers red bulb color, is a member of the group of large flavonoid compounds. Although their biological roles and health benefits vary depending on structure, in general flavonoids are involved in UV protection, fertility, and pigmentation in plants (Shirley 1996). In addition, flavonoids have attracted interest since they were shown to have

antioxidant activities when consumed by humans (Cook and Samman 1996; Keli et al. 1996; Knekt et al. 1996; Braca et al. 2002, Lotito and Frei 2006).

In addition to studies on the chemical structure of diverse flavonoids, the structure and function of enzymes involved in the flavonoid biosynthesis pathway have been well characterized (Ferrer et al. 2008). Structural genes encoding enzymes in this pathway and regulatory genes controlling the expression of these structural genes have been cloned in many plant species (Goodrich et al. 1992; Quattrocchio et al. 1993; Holton and Cornish 1995; Spelt et al. 2000; Yamazaki et al. 2003). In onions, the major genes encoding enzymes in the anthocyanin biosynthesis pathway were recently isolated (Kim et al. 2004a, b) and their chromosomal locations were identified (Masuzaki et al. 2006a, b). Mutations in structural and regulatory genes that result in a variety of color variants have been reported in many plant species (Holton and Cornish 1995; Yamazaki et al. 2003). Furthermore, isolation of key genes involved in flavonoid synthesis was facilitated by analysis of naturally occurring color variants within a plant species.

Several color variants exist in onions, and previous inheritance studies revealed five major loci determining bulb color (Reiman 1931; Clarke et al. 1944; El-Shafie and Davis 1967). The homozygous dominant genotype of the *I* locus, the color-inhibiting factor, is associated with a white color. This white color phenotype is incompletely dominant over other bulb colors since heterozygous phenotypes are creamy or buff. On the other hand, the homozygous recessive genotype of the *C* locus, the basic color factor, produces white bulb color regardless of the genotypes of other loci. In contrast to the dominant white phenotype resulting from the *I* locus, the white bulb color caused by homozygous recessive genotype of the *C* locus is recessive to other bulb colors. The homozygous recessive genotype of the *G* locus is responsible for a chartreuse color when the genotypes of the *I* and *C* loci are *iiC-*. Finally, the *R* and *L* loci are complimentarily involved in red color production when the genotypes of the *I*, *C*, and *G* loci are *iiC-G-*. At least one dominant allele of both *R* and *L* loci is required to produce anthocyanin (El-Shafie and Davis 1967).

Koops et al. (1991) suggested that these five loci might be closely related to genes encoding enzymes in the anthocyanin synthesis pathway on the basis of their epistatic interaction. Indeed, a deletion in the gene coding for dihydroflavonol 4-reductase (DFR), an enzyme converting dihydroquercetin into leucocyanidin in the anthocyanin synthesis pathway, is responsible for color difference between US-type yellow and red onions (Kim et al. 2004b, 2005b). In addition to an active *DFR* gene, designated as *DFR-A*, two additional homologous pseudogenes, *DFR-B* and *DFR-C* were also identified in onions (Kim et al. 2005b). A critical point mutation on the gene encoding anthocyanidin synthase (ANS) leads to lack of anthocyanin in Brazilian yellow onions (Kim et al. 2005c). The enzyme, ANS, is involved in conversion of colorless leucocyanidin into red-colored cyanidin in the pathway. Two additional alleles of the *ANS* gene containing insertion mutations in the promoter region were also identified (Kim et al. 2004a, 2006). Simple PCR-based molecular markers for allelic selection of the genes encoding DFR (Kim et al. 2005a) and ANS (Kim et al. 2005b) were developed. Since these markers were designed on the basis of the polymorphic sequences of genes that are directly involved in phenotypic variation, the accuracy of indirect selection using these molecular markers is identical to that of direct phenotypic selection. These molecular markers can be considered 'functional markers', which were suggested to be the ultimate molecular markers by Andersen and Lübberstedt (2003).

In the present study, we describe two novel mutant alleles of the *DFR* gene that are predominantly distributed in onion cultivars grown mainly in Korea and Japan, and demonstrate that these two mutant alleles are responsible for the color difference between yellow and red onions. In addition, a new molecular marker for selection of the variant alleles was developed for use in breeding programs for the development of red onion cultivars.

Materials and methods

Plant materials

Onion varieties that are currently cultivated in Korea and Japan were used in this study. A total of 50 cultivars and breeding lines were analyzed (Supplementary Table 1). 'Texas Grano 1015', a leading variety in regions of southern Texas that contains a deletion mutation in the 3′end coding sequence of the *DFR-A* gene (Kim et al. 2005a), was used as a control. After analyzing *DFR-A* genes of 11 F_2 populations originating from crosses between yellow and red onions, two representative populations containing two new *DFR-A* alleles were selected for further genetic analysis.

DNA extraction, PCR amplification, and sequencing of PCR products

Total genomic DNA was extracted from leaves, bulb scales, or peduncles of breeding lines and cultivars using a cTAB method (Murray and Thompson 1980). PCR was performed in a 10 μL reaction mixture containing 0.05 μg template, 1 μL 10x PCR buffer, 0.2 μM forward primer, 0.2 μM reverse primer, 0.2 mM dNTPs, and 0.1 μL polymerase mix (Advantage 2 Polymerase Mix, Clontech, Palo Alto, CA,

Table 1 Sequences of PCR primers used in this study

Primer names	Primer sequences (5′–3′)	Tm
DFR-F1	TCGGTATAACAAACACGGCCCACATCA	65.0
DFR-R1	ATTAATGGCGGCCGTCCTGTTAACCAT	65.0
DFR-F2	CGCGTGCGCACATACGCATACATACAT	66.5
DFR-R2	CCATTGGGGTAGCGACATGGAAAACAG	66.5
DFR-F3	ATGCCAGTGGAGTGCATGTTGAATGGT	65.0
DFR-R3	TTGCAAACTCCCATGCAGCTTTCTCTG	65.0
DFR-F4	GAGGAGCACCAAAAGCCCGAATACGAT	66.5
DFR-R4	TGGGTAGCGATTGGTTCATTCTCTTCA	63.4
DFR-R5	GAGTCGCAACAACGTTAAACGGGTCGT	66.5
ANS-F	TTTGCTCGACGTTTAGCRGAAGAAGA	62.4
ANS-R	TGAGGATGATGACAAAGTTAGCGGAGCA	65.1
Tubulin-F	GGAAGCATGTGCCCCGTGCTATATTTG	66.5
Tubulin-R	ACAATCTGGATCGTGCGCTTCGTCTTT	65.0
MK-F	CGGAACACGTGGTTAAAATCGGAGGAT	65.0
MK-R	TGTGGTTAAAATCAARGGGATTTGAGGTG	63.1

The positions of primers used for *DFR-A* gene amplification are depicted in Fig. 1

USA). PCR amplification for sequencing was carried out with an initial denaturation step at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 60–68°C for 30 s, and 72°C for 2 min, and a final 10-min extension at 72°C. The primer sequences used in this study are provided in Table 1, and their positions on the *DFR-A* gene are shown in Fig. 1.

For DNA sequencing, PCR products were purified using the QIAquick PCR Purification kit (QIAGEN, Valencia, CA, USA). The purified PCR products were sequenced directly or after cloning into TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA). Sequencing reactions were carried out using Big Dye (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol, and sequences were obtained using an ABI 3700 Genetic Analyzer (Applied Biosystems).

RNA extraction, cDNA synthesis, and RT-PCR

Total RNA was extracted from fresh scales of F_2 bulbs using an RNA extraction kit (RNeasy Plant Mini Kit,

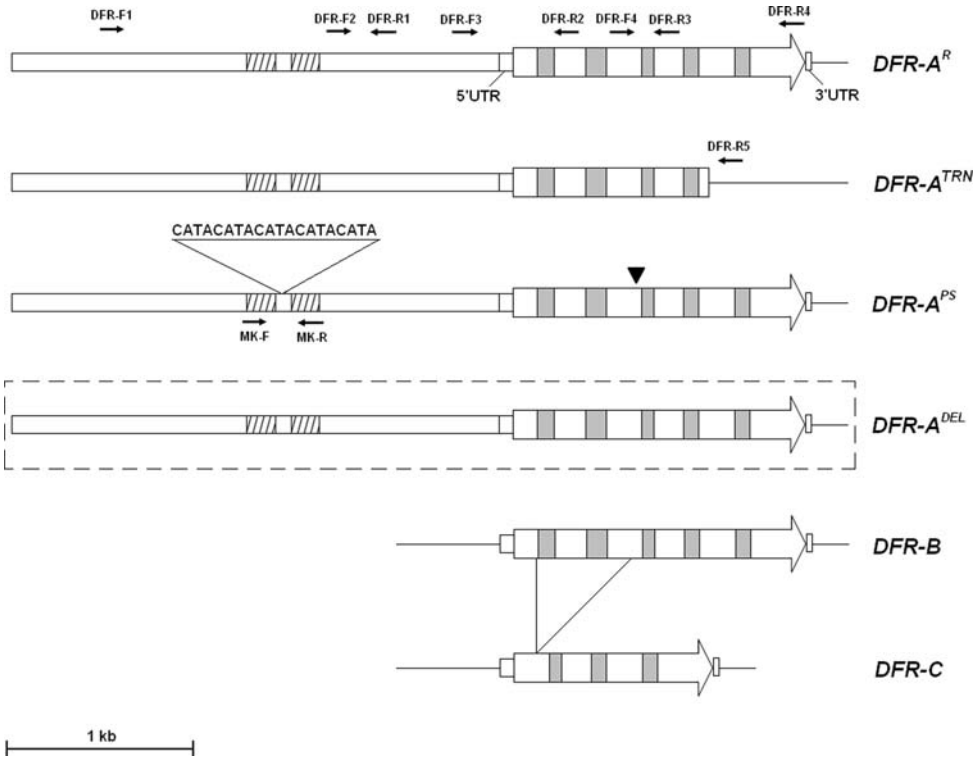

Fig. 1 Organization of multiple alleles of the *DFR-A* gene and two other homologous genes isolated from onions. The hatched boxes in the promoter region indicate duplicated sequences. The *gray boxes* in the coding regions indicate introns. The *filled triangle* in the coding region of the *DFR-A*^{PS} allele indicates the position of the premature stop codon. The 20 bp nucleotide sequence *above the empty triangle* indicates the deleted sequence in the *DFR-A*^{PS} allele. The *broken lines* around the *DFR-A*^{DEL} allele indicate that the entire sequence is deleted. *Arrow-shaped boxes* indicate the 5′–3′ direction. *Horizontal arrows* indicate primer-binding sites

QIAGEN) following the manufacturer's instructions. The cDNA was synthesized from total RNA using a commercial cDNA synthesis kit (SuperScript™ III first-strand synthesis system for RT-PCR, Invitrogen). RT-PCR amplification was performed with an initial denaturation step at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 2 min, and a final 10-min extension at 72°C. The onion/tubulin sequence obtained from EST sequences (TC125) from the DFCI *Allium cepa* Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi) was used as a control.

Results

Analysis of allelic variation of the *DFR-A* gene in yellow onion cultivars developed in Korea and Japan

To provide a molecular marker for the *DFR-A* gene, which is responsible for the color difference between US-type yellow and red onions, for use in onion breeding programs, the previously reported marker (Kim et al. 2005b) was tested in diverse onion cultivars and breeding lines grown mainly in Korea and Japan. This molecular marker is based on the 3′ partial deletion of coding sequences in the yellow *DFR-A* allele (Fig. 1). Hereafter, this inactive allele and active *DFR-A* alleles are designated as *DFR-A*^{TRN} and *DFR-A*^{R}, respectively. Three primers (DFR-F3, DFR-R4, DFR-R5) were used for PCR amplification of the molecular marker.

Unexpectedly, PCR amplification gave products representing the active red *DFR-A* allele (*DFR-A*^{R}) in 12 yellow onion cultivars and breeding lines, and the smaller product, presumed to be that of the *DFR-A*^{TRN} allele, was not detected in any of the yellow onions tested (Fig. 2). This means either that gene(s) other than *DFR-A* are responsible for the absence of anthocyanin production or that other inactive *DFR-A* alleles might be present in these yellow onions. Moreover, some yellow cultivars and breeding lines gave no PCR product derived from *DFR-A* alleles (3, 5, 7, 11, 13, 14, 16 in Fig. 2). At first, we thought that this was because of unsuccessful PCR amplification or inferior quality of DNA templates; however, repeated DNA preparation and PCR amplification gave the same result of no PCR product for those yellow onions. PCR amplification of other genes such as the ANS-coding gene was successful in these onions (Fig. 3), excluding the possibility of low quality DNA templates. This suggests the presence of critical mismatches or deletion mutations at the binding positions of the primers for the molecular marker in these yellow onions.

Identification of a novel inactive *DFR-A* allele containing a premature stop codon

To verify the existence of critical mutations in the *DFR-A* gene of yellow onions showing the band patterns of the red *DFR-A* allele in Fig. 2, the PCR products of a red control cultivar, 'Red Ring Ball', and a yellow breeding line, BT594, were sequenced. The nucleotide sequences of the *DFR-A*^{R} allele amplified from 'Red Ring Ball' were not identical to that of the previously reported *DFR-A*^{R} allele (GenBank accession: AY221250): nine single nucleotide polymorphisms existed between the two *DFR-A*^{R} alleles. The cladogram showed that the DFR-*A*^{R} allele (AY221250) was more closely related to the *DFR-A*^{TRN} allele isolated from US-type yellow onions than to the *DFR-A*^{R} allele isolated from 'Red Ring Ball' (Fig. 4). There were only three polymorphic nucleotides between the coding sequences of red and yellow *DFR-A* alleles isolated in this study. When the deduced amino acid sequences were compared, we found that a point mutation in exon 3 created a premature stop codon in the middle of the gene (Fig. 1), implying that this premature stop codon inactivates *DFR-A* gene function in these yellow onions.

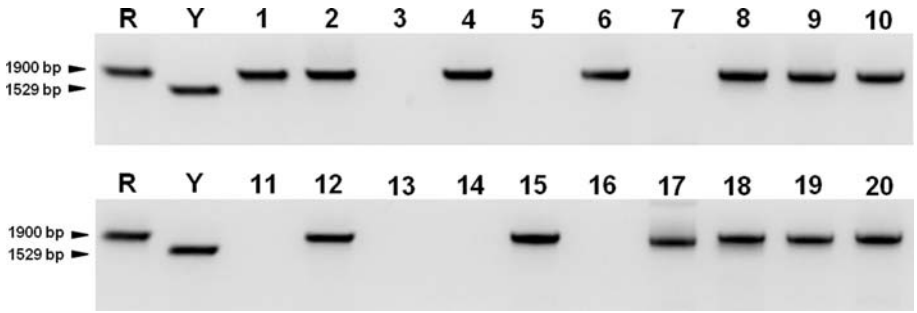

Fig. 2 PCR products of diverse onion germplasm amplified using the previously reported molecular marker for *DFR-A* allelic selection (Kim et al. 2005b). Three primers (DFR-F3, DFR-R4, DFR-R5) were used in PCR amplification. *R* red control cultivar, 'Red King Ball', *Y* yellow control cultivar, 'Texas Grano 1015'. *1* Nuriwhang, *2* Rucy, *3* Umdamaru, *4* Gangsukwhang, *5* Chilbo, *6* Sasuki, *7* Thridama, *8* Hantur, *9* 204sp, *10* MOM8-1, *11* TB538, *12* TB540, *13* TB590, *14* TB591, *15* TB594, *16* TB611, *17* Choonryukkwon, *18* Ocean, *19* Wolgwang, *20* Katamaru

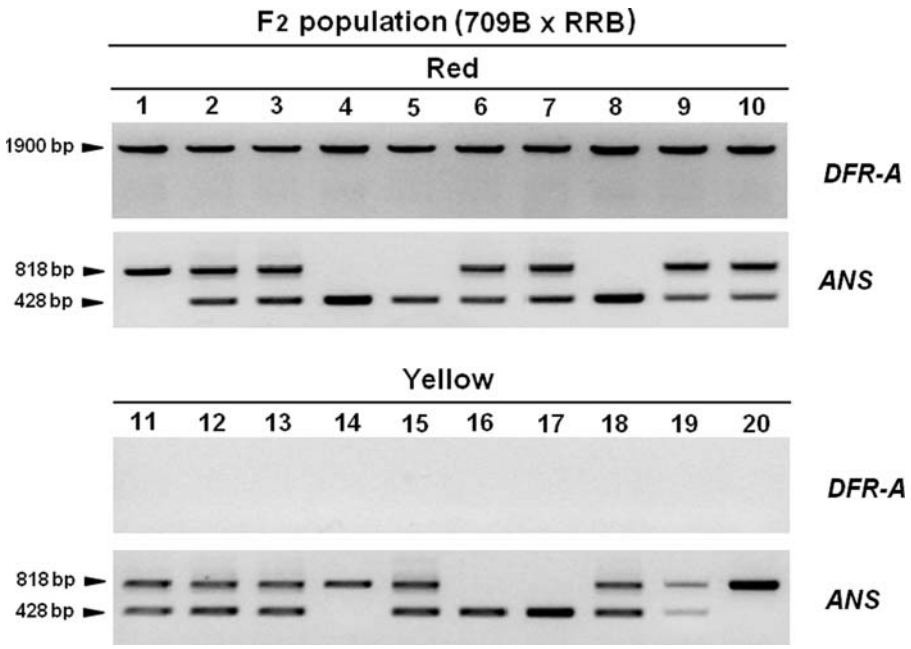

Fig. 3 Co-segregation of the *DFR-A* allele and color phenotypes in a F_2 population originating from the cross between red ('Red Ring Ball') and yellow (709B) parents. A marker for *ANS* allele selection was included as a control that does not show co-segregation of the *ANS* gene and color phenotypes in this F_2 population. The primer set (ANS-F, ANS-R) was used in PCR amplification

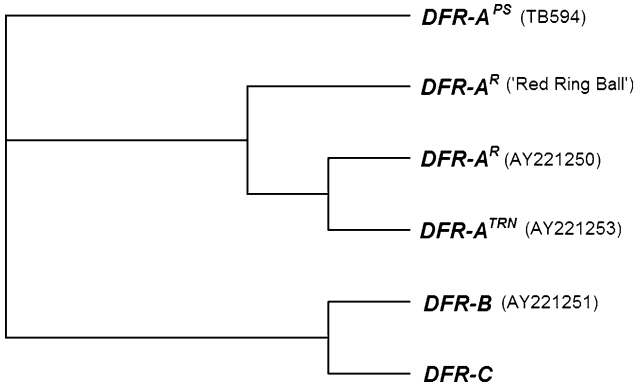

Fig. 4 Cladogram of multiple alleles of the *DFR-A* gene and homologous *DFR* pseudogenes. The cladogram was produced using ClustalX and TreeView software. The GenBank accession numbers of the previously reported DFR genes are shown in *parentheses*

To investigate the relationship between inactivation of the *DFR-A* allele and the color phenotypes, co-segregation of this variant allele with color phenotypes was analyzed in a F_2 segregating population originating from the cross between a red onion cultivar, 'Red Ring Ball', and a yellow breeding line, TB594. The 3:1 segregation ratio of red to yellow phenotypes showed a single-gene inheritance of red color in this population (Table 2). PCR products of the *DFR-A* gene from five red and five yellow F_2 plants were directly sequenced. The nucleotide sequences showed perfect co-segregation of the *DFR-A* alleles and color phenotypes: all five yellow F_2 plants contained the variant allele, whereas the red F_2 plants were either homozygous for the red *DFR-A* allele or heterozygous (data not shown), supporting the hypothesis that this variant *DFR-A* allele is responsible for the color difference between yellow and red onions. The newly identified *DFR-A* allele harboring a premature stop codon is designated *DFR-A*^{PS}.

To confirm transcription of this variant *DFR-A* allele, RT-PCR performed on the same F_2 populations ('Red Ring Ball' × TB594) showed that single bands of the same size were amplified in red and yellow F_2 plants, but the transcript level of the *DFR-A* gene was significantly lower in yellow plants (Fig. 5a). Sequencing of the RT-PCR products from yellow F_2 plants revealed only the mutant alleles.

Identification of the third mutant *DFR-A* allele from yellow onions

We previously showed that no PCR products of the *DFR-A* gene were observed in some yellow cultivars and breeding

Table 2 Chi-square test of the segregation ratio of colors in the F_2 population originating from crosses between yellow and red breeding lines

Populations	Expected (*R*:*Y*)	Observed (*R*:*Y*)	χ^2	*P*
Red Ring Ball (red) × TB594 (yellow)	3:1	110:28	1.63	0.20
709B (yellow) × Red Ring Ball (red)	3:1	127:42	0.002	0.96

R red, *Y* yellow

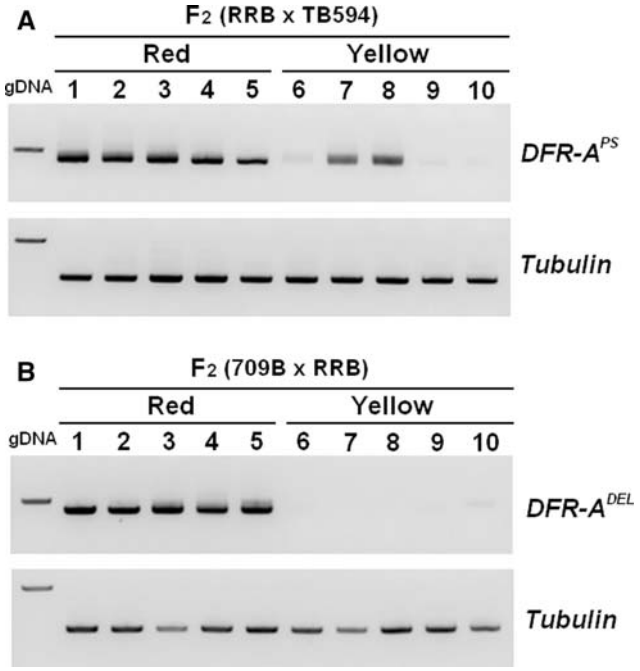

Fig. 5 RT-PCR amplification of the *DFR-A* gene from F_2 plants originating from crosses between red and yellow parents. Primer sets were designed to include at least one intron. **a** The F_2 population from the cross between a red cultivar ('Red Ring Ball') and a yellow breeding line (TB594) containing the *DFR-A*^{PS} allele. **b** The F_2 population from the cross between a red cultivar ('Red Ring Ball') and a yellow breeding line (709B) containing the *DFR-A*^{DEL} allele

lines (Fig. 2). To examine whether the failure of PCR amplification was simply because of mismatches at the primer binding positions, three additional primer pairs (DFR-F1 + DFR-R1, DFR-F2 + DFR-R2, DFR-F3 + DFR-R3), covering 3,804 bp including the promoter and coding sequences, were tested in these yellow onions. No PCR products were amplified from the promoter region of the yellow onions, but a single band of the expected size was produced with the primer pair amplifying the 3′end of *DFR-A* gene (DFR-F4 and DFR-R4) (Fig. 6). However, this PCR product was shown to be *DFR-B* (GenBank accession number: AY221251), a homologous pseudogene. The promoter region of the *DFR-B* gene is completely different from the *DFR-A* gene (Fig. 1), but there is 95% homology in the coding regions (Kim et al. 2005b). Therefore, no *DFR-A* PCR products were obtained from these yellow onions, implying that the *DFR-A* gene might be completely deleted.

To examine the relationship between the absence of the *DFR-A* gene and color phenotypes, we tested co-segregation in another F_2 population originating from the cross between a yellow breeding line, 709B, in which no PCR products of the *DFR-A* gene were observed, and a red onion cultivar, 'Red Ring Ball'. This F_2 population also showed a single-gene inheritance of color phenotypes (Table 2). No

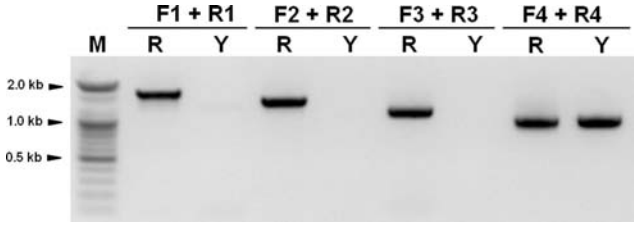

Fig. 6 PCR products amplified using a series of primer pairs that bind to the promoter and coding sequences of the *DFR-A* gene. The positions of each primer are indicated in Fig. 1. *R* red onion cultivar ('Red Ring Ball'), *Y* yellow breeding line (709B). *M* 100 bp ladder; *F1–F4* DFR-F1, DFR-F2, DFR-F3, and DFR-F4 primers, respectively; *R1–R4* DFR-R1, DFR-R2, DFR-R3, and DFR-R4 primers, respectively

DFR-A PCR products were observed in any of the 10 yellow F_2 plants, whereas PCR products were detected in all 10 red F_2 individuals (Fig. 3). This result proves co-segregation of the absence of the *DFR-A* gene and color phenotypes in a F_2 segregating population. In contrast, the previously reported molecular marker for allelic selection of the *ANS* gene (Kim et al. 2006) showed segregation of two different alleles of the *ANS* gene independent of color phenotypes (Fig. 3). Furthermore, no transcript of the DFR-A gene could be detected by RT-PCR in yellow F_2 plants (Fig. 5b). A faint large band detected in one of the yellow F_2 plants might have been amplified from the *DFR-B* gene on residual genomic DNA. Although we failed to obtain any flanking sequences of the putative *DFR-A* gene deletion in these yellow onions, our results suggest that the *DFR-A* gene is completely deleted, and that the deletion mutation leads to lack of anthocyanin in these yellow onions. This third *DFR-A* allele is designated *DFR-A*^{DEL}.

Development of a molecular marker for detection of the *DFR-A*^{PS} allele

In previous experiments, the presence of the *DFR-A*^{PS} allele was identified by direct sequencing; however, selection by direct sequencing is not amenable for large-scale analysis of breeding lines. To search for additional polymorphic sequences, approximately 2 kb of the promoter sequence of both red and yellow alleles was sequenced. A 20 bp deletion of 'CATA' repeat sequences was identified between two homologous tandem repeat sequences in the promoter region of the yellow allele (Figs. 1, 7a). By designing a pair of primers (MK-F and MK-R) in the sequences flanking the 20 bp deletion, a simple PCR-based molecular marker for selection of the *DFR-A*^{PS} allele could be developed. Homozygous red F_2 individuals were clearly distinguished from heterozygous red F_2 plants using this molecular marker (Fig. 7b).

Using this simple PCR-based marker, we analyzed the genotype of the *DFR-A* gene in all 138 F_2 plants and found

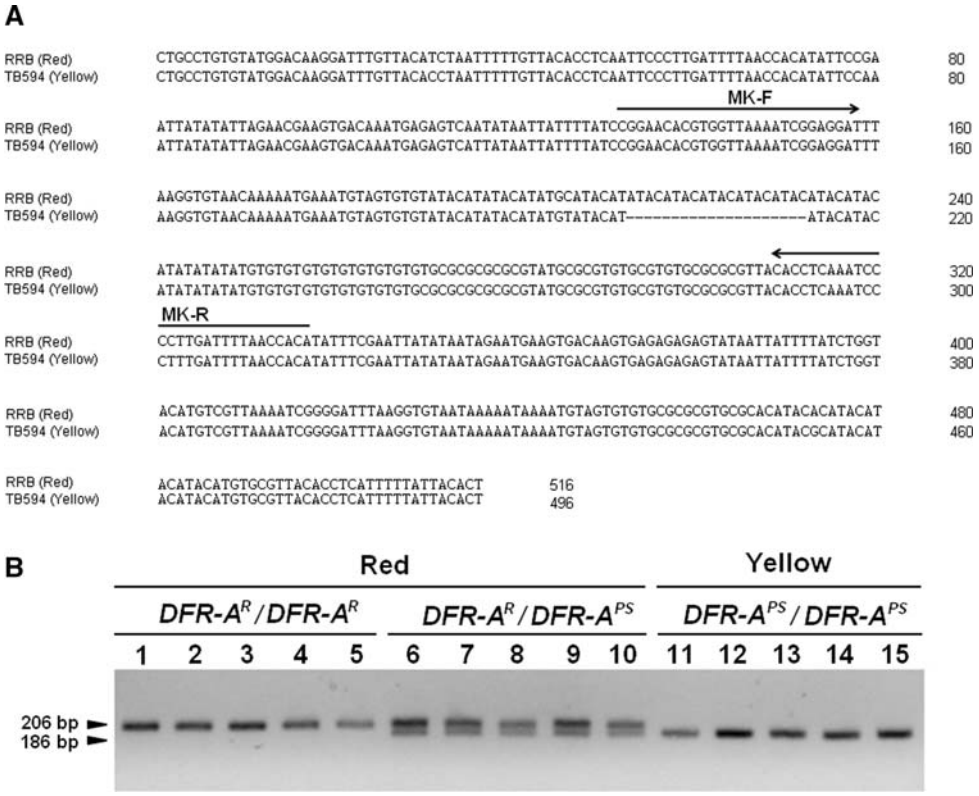

Fig. 7 A simple PCR-based marker developed for selection of the *DFR-A*^{PS} allele. **a** Alignment of nucleotide sequences of the promoter regions flanking the 20-bp deletion. *RRB* red onion cultivar, 'Red Ring Ball'. *TB594* a yellow breeding line. *Horizontal arrows* on the sequences show the position of primers used for molecular marker tests. **b** PCR products amplified using primers shown in Fig. 7a from F_2 plants originating from the cross between red ('Red Ring Ball') and yellow (TB594) parents

perfect co-segregation of the molecular marker and the color phenotypes (data not shown). Since this molecular marker was designed on the basis of the polymorphic sequence of the gene responsible for the phenotype of interest, prediction of genotypes using this molecular marker is perfectly accurate without errors or recombinants between the target gene and molecular markers. Therefore, this molecular marker will be a useful tool in breeding programs for the development of red onion cultivars.

Discussion

Identification of two inactive alleles of the *DFR-A* gene responsible for bulb color difference

A novel allele of the *DFR-A* gene containing a premature stop codon was identified in the majority of yellow onion cultivars and breeding lines used in this study. Perfect co-segregation of the *DFR-A* alleles and color phenotypes in the F_2 population indicated that inactivation of the *DFR-A* gene led to lack of anthocyanin in yellow onions of that population (Fig. 7). Although the presence of a premature stop codon is the most plausible cause of *DFR* gene inactivation, it may not be the sole defect resulting in inactivation, since the transcript level of the *DFR-A* gene in yellow F_2 onions was significantly reduced compared with that of red F_2 onions (Fig. 5a). We identified a 20 bp deletion of 'CATA' repeat sequences in the putative promoter region of the *DFR-A*^{PS} allele, suggesting that this repeat sequence may be required for proper transcription of the *DFR-A* gene. However, the *DFR-A*^{R} allele isolated from US-type red onions was found to have the same 20-bp deletion, but its transcription level and color phenotype were normal (Kim et al. 2004b), indicating that this deletion may not be critical. The reduced transcript level is more likely the result of non-sense-mediated mRNA decay (NMD). It is well known that faulty mRNAs containing a premature stop codon are degraded by the NMD mechanism to prevent any dominant-negative effects caused by truncated protein products (Hentze and Kulozik 1999; Neu-Yilik et al. 2004; Chang et al. 2007). Since NMD is conserved in all eukaryotes, it is probably acting on the transcript of the *DFR-A*^{PS} allele in onions.

We failed to amplify any *DFR-A* sequences from the *DFR-A*^{DEL} allele. In addition to the four primer combinations presented in Fig. 6, more than 15 primer pairs covering the promoter and coding regions of the *DFR-A* gene were also tested, but no PCR products corresponding to the *DFR-A* gene were obtained. This implies that a relatively large genomic region containing the entire *DFR-A* gene might be deleted in the *DFR-A*^{DEL} allele. Although the exact arrangement of the chromosomal region at the *DFR-A* locus is not known at this time, homologous recombination

between *DFR* homologs might facilitate dynamic genomic rearrangement. Two homologous *DFR* genes (*DFR-B*, *DFR-C*) were previously reported (Kim et al. 2005b).

Because we failed to obtain the sequences flanking the deleted *DFR-A* gene in the *DFR-A*^{DEL} allele, only a dominant molecular marker showing the presence or absence of the *DFR-A* gene could be developed in this study. Therefore, heterozygous and homozygous dominant red onions cannot be distinguished using this dominant marker. The efficacy of molecular markers for bulb color selection is significantly reduced if homozygous dominant individuals cannot be selected; therefore, we are currently attempting to isolate the flanking sequences so that we can develop a co-dominant marker for selection of the *DFR-A*^{DEL} allele.

Development of a molecular marker for selection of allelic selection of the *DFR-A* gene and its application in onion breeding programs

This study identified two new inactive alleles of the *DFR-A* gene in onion cultivars grown in Korea and Japan. Interestingly, the putative critical mutations responsible for inactivation of the *DFR-A* gene are completely different in the three inactive *DFR-A* alleles described to date (*DFR-A*^{TRN}, *DFR-A*^{PS}, *DFR-A*^{DEL}) although their yellow color phenotypes are indistinguishable. The cladogram of multiple *DFR-A* alleles showed that two inactive alleles (*DFR-A*^{TRN}, *DFR-A*^{PS}) might originate from independent mutations of active *DFR-A* alleles.

Since anthocyanin is unlikely to be indispensable for plant survival (Holton and Cornish 1995), accumulation of many critical mutations on the coding sequence might be expected. However, the geographical distribution of specific *DFR-A* alleles would be restricted to small areas; for example, the *DFR-A*^{TRN} allele was exclusively found in yellow onion germplasm maintained in Southern Texas (Kim et al. 2005b), and none of the cultivars grown in Korea that were analyzed in this study contained the *DFR-A*^{TRN} allele. Regional isolation of specific alleles might reflect infrequent genetic exchange between cultivars adapted to specific regions and germplasm introduced from remote areas. Sensitive and differential bulbing responses of onion cultivars to specific day-length would be a major barrier to such crosses. In addition, bulb color itself is also a barrier to crosses, since onion breeders have hesitated to make crosses between US-type and Brazilian yellow onions because their F_1 hybrids become an undesirable light pink owing to complementation of genes encoding DFR and ANS (Kim et al. 2005c).

Nonetheless, many crosses between distantly related breeding materials have recently been made to introduce useful traits from landraces or wild species. Advances in genomics and marker-assisted backcrossing techniques have made it practically feasible to introgress traits such as disease resistance from landraces (Chen et al. 2001; Neeraja et al. 2007). Therefore, knowledge of the diverse allelic composition of the *DFR-A* gene will provide valuable information to onion breeding programs to facilitate wide crosses between onion breeding materials. Indeed, the functional marker for bulb color selection developed in this study will be used as a foreground selection marker in marker-assisted back-crossing programs to develop red onion cultivars with enhanced shelf-life during storage.

Acknowledgments This study was supported by the "Specific Joint Agricultural Research-promoting Projects (Project No. 200803A010 81219)", RDA, Republic of Korea, and partially supported by the Biotechnology Research Institute, Chonnam National University, Republic of Korea. We thank Seung-Hee Kim and Seon Chong for their dedicated technical help.

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