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Identification of two novel mutant *ANS* alleles responsible for inactivation of anthocyanidin synthase and failure of anthocyanin production in onion (*Allium cepa* L.)

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Abstract Two genes (*DFR-A* and *ANS*) encoding dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS) enzymes in the anthocyanin biosynthesis pathway, respectively, are complementarily involved in anthocyanin production in onion (*Allium cepa* L.). Eleven inactive *DFR-A* alleles have been reported, with only a single inactive *ANS* allele previously identified. Two additional inactive *ANS* alleles are reported in this study. A mutant *ANS* allele containing a 4-bp insertion at the end of exon1 was identified from yellow bulbs of the F_2 population in which the *DFR-A* genotype was homozygous for an active allele. The 4-bp insertion caused a frame-shift mutation and resulted in creation of a premature stop codon at the start of exon2. This mutant *ANS* allele was designated ANS^{PS} allele. RT-PCR results showed that transcripts of the ANS^{PS} allele were almost undetectable in yellow F_2 bulbs, implying the involvement of nonsense-mediated mRNA decay. A cleaved amplified polymorphic sequence marker was developed for detection of the ANS^{PS} allele. Another inactive *ANS* allele was identified from the light-red F_1 populations showing complementation between *DFR-A* and *ANS* genes. A critical amino acid change of the strictly conserved serine residue into leucine was found in this mutant allele designated ANS^{S188L}. In addition, seven variants of active *ANS* alleles were identified from diverse onion germplasm. A stepwise process consisting of PCR amplification and sequencing of PCR products was devised to identify three inactive (ANS^{PS}, ANS^{S188L}, and ANS^{G229R}), one leaky (ANS^{P}), and two active *ANS* alleles (ANS^{L} and ANS^{h1}).

Keywords Onion · *Allium cepa* · Bulb color · Anthocyanidin synthase · Molecular marker

Introduction

Onion (*Allium cepa* L.) one of the most important vegetable crops that is cultivated worldwide from tropical to boreal regions. It has medicinal and health-promoting benefits, and has been consumed for salad or to add flavor in foods for thousands of years (Brewster 1994). Numerous studies on the health-promoting effects of functional compounds, such as flavonoid and organosulfur, have been carried out (Cook and Samman 1996; Herman-Antosiewicz and Singh 2004; Lotito and Frei 2006; Clere et al. 2011;

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Cushnie and Lamb 2011; Nishiumi et al. 2011; Russo et al. 2012). However, relatively few studies have been performed on onion genetics and genomics (Khosa et al. 2015). Biennial life cycle, severe inbreeding depression, and huge genome size (approximately 15,876 Mb) of onion (Ricroch et al. 2005) might be responsible for the paucity of genetic and genomic information. Differential requirements of temperature and day-length for bulbs and inflorescence formation of onion germplasm have also hampered maintenance of diverse germplasm in limited regions.

Although onion cultivars are mainly classified into groups depending on responses to day-length, bulb color (predominantly red, yellow, and white) is also a major criterion for grouping of onion cultivars. Flavonoid compounds are responsible for bulb coloration in onions. Flavonoids are plant secondary metabolites. There are more than 8000 derivatives (Veitch and Grayer 2011), which function in roles like ultraviolet protection and pigmentation in plants (Shirley 1996; Fini et al. 2011). Fifty-four flavonoid derivatives have been characterized in onions (Fossen et al. 1996; Rhodes and Price 1996; Slimestad et al. 2007). Among them, the concentration of quercetin is exceptionally high in red and yellow onions, and anthocyanin is the pigment for a red bulb color. However, no flavonoid compound is detected in white onions (Griffiths et al. 2002; Kim et al. 2005b).

Since mutations in color phenotypes can be easily detected, and they are not lethal in most plants, these color mutants in plants like maize and petunia have been used to isolate genes involved in the flavonoid and anthocyanin biosynthesis pathways (Goodrich et al. 1992; Quattrocchio et al. 1993; Holton and Cornish 1995; Spelt et al. 2000; Yamazaki et al. 2003). In addition to most structural genes encoding enzymes in the pathway (Holton and Cornish 1995; Ferrer et al. 2008; Vogt 2010), regulatory genes controlling transcription of structural genes have been cloned in many plant species. Although three families of transcription factors (R2R3 MYB, bHLH, and WD40) are known to be involved in regulation of the pathway, subsets of target structural genes may vary depending on plant species (Ramsay and Glover 2005; Petroni and Tonelli 2011; Czemmel et al. 2012). Almost all structural genes encoding enzymes in the anthocyanin biosynthesis pathway were cloned in onions (Kim et al. 2004a, 2005a), but no transcription factor controlling the pathway has yet been characterized.

Five loci (*I*, *C*, *G*, *L*, and *R*) determining different bulb colors were previously reported in onion (Reiman 1931; Clarke et al. 1944; El-Shafie and Davis 1967). The *I* locus, known as a color-inhibiting factor, makes a white bulb color regardless of genotypes of other loci when the *I* locus genotype is homozygous dominant. A white bulb color also appears if the genotype of the *C* locus is homozygous recessive. Transcripts of two homologous genes (*CHS-A* and *CHS-B*) encoding chalcone synthase were significantly reduced in white onions containing homozygous recessive *C* locus. A regulatory gene controlling transcription of the *CHS-A* and *CHS-B* genes is likely to be a candidate gene for the *C* locus (Kim et al. 2005b). A chartreuse bulb color appears when the *G* locus genotype is homozygous recessive, and genotypes of the *I* and *C* loci do not produce a white bulb color (El-Shafie and Davis 1967).

When the *I* locus is homozygous recessive, and at least one dominant allele of both *C* and *G* loci are present, yellow and red bulb colors can be formed. The *R* and *L* loci are involved in determination of color difference between red and yellow onions. If either the *R* or *L* locus is homozygous recessive, no anthocyanin is produced, resulting in a yellow bulb color. It was proposed that the genes encoding dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS) enzymes in the anthocyanin biosynthesis pathway corresponded to the *R* and *L* locus, respectively (Kim et al. 2005a, c). In addition, another locus (*L2*) linked to the *L* locus with a distance of 6.3 cM was reported to control the complementation with the *R* locus (Khar et al. 2008; Duangjit et al. 2014). A total of 11 inactive alleles of the *DFR-A* gene encoding the DFR enzyme were identified from diverse onion germplasm (Song et al. 2014; Kim et al. 2015). A variety of mutations, such as insertion, deletion, or critical amino acid changes, were found in 11 inactive *DFR-A* alleles. Three of them are inactivated by insertion of a long terminal repeat retrotransposon or DNA transposons (Kim et al. 2015).

A pink or light-red bulb color is caused by significantly reduced transcripts of the *ANS* gene (Kim et al. 2004b). Reduced transcription of the *ANS* allele in pink bulbs is likely to be caused by insertion of a 6258-bp non-autonomous DNA transposon (*AcPINK*) in the promoter region of the *ANS* allele (Kim et al. 2015). Meanwhile, the first inactive *ANS* allele was identified from a Brazilian yellow cultivar

of which F_1 hybrids produced a light-red bulb color when it had been crossed with a yellow breeding line containing an inactive *DFR-A* allele. A putative critical amino acid change was assumed to be responsible for inactivation of the mutant *ANS* allele (Kim et al. 2005a). In this study, in addition to the first inactive *ANS* allele, two novel inactive *ANS* alleles containing different mutations are characterized, and a systematic process for identification of major active and inactive *ANS* alleles from diverse onion germplasm is developed.

Materials and methods

Plant materials

A F_2 population originating from the cross between white (436W) and yellow (503Y) breeding lines was used for identification of an inactive *ANS* allele containing a premature stop codon. Four F_1 populations originating from the crosses between a downy mildew resistant cultivar 'Santero' and four yellow breeding lines (OB814, OB251, OB252, and OB870) were used to identify another inactive *ANS* allele containing a critical amino acid change. Bulb color phenotypes of these F_2 and F_1 populations were observed after bulb harvest. A F_2 population originating from the cross between 'Santero' and a red breeding line (H6) was used to select yellow F_2 individuals containing the homozygous inactive *ANS* allele which was derived from the 'Santero'. Color phenotypes of this F_2 population were evaluated at the four-leaf seedling stage.

A total of 116 onion accessions introduced from diverse countries were used to find variants of the *ANS* alleles (Supplementary Table 1). DNAs isolated in a previous study (Song et al. 2014) were used in this study. Fifty-three accessions introduced from 16 countries were requested from the National Plant Germplasm System, Agricultural Research Service, Baltimore, MD, USA. Leaf tissue samples of 28 and 35 accessions were collected from National Institute of Horticultural and Herbal Science (Muan, Korea) and Onion Research Institute (Changnyeong, Korea), respectively. Accessions collected from two Korean institutes have been introduced from other countries, though the detailed history of their introduction is not available.

DNA extraction, PCR amplification, and sequencing of PCR products

Total genomic DNAs were extracted from leaves of seedling or sprouted bulbs using a cetyl trimethylammonium bromide method (Doyle and Doyle 1987). PCR was performed in 25-μL reaction mixtures containing 0.05 μg template, 2.5 μL 10× PCR buffer, 0.2 μL forward primer (10 μM), 0.2 μL reverse primer (10 μM), 0.2 μL dNTPs (10 mM each), and 0.25 μL polymerase mix (Advantage 2 Polymerase Mix; Clontech, Palo Alto, CA, USA). The primer sequences used in this study are listed in Supplementary Table 2. Full-length *ANS* sequences were obtained by assembling three fragments which were amplified using three primer pairs (Supplementary Table 2).

In case of primers with lengths ranging from 25 to 28 base pairs, PCR amplification was performed with an initial denaturation step at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 3 min, and a final 10 min extension at 72 °C. When using 20–22 bp primers, PCR amplification procedure consisted of an initial denaturation step at 95 °C for 4 min; 10 cycles at 95 °C for 30 s, 65 °C (0.8 °C decrements in each cycle) for 30 s and 72 °C for 1 min; 35 cycles at 95 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min, and a final 10 min extension at 72 °C.

The PCR products were visualized on a 1.5 % agarose gel after ethidium bromide staining. For sequencing of PCR products, they were first purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and then sequenced using Big Dye (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Subsequently, the sequences were obtained using an ABI PRISM 3730XL analyzer (Applied Biosystems). In the case of the cleaved amplified polymorphic sequence (CAPS) marker, PCR products were digested with the *Sna* BI restriction enzyme at 37 °C for 1 h. The digested PCR products were visualized on 1.5 % agarose gels after ethidium bromide staining.

RNA extraction, cDNA synthesis, and RT-PCR

Total RNAs were extracted from fresh leaf sheaths of sprouted bulbs using a RNA extraction kit (RNeasy plant mini kit, Qiagen) following the manufacturer's instructions. cDNAs were synthesized from the

extracted RNA using a commercial cDNA synthesis kit (SuperScript™ III first-strand synthesis system for RT-PCR; Invitrogen, Carlsbad, CA, USA). RT-PCR amplification was performed with an initial denaturation step at 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 3 min, and a final 10-min extension at 72 °C. The primer pair of PS-L and PS-R was used in RT-PCR (Supplementary Table 2). The onion tubulin sequence obtained from EST sequences (TC125) from the DFCI *A. cepa* gene index (Antonescu et al. 2010) was used as a control.

Construction of phylogenetic trees

Nucleotide sequences from 5′ to 3′-UTRs of the *ANS* alleles and amino acid sequences of 53 ANS enzymes isolated from different species were respectively aligned using BioEdit software (Hall 1999). The species names and accession numbers of the ANS enzymes are shown in Supplementary Fig. 1. The gaps were removed using Gblocks software (Castresana 2000). Phylogenetic trees were constructed by *MEGA* version 4 (Tamura et al. 2007) using a neighbor-joining method. Node support of the phylogenetic tree was assessed by 1000 bootstrap replicates.

Nomenclature of onion *ANS* alleles

Previously, we first designated the active and inactive *ANS* alleles as *ANS-L* and *ANS-l*, respectively (Kim et al. 2005a), and the leaky mutant and another active alleles were designated *ANS-p* and *ANS-h1*, respectively (Kim et al. 2006). However, this nomenclature is inconsistent with that of the *DFR-A* gene and general genetic convention. In addition, it makes confusion between alleles and homologous genes. Since three homologous *DFR* genes were identified in onion genome, they were designated as *DFR-A*, *DFR-B*, and *DFR-C*, respectively (Kim et al. 2005c). For unification of nomenclature of genes responsible for onion bulb colors, the format used in naming of the *DFR-A* alleles (Song et al. 2014) is employed in this study to avoid any confusion. Hereafter, the *ANS-L*, *ANS-l*, *ANS-p*, and *ANS-h1* alleles are renamed as ANS^{L}, ANS^{G229R}, ANS^{P}, and ANS^{h1}, respectively.

Results

Identification of an inactive *ANS* allele harboring a premature stop codon in onion

In a F_2 population originating from the cross between white (436W) and yellow (503Y) breeding lines, unusual segregation of bulb colors was observed. Dark-red onions appeared together with yellow, white, and reddish-white onions (Fig. 1). Segregation ratio of bulb colors in the F_2 population implied that the white parental line might be homozygous dominant for the *I* locus (Table 1). The reddish-white bulb color was assumed to appear when the *I* locus genotype was heterozygous, and at least one active alleles of both *L* and *R* loci were present (Table 1). To prove this hypothesis, genotypes of the *DFR-A* gene corresponding to the *R* locus were analyzed first, since the

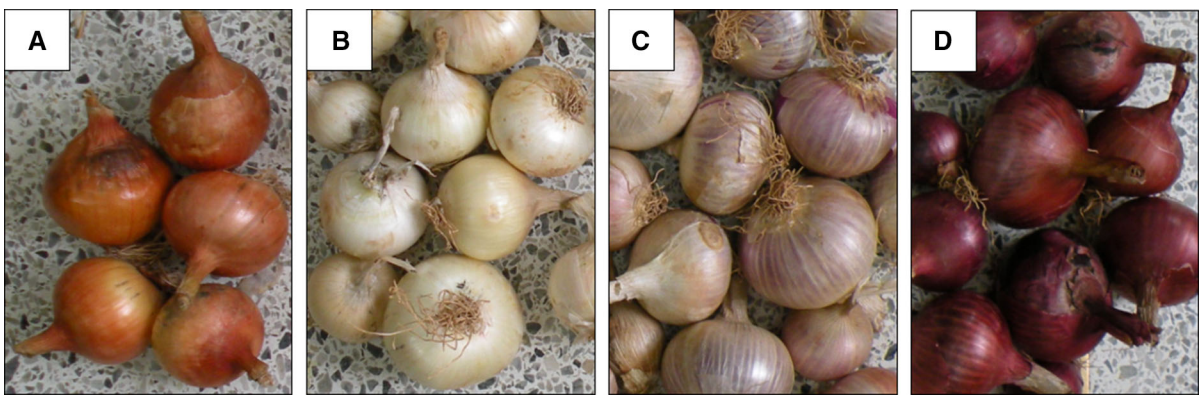

Fig. 1 Harvested onion bulbs of the F_2 population originating from the cross between *white* (436W) and *yellow* (403Y) breeding lines. **a** *Yellow* bulbs, **b** *white* bulbs, **c** *reddish-white* bulbs, and **d** *red* bulbs. (Color figure online)

Table 1 Inheritance pattern of onion bulb colors in the F_2 population originating from the cross between *white* (436W) and *yellow* (503Y) breeding lines

Phenotypes	Expected genotypes	Number of bulbs	
		Observed	Expected
Red	*ii RR L-*	12	12.37
Yellow	*ii RR ll*	6	4.13
White	*II RR-*, *Ii RR ll*	21	24.75
Reddish-white	*Ii RR L-*	27	24.75
Total		66	66

majority of yellow breeding lines contain inactive *DFR-A* alleles (Park et al. 2013). Genotyping of the *DFR-A* gene was performed following the process reported previously (Song et al. 2014). Unexpectedly, the *DFR-A* genotype of F_2 individuals were all homozygous for the active *DFR-A*^{R1} allele regardless of bulb colors. This result suggested that the *L* locus corresponding to the *ANS* gene might be inactive in this population.

After full-length *ANS* genomic regions were sequenced from yellow and red F_2 bulbs, a novel *ANS* allele was identified in the yellow F_2 bulbs. The coding sequence of this *ANS* allele was identical to that of the active *ANS*^{L} allele, except for the 4-bp ('GTAC') insertion at 3′-end of the exon1 (Fig. 2). In the 2864-bp promoter sequences, only two SNPs were identified between the active *ANS*^{L} and mutant alleles. Since the 4-bp insertion in the exon1 caused a frame-shift and created a premature stop codon at 5′ end of the exon2, this mutant *ANS* allele was probably inactive due to lack of the exon2 region. In addition, RT-PCR results showed that the *ANS* transcripts were almost undetectable in yellow F_2 individuals compared with those of red F_2 bulbs (Fig. 3a). Hereafter, this novel mutant *ANS* allele was designated the *ANS*^{PS} allele, representing the presence of a Premature Stop codon. The sequences of the *ANS*^{PS} allele was deposited into the GenBank under the accession number of KU866449.

To detect this mutant allele, a CAPS marker was developed on the basis of polymorphic recognition sequences of the *Sna* BI restriction enzyme. Since the 4-bp insertion in the *ANS*^{PS} allele created a recognition site for the *Sna* BI enzyme, the PCR products amplified from yellow F_2 bulbs were completely digested (Fig. 3b). This CAPS marker was designated ANS-PS. *ANS* genotypes of all F_2 bulbs were identified using the ANS-PS marker. As expected, all yellow onion contained the homozygous *ANS*^{PS} allele, and red and reddish-white were heterozygous or homozygous *ANS*^{L} allele (Supplementary Table 3). In case of white onions, all three possible genotypes were identified. Even though both *DFR-A* and *ANS* genes were active, white bulb colors appeared in five white F_2 bulbs. The segregation ratios of bulb colors and *ANS* genotypes of this F_2 population indicated that the white parental line probably contained the homozygous dominant *I* locus.

Identification of another inactive *ANS* allele containing a critical amino acid change

For the purpose of introgression of downy mildew resistance into domestic breeding lines, a resistant yellow cultivar, 'Santero', was crossed with four yellow breeding lines. Although bulb colors of both parental lines were yellow, all four F_1 populations showed light-red bulb colors (Supplementary Fig. 2). The light-red bulb color in the F_1 populations was assumed to appear by complementation between the *R* and *L* loci. To prove the complementation between the *DFR-A* and *ANS* genes, the *DFR-A* genotypes of 'Santero' and four breeding lines were first analyzed. Whereas 'Santero' possessed the active *DFR-A*^{R3} allele, four breeding lines contained the inactive *DFR-A*^{PS} alleles harboring a premature stop codon (Kim et al. 2009).

Regarding the *ANS* genotypes, coding sequences of four breeding lines were identical to those of the active *ANS*^{L} allele. However, the *ANS* genotype of 'Santero' identified by sequencing of the PCR products showed heterozygous patterns of sequence peaks (data not shown). To obtain homozygous *ANS* alleles present in 'Santero', yellow F_2 individuals originating from the cross between 'Santero' and red breeding lines (H6) were analyzed. Another novel *ANS* allele was identified together with the *ANS*^{PS} allele in the yellow F_2 plants. The sequences of exons and intron of this mutant allele were identical to those of the active

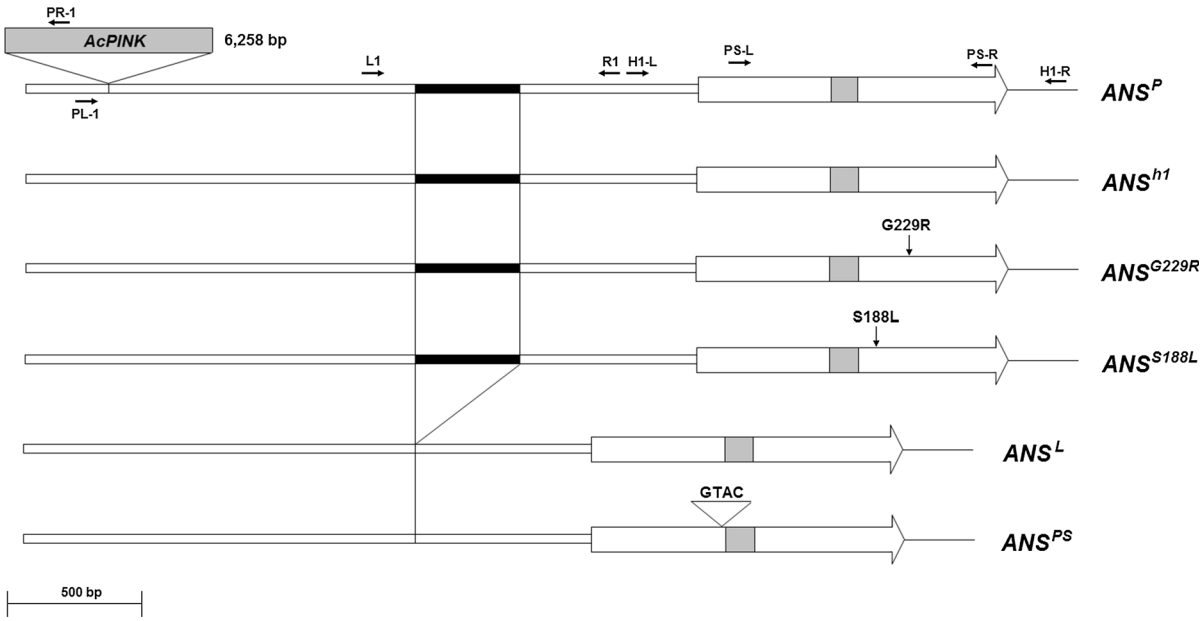

Fig. 2 Organization of the major *ANS* alleles isolated from onions. *Arrow-shaped boxes* indicate coding regions and the 5′-to-3′ direction. The *gray* and *empty boxes* in the coding regions indicate introns and exons, respectively. The *gray box* on the *inverted triangle* in the ANS^P allele indicates the DNA transposon. Size of the DNA transposon is indicated at the *right side* of the *box*. The *filled boxes* in the promoter sequences indicate large-sized inserted or deleted sequences. The 4-bp insertion in the ANS^{PS} allele is shown on the *inverted triangle*. The positions of critical amino acid changes in the ANS^{G229R} and ANS^{S188L} alleles are shown with *vertical arrows*. *Horizontal arrows* on the ANS^P allele indicate primer-binding sites. The primer sequences are shown in Supplementary Table 2

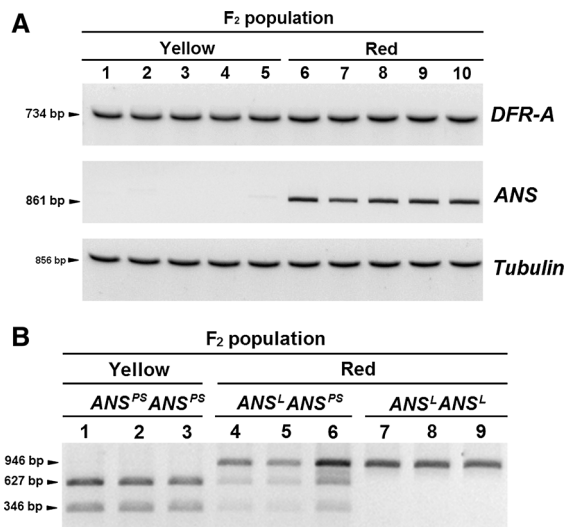

Fig. 3 RT-PCR analysis of the ANS^{PS} alleles and development of a molecular marker for identification of the ANS^{PS} allele. **a** RT-PCR amplification of the F_2 population originating from the cross between white (436W) and yellow (403Y) breeding lines. **b** *ANS* genotyping of the F_2 plants by the CAPS marker, ANS-PS. The PCR products amplified using a primer pair of PS-L and PS-R were digested with the *Sna* BI restriction enzyme

ANS^{h1} allele except for a single SNP (C → T) in the exon2 (Fig. 2).

Sequences of the 3168-bp promoter region were completely identical between the mutant and ANS^{h1} alleles. Therefore, the SNP in the exon2 might be responsible for inactivation of this mutant allele. This SNP caused an amino acid change from serine to leucine at the 188th residue in the onion ANS enzyme. To assess the importance of this serine residue in the activity of ANS enzymes, amino acid sequences of the 53 ANS enzymes were retrieved from diverse monocot and eudicot species (Supplementary Fig. 1). Alignment of ANS amino acid sequences showed that the serine residue in that position was strictly conserved in all ANS enzymes (Fig. 4), implying that this serine residue might be essential for ANS enzyme activity. However, no known active sites of ANS enzymes were identified around this residue (Supplementary Fig. 3). This mutant allele was designated ANS^{S188L} allele, and nucleotide sequence was deposited into GenBank under the accession number of KU866450.

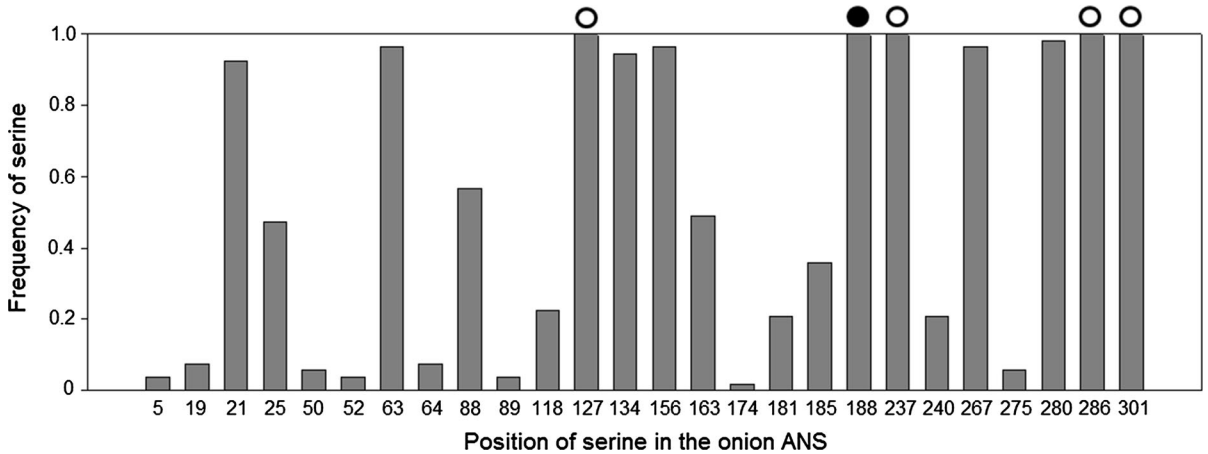

Fig. 4 Level of conservation of serine residues in the onion ANS enzyme among 53 ANS enzymes isolated from different plants species. Five *empty* or *filled circles* indicate the positions of completely conserved serine residues. The *filled circle* indicates the position of the serine residue that is changed to a leucine residue in the ANS^{S188L} allele

Detection of variants of the *ANS* alleles from diverse onion germplasm

To find additional mutant *ANS* alleles, 116 onion accessions introduced from diverse countries were analyzed (Supplementary Table 1). First, the accessions containing the ANS^{P} allele harboring the DNA transposon (*AcPINK*) in the promoter region was screened by PCR amplification using a primer pair of PL-1 and PR-1 (Fig. 2). For the rest of accessions, full-length *ANS* genomic regions were amplified and sequenced using a primer pair of H1-L and H1-R (Fig. 2). Accessions showing heterozygous patterns of sequence peaks were excluded from further analysis due to unavailability of segregation populations of each accession. Several variants of the known alleles containing a couple of SNPs were identified (Supplementary Table 4).

Two variants of the ANS^{L} allele designated ANS^{L-1} and ANS^{L-2} alleles contained 1-bp insertion in the 3′-UTR and two SNPs in the intron, respectively, but no mutation was found in the exon sequences (Supplementary Table 4). However, the 3242-bp promoter sequence of the ANS^{L-2} allele was identical to that of the ANS^{h1} allele. Four variants of the ANS^{h1} allele designated from ANS^{h1-1} to ANS^{h1-5} were identified. Three variants (ANS^{h1-1}, ANS^{h1-2}, and ANS^{h1-3}) produced no amino acid change, but the ANS^{h1-4} contained single amino acid changes from leucine to phenylalanine at the 253rd residue. However, this amino acid changes might not affect ANS enzyme activity since these positions were not conserved among 53 ANS enzymes (Supplementary Fig. 4), and even the active ANS^{P} allele contain the same amino acid change. Interestingly, the exon sequences of the ANS^{h1-4} allele were identical to those of the ANS^{h1} allele except for a single SNP, but the intron sequence was identical to that of the ANS^{P} allele (Supplementary Fig. 5), indicating occurrence of intragenic recombination between two alleles. One variant of the ANS^{P} allele designated ANS^{P-1} was identified. The sequence of genic regions of this variant allele was identical to that of the ANS^{P} allele, except for a single SNP. Although the SNP changed a threonine residue into proline at the second position, this position was not conserved (Supplementary Fig. 4), and the same amino acid change was present in the active ANS^{L} allele. Despite high homology in the coding sequences, this variant did not contain the DNA transposon in the promoter region. The 2856-bp promoter sequence of the ANS^{P-1} was identical to that of the ANS^{L} allele except for a single SNP, indicating that this variant allele might be created by recombination between ANS^{L} and ANS^{P} alleles.

Overall, the phylogenetic tree showed that the *ANS* alleles and variants were grouped into three clades based on sequence polymorphism (Fig. 5). In respect to the activity of *ANS* alleles, three inactive (ANS^{PS}, ANS^{G229R}, and ANS^{S188L}) and one leaky mutant (ANS^{P}) alleles have been identified together with two major active alleles (ANS^{L} and ANS^{h1}). Seven variants identified in this study were assumed to have

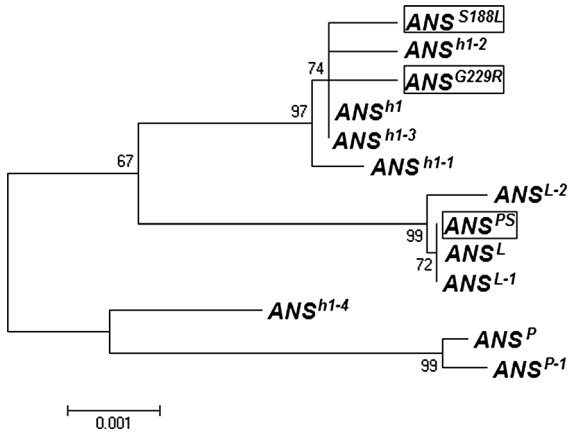

Fig. 5 Phylogenetic tree constructed using genic sequences of the 13 *ANS* alleles. Three inactive *ANS* alleles are enclosed with *empty rectangular boxes*. The *numbers* at the *nodes* are the bootstrap probability (%) with 1000 replicates. The *scale bars* indicate nucleotide substitutions per site

normal activity. Therefore, to identify six major *ANS* alleles from diverse onion germplasm, a process consisting of sequential PCR amplification and sequencing of the PCR products was developed (Fig. 6). Using this process, specific *ANS* alleles of any homozygous breeding lines can be systemically identified. In addition, any potential unidentified inactive *ANS* alleles can be detected by this process.

Discussion

Identification of a novel inactive *ANS* allele containing a premature stop codon in onion

Two additional inactive *ANS* alleles (ANS^{PS} and ANS^{S188L}) responsible for lack of anthocyanin in onions were identified in this study. The first inactive allele (ANS^{G229R}) had been identified from a Brazilian yellow cultivar in the previous study (Kim et al. 2005a). Unlike ANS^{G229R} and ANS^{S188L} alleles containing putative critical amino acid changes, the ANS^{PS} allele harbored a more apparent mutation. The 4-bp insertion in the exon1 shifted reading frames and created a premature stop codon at the start of exon2. Without the exon2 region, ANS enzymes may not function properly because 15 out of 20 active sites reported by Wilmouth et al. (2002) were positioned in the exon2 region of ANS enzymes. In addition, since there is only a single copy of the *ANS* gene in onion genome (Khar et al. 2008), this mutant *ANS* allele is

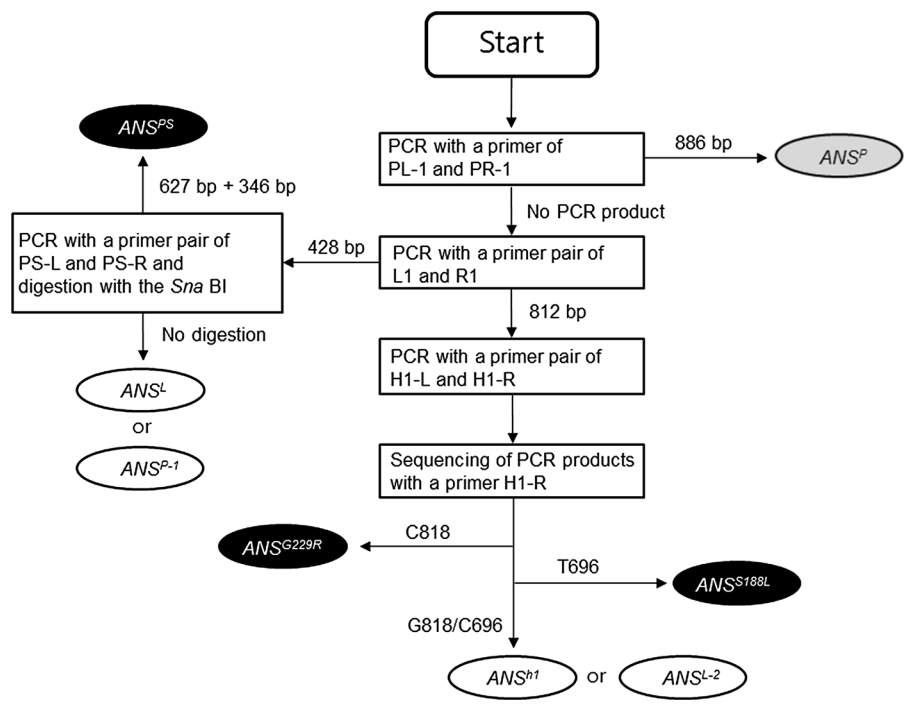

Fig. 6 Flowchart showing the process for identification of major *ANS* alleles. The three inactive and one leaky *ANS* alleles are shown in *filled* and *gray oval-shaped circles*, respectively. Four active *ANS* alleles are shown in *empty oval-shaped circles*

most likely to be responsible for failure of anthocyanin production in this population.

Stronger evidence came from the RT-PCR results. Transcripts of the ANS^{PS} allele in the yellow F_2 plants were almost undetectable compared with those of red F_2 plants. In addition, only a couple of reads mapped to the *ANS* gene were detected by RNA-Seq analysis of another yellow breeding line containing the ANS^{PS} allele (unpublished data). Since there were only two SNPs between active ANS^{L} and inactive ANS^{PS} alleles in the 2864-bp promoter region, transcription of the ANS^{PS} allele was assumed to occur normally. Therefore, transcripts of the ANS^{PS} allele were likely to be degraded by the nonsense-mediated mRNA decay (NMD) mechanism. NMD, a RNA surveillance mechanism by which faulty mRNAs containing premature stop codons are degraded to prevent negative effects of aberrant proteins, is prevalent in all eukaryotes (Hentze and Kulozik 1999; Neu-Yilik et al. 2004; Chang et al. 2007; Shaul 2015). Similarly, the NMD was assumed to affect one of the mutant *DFR-A* alleles. Transcripts of the inactive $DFR\text{-}A^{PS}$ allele containing a premature stop codon were significantly reduced in yellow F_2 onions (Kim et al. 2009). The majority of yellow onion cultivars developed in Korea contain the $DFR\text{-}A^{PS}$ allele (Park et al. 2013).

A CAPS marker, ANS-PS, was developed to detect the ANS^{PS} allele in this study. *ANS* genotypes of the F_2 population originating from the cross between white and yellow breeding lines could be easily identified using the ANS-PS marker. Genotypes of the ANS-PS marker perfectly co-segregated with bulb color phenotypes (Supplementary Table 3). The segregation ratio of bulb colors in this F_2 population indicated that the white parental line might contain homozygous dominant *I* locus. If this white parental line were a recessive-white onion containing homozygous recessive *C* locus, the segregation ratio of red, yellow, and white should be 9:3:4. However, the segregation ratio of red, yellow, white, and reddish-white fitted into a 3:1:6:6 model. The reddish-white bulb color was assumed to appear when the genotype of the *I* locus was heterozygous, and at least one active allele of both *DFR-A* and *ANS* genes was present. El-Shafie and Davis (1967) reported that buff or creamy bulb colors appeared when the *I* genotype was heterozygous. Therefore, the white bulb color conferred by the *I* locus might be incompletely dominant over yellow or red colors. When the genotype of the *I* locus is heterozygous, creamy or buff colors would be expressed if either *DFR-A* or *ANS* genes were inactive. A reddish-white color would appear if at least one alleles of both *DFR-A* and *ANS* genes were active. Few studies about white bulb colors have been performed in onions. This F_2 population would be a good material for isolation of the gene responsible for the dominant-white bulb color.

Identification of an inactive *ANS* allele containing a critical amino acid change in onion

In regard to the ANS^{S188L} allele, only a single amino acid change from serine to leucine was identified compared with the active ANS^{h1} allele. Since 'Santero' possesses a yellow bulb color and active $DFR\text{-}A^{R3}$ allele, the ANS^{S188L} allele was likely to be inactive. The ANS enzyme encoded by the active ANS^{h1} allele contained 26 serine residues, and 5 of them were strictly conserved among 53 ANS enzymes isolated from different plant species (Fig. 4). The serine residue, which is changed into leucine in the ANS^{S188L} allele, is one of the five strictly conserved serine residues. This strictly conserved serine is not positioned around any active sites reported by Wilmouth et al. (2002), but this residue probably plays an important role in stability of protein structure or might be an unidentified active site. This mutant allele will be an ideal material for studying structure of the ANS enzyme and its catalytic activity in the future studies.

In case of the first reported inactive ANS^{G229R} allele, the importance of the 229th glycine residue in ANS enzyme activity was shown by Abrahams et al. (2003). They found an *Arabidopsis* mutant line (*tds4-1*) containing an amino acid change of the glycine into an aspartate residue at the same position as the G229 of the onion ANS enzyme. This mutant failed to produce anthocyanin in the cotyledon of *Arabidopsis*. A critical role of this glycine residue in the anthocyanin production was also confirmed by complementation of the *tds4-1* mutant with a normal *ANS* gene introduced by *Agrobacterium*-mediated transformation (Abrahams et al. 2003).

Establishment of a system for identification of diverse onion *ANS* alleles

Altogether, three major active *ANS* alleles (ANS^{L}, ANS^{h1}, and ANS^{P}) and their seven variants have been

identified together with three inactive alleles (ANS^{PS}, ANS^{G229R}, and ANS^{S188L}) from diverse onion germplasm. Since these diverse *ANS* alleles could not be identified by any single molecular marker, a process consisting of serial PCR amplification and sequencing of the PCR products were developed in this study (Fig. 6). Similarly, a process for identification of 11 inactive and four active *DFR-A* alleles was developed in the previous studies (Song et al. 2014; Kim et al. 2015). Because red and yellow bulb colors in onion are determined by complementation of *DFR-A* and *ANS* genes, both *DFR-A* and *ANS* genotypes should be considered at the same time to pinpoint the blocked step in the anthocyanin biosynthesis pathway. If specific *DFR-A* and *ANS* alleles of both parental lines were identified, unwanted light-pink coloration of F_1 hybrids by complementation of these two genes could be avoided. In addition, appropriate molecular markers can be employed for marker-assisted selection of desirable plants in segregation populations. Two processes for systematic identification of the *DFR-A* and *ANS* alleles will be an essential tool for breeding of onion cultivars with diverse bulb colors.

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