Genes Genet. Syst. (2007) **82**, p. 271

Corrigendum

Genes Genet. Syst. 81, 255–263 (2006)

Complete assignment of structural genes involved in flavonoid biosynthesis influencing bulb color to individual chromosomes of the shallot (*Allium cepa* L.)

Shin-ichi Masuzaki, Masayoshi Shigyo and Naoki Yamauchi

The second to fifth sentences in the third paragraph of DISCUSSION on page 261 included several errors, and they should be replaced by the following sentences:

The multiple addition line $S_2$27 that fulfilled this condition had a red outer scale. However, the outer scales of U138 and U83 that did not fulfill the prerequisite condition also turned red.

Genes Genet. Syst. (2006) **81**, p. 255–263

Complete assignment of structural genes involved in flavonoid biosynthesis influencing bulb color to individual chromosomes of the shallot (*Allium cepa* L.)

Shin-ichi Masuzaki[1,2], Masayoshi Shigyo[2]* and Naoki Yamauchi[2]

[1]*The United Graduate School of Agricultural Sciences, Tottori University, Tottori 680-8553, Japan*
[2]*Department of Biological and Environmental Sciences, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan*

(Received 15 April 2006, accepted 8 August 2006)

We analyzed Japanese bunching onion (*Allium fistulosum* L.) – shallot (*Allium cepa* L. Aggregatum group) alien chromosome addition lines in order to assign the genes involved in the flavonoid biosynthesis pathway to chromosomes of the shallot. Two complete sets of alien monosomic additions ($2n = 2x + 1 = 17$) were used for determining the chromosomal locations of several partial sequences of candidate genes, CHS, CHI, F3H, DFR, and ANS via analyses of PCR-based markers. The results of DNA marker analyses showed that the CHS-A, CHS-B, CHI, F3H, DFR, and ANS genes should be assigned to chromosomes 2A, 4A, 3A, 3A, 7A, and 4A, respectively. HPLC analyses of 14 *A. fistulosum* – shallot multiple alien additions ($2n = 2x + 2 - 2x + 7 = 18 - 23$) were conducted to identify the anthocyanin compounds produced in the scaly leaves. A direct comparison between the genomic constitution and the anthocyanin compositions of the multiple additions revealed that a 3GT gene for glucosylation of anthocyanidin was located on 4A. Thus, we were able to assign all structural genes involved in flavonoid biosynthesis influencing bulb color to individual chromosomes of *A. cepa*.

Key words: alien chromosome addition line, *Allium cepa*, *Allium fistulosum*, chromosomal location, flavonoid biosynthetic gene

INTRODUCTION

Onions (*Allium cepa* L. Common onion group) are known to contain a large amount of flavonoids, which are present in the colored scales of the edible part (Chu et al. 2000; Bahorun et al. 2004). Quercetin-4'-glucoside and 3,4'-diglucoside account for 80% of all flavonoids in onions (Tsushida and Suzuki 1995; Rhodes and Price 1996). Red onions also contain anthocyanins, which are the largest subclass of plant flavonoids. The major anthocyanins in red onions are cyanidin 3-(6"-malonylglucoside), cyanidin 3-(6"-malonyl-3"-glucosylglucoside), cyanidin 3-(3"-glucosylglucoside), and cyanidin 3-glucoside (Terahara et al. 1994; Fossen et al. 1996; Donner et al. 1997). Cyanidin 3-(6"-malonylglucoside) constitutes more than 50% of the total anthocyanin content in three different cultivars of red onion (Fossen et al. 1996). Although the level of the anthocyanin content of red onions is <10% with respect to flavonols such as quercetin (Rhodes and Price 1996), the simultaneous intake of flavonols and anthocyanins could be regarded as particularly healthy (Gennaro et al. 2002).

The study of a complete set of Japanese bunching onion (*Allium fistulosum* L.) – shallot (*A. cepa* L. Aggregatum group) monosomic addition lines (Shigyo et al. 1996) has revealed that the genes related to the production of flavonoids are located on chromosome 5A of shallots (Shigyo et al. 1997), and there is also a possibility that their genes are located on other chromosomes. Masuzaki et al. (2006) demonstrated that the F3'H gene is located on

Edited by Kiichi Fukui
* Corresponding author. E-mail: shigyo@yamaguchi-u.ac.jp
Note: Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number, DQ394303

Abbreviations: 3GT, UDP glucose:flavonoid 3-*O*-glucosyltransferase; 3MaT, malonyl-coenzyme A:anthocyanidin 3-*O*-glucoside-6"-*O*-malonyltransferase; ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; FLS, flavonol synthase; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; SCAR, sequence-characterized amplified region

chromosome 7A, as shown by HPLC analyses using *A. fistulosum* – shallot multiple alien addition lines carrying chromosome 5A and the other chromosome(s) and that the F3'H and FLS genes are located, respectively, on chromosomes 7A and 4A, as shown by SCAR analyses using the monosomic additions. Recently, studies of the relationship between bulb color and the structural genes in the flavonoid biosynthesis pathway have revealed that white onions resulted from a reduced level of transcripts of CHS genes (Kim et al. 2004c, 2005c), gold onions from mutation of the CHI gene (Kim et al. 2004c), yellow onions from inactivation of DFR gene transcription (Kim et al. 2004b), and pink onions from mutation of the ANS gene (Kim et al. 2004a, 2005d). One sequence (GenBank™ accession number, CF445052) was selected from 10 onion sequences similar to the sequence of 3GT and used for examining the expression level of 3GT (Kim et al. 2004a, 2004c, 2005c, 2005d), as the deduced amino acid sequence showed the highest homology with 3GTs of other plant species (Kim et al. 2004a). However, whether the presumed protein transfers a glucoside residue from UDP-glucose to the 3-OH position of anthocyanidin has not been demonstrated. HPLC analysis of the above-mentioned *A. fistulosum* – shallot multiple additions is a more valid method for the determination of the chromosomal location than SCAR analysis in the monosomic additions, because no 3GT gene has been cloned yet in *A. cepa,* nor the copy number described. In *A. cepa*, SCAR analyses have so far been used for genetic mapping of alliinase (van Heusden et al. 2000a) and chromosomal assignment of 6GFFT (McCallum et al. 2006). To utilize flavonoids effectively for human health and to achieve uniformity of the bulb colors in breeding onion cultivars, it is necessary to accumulate more genetic data regarding flavonoid biosynthesis.

We report here the chromosomal locations of seven genes involved in flavonoid biosynthesis, i.e., CHS-A, CHS-B, CHI, F3H, DFR, ANS, and 3GT, which we assigned by using *A. fistulosum* – shallot alien chromosome additions.

MATERIALS AND METHODS

Plant materials Two complete sets of *A. fistulosum* – shallot monosomic additions (Shigyo et al. 1996) were used to assign a segment of the coding region for flavonoid genes to the chromosomes of shallots. The sets were analyzed together with their parents: the shallot (*A. cepa* L. Aggregatum group) and the Japanese bunching onion (*A. fistulosum* L. cv. 'Kujo Hoso'). Total genomic DNA was isolated from fresh leaf tissue using a mini prep DNA-isolation method (van Heusden et al. 2000a).

A. fistulosum – shallot multiple additions carrying chromosome 5A and the other chromosome(s) ($2n = 2x + 2 - 2x + 7 = 18 - 23$) (Masuzaki et al. 2006) were subjected to HPLC analyses in which we detected anthocyanins to reveal the chromosomal locations of the genes related to the anthocyanin biosynthesis.

SCAR primer design Outside- and inside-primer sets to amplify a segment of the gene-encoding enzymes from CHS to DFR, with the exception of F3'H, in a possible flavonoid biosynthesis pathway in *A. cepa*, were designed with the software GENETYX® 6.1.3 (Genetyx, Tokyo, Japan) based on the GenBank™ accession numbers of AY221244 (a full-length cDNA sequence of CHS-A), AY221245 (a full-length cDNA sequence of CHS-B), AY700850 (a full-length genomic sequence of CHI), AY221246 (a full-length cDNA sequence of F3H), and AY221250 (a full-length genomic sequence of DFR) (Table 1). The primer set developed by Kim et al. (2005a) was used for ANS. The primer set was as follows: forward, 5' – TTT GCT CGA TCG TTT AGC RGA AGA AGA – 3'; reverse, 5' – TGA GGA TGA TGA CAA AGT TAG CGG AGC A – 3'.

Optimization of PCR conditions and digestion of PCR products All PCR amplifications were done with template DNA [about 100 ng of genomic DNA for the first PCR and 5 µl of diluted template DNA (the products of the first PCR diluted 1/50 with sterile water) for the second PCR, except in the cases of CHI and F3H, in which case, 5 µl of the first PCR product was used for the second PCR], each of the primers at 1 µM, 0.25 mM dNTPs, 1 × Ex *Taq* buffer, and 0.5 U of Ex *Taq* polymerase (Takara, Shiga, Japan) in a volume of 25 µl. All PCRs were performed as follows: an initial denaturation of 3 min at 94°C; 25 cycles of PCR amplification (a 1-min denaturation at 94°C, a 1-min annealing, and a 1-min primer extension at 72°C); and a final extension for 7 min at 72°C on a Program Thermal Cycler iCycler™ (Bio-Rad, Hercules, CA, USA). The ramp times were carried out in the default conditions that adjusted temperatures at the maximum ramp rate with the minimum ramp time. The annealing temperature gradient included the temperatures between 54.0° and 68.0°C. The optimum annealing temperatures of the primer sets are given in Table 1. The second PCR products were separated by electrophoresis on 2.0% agarose gels. The analysis for ANS was done according to the procedure of Kim et al. (2005a).

If the PCR products were monomorphic on agarose gels, they were subjected to restriction analysis. Nine microliters of the second PCR products were incubated for 2 h at 37°C in a volume of 15 µl using 2 U of a restriction enzyme, and subsequently resolved by 5% denaturing polyacrylamide gel electrophoresis (PAGE) with silver staining according to the procedure of Martin et al. (2005). Restriction digestion with two enzymes, *Bam*H I and *Hae* III (Toyobo, Osaka, Japan), was used in an attempt to generate polymorphisms. The two enzymes were selected using GENETYX® 6.1.3, based on the onion

Table 1. Primer sequences and amplification conditions used in this study

Gene	PCR	Primer sequences (5' to 3')		Expected PCR	Annealing
		Forward	Reverse	product size (bp)	temperature (°C)
CHS-A	first	CGATACATGCACGTAAACGAAC	ATGCGCTCGACATATTCCC	440	68
	second	TCAACCGCTTCATGATGTACC	CCAAAACACCTCGTTATAGTCCT		
CHS-B	first	CACCTGTCCGAAGACATCC	CCCTCCTTACTTGAGTTCTTCC	452	68
	second	GTGAAGCGCTTCATGATGTACC	GGATGCGCTATCCAAAACACC		
CHI	first	TGCCTTTGATTCAGTCATC	AATAATCGACTCCAATACGG	461	67
	second	TGTAAGGGGTTTGGAAATAGGAG	GGGGTATGAGTGAAGAGAATGG		
F3H	first	AGAGAGGGGAAATATGTAGG	GGCTCCTCTAATATCGGTT	471	65
	second	TGGAAAGAAGGGCGGTTTC	TAATGGCCATGGTCACCAAG		
DFR	first	CAAAAGCCCGAATACGATG	CGGTTCATTTGGATGATGG	625	65
	second	ACAGATGTACTTTGTGTCCA	GCTTCATCGAACATACTTTCC		

sequence, which corresponded to the fragment of shallot amplified using the primer sets used in this study.

DNA sequencing The reliability of PCR amplification was evaluated by direct sequencing of the PCR products. Homology searches were performed in the GenBank™ database using the BLAST program.

HPLC analyses of anthocyanins The outer scale of the basal part of the leaf sheath was collected from the 14 multiple additions, along with the monosomic addition of *A. fistulosum* carrying shallot chromosome 5A (FF + 5A), and shallots, used as controls. Anthocyanins were extracted by the method described by Yoshitama and Yamaguchi (2004) with modification. One gram of the scale was extracted with 10 ml of 50% acetic acid for 3 days at 4°C. The extract was concentrated about 50-fold by vacuum concentration. The concentrate was filtered through a 0.5-μm filter (Katayama Chemical, Osaka, Japan) to remove pigments and used for the HPLC analyses. The HPLC separation was carried out at a flow rate of 0.8 ml per min using a LiChrospher C18 column (Merck, Darmstadt, Germany) of dimensions 4 × 250 mm. The column was equilibrated in 75% solvent A (1.5% phosphate) and 25% solvent B (1.5% phosphate, 20% acetic acid, and 25% acetonitrile). After an aliquot of filtrate (20 μl) was injected into the HPLC apparatus, a linear gradient up to 85% solvent B was applied during 40 min to elute the anthocyanin compounds (monitored at 520 nm). The apparatus for the HPLC consisted of an L-7100 pump (Hitachi, Tokyo, Japan), L-7300 column oven (Hitachi), L-7420 UV/visible detector (Hitachi), and L-7610 Degasser® (Hitachi) to degas the filtrate.

RESULTS

Assignment of genes involved in flavonoid biosynthesis to chromosomes

1) CHS The primer sets for CHS-A amplified a single PCR product with the template DNA of the shallot but not that with *A. fistulosum* (Fig. 1a). The amplicon of CHS-A was the same size as expected. An amplicon of the same size as in the shallot was observed only in the monosomic addition FF + 2A. The amplicon of shallot was sequenced. The BLASTn of the sequences showed 100% homology with the original sequence (AY221244). The primer set specific for the CHS-A gene of *A. cepa* designed in this study could not amplify a CHS-A gene of *A. fistulosum*. In the monosomic additions, the primer set could not amplify a CHS-A gene in the monosomic additions, except for FF + 2A. These results indicated that only FF + 2A had the CHS-A gene derived from the shallot. Therefore, we assigned the CHS-A gene of the shallot to chromosome 2A.

The primer sets of CHS-B amplified a single fragment of the expected size with the DNA of the shallot, and a comigrating fragment in *A. fistulosum* as well (data not shown). When the fragments of *A. fistulosum* and shallot were digested with the enzyme *Bam*H I, they both showed the same digestion patterns on 5% denaturing PAGE (data not shown). After both PCR products were digested with *Hae* III, a polymorphism between *A. fistulosum* and the shallot was detected via 5% denaturing PAGE. In the two complete sets of the monosomic additions, the same restriction fragment length polymorphism (RFLP) patterns as in the shallot were present only in FF + 4A (Fig. 1b). BLASTn of the sequence data from the amplicon of shallot had homology of 99% with the original sequence (AY221245). The primer set specific to the CHS-B gene of *A. cepa* amplified CHS-B genes in both *A. fistulosum* and shallot. The *Hae* III – RFLP patterns showed that FF + 4A had the CHS-B gene from the shallot genome and that the other monosomic additions had only a CHS-B gene from *A. fistulosum*. These results revealed that the CHS-B gene of the shallot was located on chromosome 4A.

2) CHI The primer sets of CHI amplified a single fragment of the expected size in the shallot and FF + 3A (Fig.

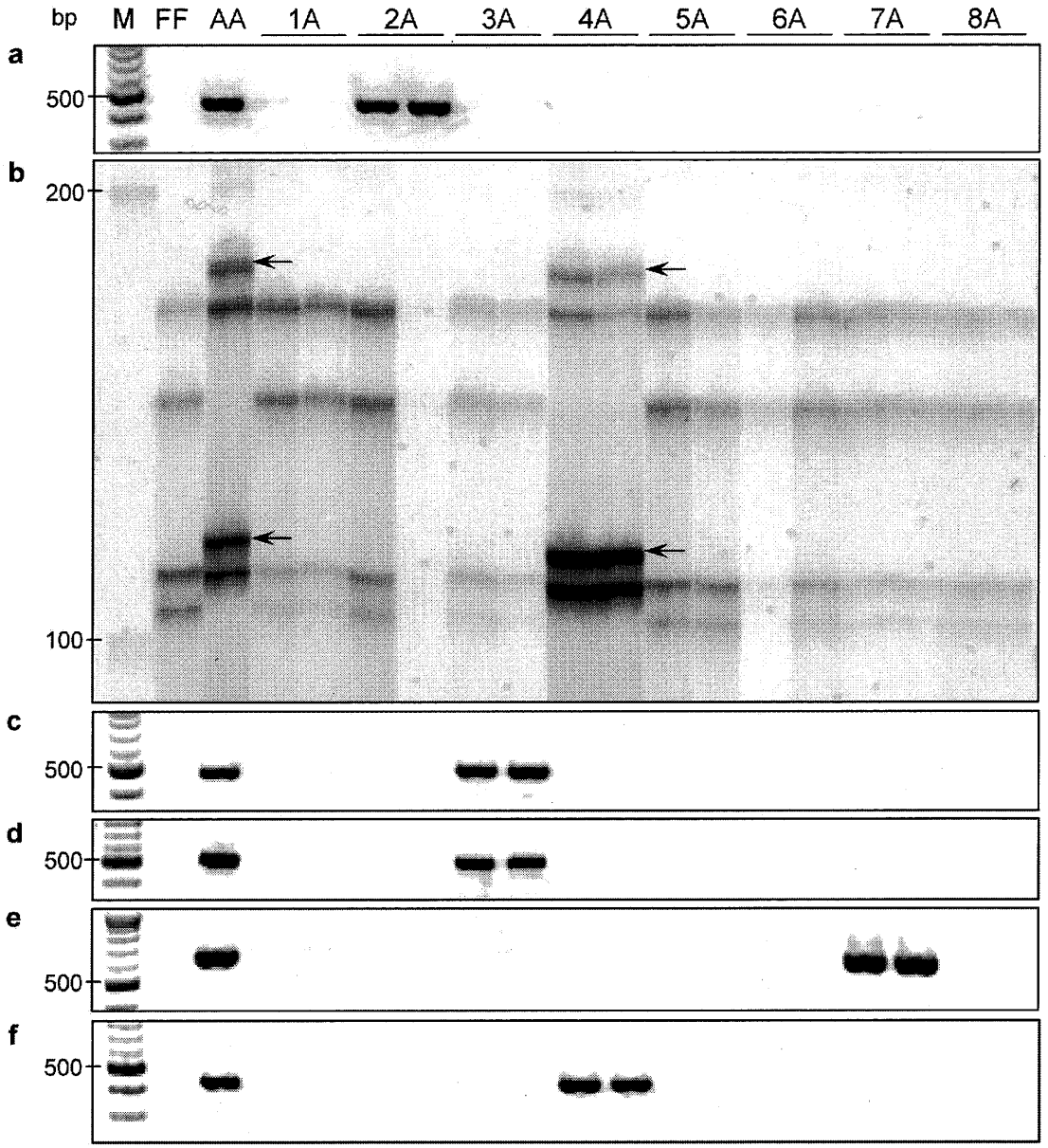

Fig. 1. Electropherograms revealing the chromosomal locations of flavonoid biosynthesis genes in shallot. FF, *A. fistulosum*; AA, shallot; 1A-8A, two complete sets of *A. fistulosum* - shallot monosomic addition lines. M, molecular size marker (100-bp DNA ladder). (**a**) CHS-A. (**b**) CHS-B. Arrows indicate shallot-specific bands. (**c**) CHI. (**d**) F3H. (**e**) DFR. (**f**) ANS.

1c). The BLASTn of the amplicon showed 100% homology with the original sequence (AY700850). The four different alleles of the onion CHI gene were designated *CHI-b* (AY700850), *CHI-t* (AY700851), *CHI-h* (AY700852), and *CHI-g* (AY700853), and unusual gold-colored onions result from *CHI-g*, which is the mutant CHI allele (Kim et al., 2004c). There was a possibility that the CHI gene of the shallot used in this study was the functional gene, as the sequence of CHI obtained in this study showed 100% homology (no gaps) with *CHI-b* and had a homology of 98% (one gap) to *CHI-g* being the mutant allele. These results revealed that the activated CHI gene might be located on chromosome 3A.

3) F3H The BLASTn of a single fragment amplified from the DNAs of the shallot and FF + 3A using the primer sets of F3H (Fig. 1d) showed 99% homology with the original sequence (AY221246). These results revealed that the F3H gene was located on chromosome 3A. The amplicon of F3H was larger than expected. Alignment between the original sequence (AY221246) and our sequence data indicated that a 159-bp indel existed as an intron (data not shown). The fact that the amplicon

was larger than the expected size resulted from this indel. This sequence has been deposited with GenBank™ (Accession number, DQ394303).

4) DFR The primer sets for DFR amplified a fragment of the expected size in the shallot and FF + 7A (Fig. 1e). The result of the homology search of the amplicon had a higher homology of 99% to a DFR gene that produces red pigment (AY221250) compared with 94% to a DFR pseudogene that does not produce red coloring (AY221251) (Kim et al. 2005b). These results indicated that the DFR gene that directs the production of red coloring might be located on chromosome 7A.

5) ANS The primer set for ANS described by Kim et al. (2005a) amplified a single PCR product with the DNAs of the shallot and FF + 4A but not with *A. fistulosum* or the other monosomic additions (Fig. 1f). The size of these amplicons was approximately 430-bp. This primer set, which was designed to distinguish between the alleles of the ANS gene, amplified an 828-bp fragment in the pink allele and a 428-bp fragment in the red (Kim et al. 2005a). This result revealed that the red allele of the ANS gene was located on chromosome 4A.

Chromosomal assignment of gene related to glucosylation of anthocyanidin based on HPLC analysis

Several peaks attributable to anthocyanins were observed in the HPLC profiles of the shallot and FF + 5A, and three compounds were identified as cyanidin 3-*O*-glucoside (Cy 3-glc), cyanidin 3-*O*-(6"-malonylglucoside) [Cy 3-(6"-Maglc)], and cyanidin aglycone (Cy) by their retention times and absorption spectra (Fig. 2a). In the shallot, all three compounds appeared, and the content of Cy 3-(6"-

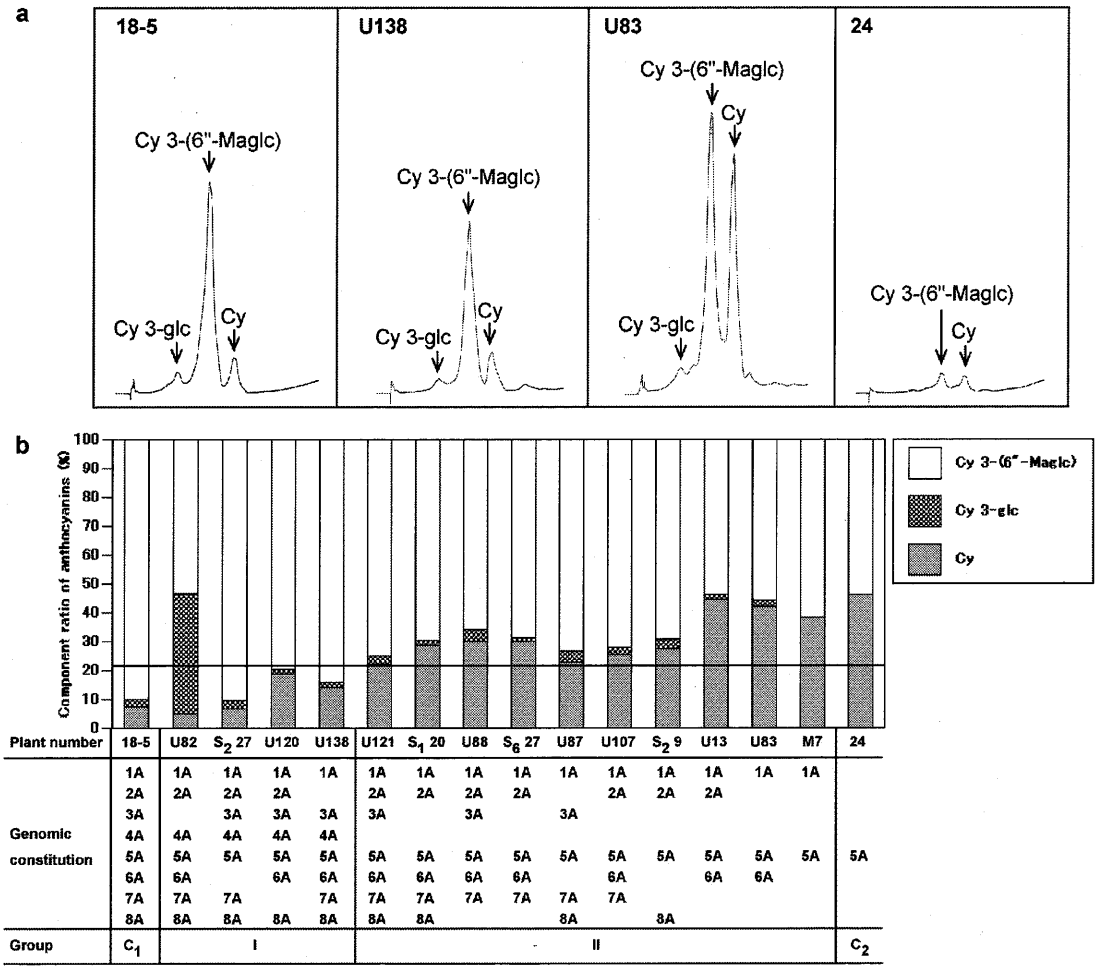

Fig. 2. High-performance liquid chromatography profiles of acetic acid extracts (detection: 520 nm) (**a**) and component ratio of anthocyanins (**b**) in shallot (18-5), a monosomic addition FF + 5A (24) and multiple alien addition lines. (**a**) Cy 3-glc, cyanidin 3-*O*-glucoside (Rt 12.14 min); Cy 3-(6"-Maglc), cyanidin 3-*O*-(6"-malonylglucoside) (Rt 18.24 min); Cy, cyanidin aglycone (Rt 23.23 min). (**b**) The genomic constitution shows the shallot chromosome which each of the plants possessed. C_1 and C_2 in the group represent control plants carrying all the chromosomes of shallot and only chromosome 5A, respectively. Group I has Cy < 21.8% as the component ratio of anthocyanins, and II has Cy > = 21.8%.

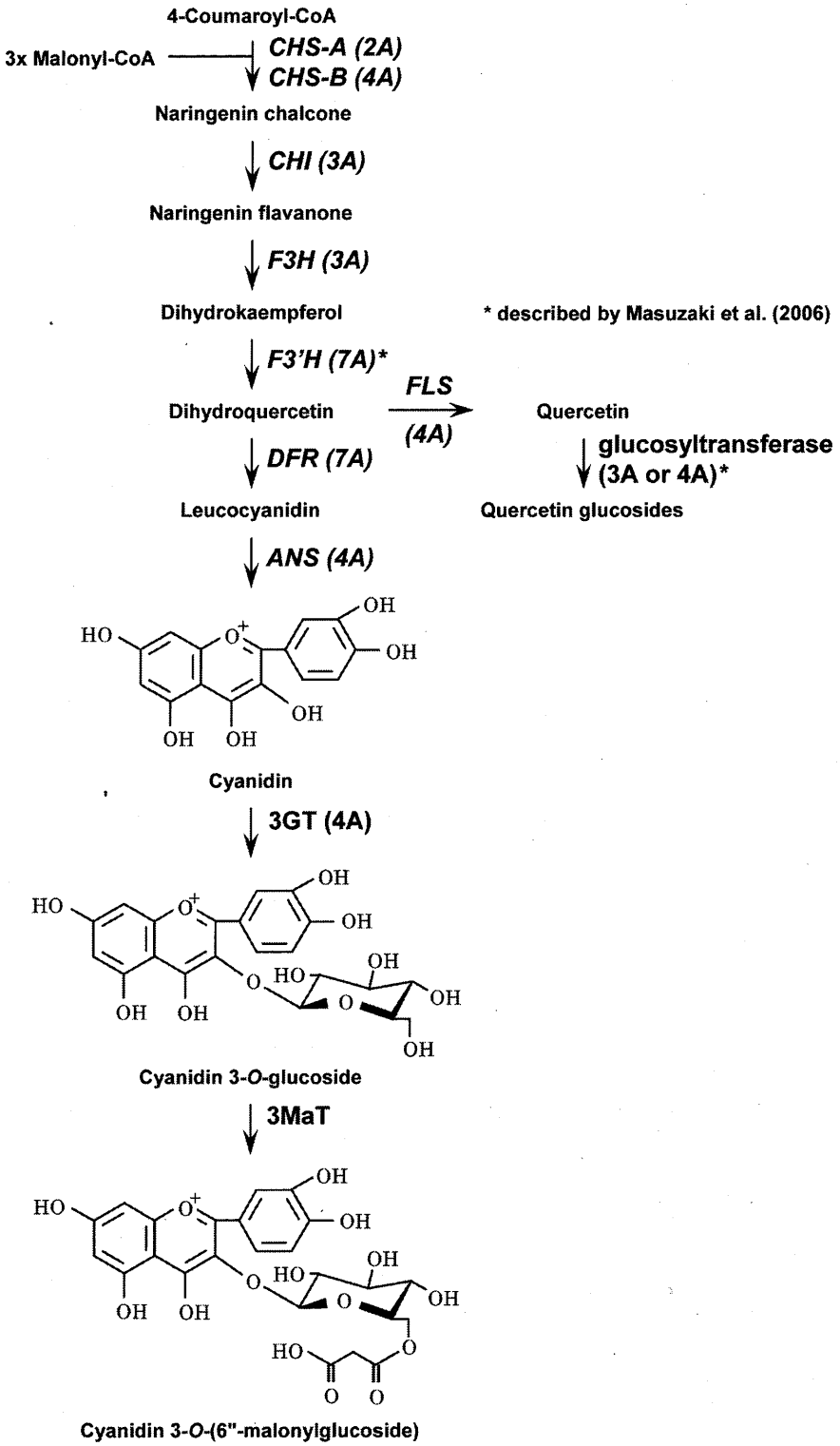

Fig. 3. Putative flavonoid biosynthesis pathway in *A. cepa* and chromosomal locations of the gene for each enzyme. The numbers in parentheses stand for the chromosome numbers of shallot on which each enzyme gene is located. The *italics* show that the chromosomal locations were determined by SCAR analyses. Asterisks indicate the genes assigned to chromosomes by Masuzaki et al. (2006). CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, UDP glucose:flavonoid 3-*O*-glucosyltransferase; 3MaT, malonyl coenzyme-A:anthocyanidin 3-*O*-glucoside-6"-*O*-malonyltransferase; FLS, flavonol synthase.

Maglc) was the highest. In FF + 5A, almost the same contents of Cy 3-(6"-Maglc) and Cy were present, but Cy 3-glc was not detected. We divided the 14 multiple additions (Fig. 2a) into two groups according to the component ratio of Cy (Fig. 2b). U121 ($2n = 2x + 7$ = FF + 1A + 2A + 3A + 5A + 6A + 7A + 8A) showed the highest component ratio of Cy (21.8%) among the five multiple additions possessing 23 chromosomes, including seven out of eight kinds of extrachromosomes. We presumed that this phenomenon was caused by an absence of one extrachromosome carrying the genes for glucosylation of Cy. Therefore, we made a demarcation at a 21.8% component ratio of Cy to evaluate whether the multiple additions had a little Cy, i.e., whether they had the genes for glucosylation of Cy. Group I had a ratio of Cy less than 21.8%, while Group II had a ratio of Cy more than 21.8%. Comparison of the genomic constitution between the two groups revealed that chromosome 4A was found only in Group I. The gene responsible for the glucosylation of anthocyanidin is 3GT, which encodes the enzyme that converts anthocyanidin into anthocyanin 3-*O*-glucoside (Kamsteeg et al. 1978; Holton and Cornish 1995) (Fig. 3). Therefore, a 3GT gene might be located on chromosome 4A.

DISCUSSION

The eight genes coding the enzymes from CHS to ANS in the flavonoid biosynthesis pathway in shallots were assigned to four different chromosomes in the previous and present studies (Fig. 3). Shigyo et al. (1997) reported that the flavonoid and anthocyanin compounds were produced only in FF + 5A out of the set of monosomic additions, but none of the flavonoid genes have thus far been allocated to chromosome 5A. In a previous report (Masuzaki et al. 2006), we demonstrated that a regulatory gene or CHS might be located on 5A. This study provides considerable support for the possibility that the regulatory gene might be located on 5A. Although the level of expression of the F3'H gene of *A. fistulosum* may be low, as indicated by an excess content of kaempferol compared with quercetin in FF + 5A (Shigyo et al. 1997; Masuzaki et al. 2006), the fact that flavonoids, including anthocyanins, were produced in FF + 5A supports the notion of a backup expression of the flavonoid genes of *A. fistulosum* in FF + 5A. Therefore, the regulatory gene for the flavonoid biosynthesis must be inactivated in *A. fistulosum*. Kim et al. (2005c) mentioned that the regulatory gene of the flavonoid biosynthesis in onions seemed to control the expression of all the structural genes. It is important to identify the structural genes controlled by the regulatory gene in *A. cepa*. We attempted to determine the copy number of the structural genes by Southern blotting with non-radioisotopes (data not shown), but no signals were detected under the genome background of 15.290 Mbps per 1C nucleus in *A. cepa* (Arumuganathan and Earle 1991). This result indicated the possibility that the structural genes involved in flavonoid biosynthesis in shallot as well as in other plants might be present as a single copy gene or a small multigene family (Sparvoli et al. 1994; Pelletier et al. 1997; Woo et al. 1999; Moriguchi et al. 2001).

Anthocyanins exist as glucosylated, acylated, and/or methylated forms in vacuoles (Strack and Wray 1994). It has been proposed that aliphatic acylation, including malonylation, of anthocyanins enhances the pigment solubility in water, protects glycosides from enzymatic degradation, stabilizes anthocyanin structures, or enhances the uptake of anthocyanins into vacuoles (Heller and Forkmann 1994). In addition, Suzuki et al. (2002) reported that malonylation of anthocyanins also prevented anthocyanins from the attack of β-glucosidase and that malonylation should enhance the pigment stability and thereby play a very important role in maintaining the color of anthocyanins in dahlia flowers. Among the three cyanidin compounds identified in this study, Cy 3-(6"-Maglc) in the outer scale of the shallot accounted for nearly 90% of the cyanidin, and among five anthocyanin compounds, two kinds of malonylated cyanidin derivatives in the skin of the shallot constituted approximately 80% of the anthocyanin (Shigyo et al. 1997). Shallots, like red onions, contain a large amount of malonylated anthocyanins, and malonylation of anthocyanins might also serve as a strategy for pigment stabilization in *A. cepa*. Cy 3-(6"-Maglc) is synthesized from Cy 3-glc by 3MaT (Yamaguchi et al. 1999; Suzuki et al. 2002) (Fig. 3). The chromosomal locations of malonylation genes will be determined by SCAR analysis in the future.

It seems that at least five different chromosomes, i.e., chromosomes 2A, 3A, 4A, 5A, and 7A, are required for the expression of red bulb color in shallot. Unfortunately, the four multiple additions ($S_2$27, U82, U120, and U138) that fulfilled this condition did not always show a red outer scale (data not shown). $S_2$27 and U138 had a red outer scale, but U120 and U82 had light red and yellow scales, respectively. The scale color of the latter two plants might result from a lower component ratio of Cy 3-(6"-Maglc) than those of the former two plants. However, in group II, which did not fulfill the prerequisite condition, the outer scale of U83 turned red. These results indicated that the color of the scale in the multiple additions might not be determined by a simple combination of extrachromosome(s) of the shallot. Consequently, to identify the major factor causing the red scale in the multiple additions, investigations must be made into correlations among the scale color, genomic constitution, flavonoid and anthocyanin constitution, and expression level of the flavonoid biosynthesis genes derived from shallot and *A. fistulosum* in each of the multiple additions.

We assigned all structural genes from CHS to ANS

involved in flavonoid biosynthesis to the chromosomes of *A. cepa*. In *A. cepa*, linkage maps based on AFLPs (van Heusden et al. 2000a, 2000b) and SSRs, SNPs, RAPDs, and RFLPs (King et al. 1998; Martin et al. 2005) have been produced. The flavonoid genes whose chromosomal locations have been revealed will be represented on these two maps hereafter, as that will be indispensable for further breeding of onion.

The authors sincerely thank Mr. Toyoshi Iwata, Shippo Seed Co., LTD, Kagawa, Japan, for providing financial support for this study. We are also grateful to Dr. Takejiro Takamura of Kagawa University for technical suggestions on HPLC analysis. We would like to acknowledge the technical expertise of The DNA Core Facility of the Center for Gene Research, Yamaguchi University, supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

Arumuganathan, K., and Earle, E. D. (1991) Nuclear DNA content of some important plant species. Plant Mor. Biol. Rep. **9**, 208–218.

Bahorun, T., Luximon-Ramma, A., Crozier, A., and Aruoma, O. (2004) Total phenol, flavonoid, proanthocyanidin and vitamin C levels and antioxidant activities of Mauritian vegetables. J. Sci. Food Agric. **84**, 1553–1561.

Chu, Y-H., Chang, C-L., and Hsu, H-F. (2000) Flavonoid content of several vegetables and their antioxidant activity. J. Sci. Food Agric. **80**, 561–566.

Donner, H., Gao, L., and Mazza, G. (1997) Separation and characterization of simple and malonylated anthocyanins in red onions, *Allium cepa* L. Food Res. Int. **30**, 637–643.

Fossen, T., Andersen, Ø. M., Øvstedal, D. O., Pedersen, A. T., and Raknes, Å. (1996) Characteristic anthocyanin pattern from onions and other *Allium* spp. J. Food Sci. **61**, 703–706.

Gennaro, L., Leonardi, C., Esposito, F., Salucci, M., Maiani, G., Quaglia, G., and Fogliano, V. (2002) Flavonoid and carbohydrate contents in tropea red onions: Effects of homelike peeling and storage. J. Agric. Food Chem. **50**, 1904–1910.

Heller, W., and Forkmann, G. (1994) Biosynthesis of flavonoids. In: The Flavonoids (ed.: J. B. Harborne), pp. 499–535. Chapman & Hall/CRC, Washington, DC.

van Heusden, A. W., van Ooijen, J. W., Vrielink-van Ginkel, R., Verbeek, W. H. J., Wietsma, W. A., and Kik, C. (2000a) A genetic map of an interspecific cross in *Allium* based on amplified fragment length polymorphism (AFLP™) markers. Theor. Appl. Genet. **100**, 118–126.

van Heusden, A. W., Shigyo, M., Tashiro, Y., Vrielink-van Ginkel, R., and Kik, C. (2000b) AFLP linkage group assignment to the chromosomes of *Allium cepa* L. via monosomic addition lines. Theor. Appl. Genet. **100**, 480–486.

Holton, T. A., and Cornish, E. C. (1995) Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell **7**, 1071–1083.

Kamsteeg, J., van Brederode, J., and van Nigtevecht, G. (1978) Identification and properties of UDP-glucose: Cyanidin-3-*O*-glucosyltransferase isolated from petals of the red campion (*Silene dioica*). Biochem. Genet. **16**, 1045–1058.

Kim, S., Binzel, M. L., Yoo, K. S., Park, S., and Pike, L. M. (2004a) *Pink* (*P*), a new locus responsible for a pink trait in onions (*Allium cepa*) resulting from natural mutations of anthocyanidin synthase. Mol. Gen. Genomics **272**, 18–27.

Kim, S., Binzel, M. L., Park, S., Yoo, K-S., and Pike, L. M. (2004b) Inactivation of *DFR* (Dihydroflavonol 4-reductase) gene transcription results in blockage of anthocyanin production in yellow onions (*Allium cepa*). Mol. Breed. **14**, 253–263.

Kim, S., Jones, R., Yoo, K. S., and Pike, L. M. (2004c) Gold color in onions (*Allium cepa*): a natural mutation of the chalcone isomerase gene resulting in a premature stop codon. Mol. Gen. Genomics **272**, 411–419.

Kim, S., Yoo, K-S., and Pike, L. M. (2005a) Development of a codominant PCR-based marker for allelic selection of the pink trait in onions (*Allium cepa*), based on the insertion mutation in the promoter of the anthocyanidin synthase gene. Theor. Appl. Genet. **110**, 573–578.

Kim, S., Yoo, K. S., and Pike, L. M. (2005b) 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*). Theor. Appl. Genet. **110**, 588–595.

Kim, S., Yoo, K-S., and Pike, L. M. (2005c) The basic color factor, the *C* locus, encodes a regulatory gene controlling transcription of chalcone synthase genes in onions (*Allium cepa*). Euphytica **142**, 273–282.

Kim, S., Jones, R., Yoo, K-S., and Pike, L. M. (2005d) The *L* locus, one of complementary genes required for anthocyanin production in onions (*Allium cepa*), encodes anthocyanidin synthase. Theor. Appl. Genet. **111**, 120–127.

King, J. J., Bradeen, J. M., Bark, O., McCallum, J. A., and Havey, M. J. (1998) A low-density genetic map of onion reveals a role for tandem duplication in the evolution of an extremely large diploid genome. Theor. Appl. Genet. **96**, 52–62.

Martin, W. J., McCallum, J., Shigyo, M., Jakse, J., Kuhl, J. C., Yamane, N., Pither-Joyce, M., Gokce, A. F., Sink, K. C., Town, C. D., and Havey, M. J. (2005) Genetic mapping of expressed sequences in onion and in silico comparisons with rice show scant colinearity. Mol. Gen. Genomics **274**, 197–204.

Masuzaki, S., Shigyo, M., and Yamauchi, N. (2006) Direct comparison between genomic constitution and flavonoid contents in *Allium* multiple alien addition lines reveals chromosomal locations of genes related to biosynthesis from dihydrokaempferol to quercetin glucosides in scaly leaf of shallot (*Allium cepa* L.). Theor. Appl. Genet. **112**, 607–617.

McCallum, J., Clarke, A., Pither-Joyce, M., Shaw, M., Butler, R., Brash, D., Scheffer, J., Sims, I., van Heusden, S., Shigyo, M., Havey, M. J. (2006) Genetic mapping of a major gene affecting onion bulb fructan content. Theor. Appl. Genet. **112**, 958–967.

Moriguchi, T., Kita, M., Tomono, Y., Endo-Inagaki, T., and Omura, M. (2001) Gene expression in flavonoid biosynthesis: Correlation with flavonoid accumulation in developing citrus fruit. Physiol. Plant. **111**, 66–74.

Pelletier, M. K., Murrell, J. R., and Shirley, B. W. (1997) Characterization of flavonol synthase and leucoanthocyanidin dioxygenase genes in Arabidopsis. Plant Physiol. **113**, 1437–1445.

Rhodes, M. J. C., and Price, K. R. (1996) Analytical problems in the study of flavonoid compounds in onions. Food Chem. **57**, 113–117.

Shigyo, M., Tashiro, Y., Isshiki, S., and Miyazaki, S. (1996) Establishment of a series of alien monosomic addition lines of Japanese bunching onion (*Allium fistulosum* L.) with extra chromosomes from shallot (*A. cepa* L. Aggregatum group). Genes Genet. Syst. **71**, 363–371.

Shigyo, M., Tashiro, Y., Iino, M., Terahara, N., Ishimaru, K., and Isshiki, S. (1997) Chromosomal locations of genes related to flavonoid and anthocyanin production in leaf sheath of shallot (*Allium cepa* L. Aggregatum group). Genes Genet. Syst. **72**, 149–152.

Sparvoli, F., Martin, C., Scienza, A., Gavazzi, G., and Tonelli, C. (1994) Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera* L.). Plant Mol. Biol. **24**, 743–755.

Strack, D., and Wray, V. (1994) The anthocyanins. In: The Flavonoids (ed.: J. B. Harborne), pp. 1–22. Chapman & Hall/CRC, Washington, DC.

Suzuki, H., Nakayama, T., Yonekura-Sakakibara, K., Fukui, Y., Nakamura, N., Yamaguchi, M-A., Tanaka, Y., Kusumi, T., and Nishino, T. (2002) cDNA cloning, heterologous expressions, and functional characterization of malonyl-coenzyme A:anthocyanidin 3-*O*-glucoside-6"-*O*-malonyltransferase from dahlia flowers. Plant Physiol. **130**, 2142–2151.

Terahara, N., Yamaguchi, M., and Honda, T. (1994) Malonylated anthocyanins from bulbs of red onion, *Allium cepa* L. Biosci. Biotechnol. Biochem. **58**, 1324–1325.

Tsushida, T., and Suzuki, M. (1995) Isolation of flavonoid-glycosides in onion and identification by chemical synthesis of the glycosides. J. Jpn. Soc. Food Sci. Technol. **42**, 100–108.

Woo, H-H., Orbach, M. J., Hirsch, A. M., and Hawes, M. C. (1999) Meristem-localized inducible expression of a UDP-glycosyltransferase gene is essential for growth and development in pea and alfalfa. Plant Cell **11**, 2303–2315.

Yamaguchi, M-A., Oshida, N., Nakayama, M., Koshioka, M., Yamaguchi, Y., and Ino, I. (1999) Anthocyanidin 3-glucoside malonyltransferase from *Dahlia variabilis*. Phytochemistry **52**, 15–18.

Yoshitama, K., and Yamaguchi, M. (2004) In: How to Study on Plant Pigment. (eds.: T. Adachi, and K. Yoshitama), pp. 3–20. Osaka Municipal University Press, Osaka. In Japanese.