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Characterization of Amino Acid and *S*-alk(en)yl-L-cysteine Sulfoxide Production in Japanese Bunching Onion Carrying an Extra Chromosome of Shallot

Noriya Masamura[1,2], Shigenori Yaguchi[3]**,***, Yasunori Ono[3], Tetsuya Nakajima[3], Shin-ichi Masuzaki[4], Shinsuke Imai[1], Naoki Yamauchi[3] and Masayoshi Shigyo[3]***

[1]*Somatech Center, House Foods Corporation, Yotsukaido 284-0033, Japan*
[2]*The United Graduate School of Agricultural Sciences, Tottori University, Tottori 680-8553, Japan*
[3]*Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan*
[4]*Forensic Science Laboratory, Yamaguchi Pref. Police Headquarters, Yamaguchi 753-8504, Japan*

Eight Japanese bunching onion (*Allium fistulosum* L.)—shallot (*Allium cepa* L. Aggregatum group) monosomic addition lines (MALs, FF+1A–FF+8A) were used to reveal the effects of single alien chromosomes of *A. cepa* on the production of amino acids and *S*-alk(en)yl-L-cysteine sulfoxides (ACSOs) in leaf tissues of *A. fistulosum*. Amino acid and ACSO contents in MAL leaf blades were determined once every 3 months from August 2005 to May 2006. The amino acid found in the greatest amount in all of the MALs throughout the year was cysteine, except for FF+7A accumulated glycine as the greatest amount of amino acid (Nov. 2005). All of the MALs contained three ACSOs in varying amounts and proportions, and all accumulated *S*-1-propenyl CSO as a major ACSO but hardly produced *S*-2-propenyl CSO throughout the year. FF+3A showed greatly increased proportions of *S*-methyl CSO in total ACSO, suggesting that anonymous genes controlling *S*-methyl CSO production are located on chromosome 3A of shallot. High accumulation of total ACSOs in the monosomic additions FF+3A, FF+4A, FF+5A, and FF+8A was observed during different growth periods. Using PCR-based marker analysis, sulfate transporter, adenosine 5'-phosphosulfate reductase (APSR), serine acetyltransferase, *O*-acetylserine thiol-lyase, glutamylcysteine synthase, glutathione synthase, and γ-glutamyl transpeptidase candidate genes related to sulfur assimilation and ACSO biosynthesis were allocated to chromosomes 7A, 2A, 7A, 7A, 2A, 7A, and 4A, respectively. This result showed little association between the chromosomal locations of these genes and ACSO accumulation, suggesting that anonymous genes controlling ACSO accumulation were dispersed on the 2A, 3A, 4A, 5A, 7A, and 8A chromosomes of shallot. APSR gene expression was inhibited by 5A chromosome additions, suggesting that one of the regulatory genes was located on a 5A chromosome and inhibited APSR expression.

Key Words: alien monosomic addition line, *Allium cepa* Aggregatum group, *Allium fistulosum*, sulfur assimilation.

Introduction

Onion (*Allium cepa* L.) and other *Allium* vegetables are valued as food and medicines, primarily for the respective flavors and medicinal properties of their sulfur compounds. There is growing interest in optimizing breeding and production to produce fresh or processed products with defined flavor and health characteristics (Griffiths et al., 2002). When their tissues are damaged, *Allium* plants produce distinct sulfur volatiles through the hydrolysis of *S*-alk(en)yl-L-cystein sulfoxides (ACSOs), flavor precursors biosynthetically derived from cysteine, by the action of alliinase (Lancaster and Kelly, 1983). The existence of four ACSOs in the *Allium* species, *S*-methyl (Me)-, *S*-propyl (Pr)-, *S*-2-propenyl (allyl, Al)-, and *S*-1-propenyl (Pe)-CSOs, has been reported (Freeman and Whenham, 1975; Granroth, 1968; Lancaster and Kelly, 1983). These ACSO compounds and their derivatives show a number of activities that are beneficial to human health, such as antidiabetic (Kumari et al., 1995), antihyperglycemic (Roman-Ramos et al., 1995), and antiplatelet activities (Ali et al., 1999).

Cysteine, an amino acid essential for biosynthesis in plants, plays a key role in the natural sulfur cycle because

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* Corresponding author (E-mail: shigyo@yamaguchi-u.ac.jp).
** These authors contributed equally to this work.
*** Present address: National Institute of Vegetable and Tea Science, National Agriculture and Food Research Organization, Tsu 514-2392, Japan. (Research Fellow of Japan Society for the Promotion of Science)

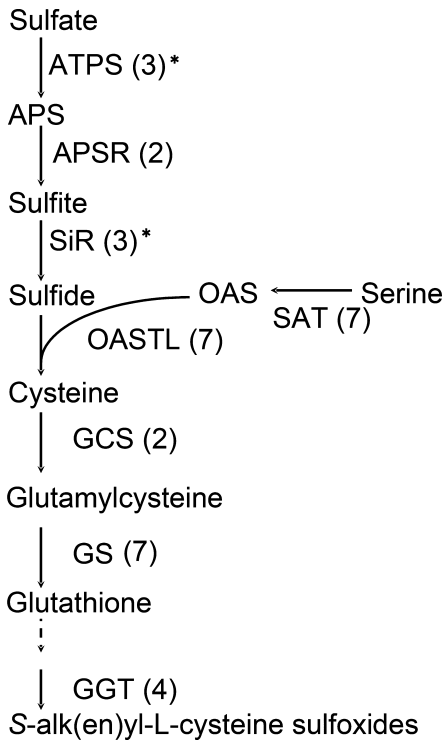

Fig. 1. Putative sulfate assimilation pathway in *Allium cepa* and chromosomal locations of the gene for each enzyme. Numbers in parentheses are the chromosome on which each enzyme gene is located. Chromosomal locations of two genes with asterisks were described by McCallum et al. (2007). APS, adenosine 5'-phosphosulfate; OAS, *O*-acetyl serine; ATPS, adenosine 5'-triphosphate sulfurylase; APSR, adenosine 5'-phosphosulfate reductase; SiR, sulphite reductase; OASTL, *O*-acetylserine thiol-lyase; SAT, serine acetyl-transferase; GCS, glutamylcysteine synthase; GS, glutathione synthase; GGT, γ-glutamyl transpeptidase.

inorganic sulfur in the environment (*e.g.*, sulfate ion in soil and sulfur dioxide in air) is fixed in cysteine, mainly through the biosynthetic pathway (Saito, 1999, 2000). Cysteine is incorporated into proteins and glutathione or serves as the sulfur donor for the biosynthesis of methionine and sulfur-containing secondary ACSOs. The cysteine and ACSO biosynthetic pathway involves several enzymatic reactions associated with the sulfur assimilation pathway in plants (Brunold and Rennenberg, 1997; Leustek and Saito, 1999) (Fig. 1). Sulfate (SO_4^{2-}) is used as the primary source of sulfur for sulfur assimilation and the Cys biosynthesis pathway. Initially, SO_4^{2-} is transported across the root plasma membrane, whereupon it accumulates within plant cells. In order for SO_4^{2-} to be utilized for Cys biosynthesis, it must first be converted to the intermediate compound 5-adenylsulfate (APS). This reaction is catalyzed by the enzyme ATP sulfurylase (ATPS) in plastids. APS can then be used by APS reductase (APSR) to form sulfite prior to its conversion to sulfide by the enzyme sulfide reductase (SiR). Next, cysteine is formed from the reaction of sulfide with *O*-acetylserine (OAS), a process catalyzed by the enzyme OAS thiol-lyase (OASTL). OAS is derived from the acetylation of serine by the action of the enzyme serine acetyltransferase (SAT). Some of the cysteine is exchanged for glutathione catalyzed by glutamylcysteine synthase (GCS) and glutathione synthase (GS). Subsequently, some of the cysteine and synthesized glutathione forms ACSOs through a chain reaction including decarboxylation, oxidation, and transpeptidation. Lancaster and Shaw (1989) suggested that the biosynthesis of ACSOs proceeds in onion via γ-glutamyl peptide intermediates. γ-glutamyl transpeptidase (GGT) can catabolize glutathione conjugates, thus producing ACSOs.

Genetic analysis in onion has been limited by a lack of genetic and genomic resources. The very large genome size of the *Allium* species (Ricroch et al., 2005) complicates genomic cloning and sequencing strategies. The recent development of an EST resource and a PCR-based map of onion (Kuhl et al., 2004; Martin et al., 2005) has provided a basis for the genetic analysis of agronomic traits; however, the biosynthesis of flavor precursors remains poorly understood (Jones et al., 2004), limiting the use of candidate gene approaches (McCallum et al., 2007).

We previously developed eight *Allium fistulosum* L. (genomes FF)—shallot (*A. cepa* L. Aggregatum group, genomes AA) monosomic addition lines (MALs; $2n = 2x + 1 = 17$, FF + 1A–FF + 8A) displaying morphological and physiological characteristics different from those of the *A. fistulosum* parent (Shigyo et al., 1997a), likely due to an alien gene or genes on the extra chromosome from shallot. Studies using these lines have revealed that several lines with alien chromosomes showed different compositions of flavonoids (Masuzaki et al., 2006a, b; Shigyo et al., 1997b), ascorbic acid (Yaguchi et al., 2008a), polyphenols (Yaguchi et al., 2009), and carbohydrates (Hang et al., 2004; Yaguchi et al., 2008b). The results of these studies indicate that the biochemical analyses of eight MALs can define the chromosomal locations of genes or QTLs affecting biochemical component production in *A. cepa*.

An investigation of the amino acids and ACSOs in *Allium* MALs should reveal changes in the contents of *A. fistulosum* and show the chromosomal locations of the shallot genes which fulfill important roles in amino acid and ACSO metabolism. In the present study, the amino acid and ACSO contents in a complete set of *A. fistulosum*—shallot MALs were evaluated to determine the effects of genes related to sulfate metabolism on a single alien chromosome from shallot on the production of these compounds in *A. fistulosum*.

Materials and Methods

Chemicals

Standard ACSO compounds (MeCSO, PeCSO, and AlCSO) were synthesized at the Somatech Center (House Foods Corporation, Yotsukaido, Japan). Amino acid standards, phenylisothiocyanate (PITC), triethylamine (TEA), sodium acetate, and trihydrate, were purchased

from Sigma Chemical Co. (St. Louis, USA). Acetonitrile and methanol (HPLC grade) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and trifluoroacetic acid was from Kanto Chemical Co., Inc. (Tokyo, Japan). High-purity water was supplied by a Milli-Q reagent water system from Nihon Millipore (Tokyo, Japan).

Plant materials

As shown in Table 1, plant materials were two complete sets of *A. fistulosum*—shallot MALs (FF + 1A–FF + 8A, Shigyo et al., 1996) and controls, Japanese bunching onion (*A. fistulosum* 'Kujyo-hoso', FF) and shallot (*A. cepa* Aggregatum group, one accession from Thailand, 'Chang Mai', AA). The MALs were grown in an experimental field at Yamaguchi University (34°N, 131°E). Cultivation and fertilization were carried out according to the procedures of Shigyo et al. (1997a).

Extraction of amino acids and S-alk(en)yl-L-cysteine sulfoxides

Extraction was performed once every 3 months (August 2005 to May 2006). Three or more leaf blade tissues (10 g) of each MAL were microwaved for 2 min (EMP-R51F, Sanyo, Tokyo, Japan) until the tissues were completely cooked to denature the alliinase. The cooked tissues were weighed to calculate the amount of evaporated water (x g) and were homogenized with (10 + x) g high-purity water for 10 min in a mortar and pestle. The homogenate was centrifuged at 2,000 × *g* for 10 min at room temperature, and the centrifuged sediments were removed. One milliliter supernatant was again centrifuged at 15,000 × *g* for 1 min and was filtered by being passed through a 0.45 μm syringe-type filter (HCL-Disk3, Kanto Chemical Co., Inc.). All extractions and subsequent determinations were performed two and three times, respectively.

Free amino acid analysis

Aliquots of filtered extracts (20 μL) and amino acid standards (20 μL) were pipetted into 1.5 mL plastic tubes and dried under a vacuum using a VD-360 centrifugal concentrator and VD-30 freeze dryer (Taitec Co., Ltd., Saitama, Japan). Ten microliters of 1 M sodium acetate : methanol : TEA (2 : 2 : 1) were added to each tube and dried again under a vacuum. Derivatization took place by the addition of 20 μL of freshly prepared methanol : H_2O : TEA : PITC (7 : 1 : 1 : 1) to the samples. The tubes were vortexed and allowed to react for 20 min at room temperature before being thoroughly dried under a vacuum. Derivatized samples and standards could be stored frozen at −20°C for over a week prior to chromatography.

Following derivatization, the residue was dissolved in 100 μL of 5 mM sodium phosphate, pH 7.6, containing 5% acetonitrile. The injection volume was 20 μL for amino acid standards and samples. The high-performance liquid chromatography (HPLC) system

Table 1. Strains of all plant materials used in this study.

Plant types	Genomes	Strains	Amino acid analysis	ACSO[z] analysis	Genomic DNA isolation	Total RNA isolation
Monosomic	FF + 1A	11	−	−	+	−
addition line		130	+	+	+	+
	FF + 2A	132	−	−	+	−
		141	+	+	+	+
	FF + 3A	5	+	+	+	+
		121	−	−	+	−
	FF + 4A	10	+	+	+	+
		50	−	−	+	−
	FF + 5A	26	+	+	+	+
		71	−	−	+	−
	FF + 6A	120	−	−	+	−
		308	+	+	+	+
	FF + 7A	76	−	−	+	−
		324	+	+	+	+
	FF + 8A	65	−	−	+	−
		240	+	+	+	+
A. fistulosum	FF	'Kujo-hoso' 4-2	+	+	+	+
Shallot	AA	'Chang Mai' 18-5	+	+	+	+
Allotriploid	FFA	22	−	−	−	+
Single alien deletion line	FFA-5A	S_2	−	−	−	+

[z] *S*-alk(en)yl-L-cystein sulfoxides.
+, used for each analysis.
−, not used for each analysis.

consisted of a model 717 Autosampler, a model 600 Controller, a model 486 Detector, and an Empower2 data collection system (Nihon Waters, Tokyo, Japan). Detection was performed by a model 486 Detector fitted with a 254 nm filter. A Waters C_{18} Pico-Tag Free Amino Acid Analysis column (3.9 mm × 300 mm), immersed in a thermostatic water bath at 46°C, was used for amino acid separation.

The following solvent system was used for HPLC analysis. Solvent A: 19 g sodium acetate trihydrate and 2 mL TEA were dissolved in 1 L high-purity water. The solution was adjusted to pH 6.08 by the addition of glacial acetic acid and then filtered through a 0.45 μm syringe-type filter. To make up 5% acetonitrile solution, 975 mL filtered solution was added to 25 mL acetonitrile. Solvent B: 60% acetonitrile and 40% high purity water (v/v) were mixed and filtered through a 0.45 μm syringe-type filter. The delivery of the gradient elution took 110 min at a flow rate of 1.0 mL·min^{-1} as follows: initially, 100% of solution A; for the next 13 min, 100% A; for another 1 min, 98% A; for 10 min, 99% A; for 1 min, 100% A; for 31 min, 72% A; for 27 min, 0% A; for 6 min, 100% A; and finally, 100% A for 21 min.

S-alk(en)yl-L-cysteine sulfoxide determination

A 50 μL filtered sample was injected into the HPLC system and quantified. The HPLC system included a pump, degasser, column oven, a diode array detector set to 220 nm, a data collection system (EZchrom Elite™, Hitachi High-Technologies Co., Tokyo, Japan), and an AQUASIL SS-1251-120 column (4.6 mm i.d. × 250 mm long, Senshu Scientific Co., Ltd., Tokyo, Japan). The solvent was 0.1% trifluoroacetic acid and flowed for 15 min at a flow rate of 0.6 ml/min. A series of standards was dissolved in high-purity water and analyzed as described above.

The ACSO peaks were confirmed by comparing peak patterns between alliinase-activated and -deactivated samples. Duplicate 10 g samples were cut from leaf blade tissues, and one part of a sample was microwaved for 2 min until the tissues were completely cooked to denature the alliinase. The other fresh part of the sample was crushed for 10 min in a mortar and pestle in order to hydrolyze the ACSOs. Both samples were then homogenized with distilled water, and the ACSOs were extracted and analyzed. Every extract was stored at −20°C until HPLC analysis.

DNA isolation

Total genomic DNA of two complete sets of *A. fistulosum*—shallot MALs and control plants was isolated from fresh leaf tissue using a mini-prep DNA-isolation method (van Heusden et al., 2000).

PCR primer design

Primer sets to amplify a segment of the gene-encoding enzymes from sulfate transporter (SULTR) to GGT in possible sulfur assimilation and the ACSO biosynthesis pathway were designed with GENETYX 6.1.3 software (Genetyx, Tokyo, Japan) based on the GenBank™ accession numbers. Primer sequences and GenBank™ accession numbers are shown in Table 2.

Table 2. PCR primer sets used in this study.

Primer set	GenBank accession No.	Putative function	PCR	Forward and reverse primers (5' to 3')	Analysis method	Chromosome
SULTR	CF443403/AF458090	Sulfate transporter	first	CATTTGCGGCAATGAAGGAC GGATTCGCCAGTAGAAGCTC		
			second	TACATGGCTGGATGCCAAAC ATTCACAACTCCTGGTACCTTTC	SCAR[y]	7A
APSR	AF212155	APS reductase	first	TGGAGGTGACATTGCAATC AACCGATCAAAATGCAGTCA		
			second	TAGCCCTTATAGAATACGCA ATCCCAAAACTAACTTCACC	*Taq* I—CAPS[x]	2A
SAT[z]	AF212156	Serine acetyltransferase		AAGGTCGGAGCAGGGTCT AACAACAAGACGATCACGTACAA	SCAR	7A
OASTL	CF451460	*O*-acetylserine thiol-lyase	first	TCACCGAGTTGATTGGAAGA TCATCACTGGAGACCGTTAG		
			second	TATTTGTTGCTGGTGTTGGA CTGGAGACCGTTAGAGTTTCAT	*Heteroduplex analysis*	7A
GCS	AF401621	Glutamylcysteine synthase		AGAAGCCATATCTGGACAGAC TCAGCCGTTATTTCAATGACAC	SCAR	2A
GS	CF451556	Glutathione synthase		GGTCTTAGCTGTCTAGTAAGTGAG CTTACATCTTCGCCATAAGTGTTG	*Hin*fI—CAPS	7A
GGT	AF401622	γ-glutamyl transpeptidase		GCTATCAGAATGAATCTTGG CCCTTGAATGATAAACTGAC	*Dra* I—CAPS	4A

[z] This primer set was designed by McManus et al. (2005).
[y] Sequence-characterised amplified region.
[x] Cleaved amplified polymorphic sequence.

PCR amplification and digestion of PCR products

PCR conditions were employed as described previously (Masuzaki et al., 2006a). If the PCR products were monomorphic on 2% agarose gel or 5% denaturing polyacrylamide gel electrophoresis, they were subjected to restriction analysis for the cleaved amplified polymorphic sequence (CAPS). Nine microliters of the second PCR product was incubated for 2 h at 37°C in a volume of 15 μL using 2 U of a restriction enzyme, and was subsequently resolved by 2% agarose gel electrophoresis. Restriction digestion was used in an attempt to generate polymorphisms. The restriction enzyme was selected using GENETYX 6.1.3 based on the onion sequence, which corresponded to the fragment of shallot amplified using the primer sets used in this study. If the restriction digestion was monomorphic, the PCR products were subjected to single strand conformation polymorphism (SSCP) analysis. SSCP conditions were employed as described previously (McCallum et al., 2001) with simple modification in a staining step. Silver staining, performed according to the procedure of Martin et al. (2005), was used for the staining step. If *A. fistulosum* and shallot were monomorphic according to SSCP analysis, heteroduplex analysis was carried out using cDNA as the PCR template.

Total RNA isolation and cDNA synthesis

Total RNA of a complete set of *A. fistulosum*—shallot MALs and control plants was isolated from fresh leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) on March 10, 2008. Using total RNA of each plant, cDNA was synthesized using a cDNA synthesis kit, 1st strand with AMV reverse transcriptase (Roche Diagnostics, Mannheim, Germany).

Heteroduplex analysis

PCR was performed in 15 μL with RapidCycler® 2 (Idaho Technology Inc., Salt Lake City, USA). The amplification mixture in capillaries was 2 mM Mg^{2+}, 50 mM Tris-HCl (pH 8.3), 10 μM EDTA, 500 mg·L^{-1} bovine serum albumin, 200 μM each deoxynucleotide triphosphate, 1 × LCGreen I Heteroduplex melting dye (Idaho Technology Inc.), 30 ng cDNA, 0.5 U of ex Taq polymerase (Takara, Ohtsu, Japan), and 0.5 μM each of two primers. The temperature cycling protocol included an initial denaturation step at 95°C for 15 s, followed by 40 cycles of denaturation at 95°C for 0 s, annealing at 65.9°C for 5 s, and extension at 72°C for 12 s. A 5-microliter aliquot of the PCR products was resolved by 4% agarose gel electrophoresis. If a single PCR product was successfully amplified, the remaining 10 μL aliquot of the PCR products was subjected to heteroduplex analysis. The PCR products in capillaries were transferred to a high-resolution melting instrument (HR-1, Idaho Technology Inc.), and melting curves were obtained by heating from 70 to 95°C at 0.3°C/s. High-resolution melting data were analyzed with HR-1 software by fluorescence normalization and temperature overlay as described previously (Gundry et al., 2003).

Microarray analysis

Using a custom array design service (GeneFrontier Co., Chiba, Japan), at least six independent 60-mer oligonucleotide probes were designed for the 11,675 *A. cepa* expressed sequence tag (Kuhl et al., 2004). Total RNA from four plants [FF, FF + 5A, *A. fistulosum*—shallot allotriploid (FFA), and an *A. fistulosum*—shallot single-alien deletion plant (FFA-5A)] was independently isolated from fresh leaf tissue on June 20, 2008. Microarray analyses were conducted once using the multiplex microarray analysis service (Roche Diagnostics K.K., Tokyo, Japan).

Analysis of gene expression data

Gene expression data were processed and analyzed using NANDEMO Analysis 1.0.1 software (GeneFrontier, Co.). Background subtraction was carried out using the average of the lowest 5% of spot fluorescence intensities. To remove non-linearities in log-log plots of different arrays, expression data of all arrays were subjected to Lowess normalization (f = 0.1) (Cleveland, 1979) to a common baseline array (average fluorescence intensity of all arrays) based on least-invariant sets of probes. The latter were defined by rank difference thresholds, with approximately 10% of the probes selected per array.

Differential expression of genes was assessed using the regularized Bayesian unpaired *t*-test CyberT (Baldi and Long, 2001) and correction of *P*-values for multiple testing by calculating the false discovery rate (Benjamini and Hochberg, 1995). Genes with *P*-values < 0.05 and a minimum of 1.5 changes of expression were considered to be differentially expressed. Fold changes were calculated from the average expression values detected between a control and a 5A chromosome addition/deletion plant. The following three combinations were analyzed: 1, FF vs. FF + 5A; 2, FFA vs. FFA-5A; 3, FF vs. FFA.

Statistical analyses

Monthly data on amino acid and ACSO contents for 4 months in the eight addition lines and *A. fistulosum* were used for one-way analysis of variance (ANOVA). Dunnett's test was employed to compare the amino acid and ACSO contents of *A. fistulosum* and each MAL. Statistical analyses of the data were performed using SPSS 11.5 software with advanced models (SPSS Japan Inc., Tokyo, Japan).

Results

Determination of amino acid content in MALs

Amino acids were separated with baseline resolution. Fourteen amino acids were identified by comparing the retention time with amino acid standards. As shown in Figure 2, the seasonal changes of five (Cys, Ser, Glu,

Gly, and Ala) and nine amino acids (Thr, Asp, Val, Leu, Phe, Met, Ile, Pro, and Asn) were separately illustrated by the difference in the average concentration for all plants. High accumulation of Cys in all MALs and *A. fistulosum* was found in every determination except for FF + 7A in Nov. 2005. Only in FF + 7A (Nov. 2005) the Gly content (153 mg/100 gFW) was higher than Cys content (136 mg/100 gFW). ANOVA revealed significant differences ($P < 0.05$) among plant materials in Cys content. Dunnett's multiple comparison test for the mean separation of Cys content between *A. fistulosum* and each MAL showed that FF + 3A and FF + 5A were Cys high-accumulation lines, whereas FF + 1A and FF + 7A were Cys low-accumulation lines. ANOVA results for the contents of Asn, Ile, Phe, and Val revealed significant differences across the sampling times.

Determination of ACSO content in MALs

The ACSOs were separated with baseline resolution. MeCSO was separated first (retention time, 6.3 min), followed by AlCSO (7.4 min) and PeCSO (8.5 min) (Fig. 3). Degradation of Me-, Al-, and PeCSO peaks in freshly ground samples confirmed the ACSO peaks (Fig. 3b); however, some ACSOs were not completely degraded within 10 min.

AlCSO, a major flavor precursor of garlic (*A. sativum* L.), was detected at a low level in all MALs and *A. fistulosum*. Several reports (Lancaster and Kelly, 1983; Yoo and Pike, 1998) have indicated that AlCSO was undetectable in shallot and *A. fistulosum*. The differences were due to the detection sensitivity of the methods of analysis.

The content of MeCSO, a major flavor precursor of Chinese chives (*A. tuberosum*) and rakkyo (*A. chinense*), ranged between 2.0 and 58.0 mg/100 gFW in

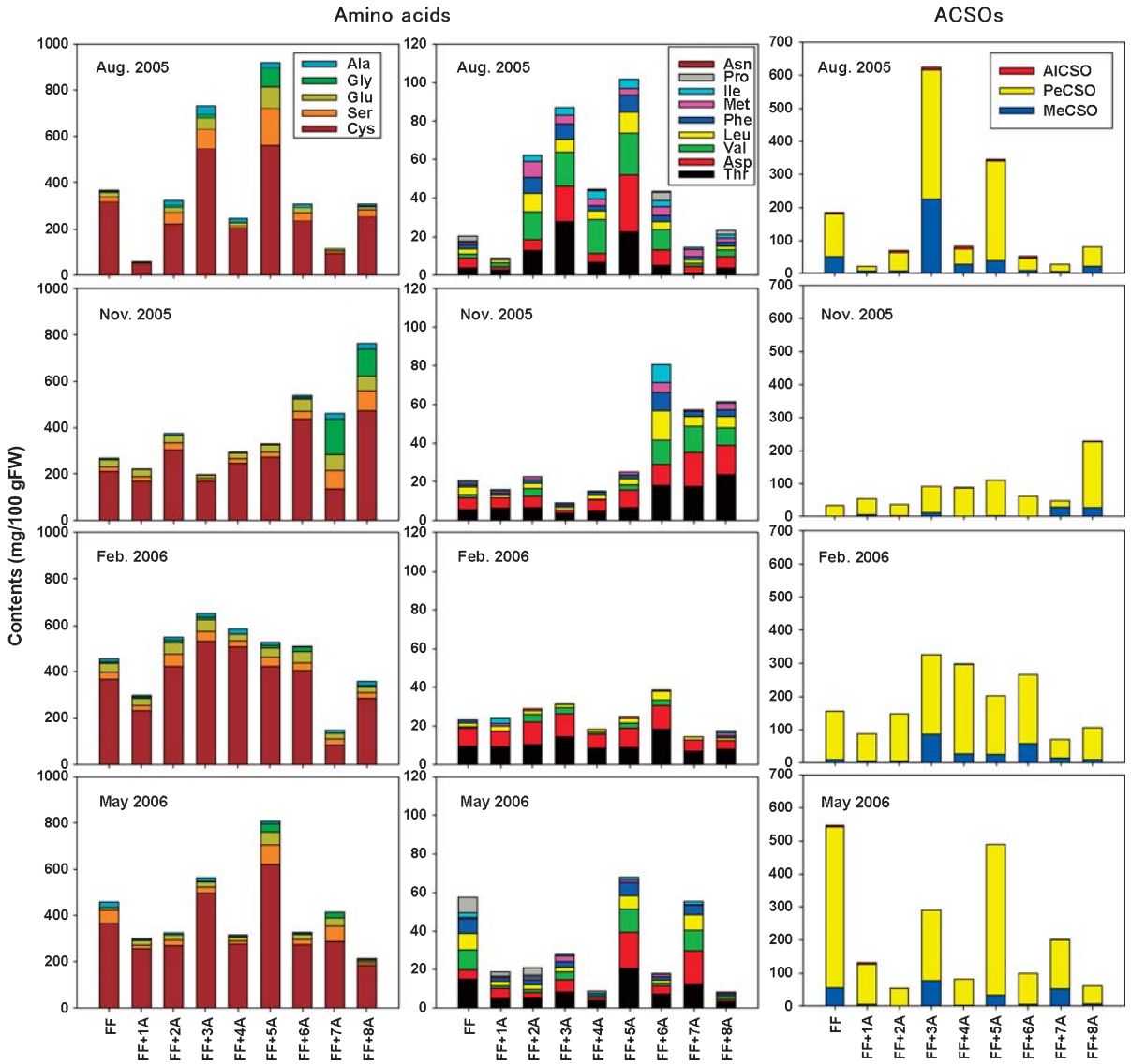

Fig. 2. Content of amino acids and *S*-alk(en)yl-L-cystein sulfoxides (ACSOs) in monosomic addition lines (FF + 1A–FF + 8A) and *Allium fistulosum* (FF). MeCSO, *S*-methyl cysteine sulfoxide; AlCSO, *S*-2-propenyl cysteine sulfoxide; PeCSO, *S*-1-propenyl cysteine sulfoxide.

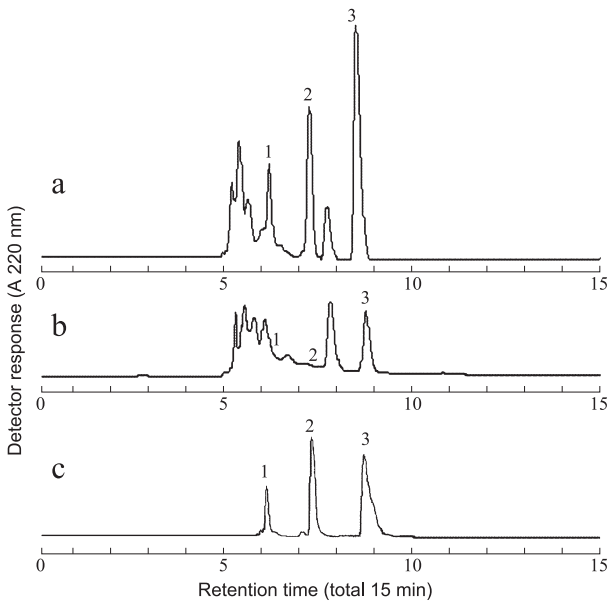

Fig. 3. Representative chromatograms of *Allium fistulosum* containing *S*-methyl-L-cysteine sulfoxide (peak 1), *S*-2-propenyl (allyl)-L-cysteine sulfoxide (peak 2), and *S*-1-propenyl-L-cysteine sulfoxide (peak 3) as major flavor precursors. Chromatograms show the different samples of enzyme-denatured tissues (ground after being microwaved, a), enzyme-reacted tissues (freshly ground, b), and standards (c).

Table 3. Analysis of variance for percentages of *S*-methyl cysteine sulfoxide contents in monosomic addition lines and *Allium fistulosum*.

Source	Degree of freedom	Sum of squares	Mean of squares	*F* value
Total	35			
Plant materials	8	783.89	97.99	3.33
Months	3	37.53	12.51	0.43**
Error	24	705.60	29.40	

** significant at the 1% level.

Table 4. Dunnett multiple-comparison test for comparison of the percentages of *S*-methyl-L-cysteine sulfoxide contents between *Allium fistulosum* and each monosomic addition line (MAL).

MALs	Mean difference
FF + 1A	–0.5 ± 3.6
FF + 2A	–3.5 ± 3.6
FF + 3A	11.2 ± 3.6**
FF + 4A	–1.3 ± 3.6
FF + 5A	–0.4 ± 3.6
FF + 6A	0.5 ± 3.6
FF + 7A	9.6 ± 3.6
FF + 8A	0.3 ± 3.6

** significant at the 1% level.

A. fistulosum (Fig. 2). The highest concentration of MeCSO in MALs was found in FF + 3A (226.8 mg/100 gFW, Aug. 2005). Except for FF + 3A and FF + 7A, the percentages of MeCSO contents in total ACSO were lower than 16.0%. The mean values for FF + 3A and FF + 7A were 26.0% and 32.3%, respectively. ANOVA using the percentages of MeCSO contents in total ACSO revealed significant differences among MALs (Table 3). Dunnett's test showed significant differences between *A. fistulosum* and FF + 3A (Table 4). Several researchers reported that the methylation of glutathione gave *S*-methyl glutathione and then converted it to MeCSO (Lancaster and Shaw, 1989; Randle and Lancaster, 2002), suggesting that several genes involved in the methylation of glutathione could be located on the 3A and 7A chromosomes.

PeCSO, a precursor of the lachrymatory factor and flavor in *A. cepa*, was a major ACSO in the plant materials used in this study, the results of which are in accord with those of a previous study by Yoo and Pike (1998), who classified shallot and *A. fistulosum* as

Table 5. Analysis of variance for the contents of *S*-alk(en)yl-L-cystein sulfoxides in monosomic addition lines and *Allium fistulosum*.

Component[z]	Source	Degree of freedom	Sum of squares	Mean of squares	*F* value
MeCSO	Total	35	0.30		
	Plant materials	8	0.10	0.01	1.51
	Months	3	0.01	0.00	0.51
	Error	24	0.19	0.01	
PeCSO	Total	35	15.50		
	Plant materials	8	3.10	0.39	0.99
	Months	3	3.05	1.02	2.61*
	Error	24	9.35	0.39	
AlCSO	Total	35	0.01		
	Plant materials	8	0.00	0.00	0.90
	Months	3	0.00	0.00	4.80*
	Error	24	0.00	0.00	

[z] MeCSO, *S*-methyl cysteine sulfoxide; AlCSO, *S*-2-propenyl cysteine sulfoxide; PeCSO, *S*-1-propenyl cysteine sulfoxide.
* significant at the 5% level.

PeCSO-dominant. In the current study, the PeCSO contents in *A. fistulosum* ranged between 31.9 and 485.9 mg/100 gFW. The highest amount of PeCSO (453.2 mg/100 gFW) among MALs was observed in FF + 5A (May 2006). Further, it was observed that the PeCSO contents in FF + 3A (Aug. 2005 and Feb. 2006), FF + 4A (Feb. 2006), FF + 5A (Nov. 2005 and May 2006), FF + 6A (Feb. 2006), and FF + 8A (Nov. 2005) substantially increased compared with those in *A. fistulosum*. The results of ANOVA analysis of the PeCSO contents revealed significant differences ($P < 0.05$) among the sampling periods (Table 5). Dunnett's test showed a decrease in three MALs (FF + 1A, FF + 2A, and FF + 7A) and an increase in FF + 5A compared with *A. fistulosum*. These results indicate that several genes related to PeCSO biosynthesis are located on at least five distinct shallot chromosomes: 3A, 4A, 5A, 6A, and 8A. These genes would be expressed in different stages of growth since high PeCSO accumulation of several MALs was observed in different months. Moreover, several factors related to the inhibition of PeCSO production might be located on at least three shallot chromosomes: 1A, 2A, and 7A.

The total ACSO contents of MALs were lower than those of *A. fistulosum* in May 2006. The probable reason for the low ACSO accumulation in MALs was the negative growth of leaf tissues in MALs during bolting and flowering. The negative growth was affected by the genomic affinity between *A. fistulosum* genomes and each shallot chromosome.

As shown in Figure 4, PeCSO content was positively correlated with Cys and Ser content in MALs. Higher correlations with PeCSO content were found in Cys than in Ser. In Cys production, which is the last step in reductive S assimilation, incorporation of S^{2-} into Cys is catalyzed by OASTL from S^{2-} and OAS. The synthesis of OAS is catalyzed by SAT (Kopriva and Koprivova, 2003; Leustek et al., 2000; Suter et al., 2000). SAT and OASTL form a multi-enzyme complex of cysteine synthase (Hell et al., 2002). Ser plays a role as a substrate for Cys production in the reaction catalyzed by SAT and OASTL. Accordingly, Cys was easily used for incorporation into ACSOs rather than Ser, and the correlation with PeCSO and Cys was higher than that of Ser.

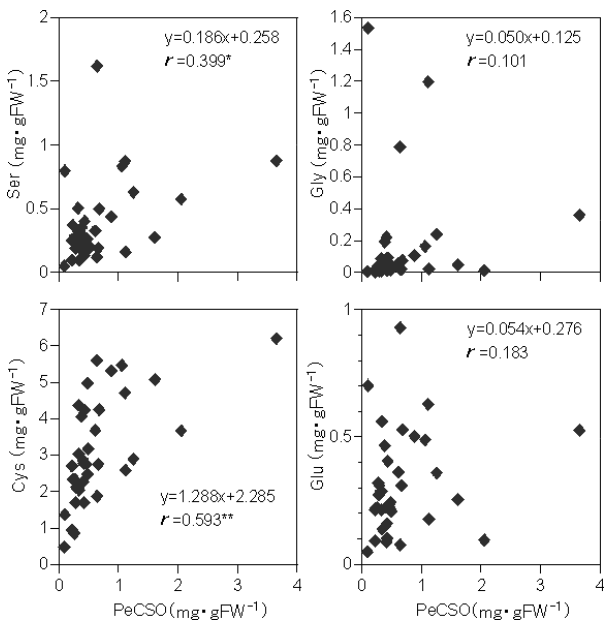

Fig. 4. Scatter plots between *S*-1-propenyl cysteine sulfoxide (PeCSO) and each amino acid content in *Allium fistulosum*. Regression expressions were calculated by simple linear regression analyses. * and ** for significance at the 5% and 1% levels, respectively.

Chromosomal assignment of candidate genes involved in sulfur assimilation and ACSO synthesis

The primer set for SULTR amplified two PCR products of approximately 520 and 480 bp in *A. fistulosum*, whereas a single PCR product of approximately 460 bp was observed in shallot. A PCR product the same size as shallot was found only in FF + 7A (Fig. 5), revealing that FF + 7A had a SULTR gene from the shallot genome and the other MALs had only a SULTR gene from *A. fistulosum*. Smith et al. (1995) showed that the SULTR is a multigene family and *Arabidopsis* possess 14 genes for SULTRs divided into five groups according to sequence similarity and function. Accordingly, one of the genes in the SULTR gene family was located on chromosome 7A of the shallot.

Chromosomal assignment was performed similarly for the following candidate genes, ASPR, SAT, GCS, GS, and GGT, as shown in Table 2. Heteroduplex analysis was carried only for OASTL using the cDNA of each plant. High-resolution melting analysis of these PCR products revealed differences in the melting curve shape correlating to plant materials. The melting curves from *A. fistulosum* and shallot had different patterns at temperatures from 83 to 86°C. The melting temperature of shallot shifted to a lower temperature than that of *A. fistulosum* and showed broader transitions because the duplexes formed were heterogeneous and included low-melting hetroduplexes. Except for FF + 7A, all MALs

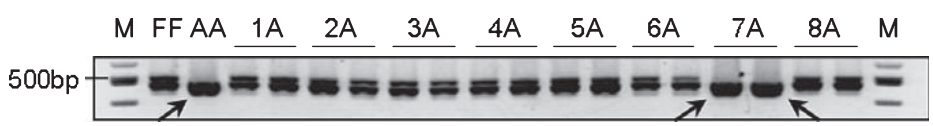

Fig. 5. Electrophoretogram revealing the chromosomal locations of sulfate transporter (SULTR) genes in shallot. FF, *Allium fistulosum*; AA, shallot; 1A–8A, two complete sets of *A. fistulosum*—shallot monosomic addition lines. M, molecular size marker (100 bp DNA ladder). Arrows point to the shallot-specific band.

showed the same melting curves as *A. fistulosum*. The melting curve of FF + 7A, which is slightly different from that of shallot, showed a heteroduplex between shallot and *A. fistulosum*. These melting curves from shallot and FF + 7A indicate that the OASTL gene is not only located on chromosome 7A of shallot but also expressed in FF + 7A.

Assesment of regulatory genes involved in ACSO biosynthesis on additional shallot chromosomes

A distinct characteristic of ACSO biosynthesis in FF + 5A was observed. In FF + 5A, ACSOs and amino acid (Ser and Cys) accumulation levels remained high through out the year, but no candidate genes involved in sulfur assimilation and ACSO synthesis were detected. Therefore, we hypothesized that anonymous regulatory genes related to the upregulation of the expression of *A. fistulosum* candidate genes in the ACSO biosynthesis pathway were located on chromosome 5A of shallot. Microarray analysis and subsequent analysis of gene expression were conducted for each combination of 5A chromosome addition/deletion plants.

All genes, including eight genes related to sulfur assimilation and ACSO biosynthesis in three combinations, are plotted in Figure 6. Gene expression levels of OASTL, GS, and GGT in each plant remained low. These three genes might not contribute to an increase in ACSO biosynthesis of FF + 5A. Two genes (APSR and ATPS) were identified as differentially expressed genes in this study. ATPS signal intensity was low in FFA (signal intensity, 4210) and FFA-5A (7455), but was identified as differentially expressed in the combination of FFA and FFA-5A (Fig. 6b). On the other hand, two combinations identified APSR, and the signal intensities were relatively high (Fig. 6a, b). In the combination of FF and FF + 5A, APSR expression decreased in FF + 5A (11809) rather than in FF (20251). A single alien addition of the 5A chromosome to FF resulted in decreased APSR

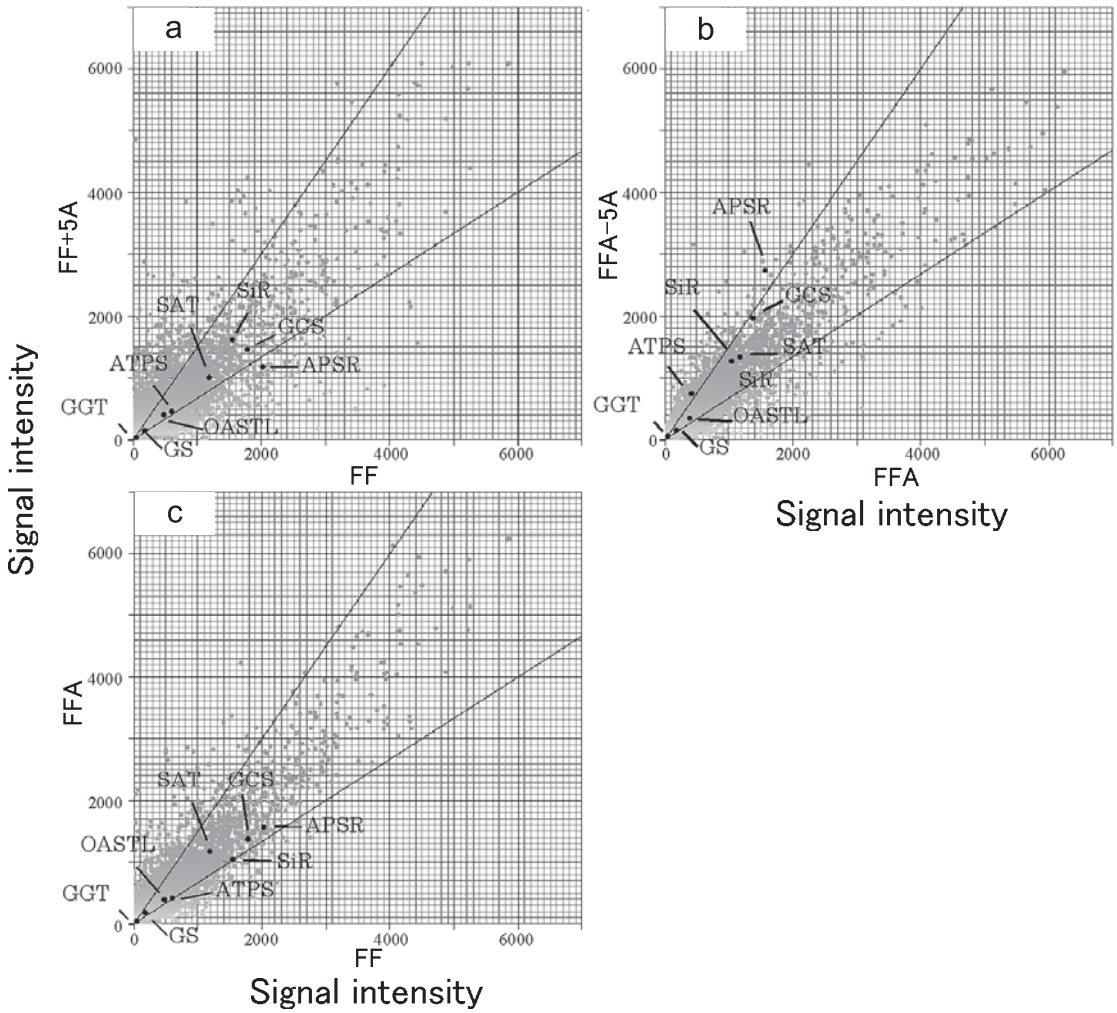

Fig. 6. Scatter plots of gene expression levels derived from microarray analyses of three combinations: a, *Allium fistulosum* (FF) and 5A monosomic addition line (FF + 5A); b, *A. fistulosum*—shallot allotriploids (FFA) and single-alien deletion plant (FFA-5A); c, FF and FFA. Diagonal lines indicate the border of an increase in gene expression by 1.5 or more times compared with the same gene in a counterpart. ATPS, adenosine 5'-triphosphate sulfurylase; APSR, adenosine 5'-phosphosulfate reductase; SiR, sulphite reductase; OASTL, *O*-acetylserine thiol-lyase; SAT, serine acetyl-transferase; GCS, glutamylcysteine synthase; GS, glutathione synthase; GGT, γ-glutamyl transpeptidase.

expression in FF + 5A, suggesting that the anonymous APSR-inhibiting gene would be located on chromosome 5A. On the other hand, in the combination of FFA and FFA-5A, an increase in APSR expression in FFA-5A (27393) compared with that in FFA (15655) was observed. It is possible that deleting chromosome 5A from FFA with anonymous factors repressing APSR expression on chromosome 5A inhibited APSR expression in FFA-5A. In the combination of FF and FFA, no genes were identified as differentially expressed genes, suggesting that the multiple addition of shallot chromosomes to FF would inhibit gene expression related to sulfur assimilation and ACSO biosynthesis by the cross-interaction of anonymous factors on each chromosome. As a result, APSR expression decreased with the 5A chromosome addition to FF and increased with the 5A chromosome deletion from FFA.

In the combination of FF and FF + 5A, 1853 ESTs were expressed more highly in FF + 5A than that in FF (condition I) and 2096 were expressed at a lower level (condition II). In the combination of FFA and FFA-5A, 33 (condition III) and 54 (condition IV) expressions were significantly increased and decreased, respectively, in FFA-5A compared to FFA. The 11 ESTs fulfilled both conditions I and IV, suggesting that the expression of these ESTs would be significantly up regulated by 5A chromosome. Putative functions could be assigned to 8 of these 11 ESTs, including pyruvate kinase (AB009049) and UDP-glucose dehydrogenase (AY222335). The opposite condition (II and III) identified 12 genes, 11 of which could be assigned putative function, including an ethylene forming enzyme-like dioxygenase-like protein (AP003019).

Discussion

The determination of ACSO contents and chromosomal assignment using monosomic addition lines revealed that FF + 2A was a low-ACSO assimilation line and APSR was located on 2A chromosome. It therefore seemed that ASPR could not influence the ACSO content of FF + 2A. Microarray analysis and subsequent analysis of gene expression revealed that the 5A chromosome seemed to negatively regulate APSR expression against the FF background. McCallum et al. (2005) reported that higher APSR activity was not associated with higher pungency of onion. From this point of view, low APSR activity in FF + 5A by inhibited APSR expression enable the high pungency in FF + 5A to contain high ACSOs. Galmarini et al. (2001) reported that the QTL affecting onion pungency was located on chromosome 5. The detection of low ACSO contents in FF + 2A and high ACSO contents in FF + 5A was recognized not as the effectiveness of upregulation of the candidate genes related to ACSO biosynthesis, but as an effect of this QTL.

From microarray analysis, about 20 ESTs were identified as up- or down-regulated ESTs by 5A chromosome. EST was closely related to sulfur assimilation and ACSO biosynthesis did not occur in these ESTs. This suggested that the regulation of expression related to ACSO biosynthesis could not be accomplished by one regulatory gene on 5A chromosome and several anonymous genes on a number of shallot chromosomes might be involved.

The mechanisms of sulfur assimilation and ACSO biosynthesis are controversial. For example, APSR activity has a diurnal rhythm, and sucrose mimics the effect of light, revealing an interaction of sulfate assimilation with carbon metabolism (Kopriva et al., 1999). Moreover, endogenous regulators of the sulfur assimilation pathway, glutathione, which represses the metabolic step, and OAS, which increases metabolite accumulation or induces the reaction, have been suggested (Leustek and Saito, 1999). Complex factors, as typified by the above, would affect the unclear relationship between the gene expression and the concentrations of amino acids and ACSOs in plants of the 5A chromosome addition/deletion lines.

Galmarini et al. (2001) reported that the QTL affecting onion bulb dry matter and pungency, as well as onion-induced in vitro antiplatelet activity, was located on chromosome 5. Several candidate genes involved in sulfur assimilation, including SAT (McManus et al., 2005), ATPS, and SiR (McCallum et al., 2005), have been mapped to chromosomes 7, 3, and 3, respectively (Fig. 1). Linkage analyses revealed that two key sulfur assimilation genes, ATPS and SiR, were closely linked on chromosome 3 (McCallum et al., 2007). Another QTL mapping study revealed that these two genes on chromosome 3 had a significant association with bulb pungency but not with bulb solid content (McCallum et al., 2007). Marker-assisted selections using the key candidate genes mapped along with several QTLs affecting carbohydrate traits and with other candidate genes for amino acids and ACSO biosynthesis offer the potential to control ACSO contents in the bulb of *A. cepa*. As a result, consumers might receive health benefits through a diet including novel onion and shallot cultivars possessing high concentrations of ACSOs.

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