

Profiling of Nondigestible Carbohydrate Products in a Complete Set of Alien Monosomic Addition Lines Explains Genetic Controls of Its Metabolisms in *Allium cepa*

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ABSTRACT. Eight kinds of Japanese bunching onion [*Allium fistulosum* L. (genomes FF)]-shallot [*Allium cepa* L. Aggregatum group (genomes AA)] monosomic addition line [MAL (FF+1A-FF+8A)] were used to study the effects of single alien chromosomes from *A. cepa* on the production of carbohydrates in the leaf tissues of *A. fistulosum*. Carbohydrate contents in green leaf blades of these MALs were measured during alternate months from May 2005 to Mar. 2006. The determination of soluble sugar content from leaf blades of each MAL and *A. fistulosum* revealed that nonreducing sugars (sucrose and fructan) accumulated in winter leaf blades. Reducing sugar (fructose) in the leaf blades of each MAL was lower than *A. fistulosum* in almost every time period. In the leaf blades of FF+4A, high fructan accumulation was observed from Nov. 2005 to Mar. 2006. A series of determinations on the pectin content showed that amounts of NaOH-soluble pectin and HCl-soluble pectin remained at low levels at all time periods. High pectin accumulations in FF+7A and FF+8A occurred in September and slightly decreased in November. A decrease in hexametaphosphoric acid-soluble pectin content was associated with the maturity of MALs from autumn to winter, whereas the water-soluble pectin content increased. The pectin methylesterase and polygalacturonase genes of shallot were assigned to chromosome 7A and 4A, respectively. These results demonstrate that important genes related to pectin metabolism in shallot are located on chromosomes 4A, 7A, and 8A of shallot.

Pectins are sources of dietary fiber and are basically galacturonic acid polymers (Ridley et al., 2001). The health effects of pectin substances are receiving increasing interest (Willats et al., 2006). It is generally accepted that a high-fiber diet is beneficial to human health and that pectin is an important soluble fiber in fruit and vegetables. There is clear evidence that pectin can lower cholesterol and serum glucose levels, and that it may also have anticancer properties (Behall and Reiser, 1986; Yamada, 1996). Pectin and pectic oligosaccharides have been shown to induce apoptosis in human colonic adenocarcinoma cells (Olano-Martin et al., 2003).

Although the health-enhancing effects of pectin substances are widely known, the study of pectin biosynthesis is challenging, and progress in this area has been slow (Ridley et al., 2001). The textures of vegetables and fruit are affected by pectin

substances (Femenia et al., 1998; Sapers et al., 1997). Several studies have shown that the degradation of pectin substances is related to the softening of vegetable tissues (Brady, 1987; Campbell et al., 1990; Huber, 1983; Sakurai and Nevins, 1993). For the reasons reported above, the amount of pectin in the leaf blades (green leaves) of Japanese bunching onion [*Allium fistulosum* (genomes FF)] greatly affects its quality in terms of texture and health-promoting effects.

We previously developed eight *A. fistulosum*-shallot [*A. cepa* Aggregatum group (genomes AA)] monosomic addition lines (MAL; $2n = 2x + 1 = 17$, FF+1A-FF+8A) that display morphological and physiological characteristics different from those of the *A. fistulosum* parent (Shigyo et al., 1997), likely due to alien gene or genes on the extra chromosome from the shallot. Studies using these lines have revealed that several lines with alien chromosomes, which possess the key regulatory and structural genes for carbohydrate metabolism, show large differences in the nonreducing sugar components (Hang et al., 2004; Yaguchi et al., 2008b).

The aims of the present study were to reveal seasonal changes in the pectin content of *A. fistulosum* and to determine chromosomal locations of shallot genes important for pectin metabolism by means of biochemical and genetic studies in *A. fistulosum*-shallot MALs. In this study, pectin contents in

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a complete set of MALs were evaluated over time to determine the effect of a single alien chromosome from the shallot on pectin accumulations in *A. fistulosum*.

Materials and Methods

PLANT MATERIALS. The plant materials were a complete set of *A. fistulosum*-shallot MALs ($2n = 2x + 1 = 17$, FF+1A-FF+8A) and a control plant, 'Kujyo-hoso' japanese bunching onion ($2n = 2x = 16$, FF). The MALs were produced via the backcrossing of allotriploids [FFA (female)], between *A. fistulosum* 'Kujyo-hoso' and shallot 'Chiang mai', to *A. fistulosum* 'Kujyo-hoso' (male) (Shigyo et al., 1996). The experimental design used single replicates of eight MALs and a control. Cultivation and fertilizer applications were carried out as described by Shigyo et al. (1997). A row (1.5×21.9 m) with silver mulching was prepared by making 288 holes (four lines \times eight columns per line) with 0.3-m spacing between each hole. Thirty-two clones of each MAL and *A. fistulosum* were grown in an experimental field at Yamaguchi University (lat. 34°N , long. 131°E) from May 2005 to Mar. 2006. Liquid fertilizer (6.5N–6P–19K; HYPONEX Japan, Osaka, Japan) $\times 1000$ solution was applied at weekly intervals with irrigation to each plant to ensure uniform growth. Healthy mature leaf blade tissue of the same growth stage in each MAL was sampled from the youngest fully expanded leaf of developing plants. Once plants were sampled, those plants were avoided being sampled again in the next sampling period, and were then used again for conforming to the growth stage of the other plants. The following extractions and determinations were replicated twice.

EXTRACTION AND PRESERVATION OF PECTIN. Tissue extraction was performed in alternate months from May 2005 to Mar. 2006. Leaf blade tissues (20.0 g) from three or more plants of one strain were sliced to reduce particle size to less than 5 mm. The tissues were combined and transferred into boiling ethanol (final concentration of 70%) for 15 min. The boiled tissues were homogenized in a Universal homogenizer (Nihon Seiki Seisakusho, Tokyo) with the boiling ethanol. The homogenate was filtered through a No. 2 filter paper (ADVANTEC, Tokyo). The filtrate was used for the determination of sugars and ethanol-soluble pectin (EtOH-P). The residue was homogenized three times in 70% ethanol and boiled for 15 min. The residue was washed with acetone and air-dried in a fume hood as alcohol-insoluble solids (AIS).

SEQUENTIAL EXTRACTION OF PECTIN WITH DIFFERENT SOLUBILITY. AIS (0.1 g) was suspended in water (50 mL), stirred for 15 min, and then left to stand one night at room temperature. The homogenate was filtered through a funnel fused-in fritted glass disc (11G-3, micropore diameter 20–30 μm ;

Sansyo, Tokyo). The filtrate was used as water-soluble pectin (WP). The water-insoluble residue was further extracted in 0.4% hexametaphosphoric acid (50 mL) for 1 h at 80°C . The filtrate was used as hexametaphosphoric acid-soluble pectin (HMP). The hexametaphosphoric acid-insoluble residue was then extracted in 0.05 N HCl (50 mL) for 1 h at 100°C . The filtrate was used as HCl-soluble pectin (HCl-P). The HCl-insoluble residue was finally extracted in 4% NaOH (50 mL) overnight at room temperature. The filtrate was used as NaOH-soluble pectin (NaOH-P).

DETERMINATION OF ALCOHOL-SOLUBLE CARBOHYDRATE AND PECTIN CONTENTS. Free fructose in 70% ethanol extracts was determined by the thiobarbituric acid method (Percheron, 1962). To determine fructan plus sucrose, free fructose was removed from extracts by heating a 20-L aliquot in 1 N NaOH at 100°C for 10 min and then assaying for released fructose. To determine fructan alone, sucrose was first removed by digestion with invertase. A 20- μL aliquot of extract was incubated with 10 μL of 2 $\text{mg}\cdot\text{mL}^{-1}$ invertase (bakers yeast; Sigma, St. Louis) and 10 μL of 25 mM ammonium acetate buffer (pH 5.5) for 5 min and was then assayed to determine released fructose. The released fructose was determined by the thiobarbituric acid method.

Before the determination of pectin content, 5 mL of EtOH-P, WP, HMP, and HCl-P was saponified in 0.02 N NaOH (5 mL) for 30 min at room temperature. The pectin contents were colorimetrically determined by the carbazol-sulfuric acid method (Filisetti-Cozzi and Carpita, 1991) as D-galacturonic acid. The carbazole-sulfuric acid method was performed in a test tube on 0.2-mL saponified samples and 0.2 mL of NaOH-P. To analyze the sample, 1.2 mL of 25 mM sodium tetraborate (in H_2SO_4) was added. The tube was placed in a boiling water bath for 10 min followed by cooling in an ice-water bath until room temperature was reached. When the mixture reached 20°C , 40 μL of the carbazole reagent [0.125% (w/v) of carbazole in EtOH] was added. The tube was placed in a boiling water bath for 15 min followed by cooling in an ice-water bath until 20°C was reached. The light absorbance of the solution at 530 nm was measured.

DNA ISOLATION. The 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).

POLYMERASE CHAIN REACTION (PCR) PRIMER DESIGN. Nested primer sets were designed with the software GENETYX 6.1.3 (Genetyx, Tokyo) to amplify regions from pectin methyltransferase (PME) and polygalacturonase (PG) based on cDNA sequences, with GenBank accession nos. CF442750 and CF451451 for PME and GenBank accession no. CF452289 for PG. The primer sequences are shown in Table 1. PME

Table 1. PCR primer sets used in this study to identify chromosomal locations of the genes related to the pectin metabolisms.

Primer set ^a	GenBank accession no.	PCR	Forward and reverse primers (5'-3')	Annealing temp. ($^\circ\text{C}$)
PME	CF442750/ CF451451	First	TGTAATTCCCTTGCCATGACC	60
		Second	AGCATTGCCGAAGATAAAGTCC	65
PG	CF452289	First	CACGGCTGCAACGATAGTGG	58
		Second	CTGACTCTCGGTAGAACTGG	58
			GGAAGAGGAGAAGCCTTGTG	
			CTGATTTTAGTCCCATTTCATCGTC	
			TCTCTTTTGTTCAGCGATTTCGAAG	
			CACGGTCGATTTCACGATCAC	

^aPME = pectin methyltransferase, PG = polygalacturonase.

Table 2. Carbohydrates content in *Allium fistulosum* (FF) and eight monosomic addition lines (FF+1A-FF+8A) from May 2005 to Mar. 2006.

Elements ^z	Plant materials	Carbohydrate content (mg·g ⁻¹ FW)						Mean ^y	SE
		2005				2006			
		May	July	Sept.	Nov.	Jan.	Mar.		
Fructose	FF	17.4	17.0	7.3	10.8	16.8	14.3	14.0	1.8
	FF+1A	6.2	10.2	8.0	5.4	6.8	9.1	7.6*	0.8
	FF+2A	7.6	11.8	7.8	4.1	12.8	6.7	8.5*	1.5
	FF+3A	7.6	7.8	4.8	6.7	4.8	8.2	6.6*	0.7
	FF+4A	7.0	12.7	8.8	6.5	14.1	9.4	9.7*	1.4
	FF+5A	3.5	9.7	2.0	7.9	6.6	5.9	5.9*	1.3
	FF+6A	8.1	13.2	8.4	13.7	9.6	6.8	10.0*	1.3
	FF+7A	8.1	8.5	5.8	4.9	5.6	9.7	7.1*	0.9
	FF+8A	8.9	8.2	9.8	7.2	7.3	9.2	8.4*	0.5
Sucrose	FF	3.2	1.6	3.1	9.0	5.3	10.0	5.4	1.5
	FF+1A	4.9	1.7	1.7	5.1	3.7	8.6	4.3	1.2
	FF+2A	2.5	1.0	2.0	4.3	0.6	1.9	2.1	0.6
	FF+3A	1.4	3.5	2.2	2.4	2.8	4.8	2.8	0.5
	FF+4A	2.4	3.2	1.0	6.3	7.5	10.8	5.2	1.6
	FF+5A	3.8	2.0	1.2	1.1	4.3	3.8	2.7	0.7
	FF+6A	11.6	3.0	4.7	9.2	7.0	19.8	9.2	2.7
	FF+7A	4.3	4.7	1.9	4.2	2.4	3.5	3.5	0.5
	FF+8A	8.7	3.0	2.6	4.1	2.8	5.3	4.4	1.1
Fructan	FF	7.1	5.9	6.6	7.5	9.0	6.4	7.1	0.5
	FF+1A	5.5	5.5	5.9	7.6	5.1	7.7	6.2	0.5
	FF+2A	0.9	3.9	1.7	1.1	1.6	3.2	2.1	0.5
	FF+3A	2.0	1.9	2.1	6.3	7.2	11.7	5.2	1.8
	FF+4A	5.3	4.0	2.7	10.9	28.7	18.6	11.7*	4.6
	FF+5A	4.5	3.4	1.6	9.0	13.2	8.0	6.6	1.9
	FF+6A	7.2	1.1	1.1	5.7	3.6	10.4	4.9	1.6
	FF+7A	3.3	2.9	1.7	1.7	3.3	2.2	2.5	0.3
	FF+8A	4.4	0.8	1.1	1.2	1.9	1.6	1.8	0.6
EtOH-P	FF	7.1	5.9	6.6	7.5	9.0	6.4	7.1	0.5
	FF+1A	5.5	5.5	5.9	7.6	5.1	7.7	6.2	0.5
	FF+2A	4.2	5.9	5.5	5.0	5.1	4.7	5.0	0.3
	FF+3A	3.5	5.9	6.0	6.1	4.3	6.5	5.4	0.5
	FF+4A	5.0	7.5	6.9	9.2	13.9	8.8	8.6	1.3
	FF+5A	4.9	5.9	6.0	7.3	7.5	5.1	6.1	0.5
	FF+6A	8.3	6.9	7.7	9.6	7.8	10.0	8.4	0.5
	FF+7A	5.7	7.3	6.1	18.0	3.4	4.1	7.4	2.4
	FF+8A	6.7	5.7	6.1	17.8	4.0	4.2	7.5	2.3
WP	FF	4.3	2.4	3.3	2.8	6.4	2.7	3.6	0.7
	FF+1A	2.6	3.2	1.7	3.5	5.7	5.8	3.7	0.8
	FF+2A	4.1	7.0	5.3	3.5	10.7	7.9	6.4*	1.2
	FF+3A	2.5	3.2	4.6	3.9	7.4	7.0	4.8	0.9
	FF+4A	3.1	5.6	2.9	2.9	7.9	8.2	5.1	1.1
	FF+5A	2.1	4.9	3.2	4.2	11.0	7.4	5.5	1.5
	FF+6A	2.5	6.1	3.5	3.1	7.4	7.5	5.0	1.0
	FF+7A	1.8	6.4	10.3	1.2	6.6	7.6	5.6	1.6
	FF+8A	3.5	4.0	7.1	1.6	6.7	4.8	4.6	0.9
HMP	FF	11.7	7.6	8.8	11.0	3.9	4.0	7.8	1.5
	FF+1A	6.5	7.2	6.3	8.1	4.6	6.0	6.4	0.5
	FF+2A	10.8	14.0	9.5	12.8	9.1	9.2	10.9	0.9
	FF+3A	9.9	4.7	9.0	13.8	4.9	5.5	8.0	1.6
	FF+4A	10.1	10.2	3.8	11.8	7.8	7.4	8.5	1.3
	FF+5A	9.4	9.9	7.5	11.9	5.5	6.0	8.4	1.1
	FF+6A	12.1	13.4	11.3	6.1	3.4	6.5	8.8	1.8
	FF+7A	7.7	12.1	21.4	3.5	4.5	6.4	9.3	3.0
	FF+8A	13.6	11.9	15.4	5.0	7.1	5.3	9.7	2.0

Continued next page

Table 2. Continued.

Elements ^z	Plant materials	Carbohydrate content (mg·g ⁻¹ FW)						Mean ^y	SE
		2005				2006			
		May	July	Sept.	Nov.	Jan.	Mar.		
HCl-P	FF	3.5	1.5	2.6	2.8	3.2	1.9	2.6	0.3
	FF+1A	2.3	1.2	3.0	3.6	2.1	2.8	2.5	0.4
	FF+2A	1.5	2.9	4.1	4.5	2.6	3.3	3.2	0.5
	FF+3A	2.0	1.3	3.9	4.1	2.3	2.1	2.6	0.5
	FF+4A	3.5	1.8	1.7	2.9	3.2	3.1	2.7	0.3
	FF+5A	2.0	2.3	4.5	3.0	1.3	3.3	2.7	0.5
	FF+6A	2.6	3.2	4.8	2.6	2.5	3.3	3.2	0.4
	FF+7A	1.4	3.3	9.3	0.9	1.9	2.9	3.3	1.4
	FF+8A	2.8	2.5	6.7	1.3	1.9	2.4	2.9	0.9
NaOH-P	FF	0.5	0.6	0.6	0.3	0.7	0.4	0.5	0.1
	FF+1A	0.3	0.6	0.5	0.4	0.6	0.6	0.5	0.1
	FF+2A	0.3	0.9	0.5	0.4	0.8	0.7	0.7	0.1
	FF+3A	0.4	0.5	0.6	0.9	0.7	0.8	0.7	0.1
	FF+4A	0.5	0.8	0.4	0.5	1.0	0.8	0.6	0.1
	FF+5A	0.4	0.9	0.7	0.9	0.7	0.8	0.7	0.1
	FF+6A	0.7	1.1	0.8	0.7	0.8	0.9	0.8	0.1
	FF+7A	0.2	0.9	1.8	0.2	0.4	0.7	0.7	0.3
	FF+8A	0.5	0.6	1.0	0.2	0.4	0.6	0.5	0.1
Insoluble residue	FF	14.4	12.3	13.3	10.9	8.3	5.8	10.8	1.4
	FF+1A	8.3	10.2	9.7	10.0	7.1	9.7	9.2	0.5
	FF+2A	11.6	31.4	14.6	12.8	13.4	11.5	15.9	3.4
	FF+3A	6.8	9.9	13.1	13.0	8.5	9.5	10.1	1.1
	FF+4A	15.3	18.5	7.4	11.0	12.4	13.2	13.0	1.7
	FF+5A	13.1	19.5	12.0	15.4	8.0	9.8	13.0	1.8
	FF+6A	14.8	23.3	13.7	8.3	9.2	9.2	13.1	2.5
	FF+7A	6.4	22.7	35.3	4.1	5.6	9.1	13.9	5.6
	FF+8A	15.9	22.8	25.3	4.6	5.2	7.3	13.5	4.1

^zEtOH-P = ethanol-soluble pectin, WP = water-soluble pectin, HMP = hexametaphosphoric acid-soluble pectin, HCl-P = HCl-soluble pectin, NaOH-P = NaOH-soluble pectin.

^yMean separation of each element between *A. fistulosum* and each monosomic addition lines by Dunnett's multiple test.

*Significant at $P \leq 0.05$.

sequence information from accession no. CF442750 was used for the forward primers, whereas accession no. CF451451 was used for the reverse primers.

PCR AMPLIFICATION AND DIGESTION OF PCR PRODUCT. PCR amplifications were conducted with template DNA [about 100 ng of genomic DNA for the first PCR and 5 μ L of diluted template DNA (the products of the first PCR diluted 1/50 with sterile water) for the second PCR], each of the primers at 1 μ M, 0.25 mM dNTPs, a 1 \times Ex *Taq* buffer, and 0.5 U of Ex *Taq* polymerase (Takara Bio, Shiga, Japan) in a volume of 25 μ L. All PCRs were performed as follows: an initial denaturation of 3 min at 94 °C and 35 cycles of PCR amplification (a 1-min denaturation at 94 °C, a 1-min annealing, and a 1-min primer extension at 72 °C) in a thermal cycler (iCycler; Bio-Rad, Hercules, CA). The ramp times were carried out under default conditions that adjusted temperature at the maximum ramp rate with the minimum ramp time. The annealing temperatures of the primer sets are given in Table 1. Nine microliters of the second PCR products was incubated for 2 h at 37 °C in a volume of 15 μ L 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 *Bgl* II (Toyobo, Osaka, Japan) was used to reveal polymorphisms.

STATISTICAL ANALYSES. Data of the pectin content with different solubility in alternate months in the eight MALs and *A. fistulosum* were used for one-way analysis of variances (ANOVA). Dunnett's test was employed to compare the series of pectin contents between *A. fistulosum* and each MAL. Data of the alcohol-soluble carbohydrate content and the pectin content were used for principal component analyses (PCA). Statistic analyses were performed using SPSS 11.5 software with advanced models (SPSS Japan, Tokyo).

Results and Discussion

DETERMINATION OF ALCOHOL-SOLUBLE CARBOHYDRATE AND PECTIN CONTENTS IN MALs. The alcohol-soluble carbohydrate content in *A. fistulosum* and each MAL are shown in Table 2 and Fig. 1. The reducing sugar (fructose) contents remained constant throughout the year for all MALs. The fructose concentration in *A. fistulosum* was significant higher than that in each MAL. The seasonal changes of nonreducing sugar (sucrose and fructan) contents in *A. fistulosum* showed that they stayed high in winter and then decreased in spring and summer. These tendencies were almost the same as our previous study for which the sugar determinations were performed from Jan. 2002 to Dec. 2003 (Yaguchi et al., 2008b). In this study,

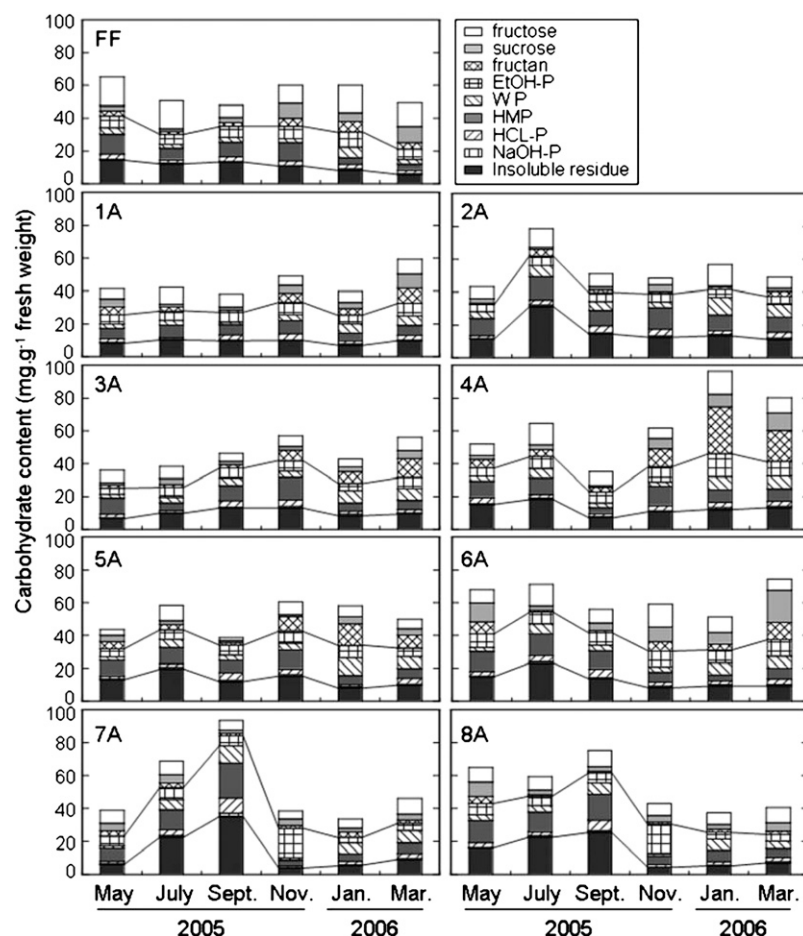


Fig. 1. The year-round variations of soluble and insoluble carbohydrate contents in a complete set of monosomic addition lines (1A-8A) compared with *Allium fistulosum* (FF) (EtOH-P = ethanol-soluble pectin, WP = water-soluble pectin, HMP = hexametaphosphoric acid-soluble pectin, HCL-P = HCl-soluble pectin, and NaOH-P = NaOH-soluble pectin). Lines show the partitions between soluble sugars and pectins (upper) and between pectins and the insoluble residue (lower).

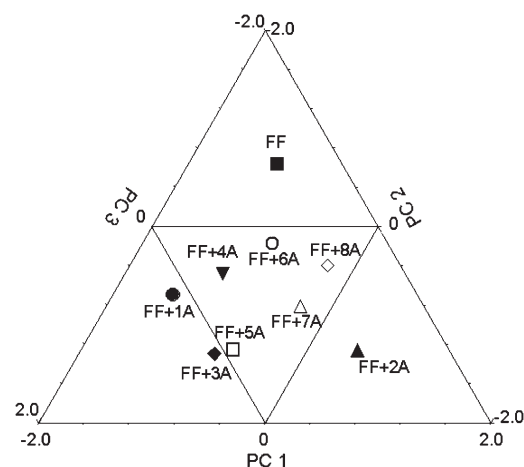


Fig. 2. Scores plot for three principal components (PC1-PC3) of the principal component analyses applied to the nine elements in the complete set of monosomic addition lines (FF+1A-FF+8A) and *Allium fistulosum* (FF).

seasonal changes of nonreducing sugar content in every MAL showed the same tendency as *A. fistulosum*, with the exception of fructan accumulation in FF+4A. The winter fructan contents

in FF+4A were significantly higher than that of *A. fistulosum*. Fructan is an important reserve carbohydrate in *Allium* L. species. In a previous study, no candidate genes related to fructan metabolism were assigned to chromosome 4A of shallot (Yaguchi et al., 2008b). This result indicates that anonymous genes controlling fructan accumulation are located on chromosome 4A of shallot.

The NaOH-P contents in *A. fistulosum* and every MAL were very low in every month of the year [average $0.6 \text{ mg} \cdot \text{g}^{-1}$ fresh weight (FW) (Table 2 and Fig. 1)]. In HCL-P contents, *A. fistulosum* remained at low levels throughout the year (average $2.6 \text{ mg} \cdot \text{g}^{-1}$ FW). All the MALs also remained at low levels of HCL-P contents in this study, except for FF+7A and FF+8A in Sept. 2005 (9.3 and $6.7 \text{ mg} \cdot \text{g}^{-1}$ FW, respectively). The seasonal change of the HMP content in *A. fistulosum* showed that this fraction was high from May to Nov. 2005 (average $9.8 \text{ mg} \cdot \text{g}^{-1}$ FW) and then decreased from Jan. to Mar. 2006 (average $4.0 \text{ mg} \cdot \text{g}^{-1}$ FW). In contrast, the seasonal change of the WP content in *A. fistulosum* showed that the WP remained at a low level from May to Nov. 2005 and then increased in January. Except for FF+7A and FF+8A, all MALs showed the same tendency of HMP and WP accumulation as *A. fistulosum*. The greater accumulations of HMP in FF+7A and FF+8A were observed in Sept. 2005 (21.4 and $15.4 \text{ mg} \cdot \text{g}^{-1}$ FW, respectively) and were twice or 1.5 times higher than that in *A. fistulosum*. In both HMP and WP contents, ANOVA revealed significant differences across the sampling times. In FF+7A and FF+8A, the total pectin content substantially increased in Sept. 2005. In Nov. 2005, pectin contents extracted from AIS in FF+7A and FF+8A drastically decreased, whereas the EtOH-P contents drastically increased. The maximum EtOH-P accumulations in FF+7A and FF+8A were observed in Nov. 2005 and then decreased in Jan. 2006. The total pectin content of FF+7A and FF+8A substantially increased in Sept. 2005. Pectin biosynthesis requires the action of at least 53 different enzymatic activities (Mohnen, 1999). Therefore, several genes related to pectin biosynthesis may be concentrated on chromosomes 7A and 8A.

In *A. fistulosum*, mucilage, which are well-hydrated gels of cellulose, hemicellulose, and pectin, accumulated in the winter leaf blade (Inden and Asahira, 1990). The biosynthesis of the mucilage is poorly understood; a large amount of uronic acid, which consists mainly of L-galacturonic acid (Ohsumi and Hayashi, 1994), is present in the mucilage (Mizuno and Kinpyo, 1955). In FF+7A and FF+8A, the maximum EtOH-P accumulations were observed in Nov. 2005 and then decreased in Jan.

2006. The degradation compounds of the pectin might be changed to mucilage by absorbing water. The mucilage content in the winter leaf blade of MALs was the highest in FF+8A (Hang et al., 2004). It was suggested that EtOH-P might be related to a change in the mucilage content in the winter leaf blade of FF+7A and FF+8A.

ESTIMATION OF VARIATION ON ALCOHOL-SOLUBLE CARBOHYDRATE AND PECTIN CONTENTS IN MALs BY PRINCIPAL COMPONENT ANALYSES. Principal component analyses were conducted on the nine fractions measured in the *A. fistulosum* and each MAL. The first three principal components (PCs) explained 86.0% of the variation (PC1 = 44.4%, PC2 = 26.0%, PC3 = 15.6%). All MALs were plotted on a different position from that of *A. fistulosum* by using scores plot for three PCs (Fig. 2). The coefficient values of each carbohydrate parameter in the PC1, PC2, and PC3 are shown in Table 3. In PC1, a series of pectin derived from AIS showed high coefficient values, and each soluble carbohydrate displayed negative coefficient values. High coefficient values for PC2 were detected in the parameters of sucrose (0.91) and EtOH-P (0.91). In PC3, a high coefficient value was detected in fructan (0.77). Several parameters showed negative coefficient values for PC3. The largest negative value of PC3 was detected for fructose (−0.56). From these parameters, PC2 and PC3 could be associated with a specific carbohydrate. On the other hand, the coefficient values of each carbohydrate parameter suggested that PC1 would be associated with alcohol-insoluble carbohydrate production, including pectin productions. Analyses of the carbohydrate composition in this study showed that the amounts of pectin in *A. fistulosum* and several MALs were comparable to the contents of soluble sugars. This result and PCA indicated that pectin would be one of the essential carbohydrates in *A. fistulosum* and that its composition was diverse in each MAL.

CHROMOSOMAL ASSIGNMENTS OF PME AND PG GENES. Primers for the PME gene of *A. cepa* amplified a single band of ≈1000 bp in *A. fistulosum* and shallot. After both PCR products were digested with the enzyme *Bgl* II, a polymorphism between *A. fistulosum* and shallot was detected. In the two complete sets of MALs, the restriction fragment length polymorphism (RFLP) from shallot was present only in FF+7A (Fig. 3a). This result revealed that the PME gene of the shallot is located on chromosome 7A. The primer set for the PG gene amplified a single band of ≈500 bp in *A. fistulosum* and 550 bp in shallot. In MALs, the amplicon derived from shallot was present only in FF+4A (Fig. 3b). This result revealed that the PG gene of the shallot is located on the chromosome 4A.

PME de-esterifies the methyl group of pectin (Moustacas et al., 1991), whereas PG hydrolyzes the glycosidic linkages in pectic substances, significantly decreasing viscosity. The high activities of these pectin degradation enzymes

increased the pectin solubilization and softening of the plant tissues (Bartolome and Hoff, 1972; Pilnik and Voragen, 1991). The decrease in the pectin content extracted from AIS in FF+7A (in Nov. 2005) could be caused by the high PME activity on chromosome 7A of shallot. A small decrease of the pectin content in FF+4A was observed in Sept. 2005. This suggested that the decrease of pectin content in FF+4A may have been caused by the increase PG activity on chromosome 4A of shallot in autumn. The EtOH-P content in FF+4A remained at a high level from Sept. 2005 to Mar. 2006 compared with the *A. fistulosum*. This indicated that the enzyme activities related to pectin degradation were high in FF+4A.

In the previous study, which observed seasonal changes of L-ascorbic acid content in MALs from Jan. 2002 to Dec. 2003, and three MALs, FF+1A, FF+2A, and FF+8A, were identified as having high ascorbic acid accumulation (Yaguchi et al., 2008a). The predominant pathway of ascorbic acid biosynthesis in plants is the D-mannose/L-galactose pathway (Smirnov and

Table 3. Coefficient values of each carbohydrate parameter in the first to third principal component (PC1-PC3) revealed by the principal component analyses on the carbohydrate contents of *Allium fistulosum* and eight monosomic addition lines.

Parameters ²	PC1	PC2	PC3
Fructose	−0.35	0.57	−0.56
Sucrose	−0.21	0.91	−0.06
Fructan	−0.29	0.32	0.77
EtOH-P	−0.09	0.91	0.07
WP	0.94	−0.12	0.25
HMP	0.90	0.04	−0.30
HCl-P	0.88	0.32	−0.18
NaOH-P	0.64	0.34	0.53
Insoluble residue	0.93	0.16	−0.13

²EtOH-P = ethanol-soluble pectin, WP = water-soluble pectin, HMP = hexametaphosphoric acid-soluble pectin, HCl-P = HCl-soluble pectin, NaOH-P = NaOH-soluble pectin.

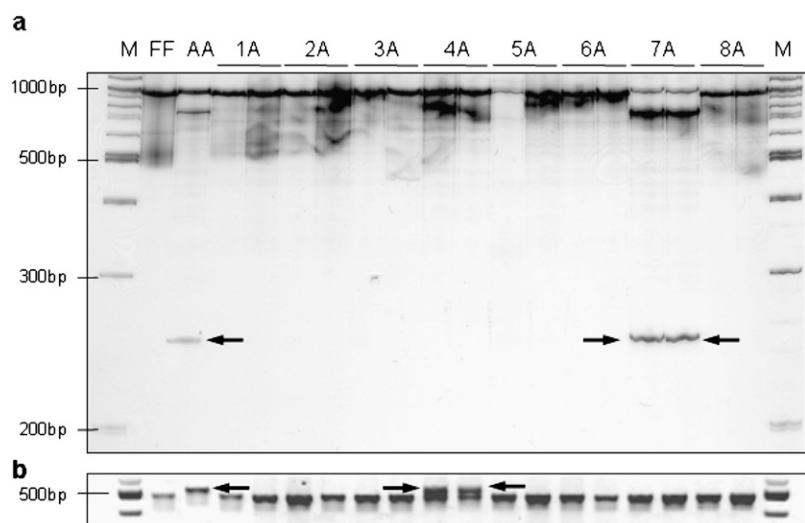


Fig. 3. Electropherograms showing the chromosomal location of pectin methylesterase (a) and polygalacturonase (b) genes in shallot [FF = *Allium fistulosum*, AA = shallot, 1A-8A = two complete sets of *A. fistulosum* - shallot monosomic addition lines, and M = molecular size marker (100-bp DNA ladder)]. Arrows point to the shallot-specific band.

Gatzek, 2004; Wheeler et al., 1998). However, any other biosynthetic pathway via uronic acid intermediates may exist. The molecular evidence for this pathway (i.e., a D-galacturonic acid pathway) was provided by the cloning and characterization of a D-galacturonic acid reductase from strawberry [*Fragaria × ananassa* Duchesne (Agius et al., 2003)]. We proposed that D-galacturonic acid derived from pectin was reduced to L-galacturonic acid, which in turn is readily converted to ascorbic acid. We hypothesize that the rapid decrease in the pectin content of FF+8A increased an endogenous ascorbic acid production via the D-galacturonic acid pathway. The carbohydrates derived from pectin degradation would be reused to produce an ascorbic acid in *A. fistulosum* and MALs, while the rapid decrease in the pectin content derived from AIS of FF+8A in November resulted in an increase in endogenous ascorbic acid production via the D-galacturonic acid pathway.

Several MALs displayed peculiar nondigestible carbohydrate composition, which could be used to breed for increasing specific pectin contents in *A. fistulosum* via the use of the MAL chromosome doubling program (Yaguchi et al., 2008a). The clarification of the pectin phenotypes in MALs and the chromosomal locations of shallot genes related to the pectin metabolism provide a more detailed understanding of the genes underlying carbohydrate phenotypes. This should be combined with an analysis of quantitative trait locus (QTL) related to a variation in *A. cepa* carbohydrate composition. An initial QTL analysis in *A. cepa* identified several genomic regions affecting dry matter content, soluble solids, and other correlated traits (Galmarini et al., 2001). A subsequent study based on this QTL analysis confirmed that two QTL affecting soluble solids and dry matter content were associated with two different RFLP markers, which are candidate genes of an acid invertase on chromosome 3 (Havey et al., 2004) and a phloem-unloading sucrose transporter on chromosome 5 (Martin et al., 2005), respectively. Marker assisted selections via the usages of the key candidate genes mapped along with several QTLs affecting carbohydrate traits together with other candidate genes for pectin metabolism would provide a possibility to control nondigestible carbohydrate contents in the bulb of *A. cepa*. As a result, consumers might receive a health benefit through a diet including novel onion (*A. cepa* Common onion group) and shallot cultivars possessing high concentrations of nondigestible carbohydrates.

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