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Chromosome Engineering Techniques Modify Contents and Constituents of Fructans in Cultivated *Allium* Species

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ABSTRACT

Onion (*Allium cepa* L.) and shallot (*A. cepa* Aggregatum group) exhibit wide variation in bulb fructan content and the *Frc* locus on chromosome 8 conditions much of this variation. To understand the biochemical basis of *Frc* we conducted biochemical and genetic analyses of *Allium fistulosum* (FF) - shallot alien monosomic addition lines (AALs; FF+1A-FF+8A), onion mapping populations and shallot - *A. fistulosum* addition lines. Sucrose and fructan levels in leaves of FF+2A were significantly lower than FF throughout the year. FF+8A showed significantly higher winter sucrose accumulation and sucrose phosphate synthase (SPS) activity. Markers for additional candidate genes for sucrose metabolism were obtained by cloning a major SPS expressed in onion leaf and exhaustively mining onion EST resources. SPS and sucrose synthase (SuSy) loci were assigned to chromosome 8 and 6 respectively using AALs and linkage mapping. Further loci were assigned, using AALs, to chromosomes 1 (sucrose phosphate phosphatase), 2 (SuSy and 3 invertases) and 8 (neutral invertase). The shallot - *A. fistulosum* AAL (AA+8F) also showed the high fructan accumulation. The concordance between chromosome 8 localization of SPS and elevated leaf sucrose levels conditioned by high fructan alleles at the *Frc* locus in bulb onion or alien monosomic additions of chromosome 8 in *A. fistulosum* and in *A. cepa* suggest that the *Frc* locus may condition variation in SPS activity.

Keywords: Japanese bunching onion, mapping, onion, shallot, sucrose
Abbreviations: AAL, monosomic alien addition line; **ACSO**, *S*-alk(en)yl-L-cysteine sulfoxide; **DP**, degree of polymerization; **HPAEC**, high-performance anion exchange chromatography; **QTL**, quantitative trait locus; **SPS**, sucrose-phosphate synthase; **SuSy**, sucrose synthase

INTRODUCTION

The most conspicuous feature of *Allium cepa* L. (onion and shallot), which distinguishes it from Welsh or Japanese bunching onion (*A. fistulosum* L.), is the formation of a well-defined bulb, where the reserve carbohydrate fructan is stored in the thickened sheaths of bladeless leaves (Darbyshire and Henry 1981). Fructan content in *A. cepa* bulbs varies very widely, comprising less than 4% of bulb dry matter in sweet and salad types to over 30% in shallots and dehydrator onions (Chope *et al.* 2006; Muir *et al.* 2007). Although *A. fistulosum* does not form bulbs (Brewster 1994), it has limited capability to accumulate the same types of soluble carbohydrate and fructan reserve as *A. cepa* in leaf bases and sheaths (Mizuno and Kinpyo 1955; Ernst *et al*. 1998). Although the enzymes involved in biosynthesis of fructan from sucrose in onion have been characterized (Vijn *et al.* 1998; Ritsema *et al.* 2003; Fujishima *et al.* 2005), the physiological and genetic basis for the wide variation in *A. cepa* fructan accumulation is only partly understood. There is a physiological basis on fructan accumulation in *A. cepa* that is mainly caused by drought stress and partially triggered by bulb abscisic acid concentration (Chope *et al.* 2006). The most notable gap in understanding of *Allium* carbohydrate metabolism is that the pathways of sucrose synthesis and degradation have been little studied (Lercari 1982; Pak *et al*. 1995; Thomas *et al.* 1997; Kahane *et al.* 2001).

The wide variation in onion carbohydrate accumulation offers opportunities for functional studies of the regulation of carbohydrate metabolism. However, unlike the forage and cereal grasses in which fructan accumulation has been intensively studied (Turner *et al.* 2006; Ruuska *et al.* 2008), genomic resources in onion are very limited. Development of the onion genetic linkage has recently allowed quantitative trait locus (QTL) analysis of loci underlying variation in onion bulb carbohydrate composition. The first-generation low-density map was used by Galmarini *et al.* (2001) to detect QTLs on chromosomes 3, 5 and 8 that affect onion bulb dry matter and solids content. Interestingly, QTLs on chromosomes 3 and 5 were associated with RFLP revealed by cDNAs encoding an acid invertase (API89; AA451557) and a phloem-unloading sucrose transporter (SUT; API66; BE205593), both candidate genes for carbohydrate metabolism. QTL analysis based on the more detailed map of Martin *et al.* (2005) revealed a major dominant gene (*Frc*) on chromosome 8 affecting fructan content and confirmed the effect of the chromosome 5 QTL on dry matter content (McCallum *et al.* 2006). Central roles for chromosomes 5 and 8 in the regulation of *A. cepa* carbohydrate metabolism were independently demonstrated using monosomic *A. fistulosum* - shallot alien monosomic addition lines (AALs)

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originally developed by Shigyo *et al.* (1996). Hang *et al.* (2004) showed that the *A. fistulosum* - shallot AALs carrying chromosome 8 from shallot also accumulated non-reducing carbohydrates in leaf blades during winter.

Because the *Frc* locus conditions a very large phenotypic effect on onion bulb composition it is desirable to develop a more detailed understanding of the gene or genes underlying it, to enable cloning and/or development of tightly linked molecular markers suitable for marker-aided breeding. However, there are limited genomic resources available in *Allium*; no relevant model system and comparative studies have shown a lack of microsynteny between onion, asparagus and rice (Jakše *et al.* 2006). Genetic studies in onion are further complicated because it is a biennial, out-crossing and highly heterozygous species. These constraints, combined with the interesting phenotypes observed in *A. fistulosum* - shallot AALs, suggest that a complementary strategy for identifying *Frc* and other major carbohydrate metabolism genes in onion is to make use of the *A. fistulosum* - shallot AALs for functional studies.

In this study we combined biochemical characterization of sucrose metabolism in *A. fistulosum* - shallot AALs and inbred onion lines differing at the *Frc* locus with candidate gene approaches shown to be highly productive for genetic dissection of carbohydrate metabolism in other species (Pflieger *et al.* 2001). This revealed concordance between chromosomal locations of candidate genes involved in onion carbohydrate metabolism and regions of chromosomes 2 and 8 revealed by QTL mapping and studies of addition lines. Furthermore, biochemical characteristics of several types of shallot - *A. fistulosum* chromosome addition plants were evaluated to determine the effect of *A. fistulosum* genes on sugar productions in shallot and to reveal the availability for using these plants in place of existing shallot varieties.

MATERIALS AND METHODS

Plant materials

The plant materials were a complete set of *A. fistulosum* - shallot AALs ($2n = 2x + 1 = 17$, FF+1A - FF+8A) and a control plant, Japanese bunching onion (*A. fistulosum* cv. 'Kujyo-hoso', $2n = 2x = 16$, FF). Moreover, one shallot - *A. fistulosum* monosomic addition plants ($2n = 2x + 1 = 17$, AA+8F) and three shallot - *A. fistulosum* single-alien deletion lines ($2n = 3x - 1 = 23$, AAF-1F, AAF-4F and AAF-8F) were used for the bulb sugar and sulfur-containing amino acid analyses. Shallot (AA) and allotriploid between shallot and *A. fistulosum* (AAF) were used as controls for bulb compositions. All the plants were grown in an experimental field at Yamaguchi University (34°N, 131°E). The *A. fistulosum* - shallot AALs were maintained over two years (January 2002 - December 2003) through vegetative propagation. The bulb compositions were analyzed in August 2005. Biochemical analyses were based on samples taken from at least three plants. Cultivation and fertilizer applications were carried out according to the procedures of Shigyo *et al.* (1997).

Sugar analysis

Sugar extraction from three or more leaf blades (2.0 g) of each strain of *A. fistulosum* - shallot AALs and three or more bulbs (10.0 g) of each strain of shallot - *A. fistulosum* single-alien deletion lines was performed as described previously (Hang *et al.* 2004). Free fructose, sucrose and fructan in extracts were determined by the thiobarbituric acid method (Percheron 1962) with a slight modification as described by Yaguchi *et al.* (2008). Fructan compositions in several extracts were analyzed by high-performance anion exchange chromatography (HPAEC) according to the procedure of Shiomi *et al.* (1997).

Enzyme assays

Enzyme extraction from three or more leaf blades (5.0 g) of each strain of *A. fistulosum* - shallot AALs was performed once a month from April 2002 to March 2003 according to the procedure of Yaguchi *et al.* (2008). Sucrose synthase (SuSy) activity as sucrose cleavage, Sucrose phosphate synthase (SPS) and Acid invertase activities were measured by the method of Pressey (1969), Nielsen *et al.* (1991) and Shono *et al.* (1997), respectively.

Biochemical analysis of high and low fructan lines from the 'W202A x Texas Grano 438' mapping population

Analytical methods and development of the 'W202A x Texas Grano 438' mapping population were described previously (McCallum *et al.* 2006). Seed of 12 inbred $F_{2:3}$ families (6 high and 6 low fructan) were direct sown on September 2005 at West Melton, Christchurch, New Zealand, in a three block row/column design of 36 plots. Plots were laid out in five rows 2.5 m long with 0.5 spacing. Each block contained six low fructan (< 20% DW fructan) and six high fructan (> 25% DW fructan) lines, based on previous analyses. Parent populations were sown in adjoining plots. The crop was managed according to standard commercial practice. Leaf tissue was sampled from the youngest fully expanded leaf of developing plants on 21 December 2005 prior to initiation of bulbing and on 13 January 2006 after commencement of bulbing.

Leaf blades and base tissue were frozen at -80°C and freeze-dried. These samples were analysed for sucrose synthase activity by the method of Dancer *et al.* (1990). Assays were duplicated.

Table 1 PCR primer sets used in this study.

Primer set	Genbank accession number	Putative function	Forward and reverse primer (5′ to 3′)	Chromosome
SUCS	CF440928	Sucrose synthase	TTTGAAGTGTGGCCTTACCTTGAG TGATGAAGTCTGTTCGATCATGGC	6
ACP013	CF452518	Sucrose synthase	TTCACCCTGAAATCGAGGAG TCGGCTTGTTCTTCCTTGTC	2
SPS3'UTR	EU164758	Sucrose phosphate synthase	AAAGGGAGATACAGACCAT ATTATACATCTCATCATGTCACA	8
SPS4	EU164758	Sucrose phosphate synthase	GAAGGCTGATATTGTTGGTGAAG TGTGTCGTAGGAGCCTGATG	8
ACP041	CF437610	Acid invertase	GGTTCAAAAGACGCATCCAA TAATCCTGCCCATTATCAGAAGT	2
ACP042	CF437950	Neutral invertase	GATTTTGTGCCCTCTGCAAT AAACTAGCTGGCATCAATCCTT	8
ACP047	CF437145	Alkaline/neutral invertase	AAGGATCTGCCGACCAAGA TCAGGCATCCATTCAACAAG	2
ACP054	CF435784	Invertase	GCTCAATGTAGGTGGTGCTG CTGCCGTCTGATTTCTTGCT	2
ACP057	CF437606	Cell wall invertase	CAGATATGCGAATGGTTTTGC TGTCTACAAAGCCTCCAGACG	2
ACP059	CF441209	Sucrose phosphate phosphatase	GAATTGTTCAGCATTCCAGATG CGTTCAGTTGCATGAATTATCCT	1

Glucose, fructose, sucrose and total fructan content were analysed by high-performance liquid chromatography (HPLC) and enzymatic methods as described previously (McCallum *et al.* 2006). Mature bulbs were hand-lifted after 90% of tops had fallen, field-cured for 10 days and stored at 4°C and 65% RH for one month before analysis. Pooled samples of 10 bulbs were analysed for total fructan.

Genetic mapping of sucrose synthase and sucrose phosphate synthase loci and assignment of genes affecting carbohydrate metabolism

PCR primer sets employed in this study are shown in **Table 1**. PCR conditions and genetic mapping populations were employed as described previously (McCallum *et al.* 2006). The assignments were conducted by using *A. fistulosum* - shallot AALs as described previously (Martin *et al.* 2005).

Determination of *S*-alk(en)yl-L-cysteine sulfoxide (ACSO) in single-alien deletion lines

ACSOs in three or more bulbs (20.0 g) of each strain of single-alien deletion lines were extracted and analyzed by HPLC according to the procedure of Yaguchi *et al.* (2009).

RESULTS

Quantitative analysis of total sugar contents in *A. fistulosum* - shallot AALs leaf blade tissues

Monthly analysis of total leaf blade carbohydrates over two years revealed increased storage of sucrose and fructan in the winter months in *A. fistulosum* and in all *A. fistulosum* - shallot AALs with the exception of FF+2A (**Fig. 1**), which hardly accumulated any sucrose and fructan. Sucrose levels were significantly higher in FF+8A. Sucrose content was correlated with fructan content in *A. fistulosum* and each *A. fistulosum* - shallot AAL (r = 0.23 - 0.85). Because of their marked differences in sucrose and fructan accumulation compared to *A. fistulosum*, the FF+2A and FF+8A lines were selected for more detailed analysis of sucrose metabolic enzymes.

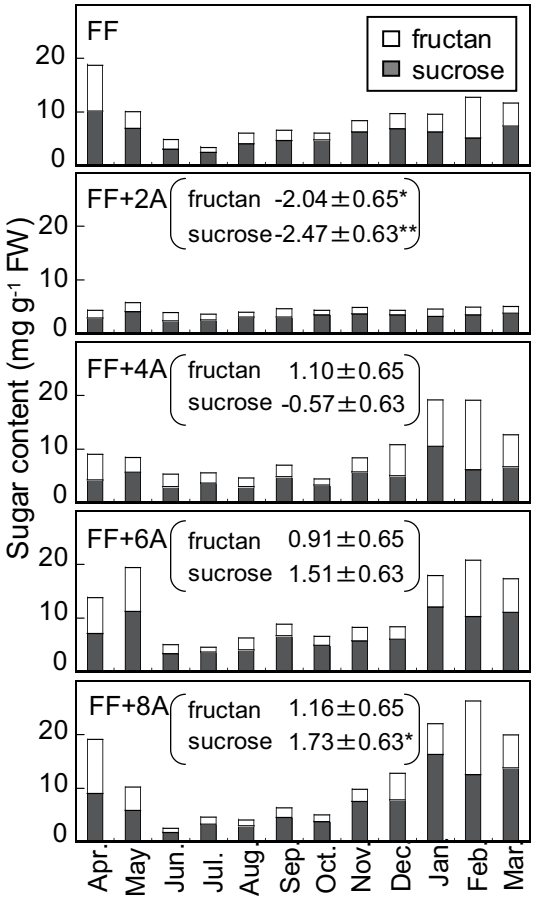

Fig. 1 Year-round variations of fructan and sucrose contents in FF+2A, FF+4A, FF+6A and FF+8A, which showed a different sugar accumulation from that of *A. fistulosum* (FF). Values denote monthly means from Jan. 2002 to Dec. 2003. Values in parentheses show the mean difference (±SE) of fructan and sucrose content between *A. fistulosum* and each *A. fistulosum* - shallot AAL. Dunnett's multiple comparison test was used to test mean separations. *, ** significant at $P < 0.05$, 0.01, respectively.

Qualitative analysis of fructan in *A. fistulosum* - shallot AALs leaf blade tissues

HPAEC analysis identified glucose, fructose, sucrose and fructan isomers (**Table 2**) in leaf blade extracts of *A. fistulosum* and *A. fistulosum* - shallot AALs (**Fig. 2**). The chromatograms of sugar extracts from four AALs (FF+1A, FF+3A, FF+5A and FF+7A) were qualitatively similar to that of the extract from *A. fistulosum*. The maximum degree of polymerization (DP) of fructan in the extract varied from DP 4 in FF+2A to DP 9 in FF+4A and the concentration of individual oligosaccharides progressively decreased with increasing DP. DP 4 fructans were barely detected in extract from FF+2A, FF+8A showed a predominance of tri-saccharides (50.3% of total fructan) and in FF+4A approximately 43% of total fructan consisted of DP higher than 5. The total content of neokestose-series saccharides (3b, 4b-c, 5b-d and 6b-d) was higher than that of 1-kestose-series saccharides (3a, 4a, 5a, 6a and 7a) in every determination.

Table 2 The structural composition of the different fructan in this study.

	Structure
1-Kestose (3a)	1F-ββ-D-fructofuranosylsucrose
Neokestose (3b)	6G-β-D-fructofuranosylsucrose
Nystose (4a)	1F (1-β-D-fructofuranosyl)2 sucrose
4b	6G (1-β-D-fructofuranosyl)2 sucrose
4c	1F, 6G-di-β-D-fructofuranosyl sucrose
5a	1F (1-β-D-fructofuranosyl)3 sucrose
5b	6G (1-β-D-fructofuranosyl)3 sucrose
5c	1F (1-β-D-fructofuranosyl)2-6G-β-D-fructofuranosyl sucrose
5d	1F-β-D-fructofuranosyl-6G (1-β-D-fructofuranosyl)2 sucrose
6a	1F (1-β-D-fructofuranosyl)4 sucrose
6b	6G (1-β-D-fructofuranosyl)4 sucrose
6c	1F (1-β-D-fructofuranosyl)3-6G-β-D-fructofuranosyl sucrose
$6d_1$	1F-β-D-fructofuranosyl-6G (1-β-D-fructofuranosyl)3 sucrose
$6d_2$	1F (1-β-D-fructofuranosyl)2-6G (1-β-D-fructofuranosyl)2 sucrose
7a	1F (1-β-D-fructofuranosyl)5 sucrose
7	1F (1-β-D-fructofuranosyl)m-6G (1-β-D-fructofuranosyl)n sucrose ($m + n = 5$)
8	1F (1-β-D-fructofuranosyl)m-6G (1-β-D-fructofuranosyl)n sucrose ($m + n = 6$)
9x	1F (1-β-D-fructofuranosyl)m-6G (1-β-D-fructofuranosyl)n sucrose ($m + n \geq 7$)

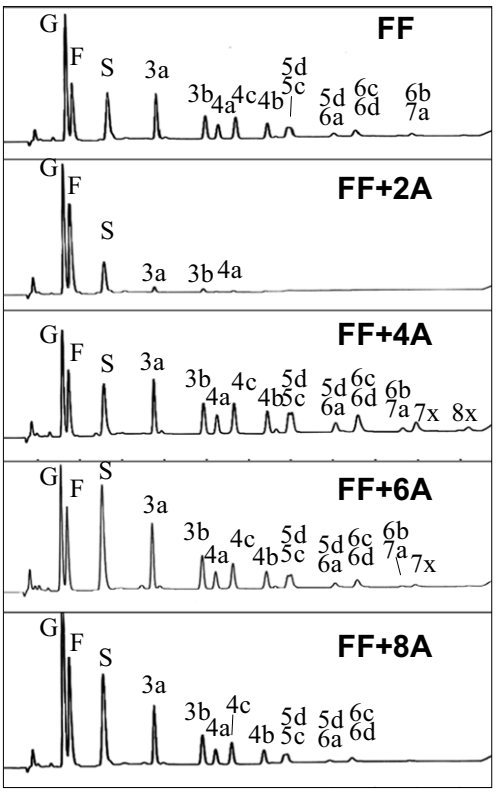

Fig. 2 High-performance anion-exchange chromatography (HPAEC) profiles of sugars extracted from the leaf blades of *A. fistulosum* (FF), FF+2A, FF+4A, FF+6A and FF+8A. Analyses were carried out by using extracts obtained on Feb. 2002. a, 1-kestose; 3b, neokestose and 1F(1-β-D-fructofuranosyl)*m*-6G(1-β-D-fructofuranosyl)*n* sucrose (4a: $m = 2$, $n = 0$; 4b: $m = 0$, $n = 2$; 4c: $m = 1$, $n = 1$; 5a: $m = 3$, $n = 0$; 5b: $m = 0$, $n = 3$; 5c: $m = 2$, $n = 1$; 5d: $m = 1$, $n = 2$; 6a: $m = 4$, $n = 0$; 6b: $m = 0$, $n = 4$; 6c: $m = 3$, $n = 1$; 6d: $m = 1$, $n = 3$ or $m = 2$, $n = 2$; 7a: $m = 5$, $n = 0$; 8x: $n + m \geq 6$).

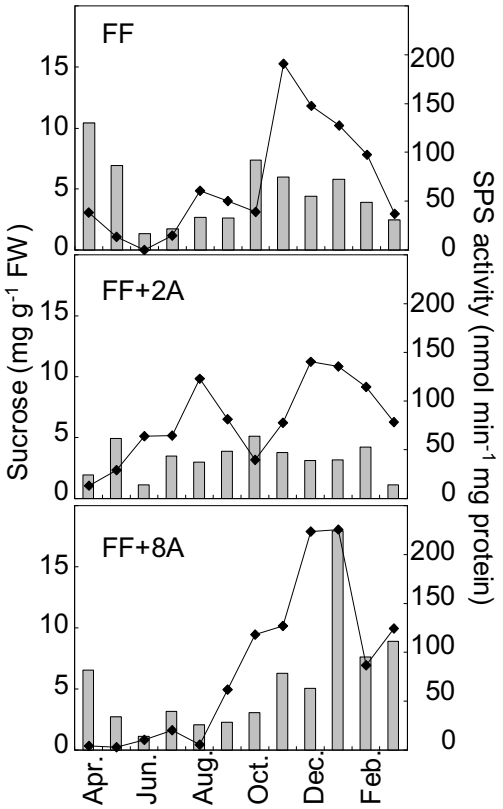

Fig. 3 Seasonal changes in sucrose phosphate synthase (SPS) activities and sucrose contents in leaf blade from Apr. 2002 to Mar. 2003 with *A. fistulosum* (FF), FF+2A and FF+8A.

Enzymology of sucrose metabolism

Enzyme activities, related to sucrose metabolisms, and sugar variations in the leaf blades of *A. fistulosum* and the *A. fistulosum* - shallot AALs FF+2A and FF+8A are shown in **Fig. 3**. Acid invertase activities of *A. fistulosum* and FF+8A were higher in summer but remained constant in FF+2A. SuSy activity, as sucrose cleavage, was constant except for a high level observed in FF+2A in April. The SPS activities in FF+8A were significantly higher in the autumn and were correlated with sucrose content ($r = 0.74$). By contrast, SPS activity and sucrose content were not correlated in *A. fistulosum* ($r = 0.15$) or FF+2A ($r = 0.05$).

Biochemical analysis of high and low fructan lines from the 'W202A x Texas Grano 438' onion mapping population

Analysis of carbohydrate contents of leaf blades and bases in developing plants revealed that sucrose content was significantly higher ($P < 0.001$ for harvest date × fructan phenotype interaction) in leaf blades and bases of high fructan lines prior to bulbing. Analysis of total fructan in mature bulbs of the selected inbred lines confirmed that mean fructan content of low fructan lines was below 20% of dry matter content while that of high lines was over 20%. Fructan levels in leaf blades and bases during development were also higher in the high fructan lines, and negatively correlated with fructose content ($r = -0.82$), as previously reported for mature bulbs (McCallum *et al.* 2006). The SuSy activity, measured as sucrose cleavage, ranged from 13-57 nmol min^{-1} mg protein in leaf blades and 117-254 nmol min^{-1} mg protein in leaf bases. Leaf blade SuSy activity was significantly higher prior to bulbing ($P < 0.001$) but was not significantly affected by fructan phenotype ($P = 0.26$). Leaf base SuSy activity increased to a small extent after bulbing and was not affected by fructan phenotype ($P = 0.78$). Leaf blade SuSy activity was correlated with leaf blade hexose content ($r = 0.75$).

Genetic mapping of sucrose synthase and sucrose phosphate synthase loci and assignment of genes affecting carbohydrate metabolism

Two marker assays were designed from SPS sequence (GenBank Accession EU164758), one spanning two exons toward the N-terminus (SPS4) and another targeting the 3′ UTR (SPS3′UTR). Both assignment using *A. fistulosum* - shallot AALs (**Fig. 4**) and mapping in an inter-specific cross placed these markers on chromosome 8 (**Fig. 5**). SPS3′UTR mapped outside the interval ACM033-ACABE58 to which *Frc* was previously mapped in 'BYG15-23 x AC43' (McCallum *et al.* 2006) but close to the dry matter QTL identified previously in this population using a partial map (Galmarini *et al.* 2001). To date we have not been able to map SPS markers in an onion pedigree due to high levels of

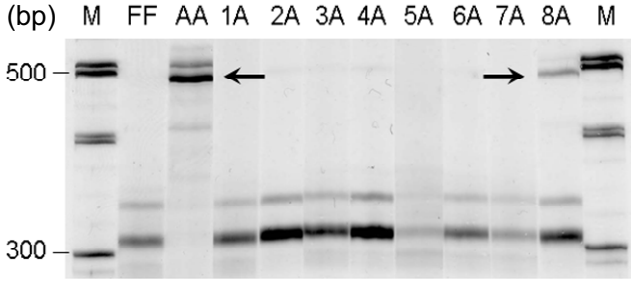

Fig. 4 Assignment of a sucrose phosphate synthase (SPS4) marker to *A. cepa* chromosomes using *A. fistulosum* - shallot AALs (1A-8A). Control lanes on left of gel contained amplicons from *A. fistulosum* (FF) and shallot (AA) donor lines. Arrows point to the shallot-specific bands. M, molecular size marker (100 bp DNA ladder).

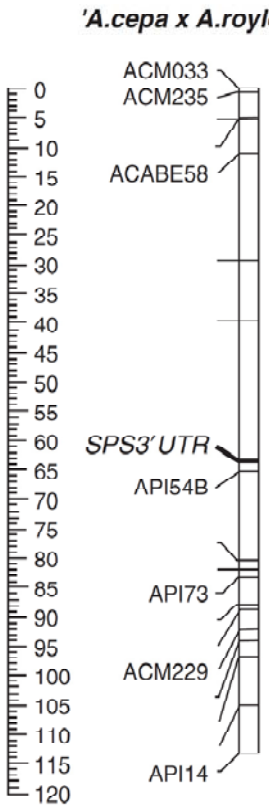

Fig. 5 Genetic mapping of the SPS locus to chromosome 8 in the '*A. cepa* x *A. roylei*' population and alignment with the onion linkage map of Martin *et al.* (2005). Scale denotes recombination distance in Kosambi units. Names of AFLP loci in the interspecific map are omitted for clarity.

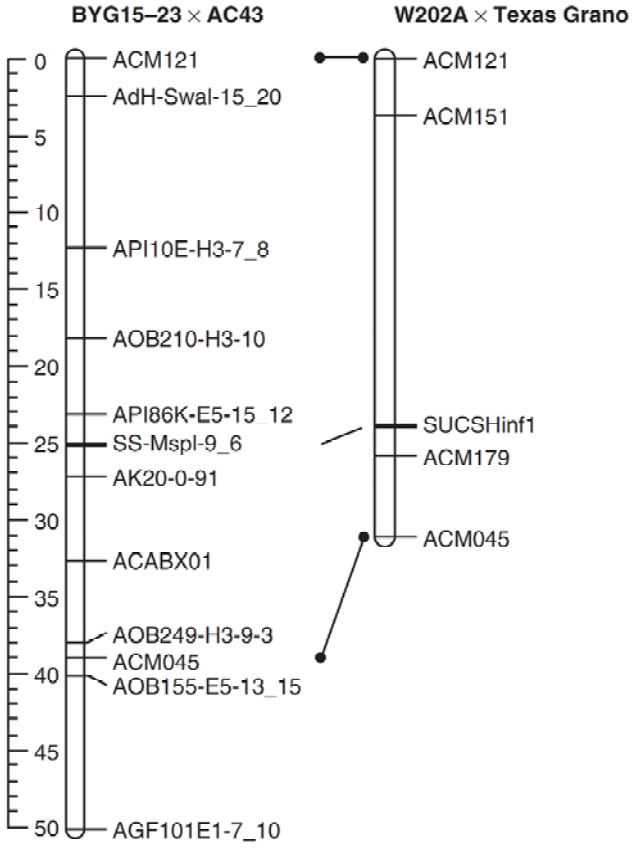

Fig. 6 Genetic mapping of a sucrose synthase locus (SUCS) to onion chromosome 6 in bulb onion mapping populations. Scale denotes recombination distance in Kosambi units. Names of AFLP loci in the interspecific map are omitted for clarity.

heterozygosity in parent lines (data not shown).

Several sucrose synthase homologs were identified in onion EST collections and two were assigned to distinct locations on chromosome 6 (CF440928; SUCS) and chromosome 2 (CF452518; ACP013) using *A. fistulosum* – shallot AALs. The SUCS marker was polymorphic across several onion populations segregating for carbohydrate composition and linked with the same markers as the sucrose synthase RFLP marker (SS-Msp1-9_6) previously reported by Martin *et al.* (2005; **Fig. 6**). Invertase homologs were identified in onion EST collections (**Table 1**). We assigned two neutral invertase homologs (ACP042 and ACP047) to chromosomes 8 and 2, a cell wall invertase homolog (ACP057) to chromosome 2, and two acid invertase homologs (ACP041 and ACP054) to chromosome 2. A sucrose phosphate phosphatase homolog was identified in onion EST collections (CF441209; ACP059) and assigned to chromosome 1 using *A. fistulosum* - shallot AALs.

Qualitative and quantitative analysis of fructan in shallot - *A. fistulosum* single-alien deletion lines

Bulb sugar analyses were conducted three shallot - *A. fistulosum* single-alien deletion lines (AAF-1F, AAF-4F and AAF-8F) and one shallot - *A. fistulosum* AAL (AA+8F) (**Table 3**). There was a significant difference in the total sugar content between shallot [73.9 mg g^{-1} fresh weight (FW)] and shallot carrying *A. fistulosum* chromosomes, i.e., single-alien deletion lines (AAF-1F and AAF-8F), AA+8F and AAF, in which sugars over 200 mg g^{-1} FW were detected. There were significant differences in the fructan content with DP 3 or higher between shallot and shallot carrying *A. fistulosum* chromosomes. While there were no significant difference in the mono- and disaccharides content between shallot and shallot carrying *A. fistulosum* chromosomes. Moreover, shallot could not produce fructan with DP 12 or more, although the single-alien deletion lines, AA+8F and AAF produced fructan with DP 20 or more, especially AA+8F, which had the longest chains (**Fig. 7**).

Determination of ACSO content in shallot - *A. fistulosum* single-alien deletion lines

There was a great difference in total contents of bulb ACSO between shallot (5.57 mg g^{-1} FW) and shallot carrying *A. fistulosum* chromosomes, i.e., AAF (1.64), the three types of single-alien deletion lines [AAF-1F (2.23), AAF-4F (2.67) and AAF-8F (2.16)] and AA+8F (3.15). The contents of PeCSO, the primary ACSO of *A. cepa* and *A. fistulosum*, were almost identical in all the examined plants. On the other hand, the shallot showed a significant increase in the contents of AlCSO, the principal ACSO of garlic (*A. sativum*), compared with each single-alien deletion lines. In addition, MeCSO, the major ACSO of Chinese chives (*A. tuberosum*) and rakkyo (*A. chinense*), had a content in shallots (3.72) that was two to five times as high as that in shallot - *A. fistulosum* single-alien deletion lines [AAF-1F (1.27), AAF-4F (1.72) and AAF-8F (1.08)], AA+8F (1.54) and AAF (0.74).

Table 3 Carbohydrate contents in leaf sheaths of shallot - *A. fistulosum* single-alien deletion lines (AAF-1F, AAF-4F and AAF-8F), shallot - *A. fistulosum* AAL (AA+8F), shallot (AA) and allotriploid (AAF).

Genomic constitution	Carbohydrate contents (mg g^{-1} FW)[z]		
	DP = 1 + 2	DP ≥ 3	Total
AAF-1F	27.6 a	267.3 b	294.9 b
AAF-4F	15.1 a	191.5 ab	215.6 ab
AAF-8F	17.8 a	217.6 b	235.4 b
AA+8F	20.9 a	231.3 b	252.2 b
AA	18.1 a	58.8 a	73.9 a
AAF	16.6 a	231.2 b	247.8 b

[z] Mean separation with each column by Tukey's multiple range test ($P < 0.05$).

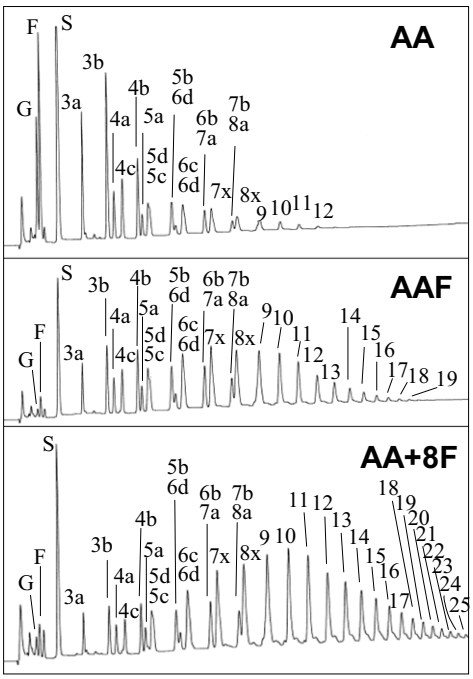

Fig. 7 High-performance anion-exchange chromatography (HPAEC) profiles of sugars extracted from the bulbs of shallot (AA), AAF and AA+8F. Analyses were carried out by using extracts obtained on Aug. 2005. 3a, 1-kestose; 3b, neokestose; 1F(1-β-D-fructofuranosyl)*m*-6G(1-β-D-fructofuranosyl)*n* sucrose (4a: $m = 2$, $n = 0$; 4b: $m = 0$, $n = 2$; 4c: $m = 1$, $n = 1$; 5a: $m = 3$, $n = 0$; 5b: $m = 0$, $n = 3$; 5c: $m = 2$, $n = 1$; 5d: $m = 1$, $n = 2$; 6a: $m = 4$, $n = 0$; 6b: $m = 0$, $n = 4$; 6c: $m = 3$, $n = 1$; 6d: $m = 1$, $n = 3$ or $m = 2$, $n = 2$; 7a: $m = 5$, $n = 0$; 8x: $n + m \geq 6$); 9 - 25, fructan with DP 9 - 25.

DISCUSSION

The present study demonstrates that important candidate structural genes for sucrose metabolism are located on chromosomes 2 and 8 of shallot and that monosomic additions of these chromosomes cause significant changes in sucrose levels and sucrose metabolic enzymes in the leaf blades compared to *A. fistulosum.* The PCR-based markers evaluated in this study were derived from an exhaustive search of existing onion EST resources. We therefore suggest that the current findings, together with earlier linkage mapping (Martin *et al.* 2005) and assignment studies (Masuzaki *et al.* 2007), have defined the chromosomal locations of major sucrose and fructan metabolism genes expressed in vegetative tissues of onion. However, since onion EST resources are relatively limited and plant glycosyltransferases share extensive sequence similarity, the functional roles of these genes are still uncertain.

We previously reported that, in multiple additions containing shallot chromosome 5, the absence of chromosome 2 was associated with increased bulb formation (Masuzaki *et al.* 2007). Since we were able to assign five candidate genes for sucrose metabolism to chromosome 2, we hypothesise that altered expression of one or more of these in FF+2A lines alters sucrose pools. Both invertases, including cell wall invertase and acid invertase (Roitsch and Gonzalez 2004) and sucrose synthases (Paul and Foyer 2001) play roles in regulating cycles of sucrose-hexose interconversion that regulate sink strength in carbohydrate-accumulating tissues (Nguyen-Quoc and Foyer 2001). The strong correlation observed between SuSy cleavage activity and hexose levels in leaf blades of onion inbreds is consistent with a key role in driving sink strength (Paul and Foyer 2001) but not in determining the *Frc* phenotype. The observation that both FF+8A addition lines and *Frc* inbred high fructan onions exhibiting higher sucrose levels suggests that a gene or genes on this chromosome also play a key role in conditioning high sucrose levels. Since SPS catalyses the rate-limiting step in sucrose biosynthesis (Huber and Huber 1996) we hypothesise that different expression of SPS may underlie the FF+8A and *Frc* phenotypes. Studies in sugarcane have shown a strong correlation between SPS activity and sucrose content within and between cultivars (Grof *et al.* 1998; 2007). It has been reported that the genes encoding sucrose:sucrose 1-fructosyltransferase (1-SST), which catalyze the first step of fructan synthesis (Vijn *et al.* 1998), were induced by high sucrose contents in barley leaves (Muller *et al.* 2000; Wang *et al.* 2000). It could be possible that the inducing expression of genes encoding 1-SST by high sucrose contents caused an increase of fructan accumulation in FF+8A and high fructan *Frc* inbred onions.

The assignment of the SuSy (SUCS) locus to chromosome 6 in this study using *A. fistulosum* - shallot AALs and linkage mapping is in agreement with previous RFLP mapping (Martin *et al.* 2005). Previous studies have also located both fructan biosynthetic genes, 1-SST (Havey *et al.* 2004) and fructan:fructan 6G-fructosyltransferase (6G-FFT) (McCallum *et al.* 2006) to this chromosome.

While the assignment of genes to chromosomes using AALs is unambiguous, interpretation of biochemical and other phenotypes is challenging since genes on the alien chromosome are expressed in the diploid genetic background of a divergent parent (Chang and de Jong 2005). Support for the idea that heterozygosity or polyploidy in sucrose metabolism genes such as SPS and SuSy can induce marked changes in carbohydrate metabolism is provided by studies in maize. Causse *et al.* (1995) observed significant heterosis for SPS activity in maize hybrids and subsequently reported co-location of the QTL for SPS activity with the structural gene (Prioul *et al.* 1999). More recent studies of gene expression in diploid (Auger *et al.* 2005) and triploid (Swanson-Wagner *et al.* 2006) maize hybrids have also revealed non-additive expression of SuSys and SPS.

In the present study, no candidate genes related to sugar metabolism were assigned on chromosome 4 using AALs. Further biochemical and genetic studies related to sugar metabolism could make clear the details of fructan accumulation in FF+4A. The clarification of the *Frc* and AAL biochemical phenotypes, combined with assignment of additional sucrose metabolism genes to the *Allium* map, now provide a more comprehensive framework for genetic and physiological analysis of economic traits in *Allium* vegetables, including consumer attributes such as sweetness as well as production traits such as bulbing, heterosis and dry matter accumulation. The observation that key candidate genes map at or near locations of several QTL affecting carbohydrate traits in onion confirms similar findings in other crops (Pflieger *et al.* 2001). In conclusion, our observation that FF+8A addition lines and high fructan *Frc* onion lines exhibit high sucrose levels suggests that targeted studies of sucrose metabolism genes, notably SPS, on this chromosome should be undertaken to determine the functional nature of *Frc*.

Shallot - *A. fistulosum* AAL (AA+8F), AAF and the three types of single-alien deletion lines (AAF-1F, AAF-4F and AAF-8F) differed widely from shallot regarding their bulb components of sugars and ACSOs. Regarding sugars, AAF, AA+8F and the single-alien deletion lines showed higher contents of fructans, which are oligosaccharides with chain lengths higher than DP 2, than shallot (**Table 3**), and the chain lengths in shallot were the shortest (**Fig. 7**). These results indicated that the chromosomes derived from *A. fistulosum* in the diploid background of shallot may contribute to an increase in the fructan production in shallot bulbs. This study revealed that the important QTL (*Frc*) and the major enzyme gene SPS related sucrose synthesis were allocated on chromosome 8A of shallot. From the point of view of a close genetic relationship between *A. fistulosum* and shallot, there is a high probability that a number of orthologues are located on a same group of chromosomes, namely homoeologous chromosomes, in these two species. The bulbs of AA+8F showed higher fructan content than shallot in this study. This indicated that anonymous factors

related to produce fructan, e.g. *Frc* and SPS, should be located on the chromosome 8F of *A. fistulosum*. The additive experiment of the enzyme activities related to fructan and sucrose biosyntheses in the shallot, the shallot - *A. fistulosum* AAL and single-alien deletion lines should reveal the gene expression event on the alien chromosome of *A. fistulosum* in shallot.

In ACSOs, Yoo and Pike (1998) reported that the total ACSO content of *A. fistulosum* was lower than that of shallot. The bulbs of AAF and the single-alien deletion lines showed much lower contents of total ACSO than shallot in this study. This result suggested that chromosomes derived from *A. fistulosum* in the diploid background of shallot might carry anonymous factors to inhibit the synthesis of ACSOs in the bulbs of shallot. Low ACSOs content correlated with low pungency of *A. cepa* bulbs (Sinclair *et al.* 1995; Lancaster *et al.* 1998; Crowther *et al.* 2005). Generally, higher soluble solid content, including fructan, correlated increase in pungency of the bulbs in *A. cepa* (Simon 1995; Sinclair *et al.* 1995; Lancaster *et al.* 1998). However, Simon (1995) noted the feasibility of independent selection for pungency and soluble solids in onion. Several shallot carrying *A. fistulosum* chromosomes showed not only the high fructan accumulation but also the low ACSOs content in this study. Accordingly, these shallot - *A. fistulosum* addition lines could be a new sweet variety of the shallot.

FUTURE PERSPECTIVES

Genetic analysis aided by a framework molecular marker map has allowed rapid advances in the understanding of variation in onion bulb composition. In this study, the effectiveness of complete set of *A. fistulosum* - shallot AALs for chromosomal assignment of genes associated with carbohydrate composition suggests that these will continue to be a key resource for functional and genetic studies of major genes. Recently, six types of shallot - *A. fistulosum* single-alien deletion lines (AAF-1F, -3F, -4F, -6F, -7F and -8F) have been produced (Hang *et al.* 2004; Yaguchi *et al.* 2009). Fifteen linkage groups based on short sequence repeats, cleaved amplified polymorphic sequences, and insertion-deletion markers of *A. fistulosum* have been allocated to a single chromosome via the use of shallot - *A. fistulosum* single-alien deletion lines (Tsukazaki et al., 2008). They have started to integrate the *A. fistulosum* linkage map with the *A. cepa* map developed by Martin et al. (2005). Those works should contribute advances in the understanding of variation in the composition of onion bulbs and in *A. fistulosum* leaves.

SMALL SUMMARY

To understand the biochemical basis of *Frc*, QTL related to the onion bulb fructan concentrations, we conducted biochemical and genetic analyses of *Allium fistulosum* (FF) - shallot alien monosomic addition lines (AALs; FF+1A-FF+8A), onion mapping populations and shallot - *A. fistulosum* addition lines. High sucrose and fructan accumulations were detected in FF+8A leaf blades correlated with high SPS activity. SPS markers obtained by cloning a major SPS expressed in onion leaf were assigned to chromosome 8 using AALs and onion linkage mapping. Moreover, the bulbs of shallot - *A. fistulosum* AAL (AA+8F) also showed the high fructan accumulation. The concordance between chromosome 8 localization of SPS and elevated leaf sucrose levels conditioned by high fructan alleles at the *Frc* locus in bulb onion or AALs of chromosome 8 in *A. fistulosum* and in *A. cepa* suggest that the *Frc* locus may condition variation in SPS activity. On the other hand, allotriploid between shallot and *A. fistulosum* (AAF) and shallot - *A. fistulosum* single-alien deletion lines showed high fructan and low ACSO levels in the bulbs. This indicated that chromosomes derived from *A. fistulosum* in the diploid background of shallot should carry several factors to promote the fructan biosynthesis and inhibit the synthesis of ACSOs in the bulbs of shallot.

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