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# Biochemical and Genetic Analysis of Carbohydrate Accumulation in *Allium cepa* L.

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**Onion and shallot (*Allium cepa* L.) 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 (*A. cepa* Aggregatum group) alien monosomic addition lines (AALs; FF+1A–FF+8A) and onion mapping populations. Sucrose and fructan levels in leaves of FF+2A were significantly lower than in FF throughout the year, and the springtime activity of acid invertase was also lower. FF+8A showed significantly higher winter sucrose accumulation and sucrose phosphate synthase (SPS) activity. Inbred high fructan (*Frc_*) lines from the 'W202A× Texas Grano 438' onion population exhibited significantly higher sucrose levels prior to bulbing than low fructan (*frcfrc*) lines. Sucrose synthase (SuSy) activity in these lines was correlated with leaf hexose content but not with *Frc* phenotype. Markers for additional candidate genes for sucrose metabolism were obtained by cloning a major SPS expressed in onion leaf and exhaustively mining onion expressed sequence tag resources. SPS and 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 three invertases) and 8 (neutral invertase). 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* suggest that the *Frc* locus may condition variation in SPS activity.**

**Keywords:** Fructan — Japanese bunching onion — Mapping — Onion — Sucrose.

Abbreviations: AAL, monosomic alien addition line; DP, degree of polymerization; EST, expressed sequence tag; HPAEC, high-performance anion exchange chromatography; QTL, quantitative trait locus; RACE, rapid amplification of cDNA ends; RFLP, restriction fragment length polymorphism; RT–PCR, reverse transcription–PCR; SPS, sucrose phosphate synthase; 1-SST, sucrose:sucrose 1-fructosyltransferase; SuSy, sucrose synthase; UTR, untranslated region.

The nucleotide sequence reported in this paper has been submitted to GenBank under accession number EU164758.

## 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 <4% of bulb dry matter in sweet and salad types to >65% in shallots and dehydrator onions. 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 not yet understood. 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 genetic 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

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chromosomes 3, 5 and 8 that affect onion bulb dry matter and solids content. Interestingly, QTLs on chromosomes 3 and 5 were associated with restriction fragment length polymorphism (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 *A. fistulosum*–shallot alien monosomic addition lines (AALs) originally developed by Shigyo et al. (1996). Hang et al. (2004) showed that the AALs carrying chromosome 8 from shallot also accumulated non-reducing carbohydrates in leaf blades during winter. Studies of bulb formation in multiple addition lines showed that lines containing *A. cepa* chromosome 5 exhibited bulb formation, but addition of chromosome 2 inhibited bulb formation (Masuzaki et al. 2007).

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*; there is 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, outcrossing and highly heterozygous species. These constraints, combined with the interesting phenotypes observed in AALs, suggest that a complementary strategy for identifying *Frc* and other major carbohydrate metabolism genes in onion is to make use of the AALs for functional studies.

In this study, we combined biochemical characterization of sucrose metabolism in 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.

## Results

### *Biochemical analysis of alien monosomic addition lines*

*Quantitative analysis of total sugar contents in leaf blade tissues*. Monthly analysis of total leaf blade carbohydrates over 2 years revealed increased storage of sucrose and fructan in the winter months in *A. fistulosum* controls and in all 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 AAL ($r = 0.23$–$0.85$). Because of their marked differences in sucrose and fructan accumulation compared with *A. fistulosum*, the FF+2A and FF+8A lines were selected for more detailed analysis of sucrose metabolic enzymes.

*Qualitative analysis of fructan in leaf blade tissues*. Qualitative analysis of total sugar content by thin-layer chromatography (TLC) revealed accumulation of fructan including 1-kestose and oligosaccharides with a degree of polymerization (DP) >4 from winter to early spring in all lines except FF+2A (data not shown).

High-performance anion exchange chromatography (HPAEC) analysis identified glucose, fructose, sucrose and fructan isomers [3a, 1-kestose; 3b, neokestose and $1_F(1\text{-}\beta\text{-D-fructofuranosyl})_m\text{-}6_G(1\text{-}\beta\text{-D-fructofuranosyl})_n$ 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: $n + m \geq 6$)] in leaf blade extracts of *A. fistulosum* and AALs (data not shown). 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 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. The fructan contents of *A. fistulosum* and four AALs, which showed a different fructan profile from that of *A. fistulosum*, are shown in Fig. 2. 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 >5. The total content of neokestose series saccharides (3b, 4b and c, 5b–d and 6b–d) was higher than that of 1-kestose series saccharides (3a, 4a, 5a, 6a and 7a) in every determination.

*Enzymology of sucrose metabolism*. Activities of acid invertase, sucrose synthase (SuSy) and sucrose phosphate synthase (SPS) in *A. fistulosum* and the 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 ($r = 0.74$). In contrast, SPS activity and sucrose content were not correlated in *A. fistulosum* ($r = 0.15$) or FF+2A ($r = 0.05$).

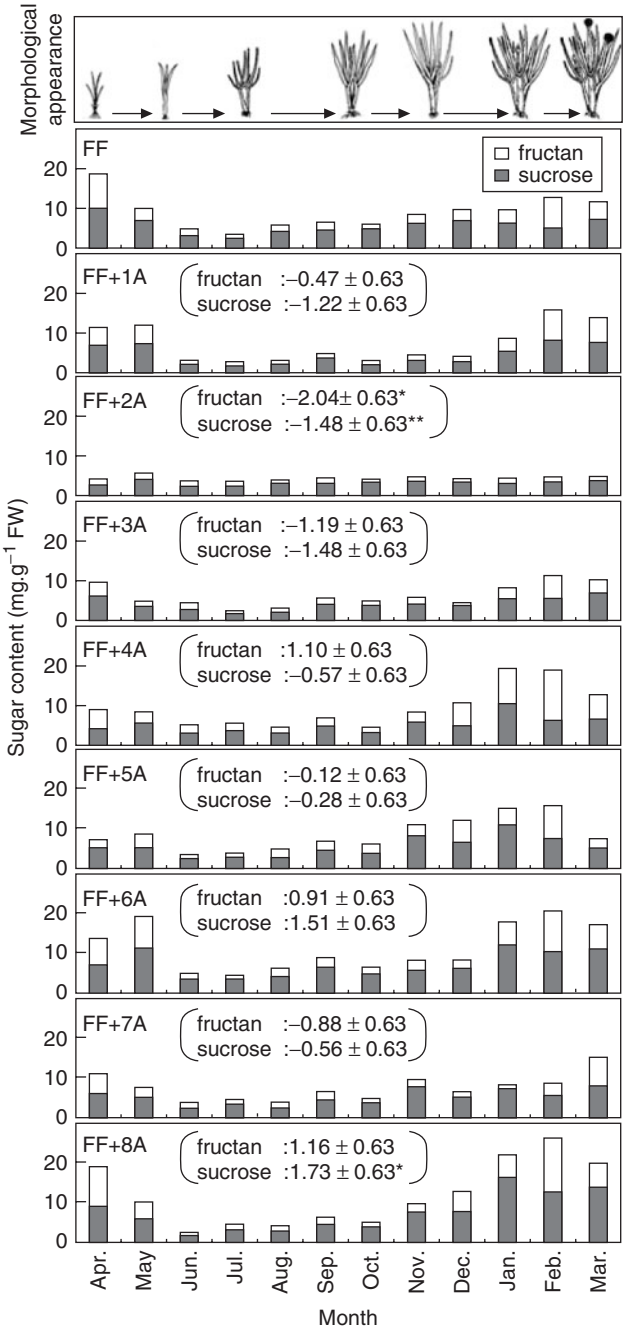


**Fig. 1** Schematic illustrations of the morphological appearance in *A. fistulosum* and the year-round variations of fructan and sucrose content from leaf blade tissues in *A. fistulosum* (FF) and each monosomic addition line (FF+1A–FF+8A). Values denote monthly means from January 2002 to December 2003. Values in parentheses show the mean difference (±SE) of fructan and sucrose content between *A. fistulosum* and each monosomic addition line. Dunnett's multiple comparison test was used to test mean separations. *, ** significant at $P \leq 0.05$, 0.01, respectively.

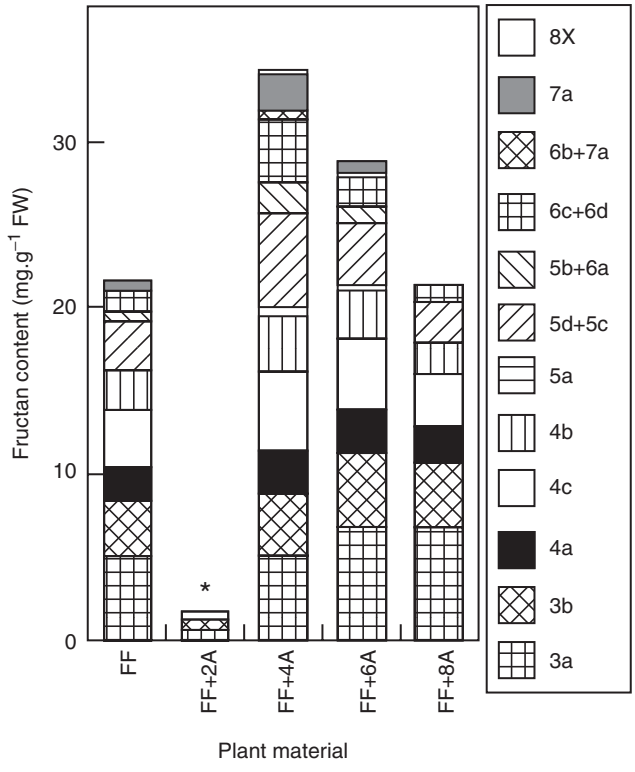


**Fig. 2** Fructan content in the leaf blades of *A. fistulosum* (FF), FF+2A, FF+4A, FF+6A and FF+8A in February 2002. 3a, 1-kestose; 3b, neokestose and $1_F$(1-β-D -fructofuranosyl)$_m$-$6_G$(1-β-D-fructofuranosyl)$_n$ sucrose (4a: $m=2$, $n=0$; 4b: $m=0$, $n=2$; 4c: $m=1$, $n=1$; 5a: $m=3$, $n=0$; 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: $n+m \geq 6$). The asterisk indicates a significant difference at the 5% level between *A. fistulosum* and the monosomic addition line by equivalence test.

*Biochemical analysis of high and low fructan lines from the 'W202A×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 (Fig. 4). Analysis of total fructan in mature bulbs of the selected inbred lines confirmed that mean fructan content of low fructan lines was <20% of dry matter content, while that of high lines was >20% (Table 1). Fructan levels in leaf blade and bases during development were also higher in the high fructan lines (Fig. 4), 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 to 57 nmol $min^{-1}$ mg protein in leaf blades and from 117 to 254 nmol $min^{-1}$ mg protein in leaf bases. Leaf blade SuSy activity was

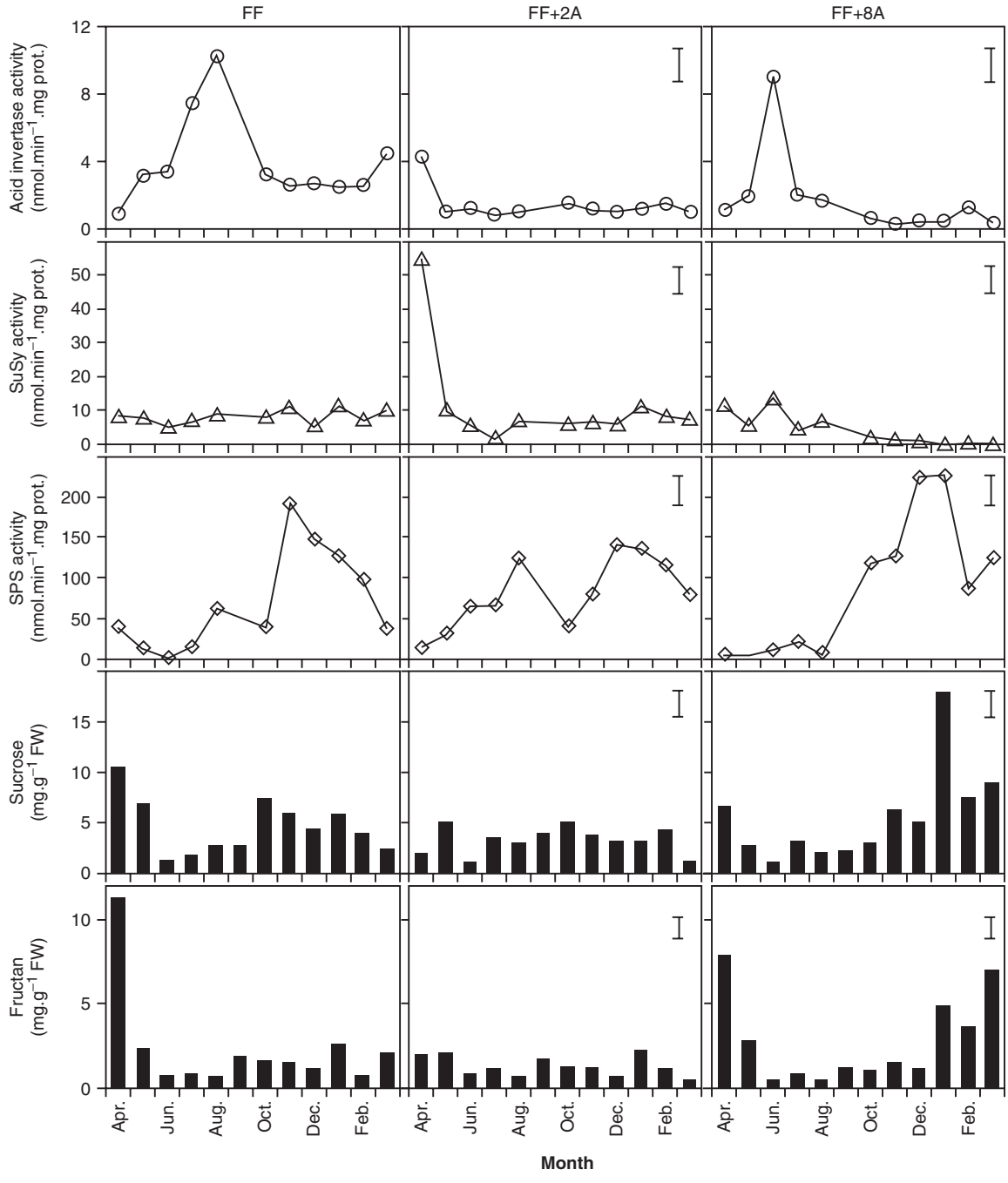


**Fig. 3** Seasonal changes in acid invertase, sucrose synthase (SuSy) and sucrose phosphate synthase (SPS) activities, and sucrose and fructan contents in leaf blade from April 2002 to March 2003 with *A. fistulosum* (FF), FF+2A and FF+8A. Open cicles, acid invertase activities; open triangles, SuSy activities for sucrose cleavage; open diamonds, SPS activities. Bars represent the least significant difference (LSD) at the 5% level between *A. fistulosum* and each monosomic addition line.

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 ($r = 0.75$) (Fig. 4).

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

An SPS gene expressed in mature onion leaf was cloned by reverse transcription–PCR (RT–PCR) and rapid amplification of cDNA ends (RACE) methods (GenBank

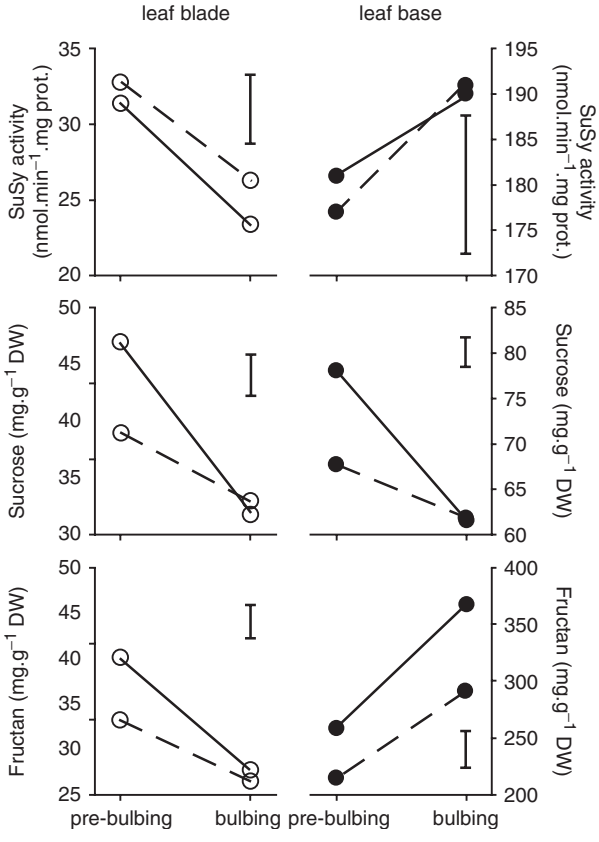


**Fig. 4** Sucrose synthase (SuSy) activity and content of sucrose and fructan in leaf blade and base tissues of developing inbred onion lines with high (solid line) and low (dashed line) bulb fructan phenotypes. Bars represent the least significant difference (LSD) at the 5% level for the harvest × fructan phenotype interaction term.

**Table 1** Fructan content of mature bulbs of parents and inbred $F_{2:3}$ lines of 'W202A × Texas Grano 438' populations

| Line(s) | Mean (mg g$^{-1}$ DW) | SEM |
|---|---|---|
| Texas Grano 438 | 128 | 9.5 |
| W202A | 230 | 9.1 |
| Low fructan lines ($n = 6$) | 163 | 5.4 |
| High fructan lines ($n = 6$) | 254 | 11.2 |

Means and SEMs were calculated from three plot replicates of each parental and inbred line.

accession No. EU164758). The hypothetical peptide translated from this sequence (ABV90637) shared 68–71% amino acid identity with Group B plant SPS proteins and contained all regulatory and structural binding motifs typical of this group (Castleden et al. 2004, Lutfiyya et al. 2007). This is the most common type of SPS cloned from monocots and is highly expressed in leaves of maize

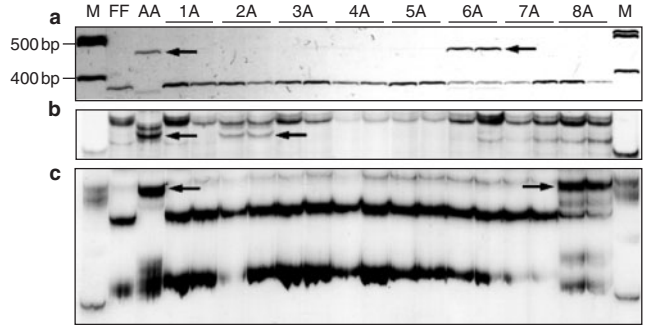


**Fig. 5** Assignment of sucrose synthase (SUCS, a; ACP013, b), and sucrose phosphate synthase (SPS3′UTR, c) markers to *A. cepa* chromosomes using a panel of independently isolated *A. fistulosum* –shallot alien monosomic addition lines (1A–8A). Control lanes on the left of the gel contained amplicons from *A. fistulosum* (FF) and shallot (AA) donor lines. Arrows point to the shallot-specific bands. M, molecular size marker (100 bp DNA ladder).

(Castleden et al. 2004). Two marker assays were designed from this sequence, one spanning two exons toward the N-terminus (SPS4) and another targeting the 3′ untranslated region (SPS3′UTR). Both assignment using AALs (Fig. 5) and mapping in an inter-specific cross (Fig. 7) placed these markers on chromosome 8. SPS3′UTR mapped outside the interval ACM033–ACABE58 to which *Frc* was previously mapped in 'BYG15-23 × AC43' (McCallum et al. 2006) but close to the dry matter QTLs 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 heterozygosity in parent lines (data not shown).

Several SuSy homologs were identified in onion expressed sequence tag (EST) collections, and two were assigned to distinct locations on chromosome 6 (CF440928; SUCS) and chromosome 2 (CF452518; ACP013) using AALs (Fig. 5a, b). The SUCS marker was polymorphic across several onion populations segregating for carbohydrate composition and linked with the same markers as the SuSy RFLP marker (SS-Msp1-9_6) previously reported by Martin et al. (2005; Fig. 6). Invertase homologs were identified in onion EST collections (Table 2). 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 (data not shown). A sucrose phosphate phosphatase homolog was identified in onion EST collections (CF441209; ACP059) and assigned to chromosome 1 using AALs (data not shown).

## Discussion

The present study demonstrates that important candidate structural genes for sucrose metabolism are located

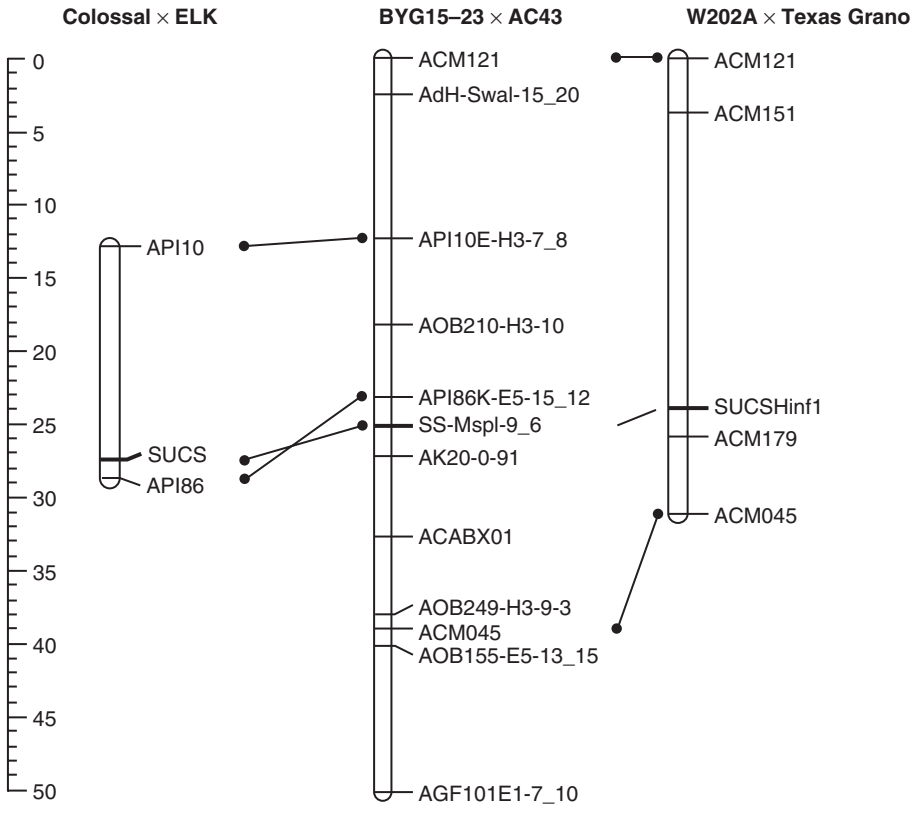


**Fig. 6** Genetic mapping of a sucrose synthase locus (SUCS) to onion chromosome 6 in bulb onion mapping populations. The scale denotes the recombination distance in Kosambi units. Names of amplified fragment length polymorphism (AFLP) loci in the interspecific map are omitted for clarity.

on chromosomes 2 and 8 and that monosomic additions of these chromosomes cause significant changes in sucrose levels and sucrose metabolic enzymes compared with *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 hypothesize that altered expression of one or more of these in FF+2A lines alters sucrose pools. Both invertases (Roitsch and Gonzalez 2004) and SuSys (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 the leaf blade 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 inbred high fructan *Frc_* onions exhibit higher sucrose levels suggests that a gene or genes on this chromosome also plays a key role in conditioning high sucrose levels. Since SPS catalyzes the rate-limiting step in sucrose biosynthesis (Huber and Huber 1996), we hypothesize 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, Grof et al. 2007). It has been reported that the genes encoding sucrose:sucrose 1-fructosyltransferase (1-SST), which catalyzes 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 induction of 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 AALs and linkage mapping is in agreement with previous RFLP mapping (Martin et al. 2005). Previous studies have also located both

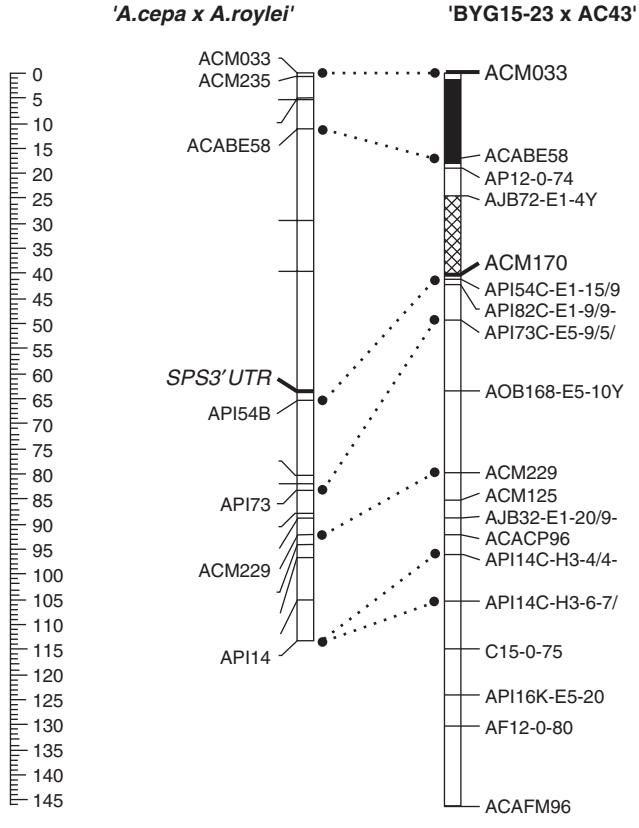


**Fig. 7** Genetic mapping of the SPS locus to chromosome 8 in the A. cepa × A. roylei population and alignment with the onion linkage map of Martin et al. (2005). The scale denotes the recombination distance in Kosambi units. Names of amplified fragment length polymorphism (AFLP) loci in the interspecific map are omitted for clarity. The highlighted intervals in the BYG1523 × AC43 denote 1 LOD confidence intervals for QTLs affecting dry matter (cross-hatched; Galmarini et al. 2001) and bulb fructan content (black; McCallum et al. 2006).

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 QTLs 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*.

## Materials and Methods

*Biochemical analysis of alien addition lines*

*Plant materials*. The plant materials were a complete set of *A. fistulosum*–shallot (*A. cepa* Aggregatum group) monosomic additions ($2n = 2x + 1 = 17$, FF+1A–FF+8A) and a control plant, Japanese bunching onion (*A. fistulosum* cv. Kujyo-hoso, $2n = 2x = 16$, FF). The monosomic additions were grown in an experimental field at Yamaguchi University (34°N, 131°E) and maintained over 2 years (January 2002–December 2003) through vegetative propagation. 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).

*Total sugar analysis*. Sugar extraction was performed as described previously (Hang et al. 2004). Free fructose in extracts was determined by the thiobarbituric acid method (Percheron 1962). To determine fructan plus sucrose, free fructose was removed from extracts by heating an aliquot in 1 N NaOH at 100°C for 10 min, 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 mg $ml^{-1}$ invertase (Bakers' yeast, Sigma, St Louis, MO, USA) and 10 µl of 25 mM ammonium acetate buffer (pH 5.5), for 5 min, then assayed to determine free plus fructan fructose.

*Analysis of sugars by HPAEC*. A 0.5 ml aliquot of 70% EtOH extracts of February 2002 was vacuum-evaporated to dryness and redissolved in 0.5 ml of Milli-Q water. The extract was filtered by passing through a 0.45 µm syringe-type filter HCL-Disk3 (Kanto Chemical Co., Inc., Tokyo, Japan) and analyzed by HPAEC according to the procedure of Shiomi et al. (1997).

*Enzyme assays*. Enzyme extraction was performed once a month from April 2002 to March 2003. All the operations for enzyme preparation were carried out at 4°C. Fresh leaf blade tissues (5.0 g) were homogenized with Polyclar AT (500 mg) and a few quartz sand grains in 20 ml of 0.1 M phosphate buffer (pH 7.5)

**Table 2** PCR primer sets used in this study

| Primer set | GenBank accession No. | Putative function | Forward and reverse primers | Chromosome |
|---|---|---|---|---|
| SUCS | CF440928 | Sucrose synthase | 5′-TTTGAAGTGTGGCCTTACCTTGAG-3′<br>5′-TGATGAAGTCTGTTCGATCATGGC-3′ | 6 |
| ACP013 | CF452518 | Sucrose synthase | 5′-TTCACCCTGAAATCGAGGAG-3′<br>5′-TCGGCTTGTTCTTCCTTGTC-3′ | 2 |
| SPS3′UTR | EU164758 | Sucrose phosphate synthase | 5′-AAAGGGAGATACAGACCAT-3′<br>5′-ATTATACATCTCATCATGTCACA-3′ | 8 |
| SPS4 | EU164758 | Sucrose phosphate synthase | 5′-GAAGGCTGATATTGTTGGTGAAG-3′<br>5′-TGTGTCGTAGGAGCCTGATG-3′ | 8 |
| ACP041 | CF437610 | Acid invertase | 5′-GGTTCAAAAGACGCATCCAA-3′<br>5′-TAATCCTGCCCATTATCAGAAGT-3′ | 2 |
| ACP042 | CF437950 | Neutral invertase | 5′-GATTTTGTGCCCTCTGCAAT-3′<br>5′-AAACTAGCTGGCATCAATCCTT-3′ | 8 |
| ACP047 | CF437145 | Alkaline/neutral invertase | 5′-AAGGATCTGCCGACCAAGA-3′<br>5′-TCAGGCATCCATTCAACAAG-3′ | 2 |
| ACP054 | CF435784 | Invertase | 5′-GCTCAATGTAGGTGGTGCTG-3′<br>5′-CTGCCGTCTGATTTCTTGCT-3′ | 2 |
| ACP057 | CF437606 | Cell wall invertase | 5′-CAGATATGCGAATGGTTTTGC-3′<br>5′-TGTCTACAAAGCCTCCAGACG-3′ | 2 |
| ACP059 | CF441209 | Sucrose phosphate phosphatase | 5′-GAATTGTTCAGCATTCCAGATG-3′<br>5′-CGTTCAGTTGCATGAATTATCCT-3′ | 1 |

containing 10 mM dithiothreitol (DTT). Homogenates were filtered through two layers of Miracloth and centrifuged for 20 min at 15,000×*g*. Supernatant (2.5 ml) was desalted on a Sephadex G-25 column equilibrated with 5 mM phosphate buffer (pH 7.0) and used as a crude enzyme. The protein content of the crude extract was determined according to the procedure of Bradford (1976).

*Sucrose synthase*. SuSy activity as sucrose cleavage was assayed by modifications of the method of Pressey (1969). Crude enzymes (500 µl) were added to 1.5 ml of 50 mM Tris-phosphate buffer (pH 7.0) supplemented with 250 mM sucrose, 10 mM UDP and 10 mM NaF. After incubation at 37°C for 1 h, the reactions were terminated by addition of 1 ml of 0.5 M dibasic phosphate and heating in a boiling water bath for 2 min. The fructose liberated was determined by the Somogyi–Nelson method (Somogyi 1952).

*Sucrose phosphate synthase*. Activity was measured by the method of Nielsen et al. (1991). Desalted homogenates (600 µl) were added to 1.2 ml of 15 mM HEPES-KOH buffer (pH 8.5) containing 15 mM fructose-6-phosphate, 2 mM UDP-glucose, 5 mM $MgCl_2$ and 1.3 mM NaF. After incubation at 37°C for 0.5 h, the reactions were terminated by addition of 250 µl of 0.4 N NaOH and heating in a boiling water bath for 20 min. The sucrose formed was then hydrolyzed by 30% HCl and the resultant ketose was determined according to the resorcinol method (Roe 1934).

*Acid invertase*. Activity was measured by the method of Shono et al. (1997) with minor modifications. Desalted homogenates (800 µl) were added to 1.2 ml of phosphate citrate buffer (pH 5.5) containing 100 mM sucrose. Reaction mixtures were incubated at 37°C for 1.5 h. The reaction was stopped by placing reaction tubes in boiling water for 2 min. The glucose and fructose formed were determined by the Somogyi–Nelson method.

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

Analytical methods and development of the 'W202A×Texas Grano 438' mapping population were described previously (McCallum et al. 2006). Seed of 12 inbred $F_{2:3}$ families (six high and six 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 m 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 December 21, 2005 prior to initiation of bulbing and on January 13, 2006 after commencement of bulbing.

Leaf blade and base tissue was frozen at –80°C and freeze-dried. These samples were analyzed for SuSy activity by the method of Dancer et al. (1990). A 20 mg aliquot of ground freeze-dried material was extracted in 1 ml extraction buffer [50 mM HEPES-KOH pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.05% (w/v)

Triton X-100], centrifuged and desalted into 20 mM HEPES-KOH pH 7.5 using BioRad Micro BioSpin six columns. A 5 µl aliquot of this solution was added to an assay mixture containing 20 mM HEPES-KOH pH 7.5, 100 mM sucrose, 4 mM UDP in a total volume of 100 µl, at 30°C for 20–30 min depending upon activity (linearity tested up to 60 min). The reaction was stopped by heating to 95°C for 4 min and cooling. A 25 µl aliquot of this solution was added to 0.2 M glycine-KOH pH 8.9, containing 5 mM $MgCl_2$, 2 mM $NAD^+$ in a total volume of 250 µl. NADH formed after the addition of 0.02 U of UDP-glucose dehydrogenase was monitored at $A_{340\,nm}$ in microplates at 30°C. Assays were duplicated. Glucose, fructose, sucrose and total fructan content were analyzed by 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 d and stored at 4°C and 65% relative humidity for 1 month before analysis. Pooled samples of 10 bulbs were analyzed for total fructan.

*Genetic marker analyses*

*Cloning of onion sucrose phosphate synthase*. A fragment of 215 bp was cloned by RT–PCR as described previously (Shaw et al. 2005) from total RNA from field-grown leaf tissue of cv 'Canterbury Longkeeper' using degenerate primers (SPS5′#4 AT GGCTGGAAAYGARTGGA; SPS3′#5 GCCAGATTCTCCA RCACATRTT) designed from GenBank accession No. M97550. Downstream sequence (~3 kb) was cloned by nested 3′ RACE with primers SPS_RACE1 (GGTAGCGTGCCAGAGCAG) and SPS_RACE3 (CTGATCTGCATAGAACCTGGATT). Upstream sequence (~480 bp) was cloned by 5′RACE with primer SPS-R (GGCCTCTTGGTTGGATCTACT). The full sequence was submitted to GenBank as accession No. EU164758.

*Genetic mapping of SuSy and SPS loci and assignment of genes affecting carbohydrate metabolism*. PCR primer sets employed in this study are shown in Table 2. PCR conditions and genetic mapping populations were employed as described previously (McCallum et al. 2006). The assignments were conducted by using *A. fistulosum*–shallot monosomic additions as described previously (Martin et al. 2005).

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