INVESTIGATION INTO THE FUNCTIONAL NATURE OF *FRC* LOCUS CONDITIONING FRUCTAN LEVELS IN ONION

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ABSTRACT

Frc, a major gene on chromosome 8, conditions fructan levels in onions (*Allium cepa* L). In order to assist genetic dissection of this locus, this study aimed to determine the factors influencing varying fructan levels in high- and low-fructan genotypes. Mapping families were developed and analysed to study the genetic architecture for the fructan trait, and to check the association of the identified variables with the *Frc* locus. To facilitate reliable and practicable sugar assays in onions, a newly-adapted high-throughput microplate enzymatic assay was validated in this study. The reliability of using leaf sugars as a representative of bulb sugars in a mapping population was studied.

Microplate enzymatic sugar assays were carried out on a segregating onion cross to validate the use of maltases in sugar analysis, and the results obtained were validated against HPLC-PAD. Sucrose measured in microplates employing maltases as the hydrolytic enzyme was in agreement with HPLC-PAD results. Maltase enzymes specifically hydrolysed sucrose in onions, providing an alternate tool in place of expensive sugar assay kits. Use of the microplate-enzymatic assay provided a rapid, cheap and practicable method for sugar analysis in onion.

Differences in carbohydrate content, sucrose metabolising enzyme activities and their expression levels were monitored in developing leaf blades and leaf bases of four high- and four low-fructan genotypes. The variation in fructan accumulation between high- and low-fructan genotypes was due to the variation in sucrose metabolism. SPS expression and activity did not vary between high- and low-fructan genotypes. Acid invertase and 1-SST showed significant variation in their activities between the two fructan groups. Post-transcriptional and translational regulation of AI and 1-SST respectively, are suggested.

Mapping populations analysed for non-structural carbohydrates showed very wide segregation for fructan (80 to 600 g kg^{-1}) and other NSC content, and were well-suited for detailed genetic and physiological analysis. Single marker analysis was carried out to study the association between the combined enzyme activity (CEA; acid invertase + 1-SST) and the *Frc* markers. Significant association between CEA and *Frc* markers has suggested genes regulating acid invertases or 1-SST or both underlie *Frc*. Leaf blade NSC did not correlate with bulb sugars and thus cannot be used as a phenotypic marker for early selection of bulb NSC traits.

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ABBREVIATIONS

^{o}C	degrees Celsius
µg	microgram
µl	microlitre
µM	micro molar
1-FFT	fructose: fructose 1-fructosyltransferase
1-SST	sucrose: sucrose 1-fructosyltransferase
6G-FFT	fructan: fructan 6G-fructosyltransferase
6-SFT	sucrose: fructan 6-fructosyltransferases
6-SST	sucrose: sucrose 6-fructosyltransferase
AC 43	Alisa Craig 43
acINV	onion vacuolar invertase
AFLP	amplified fragment length polymorphism
AgCl	silver chloride
AI	acid invertase
ANOVA	analysis of variance
AOAC	Association of Official Agricultural Chemists
ATP	adenosine-tri-phosphate
BGY15-23	Brigham yellow globe 15-23
bp	base pair
C	carbon
C x P12	Colossal grano x Early longkeeper 'P12'
Ca	calcium
CAPS	cleaved amplified polymorphic sequences
cDNA	complementary DNA
CEA	combined enzyme activity (1-SST +AI)
cM	centimorgan
C-S lyase	cysteine-sulfoxide lyase
C_T	threshold cycle
CVA	canonical variate analysis
DEPC	diethylpyrocarbonate
DM	dry matter
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DPs	degrees of polymerisation
DTT	dithiothreitol
DW	dry weight
EC	enzyme commission
EDTA	ethylene diamine tetraacetic Acid
ELK 'p12'	Early long keeper 'p12'
EST	expressed sequence tag

F	fructose
F1	first filial generation
F2	second filial generation
F3	third filial generation
FAO	Food and Agriculture Organisation
FEH	fructan exohydrolase
FF line	*Allium fistulosum* line
FOS	fructo-oligosaccharide
FW	fresh weight
g	gram
G	glucose
G/F	glucose/fructose
G6PDH	glucose-6-phosphate dehydrogenase
GC	gas chromatography
GH32	glycoside hydrolase family 32
GOI	gene of interest
GTPases	guanosine-5'-triphosphatases
h	hours
ha	hectare
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HK	hexokinase
HPAEC	high-performance anion-exchange chromatography
HPLC	High-performance liquid chromatography
HPLC-PAD	HPLC-pulsed amperometric detection
ID	identity
InDel	insertion and deletion
IU	international unit
Kcal	kilo calorie
kg	kilo grams
KOH	potassium hydroxide
LG	linkage group
LNA	locked nucleic acid
LoA	level of agreement
LOD	logarithm of odds
m	metre
M	molar
Mg	magnesium
mg	milligrams
$MgCl_2$	magnesium chloride
min	minute
ml	millilitre
mm	millimetre
mM	millimolar

mRNA	messenger RNA
n	number
Na	sodium
$NaBH_4$	sodium borohydride
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NaOH	sodium hydroxide
ng	nano grams
NI	neutral invertase
nm	nanometre
nmol	nanomoles
NSC	non-structural carbohydrate
NZD	New Zealand dollars
NZDT	New Zealand day time
pAcN2	onion 1-SST protein
pAcT1	onion acid invertase protein
PAHBAH	4-hydroxy benzoic acid hydrazide
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
pg	picogram
PGI	phosphoglucose isomerase
PKs	protein kinases
PMSF	phenylmethylsulfonyl fluoride
PPs	protein phosphatases
PVP	polyvinylpyrrolidone
qRT-PCR	quantitative real-time PCR
QTL	quantitative trait loci
r	correlation
r^2	regression
RDA	recommended daily intake
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RQ	relative quantity
rRNA	ribosomal RNA
RT	real time
s	seconds
saSST	festuca 1-SST
SD	standard deviation
SED	standard error deviation
SNP	single nucleotide polymorphism
SPS	sucrose phosphate synthase
SS	soluble solid
SSCP	single-strand conformation polymorphism

SSR	simple sequence repeats
SuSy	sucrose synthase
SWG-N96	Southport White Globe N96
Taq	*Thermus aquaticus*
TBA	thibarbituric acid
U	units
UGPase	UDP-glucose pyrophosphorylase
UKS	unknown sample
USD	United states dollar
USDA	United state department of agriculture
USDA-ARS	USDA-agriculture research station
v/v	volume per volume
V_{max}	maximum reaction rate
W x H	W2021 x Houston grano
W x T	W202A x Texas grano 438
w/v	weight per volume
x g	force of gravity
YI	yeast invertase

1. INTRODUCTION

1.1 Background

Onion (*Allium cepa* L.) is one of the most important vegetable crops grown world-wide. Onions store a functional food ingredient – the fructans - as their main carbon reserve and are known to benefit human health. Fructan, along with other non-structural carbohydrates (NSC), glucose, fructose and sucrose, form a substantial part of bulb dry matter, contributing as much as 65-80% of the total dry weight. The bulb fructan content varies among onion cultivars, from <4% of bulb dry matter in fresh market types to >65% in dehydrator onions, and this is reported to be correlated with the bulb dry matter (McCallum *et al.*, 2006).

Non-structural carbohydrate content and composition in onions are quantitative traits and are known to be under the control of many genes. Through genetic engineering some of the enzymes involved in onion fructan biosynthesis from sucrose have already been characterised, but inadequate knowledge and information on its physiological and genetic complexity with other carbohydrate metabolic genes has made it difficult to dissect the variation in fructan accumulation. Understanding the biochemical and genetic networks of carbohydrate metabolic pathways in high- and low-fructan lines, especially in the least-studied areas of sucrose synthesis and degradation pathways, will offer opportunities to dissect variation in carbohydrate metabolism.

Quantitative trait locus (QTL) mapping and chromosomal assignment of some of the candidate gene markers (using monosomic alien addition lines) has provided a means to identify some of the genes affecting carbohydrate traits in onions. McCallum *et al.* (2006) identified a key region on chromosome 8 carrying a single locus (*Frc*) that conditioned 93% of phenotypic variation in bulb fructan content. Biochemical and genetic characterisation of sucrose metabolism in alien addition lines further showed that chromosome 2 and 8 possessed allele/genes that had a major influence on the level of sucrose and fructans (Yaguchi *et al.*, 2008). Fine mapping of this region may allow us to tag closely linked markers to the gene, facilitating marker assisted selection of divergent lines in onion breeding programs. However, most of the QTL mapping studies in onions have so far been performed on small segregating progenies, limiting us in our ability to increase the resolution of the map, to tag closely linked markers to the desired QTL alleles and to detect any epistasis for a

given trait. There is need to develop and analyse large segregating families to understand the genetic networks of carbohydrate metabolism in onion, and this is the focus of this thesis.

1.2 An overview of onion

1.2.1 Area and production

Onion (*Allium cepa* L.) is among the most valued and cultivated horticultural crops in the world. Among the vegetable crops, it ranks second in terms of production and is surpassed only by tomato. Globally, onions are cultivated in more than 175 countries. According to the FAO 2010 reports, there is an estimated 3.91 million ha of onions in the world, producing about 77.84 million tonnes of onions each year (includes shallots, dry and green onions). New Zealand ranks sixth in terms of onion production (includes shallots and green onions, but excluding dehydrating onions) growing approximately 4800 ha of onions with a production of 204,000 tonnes each year. The New Zealand onion industry accounts for 2.42 percent of the world green onion acreage and over 5.71 percent of the world green onion production (FAO, 2010). In 2011, 110.2 million NZD freight on board worth of onion (ranking fourth in terms of horticultural exports from NZ) was exported from New Zealand to Japan, United Kingdom, Netherlands and other countries (Fresh Facts 2011, http://www.freshfacts.co.nz/).

1.2.2 Importance of onion

Onions have been cultivated for over 4000 years and have been valued for their culinary qualities and medicinal value. The nutritional components of the edible portion of the raw onion bulb are presented in the Table 1.1. Onions are recognised as a very good source of dietary fructans, flavonoids and organosulfur compounds.

The dietary fructan requirement of 10-40 g per day is usually met from the most common sources such as wheat, onions, garlic and banana. Moshfegh *et al.* (1999) reported that 25% of the dietary fructan intake of the average US citizen is met by onions alone. Inulin and inulin neo-series fructans present in onion and other allium species are an important functional food ingredient benefitting human health. The health benefits of fructans in general are discussed in Section 1.3.

Table 1.1. Nutrient values per 100 g of raw onion (*Allium cepa*).

Principle	Nutrient value	Percentage of RDA
Energy	40 Kcal	2%
Carbohydrates	9.34 g	7%
Protein	1.10 g	2%
Total Fat	0.10 g	0.5%
Dietary Fibre	1.7 g	4.5%
Cholesterol	0 mg	0 %
Minerals		
Calcium	23 mg	2%
Copper	0.039 mg	4%
Fluoride	1.1 µg	--
Iron	0.0.21 mg	3%
Magnesium	10 mg	2.5%
Manganese	0.129 mg	5.5%
Phosphorus	29 mg	4%
Potassium	146 mg	3%
Sodium	4mg	0%
Selenium	0.5 µg	--
Zinc	0.17 mg	1.5%
Vitamins		
Folates	19 µg	5%
Niacin	0.116 mg	1%
Pantothenic acid	0.123 mg	2.5%
Pyridoxine	0.120 mg	9%
Riboflavin	0.027 mg	2%
Thiamine	0.046 mg	4%
Vitamin A	2 IU	0%
Vitamin B-6	0.120 mg	--
Vitamin C	7.4 mg	12%
Vitamin E	0.02 mg	0%
Vitamin k	0.4 µg	--
Phyto-nutrients		
Carotene, ß	1 µg	--
Cryptoxanthin, ß	0 µg	--
Lutein + zeaxanthin	4 µg	--

Source: USDA National Nutrient Database for Standard Reference, Release 23 (2010)

In addition to fructans onions possess other compounds considered to have health benefits. Anthocyanins and flavonols present in red and yellow skinned onions are powerful antioxidants capable of reacting with free radicals and chemically neutralising them before they cause any cellular damage. Onions, particularly red and yellow varieties, are the richest

source of quercetin (350 mg kg^{-1} fresh weight) (Griffiths *et al.*, 2002; Slimestad *et al.*, 2007) among vegetables and have the potential to raise the overall antioxidant level in blood plasma following ingestion.

Onions possess special culinary qualities (flavour) that make them an indispensable ingredient in various dishes. The distinct flavour of onions and other related *Allium* species are mainly due to thiosulfinates and other sulphur-containing volatile compounds, formed in the presence of the C-S lyase enzyme allinase (EC 4.4.1.4) after cellular disruption. It has been noted that more than 80 different compounds are formed from the initial thiosulfinates in fresh and steam distilled extracts of *Allium* species (Block *et al.,* 1997).

Due to their distinct flavour and health benefits, onions have been extensively used in the food and pharmaceutical industries. Some of the manufactured food products containing onions (whole, extracts, powder or flakes) include onion flavoured oils, chips, soups, ketchups, sauces, mayonnaises, onion salt, pickles, instant noodles, onion rings etc.

1.3 Importance of fructans

A food is regarded as a functional food if it is naturally occurring and its addition in conventional/everyday food intake enhances human health beyond nutrition. Fructans or polyfructosylsucroses are one such naturally occurring functional food ingredient that has been satisfactorily demonstrated to have beneficial effects on human health (Gibson, 1998; Ernst & Feldheim, 2000; Abrams *et al.*, 2005; Roberfroid, 2007). Addition of fructans to the human diet has been shown to have the following targeted functions: fructans resist digestion in the upper gastrointestinal track and function as dietary fibre (Mussatto & Mancilha, 2007); fructans act as prebiotics stimulating the growth of potentially health promoting gut flora (Hidaka *et al.*, 1991; Gibson & Roberfroid, 1995; Harmsen & Welling, 2002; Kelly, 2008; Yen *et al.*, 2011); fructans enhance colonic functions– they regularise bowel habit (Gibson & Roberfroid, 1995; Marteau *et al.*, 2011), increase mineral absorption, mainly Ca and Mg (Coudray *et al.*, 1997; Griffin *et al.*, 2003; Lavanda *et al.*, 2011; Legette *et al.*, 2011), target bone health in terms of bone mineralisation, bone density, bone accretion and resorbtion (Coxam, 2005) and beneficially affect the functioning of the intestinal immune system, reducing the risk of irritable bowel disease and colon cancer (Pool-Zobel, 2005; Watzl *et al.*, 2005; Rafter *et al.*, 2007); fructans maintain lipid homeostasis by reducing triglyceridemia

and cholesterolemia in hyperlipidemic individuals (Delzenne & Williams, 2002; Letexier *et al.*, 2003) and indirectly reduce the risk of cardiovascular disease and arteriosclerosis; and recently fructan has also been shown to have a role in appetite control and weight management in obese patients (Alexiou, 2010). Because fructans are also a low-calorie carbohydrate, they are extensively used as alternate sweetener in various products (Franck, 2002; Aykan *et al.*, 2008; Isik *et al.*, 2011).

Fructans are the primary storage carbohydrate in >400,000 plant species. Apart from their role as a major carbon source, fructans are reported to be involved in membrane stabilisation and abiotic stress tolerance in plants. Fructan hydrolysis contributes to osmoregulation in plant tissue and helps them to overcome freezing injury and dehydration stress (Pilon-Smits *et al.*, 1995; Hisano *et al.*, 2004). Hexose sugars produced as a result of fructan hydrolysis have several functions during the mechanism of abiotic stress tolerance: they lower the freezing point of plant liquids and protect plant tissues from freezing injury (Johansson & Krull, 1970); plants can use these sugars to resist plasmolysis by increasing osmotic pressure within cells which otherwise leads to freezing damage to individual cells and, in the case of dehydration stress, these sugars have the ability to lower the water potential of intracellular liquid and allow continued leaf expansion. The presence of increased levels of apoplastic fructans and hexoses during plant stress indicate direct involvement of fructans in protecting tissues from freezing and dehydration injury (Livingston & Henson, 1998). Localisation of fructans outside the cells (under unstressed condition) of different tissue and organs, including xylem (Vieira & Figueiredo-Ribeiro, 1993; Vilhalva *et al.*, 2011), phloem sap (Wang & Nobel, 1998), mesophyll and parenchyma cells of the vascular bundle sheath (Koroleva *et al.*, 2000), cortical parenchyma and medulla of tuberous roots (Vilhalva *et al.*, 2011) suggests possible roles for fructans in cell membrane interaction and membrane stabilisation (Hincha *et al.*, 2007) in combating abiotic stress. Fructan syntheses in vacuoles lower sucrose concentrations in vacuoles. Lowering of sucrose concentration in cell has been reported to prevent sugar-induced feedback inhibition of photosynthesis in plants (Pollock 1986). Fructan in onion bulb tissues also form a transient store of carbon that can be catabolised during re-growth and sprout development of bulbs (Darbyshire, 1978).

Owing to their beneficial role in human and plant health, interest in fructans has increased tremendously over recent years, leading to rapid advances in understanding the biochemistry and genetics of the various plant sources that produce fructans. Better understanding of the

metabolic pathways and the genomic regions controlling the fructan content in fructan-storing crop plants will provide new opportunities to exploit them.

1.3.1 Occurrence of fructans

Fructans are synthesised in about 15% of flowering plants, in some bacterial genera including *Bacillus, Streptococcus, Pseudomonas, Erwinia* and *Actinomyces* (Hendry & Wallace, 1993) and to some extent in algae, fungi and liverworts.

In plants, fructans are found in a number of mono- and dicotyledonous families such as the Liliaceae, Amaryllidaceae, Asparagaceae, Gramineae, Agavaceae and Compositae. Some of the economically important plants storing fructans include cereals crops (e.g. *Avena sativa, Hordeum vulgare* and *Triticum aestivum*), vegetables (e.g., *Allium cepa, Allium sativum, Asparagus officinalis, Cynara cardunculus, Cichorium intybus* and *Lactuca sativa*), ornamentals (e.g. *Dahlia coccinea, Tulipa* sp. and *Agave* sp.) and forage grasses (e.g. *Lolium* sp. and *Festuca* sp.). Currently, agave (*Agave tequilana*), Jerusalem artichoke (*Helianthus tuberosus*) and chicory (*Cichorium intybus*) are being used for commercial production of the inulin type of fructans.

1.3.2 Chemical nature of fructans

Fructans are a class of naturally occurring oligo- and polysaccharides formed by the cumulative accumulation of fructose to a sucrose molecule either by β- (2→1) or β- (2→6) glycosidic linkages (Ernst *et al.*, 1998). In nature, there exists a great variability in the linkage of these fructosyl residues to a sucrose molecule, giving rise to structurally different fructans (Table 1.2). The major classes of structurally different fructans found in higher plants are inulin, levan, mixed levan, inulin neoseries and levan neoseries (Vijn *et al.*, 1999). Apart from structural differences, fructan also varies widely in the degree of polymerisation (DPs). Bacteria and fungi have the capacity to produce higher DP fructan chains of up to 100,000, whereas plants produce much shorter chains of up to 200 DPs. Kestotriose is the shortest and simplest form of fructan with a DP of 3.

1.3.3 Fructan biosynthetic pathway in plants

Fructans are mainly synthesised in the plant vacuole (Figure 1.1)by the action of one or more types of fructosyltransferases (Frehner *et al.*, 1984). A simple inulin series of fructan is formed by the action of two distinct enzymes, sucrose: sucrose 1-fructosyltransferase (1-SST) and fructan: fructan 1-fructosyltransferase (1-FFT) (Edelman & Jeeeord, 1968; Koops & Jonker, 1996; Luscher *et al.*, 1996; Van den Ende *et al.*, 1996). 1-SST initiates the process of fructan synthesis by catalysing the transfer of fructosyl residue from a sucrose molecule to another sucrose molecule forming a triglucoside, 1-kestose and releasing glucose as shown by:

1-SST: G1-2F + G1-2F ⟶ G1-2F1-2F + G

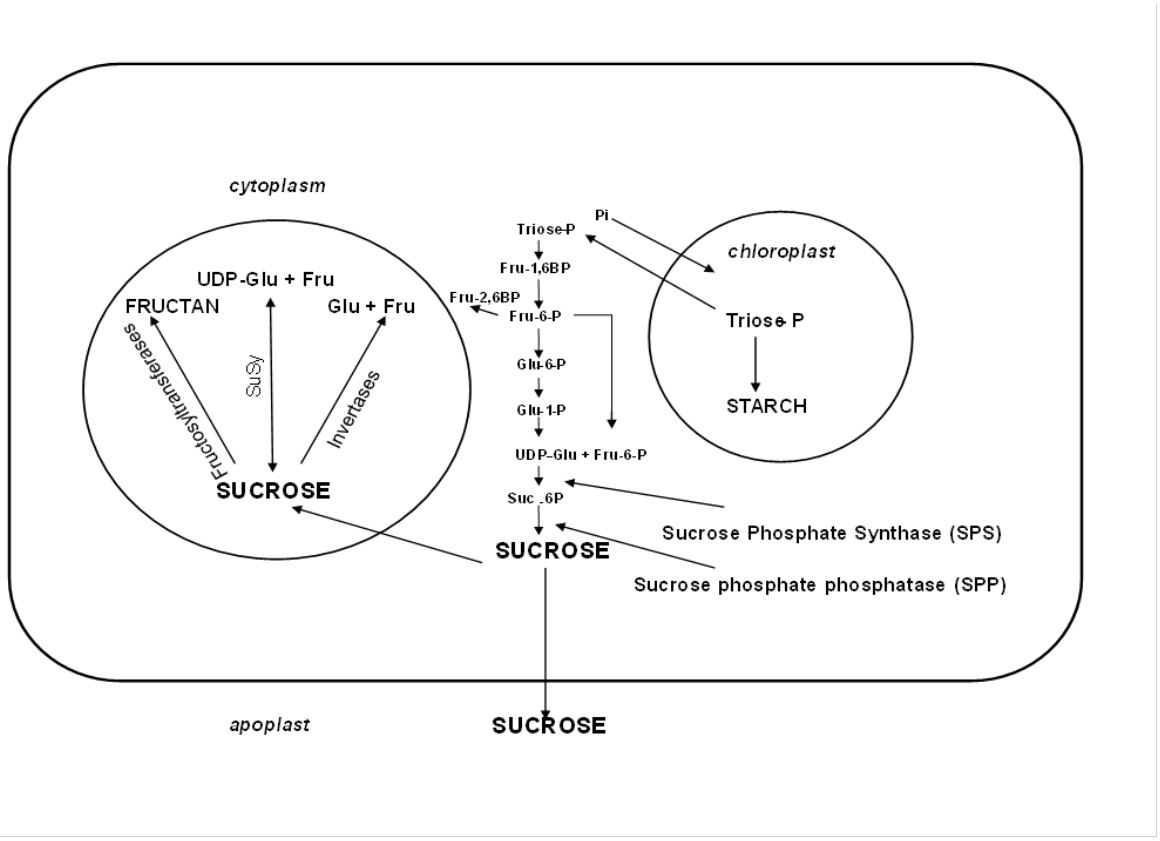

Figure 1.1. Compartmentation of major NSC metabolic pathways and NSC-correlated enzyme families in fructan storing plants. (Source: Vijn & Smeekens, 1999).

Table 1.2. Plant fructans with their linkage groups.

Type	Linkage	Plant source e.g.
Inulin	Linear (2-1)-linked β-D-fructosyl units	Chicory , Jerusalem artichoke
Levan	Linear (2-6)-linked β-D-fructosyl units	Timothy, orchard grass, big bluegrass
Mixed levan	(2-1)-linked β-D-fructosyl units and (2-6)-linked β-D-fructosyl units	Wheat, barley
Inulin neoseries	Linear (2-1)-linked β-D-fructosyl units linked to both C1 and C6 moiety of a sucrose molecule	Onions, garlic, banana, asparagus
Levan neoseries	Linear (2-6)-linked β-D-fructosyl units on either end of glu moiety of a sucrose molecule	Oats, *Lolium*

Source: (Vijn & Smeekens, 1999)

Later, 1-FFT elongates the chain by transferring fructosyl residues from one fructan molecule (≥3 DP) to another fructan molecule or sucrose as shown by:

1-FFT: $G1\text{-}2F\ (1\text{-}2F)_m + G1\text{-}2F\ (1\text{-}2F)_n \longrightarrow G1\text{-}2F\ (1\text{-}2F)_{m-1} + G1\text{-}2F\ (1\text{-}2F)_{n+1}$

Transformation of non-fructan-producing plants with cDNA encoding 1-SST and 1-FFT from fructan-producing plants has confirmed their role in producing 1-kestoses and inulins in transgenic plants (Hellwege *et al.*, 1998; Sevenier *et al.*, 1998; Li *et al.*, 2007; Pan *et al.*, 2009).

Some of the cool-season grass species accumulate the levan type of fructans (Chatterton *et al.*, 1993; Bonnett *et al.*, 1997; Chatterton & Harrison, 1997). Levans are formed by the activity of sucrose: sucrose 6-fructosyltransferase (6-SST) and sucrose: fructan 6-fructosyltransferase (6-SFT) which catalyses the formation and extension of (2-6)-linked fructan chain. Characterisation of fructan biosynthesis in big blue grass accumulating levan confirmed major activity of 6-SST and 6-SFT in the leaves (Wei *et al.*, 2002) as outlined:

6-SST: $G1\text{-}2F + G1\text{-}2F \longrightarrow G1\text{-}2F6\text{-}2F + G$

6-SFT: $G1\text{-}2F\ (6\text{-}2F)_m + G1\text{-}2F\ (6\text{-}2F) \longrightarrow G1\text{-}2F\ (6\text{-}2F)_{m-1} + G1\text{-}2F\ (6\text{-}2F)_{n+1}$

The mixed levan type of fructans, as seen in wheat, barley and some grasses, contain both (2-1)- and (2-6)-linked β-D-fructosyl units. 1-SST, 1-FFT, 6-SST and 6-SFT are the major enzymes involved in the synthesis of mixed levans. 6-SFT activity has been extensively studied and functionally characterised in economically important plants like barley (Duchateau *et al.*, 1995; Sprenger *et al.*, 1995), wheat (Kawakami & Yoshida, 2002; Gao *et al.*, 2010), Timothy (Tamura *et al.*, 2009), *Lolium perenne* (Gallagher *et al.*, 2004) and *L. temulentum* (Hisano *et al.*, 2008).

In addition to the simple fructan synthesising enzymes (as seen in the inulin and levan type), some plant species like onion, asparagus and oat also harbour an additional fructosyltransferase called fructan: fructan 6G-fructosyltransferases (6G-FFT). 6G-FFT catalyses the transfer of a fructose residue from one fructan to the C6 of a glucose molecule of another fructan or sucrose, producing inulin neo-series or levan neo-series type of fructans (as shown below). Molecular characterisation of the gene encoding 6G-FFT in onion and asparagus has confirmed its functionality (Vijn *et al.*, 1997; Ueno *et al.*, 2005; Gadegaard *et al.*, 2008).

6G-FFT: G1-2F + G1-2F1-2F ⟶ F2-6G1-2F + G1-2F

1.3.4 Fructan metabolism in onions

Onion bulbs store inulin and inulin neoseries types of fructan. These fructooligosaccharides (FOS) are synthesised by the activity of two different fructosyltransferases namely, 1-SST (EC 2.4.1.99) and 6G-FFT (EC 2.4.1.243).

Henry and Darbyshire (1980) were the first to separate and study the activities of 1-SST and fructan: fructan fructosyltransferase in onions. It was reported that incubation of sucrose with 1-SST produced only one type of trisaccharide, 1^F-fructosylsucrose and fructan: fructan fructosyltransferase was only involved in the production of tetrasaccharide and higher polymers from trisaccharides. Shiomi *et al.* (1985) successfully purified 1-SST from onion seeds and reported that 1-SST catalyses fructosyltransfer only between two sucrose molecules to form 1-kestose. Further, cloning and functional characterisation of onion cDNA encoding 1-SST in tobacco protoplasts confirmed 1-SST activity. It was reported that incubation of protein extracts of tobacco protoplasts (transformed with onion 1-SST) with 100 mM sucrose formed only 1-kestoses (Vijn *et al.*, 1998).

6G-FFT is the key enzyme involved in the formation of the inulin neoseries type of fructan in onions. It catalyses the following reactions: i) the transfer of fructosyl residues from one fructan to the C6 of the glucose moiety of sucrose, producing neokestose; ii) extension of 1-kestoses or neokestoses producing high DP fructans of inulin neoseries. Vijn et al. (1997) isolated cDNA encoding 6G-FFT from onions and studied its functionality in tobacco and chicory plants. Transgenic tobacco plants produced active protein of 6G-FFT but did not store any fructans because of the absence of substrates (1-kestose and inulins). In contrast, transgenic chicory plants, which usually synthesise linear inulins, also produced fructan of the inulin neoseries. 6G-FFT in onions is bi-functional in nature. *In vitro* studies have revealed that 6G-FFT not only has the capacity to initiate and produce inulin neoseries from 1-kestoses or linear inulins but also exhibit 1-FFT-like activity, producing nystoses and higher DP inulins (Vijn *et al.*, 1997; Vijn *et al.*, 1998; Ritsema *et al.*, 2003). So far no 1-FFT enzyme or clone has been isolated from onions and it is proposed that only two enzymes, 1-SST and 6G-FFT, are involved in fructan synthesis in onions.

1.4 Non-structural carbohydrate in Onion

Onion carbohydrates include both structural and non-structural carbohydrates. NSC are of much interest as they form a substantial part of onion bulb dry matter (DM) (Darbyshire & Henry, 1979), contributing as much as 65-80% of the total dry weight. The principal NSC contents of onions are glucose, fructose, sucrose and fructans (Darbyshire & Henry, 1978). In *Allium cepa* L. (onions and shallots), fructans are the main reserve carbohydrate. They are mainly synthesised in the vacuoles of plant cell, and in onions are found to be predominantly stored in the thickened sheaths of bladeless leaves (bulbs). Based on the dry matter (DM) and soluble carbohydrate content, onions have been classified into fresh market onions (<80 g kg^{-1} DM, 5% to 9% bulb solids), storage onions (100-150 g kg^{-1} DM, 8% to 12% bulb solids) and dehydrator onions (inc. shallots) (100-150 g kg^{-1} DM, ≥18% of bulb solids) (Darbyshire & Steer, 1990; Sinclair *et al.*, 1995b).

The distribution and composition of FOS in onions were initially investigated by Bacon (1957) and Darbyshire & Henry (1978). It was reported that the onion stored fructans of up to DP 11 and that the overall content of fructans and their DP declined from the innermost tissue to the outer leaf bases. Studies on structural composition of FOS fractions from onions leaves and bulbs revealed that they were comprised of 1^{F}–β-D- fructofuranosylsucrose (1-kestose,

Figure 1.2A); 6^{G}–β-D- fructofuranosylsucrose (neokestose, Figure 1.2B); 1^{F}(1–β-D-fructofuranosylsucrose)$_2$ sucrose (4a, 1-nystose) and high DP fructans of inulin neo-series (Figure 1.2C) (Shiomi *et al.*, 1991; Shiomi *et al.*, 1997; Stahl *et al.*, 1997). Among the different FOS fractions it is noted that the relative proportion of C6-linked neokestose derivatives predominated over C1-linked kestoses (Darbyshire and Henry 1978; Shiomi et al. 1997).

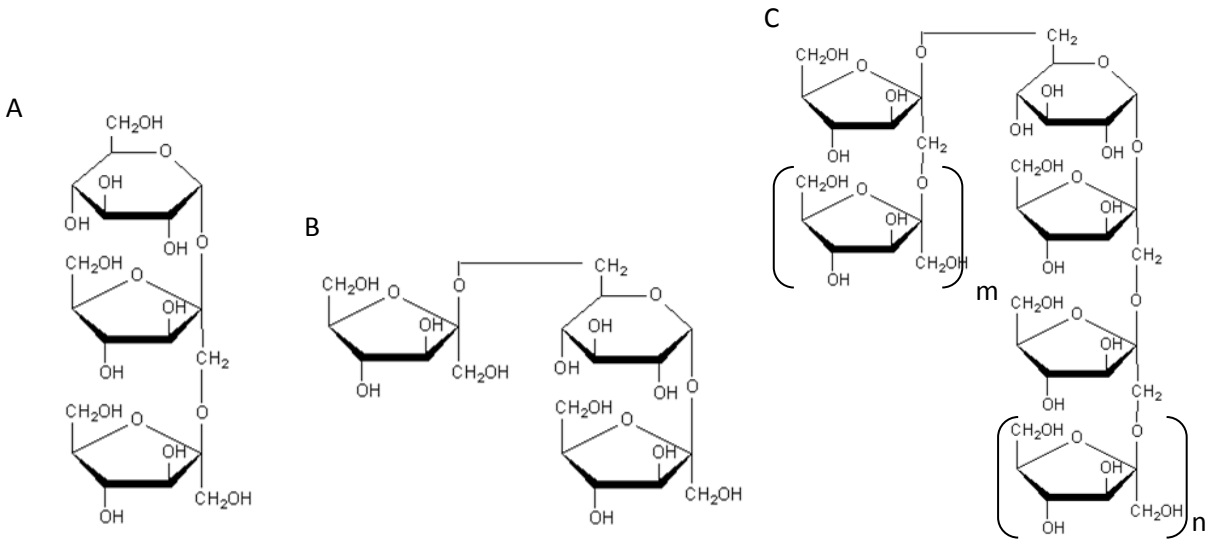

Figure 1.2. Structures of the onion fructans. A) 1-Kestose B) neokestose and C) inulin-neoseries.

1.4.1 Non-structural carbohydrate measurement in onions

Onion sugars are generally measured using chromatographic techniques, such as GC (Salama *et al.*, 1990), HPLC (Jaime *et al.*, 2001b; Kahane *et al.*, 2001; Benkeblia *et al.*, 2004; Shiomi *et al.*, 2008) and HPAEC (Ernst *et al.*, 1998; Yaguchi *et al.*, 2008). However, use of these techniques is not always practicable as they require expensive apparatus, columns and also demand for a considerable amount of time per analysis. Enzymatic methods used for sugar analysis in plant species including onions are simple, rapid and only require a spectrophotometer that is readily available in most laboratories (Velterop & Vos, 2001). Glucose and fructose levels can be directly measured using an enzymatic assay developed by Boehringer-Manheim, but sucrose and fructan measurements in onions requires the use of

expensive Megazyme enzyme kits. This is mainly because invertase enzymes used in the Boehringer-Manheim enzymatic sucrose assay not only act on sucrose but also break fructosylsucroses (McCleary *et al.*, 2000), resulting in overestimation of sucrose in fructan accumulating plants species.

Enzymatically, fructans are best measured after hydrolysis of fructans to fructose. This first requires hydrolysis of sucrose and then destroying the resulting glucose and fructose along with the pre-existing monosaccharides by boiling with sodium hydroxide. Since invertases hydrolyse fructosylsucroses and thus also reduce the fructan levels, commercially available Megazyme fructan-assay kits (developed based on AOAC method 999.03), which make use of a specific sucrase enzyme, are used to hydrolyse sucrose in fructan-accumulating plant species. Megazyme fructan-assay kits are expensive to use on a large scale and thus more economical assay methods are required for NSC analysis in onions.

Maltose is the main substrate for maltases, but these are also reported to hydrolyse sucrose (Khan *et al.*, 1973). Since onions contain no maltose, commercially available maltase can be used instead of expensive kits to measure sucrose and fructan levels in onions. Microplate enzymatic assays have been employed to measure NSC in many plant species (Velterop & Vos, 2001; Gomez *et al.*, 2007; Zhao *et al.*, 2010). This method is highly efficient to measure a large number of samples per day and is easily amenable to automation. It would, therefore, be useful to make use of these microplates to analyse large numbers of onion samples. In addition, validating the use of maltase in onion sucrose and fructan assays might further reduce input costs.

1.5 Variation in dry matter, soluble solids and NSC content in onions

In nature, there exists wide genetic variation in the amount of bulb DM, soluble solids (SS) and the proportion of NSC content present in onions. The dry matter content of onion bulb ranges from <50 g kg^{-1} (5%) in fresh market types to over 200 g kg^{-1} (20%) in dehydrating varieties. Darbyshire and Henry (1979) reported that high percentage dry weight cultivars are associated with high amounts of higher molecular weight fructans and lower concentrations of monosaccharides. Further studies by Sinclair *et al.* (1995a) on the relationship between bulb DM, SS and NSC composition in onions revealed a strong linear relationship between

DM and SS. Total fructose (free and combined) concentration was found to be strongly correlated with SS in both fresh and dehydrating varieties. Reducing sugars concentration in both high- and low-DM varieties declined with an increase in SS, in agreement with Darbyshire and Henry (1979). Non-fructose soluble solids concentration (free and combined glucose) showed no correlation to SS in dehydration varieties, while in fresh market varieties glucose concentration was strongly correlated with SS. Soluble solid concentration has also been reported to have significant correlation with other quantitative traits such as pungency and bulb size (Lin *et al.*, 1995; Simon, 1995). Strong correlation of SS with bulb DM and other quality traits, along with their ease of measurement using a refractometer or near-infrared spectrometer, makes an SS content-based selection method still the most feasible way to select/breed onion varieties for dehydration purposes.

Soluble solids content, which accounts for most of the NSC in onions, is highly heritable in nature. Several studies on the heritability pattern of SS in onions predominantly indicated additive genetic variance over dominance effect. Broad sense heritability was estimated to be 0.6-0.83 (Lin *et al.*, 1995; Simon, 1995).

Studies on the NSC composition of various high- and low-DM onion cultivars revealed that these two groups follow a different and specific NSC storage pattern during bulbing (Kahane *et al.*, 2001). Low DM onion cultivars contained higher quantities of monosaccharides (glucose and fructose) and reasonably small amounts of low DP fructans throughout the bulbing stage. In contrast, high-DM onion varieties predominantly stored longer chained fructans with lower amounts of fructose, sucrose and glucose units (Darbyshire & Steer, 1990; Kahane *et al.*, 2001; Vagen & Slimestad, 2008). It was noted the DM and NSC of these two groups remained similar before bulbing and changed markedly with bulbing. The pronounced differences between these two major groups were in the accumulation of fructans. Thus, it has been proposed that there exist genetic differences in the regulation of carbohydrate metabolism in high- and low-fructan onions.

1.6 Factors affecting differential carbohydrate accumulation in onions

Carbohydrate levels in fructan accumulating plants are influenced by both genotypic and environmental factors. Variables, such as variety/genotype, developmental stage and the

environmental conditions during the plant growth such as temperature, light level, water availability, pests and diseases, affect sugar levels mainly by influencing the activity of sucrose and fructan metabolic enzymes. The important variables that may have a possible affect on differential carbohydrate composition and fructan accumulation in high- and low-fructan cultivars of onions are discussed below.

1.6.1 Sucrose

Sucrose is the major photosynthetic product and transport metabolite for long distance transport in most of the plant species. It is synthesised in the cytoplasm via pathways involving UGPase, sucrose phosphate synthase (SPS) and sucrose phosphatase. Once sucrose is synthesised it is either compartmentalised within the cell vacuoles for storage or translocated to sink tissues for distribution of assimilated carbon.

In many fructan storing plants, apart from fructan and other oligosaccharides, sucrose is also accumulated as a storage carbohydrate in vacuoles of photosynthetic tissue/ organs (Wilson and Bailey, 1971; Riens et al., 1994; Gebbing 2003). It is reported that the amount of sucrose and fructan stored in fructan storing plants, follow a divergent distribution pattern between the organs and tissues. The amount of sucrose predominates the photosynthetically active tissues, while fructan storages are predominated in the heterotrophic tissues (Pollock and Cairns, 1991; Riens *et al*., 1994; Morvan-Bertrand *et al*., 2001). This contrast in carbohydrate accumulation has been suggested to be due to the variation in sucrose utilization in vacuolar sucrose storage and fructan synthesis (Lattanzi *et al*., 2007).

Radioautographic studies on carbon translocation patterns showed that in younger onion plants, actively growing leaves serve as a carbon source for un-emerged leaves and roots. As the plants grow older, the young emerged leaves translocate synthesised sucrose basipetally to the leaf base; and the un-emerged leaves receive their carbon supply from the mature onion leaves (Steer & Darbyshire, 1979).Sucrose concentration is important in regulating fructosyltransferases in onions. Sucrose feeding (5%, w/v) and continuous illumination of onion shoots has been shown to induce 1-SST and 6G-FFT gene expression and activity, resulting in fructan accumulation in non-fructan storing onion shoots (Vijn *et al.*, 1998). Sucrose feeding induced 6G-FFT mRNA accumulation after only 4 h of continuous illumination. However, its activity, along with an increase in mRNA levels, was seen only after 16 h of continuous illumination. This was mainly because the enzyme required

attainment of a certain threshold level of trisaccharide along with an increase in its own expression levels before commencing its activity (Vijn *et al.*, 1998). Similar studies on transgenic chicory plants have confirmed the role of sucrose in inducing fructosyltransferases.

It is reported that 6G-FFT activity is affected by the relative concentration of sucrose and 1-kestoses. When sucrose levels are higher than 1-kestoses, high DP fructan production is very much delayed (Lasseur *et al.*, 2006). *In vitro* studies have shown that, when sucrose levels are lower than 1-kestoses, the enzyme favours fructosyl transfer to 1-kestoses instead of sucrose, forming nystoses and high DP fructans (Vijn *et al.*, 1997). Apart from substrate levels, enzyme concentration is also important in oligofructan synthesis. It was previously determined that under low enzyme concentration (1-SST and FFT), only trisaccharides are formed, while under high enzyme concentration and identical conditions, fructans with ≥3 DP can be detected (Cairns, 1995). This is evident from the studies conducted by Vijn *et al.* (1997) and Vijn *et al.* (1998) on onion shoots, where high DP (>3) fructans were formed only when 6G-FFT mRNA expression levels (indicative of 6G-FFT enzyme concentration) had reached a certain limit.

Masuzaki *et al*., 2007 reported increased bulb formation in multiple additions containing shallot chromosome 5 and in the absence of chromosome 2. Since, sucrose metabolic genes SuSy and invertases were assigned to chromosome 2 in onions, it is suggested that altered expression of these genes may have regulatory role in sucrose-hexose interconvertions thus regulating sink strength in onions. High correlation noticed between SuSy activity and hexose levels in high- and low-fructan onion inbreds was consistant in driving sink strength but not in determing the fructan phenotype in onion (Yaguchi *et al.*, 2009). Since alien addition line FF+8A and high *Frc_* lines exhibited high sucrose levels, it is suggested that genes on chrosomosome 8 may have significant role in conditioning high sucrose levels in onions (Yaguchi *et al.*, 2009), and that fructan storage in onions is possibly sink- limited as noticed in artichokes (Schubert & Feuerle,1997). Sucrose not only acts as a substrate for fructan syntheses, but on degradation into hexoses (and its derivates), is also used in various plant metabolic and biosynthetic process facilitating plant growth and development. Since sucrose plays such a varied role in plants, studies on sucrose accumulation and its association with other NSC, SPS, fructosyltransferases and sucrose hydrolytic enzymes in a wide range of

cultivars varying in fructan content is important to understand the biochemistry behind varying fructan levels and NSC composition in onions.

1.6.2 Sucrose phosphate synthase

Sucrose-phosphate synthase (SPS; EC.2.4.1.14) is the key enzyme involved in sucrose synthesis. It catalyses the formation of sucrose-6-phosphate from fructose-6-phosphate and UDPGlucose (Leloir & Cardini, 1955). Though this reaction is reversible *invitro*, rapid removal of sucrose-6-phosphate by sucrose-phosphate phosphate (EC 3.1.3.24) and low concentration of cytosolic sucrose-6-phosphate under *invivo* conditions has rendered this SPS reaction as irreversible (Echeverria *et al.*, 1997).

Variation in sucrose concentration in many plant species has been linked to SPS activity, apart from other sucrose metabolic enzymes (Hubbard *et al.*, 1989; Miron & Schaffer, 1991; Lester *et al.*, 2001). High-sucrose accumulating *Lycopersicon hirsutum* (green fruited wild tomato species) has been reported to exhibit high SPS activity than low-sucrose accumulating *Lycopersicon esculentum* affecting the fruit quality in tomato (Miron & Schaffer, 1991). High- and low-sucrose accumulating sugarcane cultivars showed significant variation in the SPS transcript levels and in the activities of SPS enzyme during all stage of development (Verma *et al.*, 2011). Similar variation in SPS activity was also reported in high- and low-sucrose accumulating watermelon lines (Yativ *et al.*, 2010).

Biochemical analysis of alien monosomic addition line by Yaguchi *et al.* (2008) reported significant correlation of sucrose accumulation and SPS activity in FF+8A line. Onion SPS gene (Genebank accession no. EU164758) has been assigned to the same chromosome as that of *Frc* (discussed in Section 1.7.2), suggesting differential expression of SPS affecting sucrose and fructan levels in onions. Genotypic variation in the activity of SPS has so far not been reported in onion. Investigation of SPS activity in high- and low-fructan cultivarscultivars is required to confirm its role in varying sucrose accumulation in onions.

1.6.3 Fructosyltransferases

Fructosyltransferases, invertases and fructan exo-hydrolases (FEHs) belong to the glycoside hydrolase family 32 (GH32) and are functionally related by mainly acting on the fructosyl residues of a substrate. Phylogenetic studies of these enzymes have shown that cell wall

invertase clusters with FEHs and fructosyltransferases clusters with vacuolarinvertases. High homology of fructosyltransferases, especially with vacuolar invertases (acid invertases), and their biochemical behavior as sucrose-hydrolytic enzymes, confirms that they evolved from vacuolar invertases. In support of this, several studies reported the possibility of inducing fructan-synthesising capability in plant invertases and hydrolytic capability in fructosyltransferases. Ritsema *et al*. (2006) was able to increase the fructosyltransferase capability of vacuolar invertases by inducing a mutation at the sucrose binding box. Mutation outside the sucrose binding box of a plant vacuolar invertase and a fructosyltransferase also altered their activity to a great extent. Replacement of a selected amino acid in onion vacuolar invertases (acINV) with an amino acid that corresponds to festuca 1-SST (saSST) and vice versa, interchanged their activity by approximately 80% (Altenbach *et al.*, 2009). In these mutation analyses, the selected amino acids (that caused changes in enzyme activity) were either located inside the sucrose binding box or outside at the acceptor substrate binding site, suggesting that these particular sites would determine the hydrolase or transferase capabilities of the GH32 enzyme.

1.6.3.1 Sucrose: sucrose 1-fructosyltransferase

1-SST is the key enzyme initiating plant fructan biosynthesis. It catalyses the transfer of fructosyl residues from one sucrose to the other sucrose, producing 1-kestotriose. Several *de novo* studies on 1-SST activity have reported that under a given substrate condition, 1-SST exhibits 1-FFT, 6-SFT, FEH or invertase activity. 1-FFT activity of 1-SST was evident when 1-SST enzyme extracts incubated with sucrose/1-kestoses produced high DP inulins (Cairns, 1995; Koops & Jonker, 1996; Luscher *et al.*, 1996; Hellwege *et al.*, 1997; Chalmers *et al.*, 2003; Nagaraj *et al.*, 2004). Incubation of asparagus 1-SST with sucrose and neokestose produced $1,6_G$-kestotetraose, confirming the 6-SFT activity (Shiomi & Izawa, 1980). The 1-SST enzyme was able to produce sucrose and fructose from 1-kestoses (Nagaraj *et al.*, 2004) and fructose from sucrose (Altenbach *et al.*, 2009; Han *et al.*, 2010) suggesting FEH and invertase activity, respectively. It is noted that in the presence of both sucrose (100 mM) and 1-kestose (50 mM), 1-SST is mainly involved in 1-kestose production and its activity as 1-FFT, 6-SFT, FEH or as invertase is either repressed or exhibits limited activity. 1-SST gene expression and activities are induced by the sucrose concentration in plant tissue (Vijn *et al.*, 1998; Martinez-Noël *et al.*, 2006; Martinez-Noël *et al.*, 2009; Joudi *et al.*, 2012).

Several reports on the characterisation of onion 1-SST activity have suggested only fructosyltransferase activity (forming 1-kestoses) in onions (Darbyshire & Henry, 1979; Shiomi *et al.*, 1985; Vijn *et al.*, 1998). However, recently Han *et al.* (2010) reported hydrolase activity in this enzyme. Onion 1-SST was expressed in *E.coli* and its biological activity was analysed using HPLC. Compared to the HPLC results of non-induced bacteria, which showed only glucose and kestose peaks, results from onion 1-SST induced bacteria showed peaks of glucose, fructose and kestose, thus reporting hydrolase activity along with its usual fructosyltransferase activity.

Isolation and sequencing an *A. cepa* cDNA clone encoding 1-SST has shown that the cDNA insert (pAcN2-2202 bp) contained one long open reading frame encoding 623 amino acid residues of the enzyme and 63 bp of 5′ -untranslated sequence. The deduced amino acid sequence of the onion 1-SST (accession no. AJ006066) protein showed 49% identity to Chicory 1-SST (accession no.U81520), 53% identity to acid invertase of tulip (accession no. X95651), 58% identity to onion 6G-FFT (accession no. Y07838) and 61% identity to that of onion acid invertase (accession no. AJ007067). This homology between onion 1-SST and acid invertases again confirms that 1-SST evolved from acid invertases.

Single amino acid mutation at several acceptor binding sites in festuca 1-SST (Altenbach *et al.*, 2009) with corresponding acid invertase amino acid caused an increase in hydrolytic capacity of 1-SST from about 20% in wild type to up to 80% in mutant lines. This study suggests that if some regions at the sucrose binding or acceptor binding site of 1-SST are conserved and are similar to that of acid invertase (from which it actually evolved), it is possible that they still exhibit some hydrolytic capability. This might be true in case of onions 1-SST exhibiting hydrolytic activity.

1-SST activity is influenced by the sucrose levels. *In vitro* studies using protein extracts of pAcN2-transformed protoplasts has showed higher levels of 1-kestoses at 100 mM sucrose than protoplasts incubated at 20 mM sucrose (Vijn *et al.*, 1998). Similar sucrose enhanced 1-SST activity has been noticed in other fructan-storing plant species (Müller *et al.*, 2000). The activity of 1-SST in the high solid-type onion “Southport White Globe High solid” was higher than that in the low solid onion cultivar “Sweet Spanish Utah Jumbo” during bulb growth and expansion phase, but showed similar activity level to that of “Sapporo yellow”, a low solid onion cultivar (Shiomi *et al.*, 1997). Since 1-SST plays such an important role in

fructan synthesis, studies on its activity level in a wide range onion cultivars are required to understand its association with varying fructan levels in high- and low-fructan cultivars.

1.6.3.2 Fructan: fructan 6G-fructosyltransferase

6G-FFT localised in the vacuole, is the key enzyme involved in the formation of the inulin neoseries type of fructan. They transfer fructosyl residues from the 1-kestose to the glucose (C6) moiety of a sucrose molecule forming neo-kestoses and further extend these neo-kestose series into high DP chains through β-(2, 6) glycosidic linkage. In onions, 6G-FFT is bi-functional in nature- catalysing the biosynthesis of both inulin and inulin neoseries type of fructan. Sequencing of *A. cepa* cDNA (pAc2) encoding 6G-FFT showed that the insert (2104 bp) contained one long open reading frame encoding 612 amino acid polypeptide and a 42 bp 5′ -untranslated sequence. The cDNA had greater identity to sequences of barley 6-SFT (48.2%), acid invertases of carrot (53%) and tulip (54%) (Vijn *et al.*, 1998).
The β-fructosidase motif of the sucrose binding box specifies the type of fructan produced by a fructosyltransferase (Ritsema *et al.*, 2004; Ritsema *et al.*, 2005). Mutation of the β-fructosidase motif of onion 6G-FFT with vacuolar invertases or 1-SST caused changes in their product formation. Point mutation of asparagine in aspargine-aspartic acid-proline-serine-glycine (of 6G-FFT) to tyrosine or glycine as in 1-FFT resulted in inactive enzyme, while its mutation with serine or alanine as in 1-SST or glutamine (glutamine-structurally similar to asparagine) resulted in active enzyme with changes in their product specificity. Mutants with serine87aspargine, as in invertases, or serine87aspartic acid, as in 1-SST, did not change product formation, indicating the importance of amino acid 84 in determining the product specificity of 6G-FFT. The importance of amino acids outside the tryptophan-methionine-aspargine-aspartic acid-proline-aspargine-glycine motif in determining the functionality of fructosyltransferases as S-type (use sucrose as donor) or F-type (use fructans as preferential donor) was further reported by Lasseur *et al.* (2009). Mutation of perennial ryegrass Lp6G-FFT at aspargine340/tryptophan343 into aspartic acid/arginine, as in Lp1-SST, resulted in S-type fructosyltransferase activity, indicating that aspargine340/tryptophan343 determines the donor specificity in grasses and most possibly in onions, as 6G-FFT (accession no, AY07838) shares very high homology with Lp6G-FFT (accession no, AF492836).

6G-FFT does not use sucrose as its substrate, but its expression and activity is noted to be influenced by sucrose and its concentration (Vijn *et al.*, 1997; Vijn *et al.*, 2000; Lasseur *et al.*, 2006). 6G-FFT activity in grasses have been reported to be post-transcriptionally regulated in grasses (Lasseur *et al.*, 2006).

1.6.4 Invertases

Invertases are the enzymes involved in the irreversible hydrolysis of sucrose to glucose and fructose. Based on the pH optima, onion invertases are classified into acid invertases (AI, optimum at pH 5.2) and neutral invertases (NI, optimum at pH 7.0) (Lercari, 1982). Acid invertases are further divided into two classes, insoluble cell wall bound AI and the soluble vacuolar form of AI. Neutral/alkaline invertases are located in cytoplasm and are called cytosolic invertases.

Acid invertases are highly homologous to fructosyltransferases at the gene level. The deduced amino acid sequence of the onion acid invertase protein (pAcT1, accession no. AJ007067)) showed 47% identity to Chicory 1-SST, 61% identity to AcN2 (1-SST of onion) and 63% identity to onion 6G-FFT (Vijn *et al.*, 1998). pAcT1 had an insert of 2438 bp and contained a poly (A^+) stretch at 3′ end. It contained one long open reading frame encoding 690 amino acid residues of the enzyme and 59 bp of 5 ' -untranslated sequence. *In vitro* studies using protein extracts of AcT1 transformed protoplasts showed that this enzyme at 20 mM sucrose concentration exhibited only invertase activity, while at 100 mM sucrose formed high DP fructans along with glucose and fructose, thus exhibiting fructosyltransferase activity (Vijn *et al.*, 1998). The fructosyltransferase nature of invertases has been noticed in many fructan-storing plants such as barley (Obenland *et al.*, 1993). Although it is evident from various *in vitro* studies, the role of invertases as fructosyltransferases under high sucrose concentration in real plant systems is still unclear. Expression studies of sucrose induced onion leaves showed that invertase mRNA levels were decreased under elevated sucrose concentration in leaves, while mRNA of 1-SST and 6-FFT were induced, forming oligofructans.

Variation in sucrose levels has been hypothesised to be due to variation in the expression and activities of SPS activity in onions (Yaguchi *et al.*, 2008). However reports by Shiomi *et al.* (1997) indicated variation in invertases and fructosyltransferases in regulating sucrose levels in onion cultivars. Low-fructan onion cultivars, such as Sweet Spanish Utah Jumbo and

Sapporo yellow have exhibited high invertase activities during bulb development, while the high-fructan cultivar Southport White Globe showed low but overlapping invertase activities. Since acid invertase plays such a significant role in sucrose accumulation in many plant species (Miron & Schaffer, 1991), comparing different cultivars for acid invertase activities is important to our understanding sucrose metabolism in onions.

Compared to acid invertases, neutral invertases are much less studied in plants. Though their specific functional role is not clear, reports have suggested their involvement in growth and development in many plant species (Lou *et al.*, 2007; Jia *et al.*, 2008; Barratt *et al.*, 2009; Welham *et al.*, 2009). Neutral invertase activities in onions grown under long day conditions has shown constant activity during the growth of leaf blades of bulbing plants, while much less to no activity was observed in leaf bases (Lercari, 1982). Two neutral invertase homologs, ACP042 (accession no, CF437950) and ACP047 (accession no, CF437145) have been identified (from onion EST collections) and assigned to chromosome 8 and 2 (Yaguchi *et al.*, 2008). Since the major gene affecting onion bulb fructan content is located on chromosome 8, and the neutral acid activity is involved in sucrose hydrolysis, their role in sucrose metabolism in high- and low-fructan cultivars requires further investigation.

1.7 Genetics of onions

Allium cepa (onions and shallots) and *A. fistulosum* (Welsh or Japanese bunching onion) are among the most widely cultivated *Allium* species in the world. They were first domesticated and cultivated in the mountainous regions of Turkmenistan and northern Iran and are regarded as the primary centers of origin. The most economically important onions belong to *A. cepa*. This species does not exist in the wild and the only wild species that has been reported to be its nearest relative is *A. vavilovii* (Klass & Friesen, 2002).

Onions are outcrossing diploids with 2n=2x=16. They have a huge genome size of 17 pg or and a guanine-cytosine content of 32% (Stack & Comings, 1979; Arumuganathan & Earle, 1991; Bennett & Leitch, 1995). On self-pollination, onions exhibit strong inbreeding depression. The protandrous nature of onions effectively prevents self-pollination, favouring outcrossing and maintaining a higher degree of heterozygosity. It is observed that most of the onion improvement programs are based on mass selection from an open-pollinated population or from segregating families, which otherwise leads to inbreeding depression.

Onions are propagated either sexually via seeds or asexually via smaller, clustered bulbs. The common onion group, which includes most of the economically important, well-defined single bulbed varieties (e.g. salad onions), is propagated from seed and is biennial in nature. The bulbs of the Aggregatum group, which produce smaller onions in clusters (e.g. shallots) are usually propagated asexually (Rabinowitch & Kamenetsky, 2002).

1.7.1 Genetic resources

Genetic resources in onions are very much limited when compared to the vegetables of other taxa. Genetic analyses in onion are difficult because of its huge genome size, biennial generation time and severe inbreeding depression that are exhibited upon selfing. Comparison studies of genetic linkage in onion with physical linkage in rice have revealed a lack of colinearity between them, making widely studied grasses an inappropriate genomic model system for studying plants belonging to the order Asparagales (Kuhl *et al.*, 2004; Jakse *et al.*, 2005; Martin *et al.*, 2005).

1.7.1.1 Molecular markers

Molecular markers have been a favourite tool among plant breeders for genetic dissection of agronomically-important traits in many plant species. They assist in visualising differences at the molecular level, providing necessary information to construct molecular maps, to identify gene locations and to develop new plant varieties by means of marker assisted selection. Initially, isozyme markers (RFLP & AFLP) were used to discriminate cultivar differences in many crop plants. However, due to lack of identifiable polymorphism in many cultivated plant species, their use was very much restricted and was subsequently replaced by hybridisation and PCR-based markers. These markers were quite useful in genome wide screening, fingerprinting, interspecific comparisons and for introgression studies. Recent advancement in marker and sequencing technology has lead to development of modern genetic markers like insertion-deletion (InDel), simple sequence repeats (SSR) and single nucleotide polymorphism (SNPs). Currently, these markers are widely being used for plant genome analysis including for onions.

1.7.1.2 Molecular maps

Several groups have reported linkage maps in *A. cepa*. King *et al.* (1998) reported the first linkage map of *Allium* based on an F_3 population of 58 plants derived from a cross between Brigham Yellow Globe15-23 (BGY15-23) and Alisa Craig 43 (AC43). This low-density linkage map comprising of 116 markers on 12 linkage groups was later used for QTL analysis of various traits, including fertility restoration (*Ms* loci), bulb colour (*Crb*-1), bulb carbohydrate content, pungency and platelet aggregation.

Based on EcoR1/Mse1 AFLP markers, van Heusden *et al*. (2000) developed a genetic map from 65 F_2 families originating from the interspecific cross, *A. cepa* var Jumbo x *A. roylei*. Monosomic addition lines of *A. fistulosum*-shallot developed by Shigyo *et al.* (1996) was used to detect chromosome-specific markers and assigning them to the respective linkage groups of *A. cepa/A. roylei*. Martin *et al.* (2005) published a medium-density linkage map of BGY15-23 and AC43, spanning 1,907 cM in a 14 linkage group. Ohara *et al.* (2005) published AFLP-based maps of two *A. fistulosum* back crossed population covering 947 cM and 775 cM in 15 and 14 linkage group respectively. McCallum *et al.* (2006) developed an intraspecific onion linkage map based on SSR and SSCP markers SSR and SSCP markers from a single F_2 population (n = 81) of W202A x Texas Grano 438 (450 cM on all eight chromosomes). Tsukazaki *et al.* (2008) published the first linkage map of bunching onion based on F_2 population (n = 225) of two bunching onion inbred lines, D1s-15s-10s and J1s-14s-23s. This map was comprised of 212 bunching onion SSR markers and 42 bulb onion (*A. cepa* L.) markers, covering 2,069 cM in 17 linkage groups.

Another bunching onion linkage map was constructed using the F_2 population (n = 115) of two bunching onion inbred lines, TO3 and SO3 (Tsukazaki *et al.*, 2011). The map was built primarily based on pre-assigned chromosomal markers, making it an efficient tool to re-evaluate and re-assign some of the linkage groups in previously built bunching onion linkage maps. Chromosome-specific markers (766 markers) identified using bunching onion-shallot monosomic addition lines and an allotriploid bunching onion single alien deletion line as testers (Hang *et al.*, 2004; Yaguchi *et al.*, 2009) provide a future means to identify new QTLs and to further enhance the genetic information on onion linkage maps.

Parental lines used to develop the available onion genetic maps are heterozygous at many loci, hindering marker development and mapping studies. Availability of homozygous

doubled haploid onion lines (Alan *et al.*, 2003; Alan *et al.*, 2004) can be a very useful tool in developing polymorphic markers in onions, in developing new mapping families of wide cross (for any quantitative trait), for developing genetic maps, QTL identification and genetic analysis of complex traits such as carbohydrate trait in onions. AlliumMap (McCallum *et al.*, 2012) a new online resource (http://alliumgenetics.org) for *Allium* crops can now be used as an integrated point to incorporate new information on onion genetic maps, sequence and marker data, further facilitating comparative genomic studies.

1.7.2 Genetic analysis of bulb carbohydrate composition

Genetic studies of various complex traits including WSC in plants are widely carried out through QTL analysis in plants. QTL analysis is a statistical method that looks for correlation between the values of quantitative trait under study to the genotype at every given marker. Based on the significant test values a QTL is located near the test markers. A large number of QTLs are usually identified during the genetic analysis of complex traits, however only a limited number of QTLs has been noticed to exhibit a larger effect on the overall genetic variation (Ming *et al*., 2001; Turner *et al*., 2006). QTL linked markers are useful in molecular plant breeding as it provides a diagnostic tool for identification and selection of superior allele content of the targeted traits (Forster *et al*., 2004). Identification of the markers closely linked to major QTLs is important particularly in out-breeding crop species, as these plants exhibit heterogeneous background, and any recombination at the marker locus and the trait gene at any cycle of crossing may result in advertent counter-selection. Identification and development of genetic markers based on functionally associated variation in candidate genes further provides an opportunity for efficient market assisted selection and for production of transgenic plants.

Water soluble carbohydrates (WSCs) content is a complex genetic trait that is extensively studied in grasses. Besides being a metabolic fuel, carbohydrates are an important factor determining traits such as: grain filling and yield in wheat, barley and oats (Bonnett & Incoll, 1992; Bingham *et al*., 2007; Ehdaie *et al*., 2006a, b; Schnyder,1993; Xue *et al*.,2008); stem sugar, grain sugar and yield in sorghum (Murray et al., 2009); volume and concentration of sucrose in sugarcane (Aitken *et al*., 2006); yield stability, stress tolerance (Volaire *et al*., 1997) and nutritional value of forage grasses- important for increasing meat and milk production (Kingston-Smith *et al*., 2013; Miller *et al*., 2001; Wims *et al*., 2013); sweetness and DM content in onion (Kahane *et al*., 2001; Havey *et al*., 2004). To improve carbohydrate

related traits, genetic variation of WSC has been characterised by QTL in many crops: barley (Teulat *et al*., 2001), maize (Thévenot *et al*., 2006; Wang *et al.*, 2010), wheat (Fu *et al.*, 2011; Rebetzke *et al*., 2008; Xue G-P *et al*., 2008; Yang *et al*., 2007), sorghum (Natoli *et al*., 2002; Ritter *et al*., 2008; Murray *et al*., 2009; Shiringani *et al*., 2010), perennial rye grass (Turner *et al*., 2006; Turner *et al*., 2010, Pembleton *et al*.,2013) , sugarcane (Ming et al., 2002). Association studies of carbohydrate metabolic enzymes and their products to the QTL markers have facilitated the identification of genes/ strongly associated genes underlying QTL affecting carbohydrate content in plants (Prioul *et al*., 1997; Prioul *et al*., 1999; Pembleton *et al*., 2013, Skot *et al*., 2007).

In onions, QTL for varying bulb composition were analysed based on the linkage map of BYG15-23 x AC43 (Galmarini *et al.*, 2001). Two QTLs on linkage groups (LG) D (50 cM) and F (16 cM) were found to be associated with SS concentration, explaining 28% and 30% of the phenotypic variation. For DM%, two QTLs detected on LG-D (50 cM) and E (38 cM) accounted for 17% and 21% of the phenotypic variation. It was noticed that the markers API89 (chromosome 3, LG D) and API66 (chromosome 5, LG E) associated with SS concentration and DM% QTL, encoded acid invertase and phloem unloading sucrose transporter genes that are important candidates in carbohydrate metabolism. Using a subset (n=48) of F_3 families of BYG15-23 x AC43, Havey *et al.* (2004) identified a region on LG-A affecting (6G,1)-nystose concentration and another region on LG-D affecting sucrose concentration in onions. These results were in agreement with previous reports (Galmarini *et al.*, 2001) affecting SS concentration.

Phenotypic evaluation of F_2 progenies from storage and sweet onion crosses: Colossal Grano x ELK 'P12' (C x P12), W202A x Texas Grano 438 (W x T) and W420A x Houston Grano (W x H), showed bimodal segregation pattern for fructose and fructan content (McCallum *et al.*, 2006). Frequency distribution analysis of these F_2 progenies clearly exhibited dominance for high fructan. Genetic analysis for bulb carbohydrate in a W x T F_2 family using EST-SSR markers showed that ACM235, an SSR marker, was significantly associated with bulb fructan, fructose and glucose content. A polymorphic SSR marker, ACM171, identified in this cross was closely linked to the sucrose transporter RFLP locus API66C-E5-6.7/9.5. Similar to previous reports (Galmarini *et al.*, 2001), this marker on chromosome 5 was significantly associated with bulb DM. API89 (on chromosome 3) was weakly associated with bulb carbohydrate composition, in contrast to the previous results on BYG15-23 x AC43

cross (Galmarini *et al.*, 2001; Havey *et al.*, 2004). Linkage mapping of ACM235 in interspecific population of *A.cepa* x *A.roylei,* revealed that this chromosome 8 marker was close linked to an SSR marker ACM033. Single marker analysis of ACM235 and ACM033 markers in C x P12 showed that these markers were significantly associated with fructan, fructose and sucrose content. Interval mapping further revealed that the region encompassed by these markers had a major QTL-*Frc* (LOD >6) (Figure 1.3) explaining 93% of phenotypic variation in the bulb fructan content. The role of *Frc* in conditioning bulb fructan content was further confirmed in the C x P12 and BYG15-23 x AC43 mapping populations.

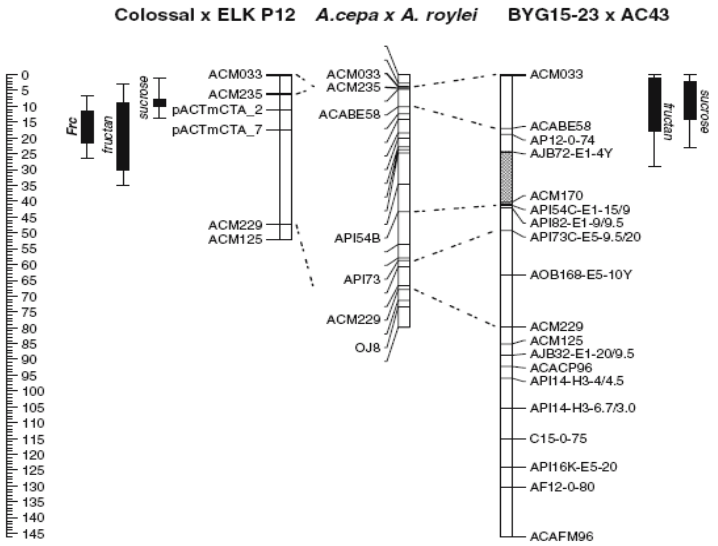

Figure 1.3 . Location of *Frc* locus on chromosome 8 of onion by QTL and linkage analysis in inter- and intraspecific *Allium* gene mapping populations. Scale denotes recombination distance in Kosambi units. (Source: McCallum *et al.*, 2006) .

Biochemical and genetic analysis of monosomic additional lines in onions showed that chromosome 2 and 8 possessed candidate genes for carbohydrate metabolism (Yaguchi *et al.*, 2008). Markers encoding sucrose synthase (ACP013), acid invertase (ACP041), neutral invertase (ACP047), invertase (ACP054) and cell wall invertase (ACP057) were assigned to chromosome 2. This chromosome, under *Allium fistulosum* background, affected sucrose and fructan accumulation (Yaguchi *et al.*, 2008), while its absence in multiple alien addition lines of *Allium* increased bulb formation (Masuzaki *et al.*, 2007). It is suggested that by regulating sucrose-hexose levels, invertases and SuSy are able to alter sucrose pools, ultimately affecting the sink strength in carbohydrate accumulating tissues. The alien addition line (FF+8A) and high fructan inbreds (*Frc_*) accumulated significantly higher levels of sucrose

than *A. fistulosum* controls and low fructan inbreds, supporting previous studies (McCallum *et al.*, 2006) that regions on chromosome 8 regulate carbohydrate composition in onions. Based on chromosomal assignment of SPS to chromosome 8, it is hypothesid that differential expression of SPS controls fructan trait in onions. This hypothesis was further supported by the previous evidence that high sucrose concentration induces 1-SST mRNA expression, resulting in the formation of 1-kestose in onions (Vijn *et al.*, 1998). In contrast to this hypothesis, genetic mapping of SPS marker (SPS3′UTR) outside the *Frc* region and close to the dry matter QTL in the BYG15-23 x AC43 mapping population (Galmarini *et al.*, 2001) led to a question of whether SPS has a major effect on bulb carbohydrate composition. So far, SPS markers have not been mapped in other onion pedigrees to support its key role in fructan regulation. It is reported the SPS markers are highly heterozygous in parental lines, hindering mapping and genetic analysis of these markers (Yaguchi *et al.*, 2008).

Since *Frc* has an LOD score of >6 and conditions 93% of phenotypic variation in fructan content, it is possible that *Frc* may possess gene/genes that on expression (either alone or in interaction with other loci) have a greater influence on fructan levels in onions. Raines *et al.* (2009) also report that the *Frc* locus (ACM033-ACABE58), along with a QTL on chromosome 5 (API66-API47), had significant effect on DW and fructan content. The high fructan parent BYG15-23 was noticed to be dominant for high DW and soluble solids at both loci. Although these regions (QTLs on chromosome 5 and 8) interacted significantly, it was noticed that they independently contributed towards soluble carbohydrate content.

We still lack knowledge on onion carbohydrate metabolic pathways and on genomic regions controlling fructan content in onions. Further research is required to genetically and biochemically dissect the carbohydrate trait in high- and low-fructan onion cultivars. Since *Frc* contributes much of the phenotypic variation in onion fructan content, understanding the functional role of this gene is necessary to dissect differential fructan accumulation. However, due to the limited availability of genetic resources and high heterozygosity in onions, developing and analysing widely segregating onion families is required for genetic analysis of carbohydrate traits in onions. Biochemical and molecular characterisation on high- and low-fructan cultivars will provide clues to the variables/candidate genes that may be involved in varying fructan levels in onions. Population-based studies and characterisation-based studies on high- and low-fructan cultivars should provide us information on the functional nature of *Frc* in Onions.

1.8 Aims and objectives

Fructan, an important quality trait associated with bulb DM% and NSC compositions, is largely governed by *Frc* locus on chromosome 8 in onions. Understanding the biochemical basis of this locus is important to develop and identify closely related molecular markers that can be used in marker-aided breeding for the fructan trait in onions. Elucidation of the variation in carbohydrate metabolism between high- and low-fructan cultivars is essential to identify the factors, which can then be checked for their association with the *Frc* locus in a reliable mapping population.

The principal aim of this project was the biochemical and molecular analysis of non-structural carbohydrate traits in high- and low-fructan onion cultivars in order to determine the factors influencing varying fructan levels in onions. Included in this was a secondary aim to develop and analyse mapping populations to study the genetic architecture for the fructan trait and to study the possible functional nature of *Frc* in onions (through association studies of identified factors with *Frc* markers).

The reliability of using leaf sugars as a representative of bulb sugars in mapping studies is also reported in this thesis. Since the project involved measurement of NSC in a large number of samples, a newly-adapted high-throughput microplate enzymatic assay was validated and used for reliable and cheap sugar assays in onion. The knowledge obtained from this project will be transferred to the New Zealand Institute for Plant and Food Research's onion genetics program, whose aim includes mapping and identification of closely linked molecular markers to carbohydrate traits in onions.

2. MATERIALS AND METHODS

2.1 Plant materials

A range of onion (*Allium cepa* L.) cultivars, landrace and F_2 lines were acquired for this project as outlined below.

2.1.1 CUDH2150

CUDH2150 is a doubled haploid onion line supplied by Cornell University (Alan *et al.*, 2003; Alan *et al.*, 2004), is characterised by low DM%, low fructan and poor storage ability

2.1.2 Nasik Red

Nasik Red (PI 271311) is a highly heterozygous Indian landrace onion with high dry matter, high fructan levels and good storage ability. The seeds were obtained from USDA-ARS plant genetics resources unit (Cornell University, Geneva, NY).

2.1.3 Southport White Globe N96

Southport White Globe N96 (SWG-N96) is an open pollinated line selected from Southport White Globe. They are most widely used as long day dehydrator onions. SWG-N96 is characterised by high fructan levels and high DM% but is poor in storage. The seeds were obtained from the New Zealand Institute for Plant and Food Research, Lincoln, NZ.

2.1.4 47AC, 47CB, 47BK and 47P

47AC, 47CB, 47BK and 47P are inbred lines of a W202A and Texas Grano 438 cross, developed by self pollination of F_2 lines (McCallum *et al.*, 2006) and provided by the New Zealand Institute for Plant and Food Research, Lincoln, NZ. 47AC, 47CB and 47P are low in fructans and have a low DM, while 47BK is considered more of a high fructan cultivar with a relatively high DM%.

2.1.5 W202A

W202A is a male sterile inbred line that is high in DM% and fructan content (Goldman, 1996). They were developed by the University of Wisconsin.

2.1.6 Texas Grano 438

'Texas Grano 438' is a mild, low fructan, low dry matter onion. Seeds were supplied by Seminis Vegetable Seeds.

2.1.7 'Nasik Red x CUDH2150' F_2 populations

Segregating F_2 populations were established by John McCallum (New Zealand Institute for Plant and Food Research, Lincoln) by crossing a male parent 'Nasik Red' with a female parent 'CUDH2150'. The F_1 seeds were obtained by crossing individual flowering plants. F_1 plants obtained were self-pollinated to generate F_2 families. Blow flies were used to facilitate the pollination. Details of the five F_2 families used as the mapping population in this study are presented in the Table 2.1.

Table 2.1. 'Nasik Red x CUDH2150' F_2 population used in experiments. Population name, seed lot number, F_1 identity number (ID), year and location grown, and the number (n) of lines sampled in each of these population is provided.

F_2 families	Seed lot	F_1 ID	Year	Environment	'n' lines
Population 1	2007052	2255	2007/2008	West Melton, NZ (43° 31′)	115
Population 2	2007057	2254	2007/2008	West Melton, NZ (43° 31′)	112
Population 3	2008064	2262	2008/2009	West Melton, NZ (43° 31′)	503
Population 4	2008076	2262	2009/2010	Kimihia, NZ (43° 31′)	197
Population 5	2008067	2262	2011/2012	Kimihia, NZ (43° 31′)	95

2.2 Experimental design, cultural practices and sampling

2.2.1 Mapping populations

Seeds of the five F_2 mapping populations of 'Nasik Red x CUDH2150' (Table 2.1) were sown in 1.5 m x 3 – 6 m beds with five rows at 215 mm spacing using an Earthway 1001-B precision garden seeder. Rows were hand thinned at the 3-leaf stage to 50-60 mm spacing between plants. Crop management practices included base dressing with Cropmaster 15 at 300 kg ha^{-1} and urea application (at 31 kg ha^{-1} and 63 kg ha^{-1}) during plant growth and development.

The youngest fully expanded leaves of population 4 were collected at the 4^{th} leaf stage (14^{th} week after sowing) between 10.00 and 12.00 h NZDT. The leaf samples were hand clipped, stored on ice and was immediately transported to the laboratory for sugar extraction by a method adapted from Antonio *et al.* (2007). Bulbs of population 1 to population 4 were lifted at 90% leaf senescence, cured for two weeks in the field, hand clipped, stored at ambient temperature and were sampled within a month of harvest as described below. While bulbs of population 5 were lifted during bulb swelling stage and sampled immediately.
One quadrant of each onion bulb (vertically dissected) from all five populations was weighed (for DM analysis) and immediately snap-frozen in liquid nitrogen. The samples were stored at -40^{o}C for about 48–60 h and were freeze-dried in a freeze-drier (VirTis Genesis-Pilot Lyophilizer, Gardiner, USA). The freeze-dried tissue was ground to a powder using a commercial Waring blender. The powdered samples were stored at room temperature in a tightly sealed container until sugar analysis.

One slice of each onion bulb (vertically dissected) from populations 3 and 5 was bagged and immediately snap-frozen in liquid nitrogen. The samples were stored at -80^{o}C (FORMA® 900 series -86^{o}C ULT freezers, Thermo Scientific) and were used for DNA extraction and enzyme analysis (population 5 only).

2.2.2 Cultivar evaluation

Eight onion cultivars (47AC, 47BK, 47CB, 47P, Nasik Red, SWG-N96, Texas Grano 438 and W202A) were grown and evaluated in field experiment conducted at West Melton (lat

43° 31'), New Zealand in 2007. Field work and sampling was carried out by John McCallum and Martin Shaw (New Zealand Institute for Plant and Food Research, Lincoln). Seed sowing, spacing and all cultural practices were followed as per Section 2.2.1. Bulbs were lifted at 90% tops senescence, cured for two weeks, hand clipped, stored at ambient temperature and were analysed within a month of harvest. Freeze dried samples (prepared as per Section 2.2.1) from three bulbs of each of the cultivars were used for sugar analysis.

2.2.3 Characterisation studies on high- and low-fructan cultivars

Four high- (Nasik Red, SWG-N96, 47BK and W202A) and low-fructan (47AC, 47CB, 47P and Texas Grano 438) onion cultivars were grown and evaluated in a field experiment conducted at Lincoln- 42° 39', New Zealand in 2009. Seeds germinated and grown in Jiffy 7's pellet trays (Jiffy Products International, The Netherlands) for five weeks were transplanted on 23/10/2009 to 1.5 m x 2 m beds, laid out in a randomised block design, with three replicates. Uniform spacing of 10 cm between plants and 20 cm between rows were maintained in all plots. Crop management practices were followed as per Section 2.2.1. Irrigation and weeding was done as and when required. Developing plants were sampled at 15 (27/11/2009), 30 (11/12/2009), 45 (24/12/2009), 60 (08/01/2010), 75 (22/01/2010) and 90 (05/02/2010) days after 4th leaf emergence. The field experienced severe cold stress early December. The mean daily temperature between 28/11/2009 and 04/12/2009 was less than or close to10°C (Appendix 8.1).

Six plants per plot per harvest were randomly selected and sampled between 10.00 and 12.00 h NZDT. The samples were stored on ice and were immediately transported to the laboratory where the leaf blades were separated from leaf base of the youngest fully emerged leaf of each plant. Tissue samples from each plot (six plants) were pooled together in a bag, snap frozen in liquid nitrogen, and stored at -80°C. Samples homogenised to a fine powder in liquid nitrogen were used for RNA isolation, NSC extraction and for enzyme activity assays.

2.3 Non-structural carbohydrate extraction

2.3.1 Water extracts

Freeze dried powder (10 mg) or fresh samples (50 mg) was dissolved in 1 ml of Milli-Q

water and incubated for 15 min at 80^{o}C. Just before plating, the samples were centrifuged for 5 min at 14000 x g. As and when required the samples were further diluted 1:2 or 1:4 with Milli-Q water.

2.3.2 Chloroform: methanol extracts

Leaf sugars were extracted using a method adapted from Antonio *et al*. (2007). A known weight of leaf sample (0.5 g) was transferred to a 15 ml Falcon tube containing 2.5 ml of ice-cold chloroform: methanol (3:7, v/v), mixed and incubated overnight at -20^{o}C to extract the water soluble sugars and sugar phosphates. To the extracted sample, 2 ml of Milli-Q water was added, vortexed and centrifuged for 5 min at 14000 x g to separate the phases. The upper aqueous-methanol phase was evaporated to dryness in a Savant (Speed vacuum concentrator, USA). The dried extract was reconstituted in 1 ml Milli-Q water just before enzymatic analysis.

2.4 Reagent and enzyme preparation for non-structural carbohydrate assays

All chemicals used in the assay were of analytical grade. Stock solutions of the buffers and reagents were prepared as per McCleary and Blakeney (1999) with highly purified Milli-Q water (Millipore, Bedford, USA). Whenever required, the solutions were made fresh. The enzyme solutions and solution 1 necessary for NSC analyses in a 96 well plate were prepared as follows:

2.4.1 Sucrase: α-glucosidase (Maltase, EC 3.2.1.20)

Sucrose assays required sucrase enzyme (Megazyme Cat. No. E-malts) at 0.4 U/well. To obtain this, 20 µl of sucrase ammonium sulphate solution (1000 U ml^{-1}) was mixed with 480 µl of 100 mM sodium maleate buffer (pH 6.5) and 10 µl enzyme solution was added per well. Fructan assays required 1.5 U of sucrase per well. This was prepared by adding 150 µl of sucrase ammonium sulphate solution (1000 U ml^{-1}) to 1850 µl of 100 mM sodium maleate buffer (pH 6.5), and 20 µl of this enzyme solution was used per reaction mixture.

2.4.2 Invertase: β-fructosidase (Yeast invertase, EC 3.2.1.26)

Invertase (Boehringer, Mannheim) was required at 0.942 U/well. 2 mg of the invertase powder (32.93 units/ mg powder) was added to 7 ml of 0.1 M citrate buffer (pH 4.6). 10 µl of the mixture contained 0.942 U of enzyme.

2.4.3 Hexokinase/ Glucose-6-phosphate dehydrogenase (HK/G6PDH, EC 2.7.1.1 / EC 1.1.1.49)

Sugar assays required HK at the concentration of 0.850U and G6PDH at 0.425 U concentration per well. To analyse one microplate of sugars, 260 µl of the enzyme suspension (340 U HK ml^{-1}, 170U G6PDH ml^{-1}, Roche, Cat. No. 10127825001), was added to 800 µl of solution 1 and 10 µl of this enzyme solution was used per reaction mixture.

2.4.4 Phosphoglucose isomerase (EC 5.3.1.9)

Phosphoglucose isomerase (PGI: Roche Cat. No. 10128139001) was required at 1.75 U/well. 30 µl of enzyme suspension (3500 U ml^{-1}) was added to a vial containing 120 µl of Milli-Q water and 450 µl of Solution 1. The enzyme solution was added at 10 µl per well.

2.4.5 Fructanase (EC 3.2.1.80)

Fructanase (Megazyme Cat. No. E-FRMXLQ) was required at 8.0 U/well. 10 µl of enzyme suspension (800 U ml^{-1}) was added to a vial containing 490 µl of Milli-Q water and 500 µl of 0.2 M Na acetate, pH 4.5. The enzyme solution was added at 10 µl per well.

2.4.6 NAD/ ATP mix (Solution 1)

25 ml of Solution 1 is required per 96 well microplate. 28 mg of NAD and 56 mg of ATP were added to 12.5 ml of 0.2 M triethanolamine buffer (pH 7.6). 100 µl of 1M $MgCl_2$ was added to the solution and the total volume was made up to 25 ml with Milli-Q water.

2.4.7 4-Hydroxy benzoic acid hydrazide working reagent

4-Hydroxy benzoic acid hydrazide (PAHBAH: Sigma Cat. No.H-9882) working Reagent was

made fresh before use (McCleary *et al.*, 2000) by adding 4 ml of Reagent A and 36 ml of Reagent B, sufficient enough to assay one 96-well plate.

To prepare Reagent A, 10 g of PAHBAH was added to 60 ml of water. The slurry was stirred and 10 ml of concentrated hydrochloric acid was added .The solution was adjusted to 200 ml with distilled water and stored at room temperature.

Reagent B was prepared by dissolving trisodium citrate (6.225 g), calcium chloride dihydrate (0.550 g) and sodium hydroxide (10.0 g) in 200 ml of distilled water. The volume was adjusted to 500 ml and stored at room temperature.

2.4.8 TBA-HCl working reagent

Thiobarbituric acid (TBA) reagent was prepared as per Puebla *et al.* (1999). TBA-HCl reagent was prepared by adding 342 mg of TBA in 100 ml of 10 mM HCl. The TBA solution diluted 1:1 times in concentrated HCl (12 M) just before use was used in the enzymatic assay.

2.5 Non-structural carbohydrate analysis

2.5.1 Enzymatic based assay for glucose, fructose and sucrose

The assays are based on the amount of NADH produced through a cascade of enzymatic reactions. The amount of NADH formed is stoichiometrically equal to the amount of glucose or fructose present in the solution, and is measured as an increase in absorbance at 340 nm (Mannheim, 1987). For the calibration curves, a glucose-fructose stock solution (10 mg ml^{-1} of each) was used to make standard solutions containing 0, 0.125, 0.250, 0.375, 0.500, 0.625, 0.750, 0.875 and 1.00 mg ml^{-1} of glucose and fructose.

Sucrose (1.0 mg ml^{-1}), Enzytec^{TM} Glucose (0.449 mg ml^{-1}) and fructose (0.50 mg ml^{-1}) were used as quality controls. The sucrose control enabled verification that the maltase or invertase enzyme activities were still optimal, while the glucose control verified the hexokinase/glucose-6-phosphate dehydrogenase was the correct activity; the fructose control did the same for the phosphoglucose isomerase activity. 10 µl of the standards and controls, each in duplicates, were dispensed into the designated wells of a 96 well microplate (Figure 2.1). The samples were dispensed as duplicated blocks of 12 unknowns each in triplicates of

10 µl. The first block of unknowns was used to determine sucrose levels, while the second block was used for fructose and glucose determination by the enzymatic reactions. Glucose levels (mg ml^{-1}) in the samples were obtained based on the standard glucose concentrations and the mean absorbance of three replicated wells. Final glucose concentrations as mg g^{-1} dry weight (DW) or mg g^{-1} fresh weight (FW) in the samples is calculated based on glucose levels (mg ml^{-1}), extract volume, dilution (1:1 or 1:2) and the actual mass of tissue sampled. The glucose level in the wells with no added maltase or invertase enzyme represented the free glucose concentration present in the sample, while the fructose levels in the sample were represented by increased glucose concentration in G/F wells after the addition of PGI and was calculated by:

$$\text{Fructose (mg ml}^{-1}\text{)} = \text{Total glucose 2 (mg ml}^{-1}\text{)} - \text{free glucose (mg ml}^{-1}\text{)}$$

Sucrose levels in the hydrolysed samples were calculated using the following equation:

$$\text{Sucrose (mg ml}^{-1}\text{)} = [\text{total glucose 1 (mg ml}^{-1}\text{)} - \text{free glucose (mg ml}^{-1}\text{)}] * 1.9$$

where:

1.9 accounts for the molecular mass of sucrose divided by the molecular mass of glucose. The overall procedure for enzymatic assay of glucose, fructose and sucrose are outlined below:

Pipette into the wells	**Blank**	**Blank**	**Sample fractions**	
	Sucrose	Glucose/fructose	----------	
			Sucrose wells	G/F wells
Maltase/invertase	10 µl	-	10 µl	-
Mix, incubate at 40 ^{o}C for 30 min				
Solution 1	250 µl	250 µl	250 µl	250 µl
HK/G6PDH	10 µl	10 µl	10 µl	10 µl
Mix, incubate at 25 ^{o}C for 20 min and read the absorbance at 340 nm				
			a	b
PGI	-	10 µl	-	10 µl
Mix, incubate at 25 ^{o}C for 20 min and read absorbance at 340 nm				
				c

G/F =glucose/fructose

a= Measuring absorbance of sucrose as total glucose1 (glucose from sucrose + free glucose)

b= Measuring absorbance of free glucose

c= Measuring absorbance of fructose as total glucose2 (fructose as glucose + free glucose)

HK/ G6PDH = Hexokinase/ Glucose-6-phosphate dehydrogenase; PGI = Phosphoglucose isomerase

	1	2	3	4	5	6	7	8	9	10	11	12
A	G/F 0.125	G/F 0.125	Blank	Sucrose UKS 1	Sucrose UKS 1	Sucrose UKS 1	Sucrose UKS 9	Sucrose UKS 9	Sucrose UKS 9	G/F UKS 5	G/F UKS 5	G/F UKS 5
B	G/F 0.250	G/F 0.250	G/F 0	Sucrose UKS 2	Sucrose UKS 2	Sucrose UKS 2	Sucrose UKS 10	Sucrose UKS 10	Sucrose UKS 10	G/F UKS 6	G/F UKS 6	G/F UKS 6
C	G/F 0.375	G/F 0.375	Sucrose control	Sucrose UKS 3	Sucrose UKS 3	Sucrose UKS 3	Sucrose UKS 11	Sucrose UKS 11	Sucrose UKS 11	G/F UKS 7	G/F UKS 7	G/F UKS 7
D	G/F 0.500	G/F 0.500	Sucrose control	Sucrose UKS 4	Sucrose UKS 4	Sucrose UKS 4	Sucrose UKS 12	Sucrose UKS 12	Sucrose UKS 12	G/F UKS 8	G/F UKS 8	G/F UKS 8
E	G/F 0.625	G/F 0.625	Glucose control	Sucrose UKS 5	Sucrose UKS 5	Sucrose UKS 5	G/F UKS 1	G/F UKS 1	G/F UKS 1	G/F UKS 9	G/F UKS 9	G/F UKS 9
F	G/F 0.750	G/F 0.750	Glucose control	Sucrose UKS 6	Sucrose UKS 6	Sucrose UKS 6	G/F UKS 2	G/F UKS 2	G/F UKS 2	G/F UKS 10	G/F UKS 10	G/F UKS 10
G	G/F 0.875	G/F 0.875	Fructose control	Sucrose UKS 7	Sucrose UKS 7	Sucrose UKS 7	G/F UKS 3	G/F UKS 3	G/F UKS 3	G/F UKS 11	G/F UKS 11	G/F UKS 11
H	G/F 1.00	G/F 1.00	Fructose control	Sucrose UKS 8	Sucrose UKS 8	Sucrose UKS 8	G/F UKS 4	G/F UKS 4	G/F UKS 4	G/F UKS 12	G/F UKS 12	G/F UKS 12

G/F = Glucose/fructose, UKS = unknown samples

Figure 2.1. Microplate map for sucrose, glucose and fructose analyses.

2.5.2 Enzymatic based assay for fructan

The fructan concentration in the plant tissue extracts were determined based on Association of Official Analytical Chemists (AOAC) method 997.08 and the Megazyme fructan-assay kit (McCleary & Blakeney, 1999). The extracted samples (20 µl) were treated with α-Glucosidase (1.5 U/well) to hydrolyse sucrose to glucose and fructose. The reducing sugars were then reduced to sugar alcohols by treatment with alkaline borohydride solution (20 µl, 10 mg ml^{-1} $NaBH_4$ in 50 mM NaOH). Dilute acetic acid (50 µl, 200 mM) was added to bring the pH to 4.5, and to remove the excess borohydride from the solution.

Three 10 µl aliquots of fructan sugar extract from each sample were then plated on to a 96 well microplate (Figure 2.2). Standards (0, 0.125, 0.250, 0.375, 0.500, 0.625, and 0.750 mg ml^{-1} of fructose), fructan flour control, negative control and samples were dispensed in triplicate onto the 96 well microplate, permitting 24 samples (unknowns) to be assayed per microplate. Fructan was hydrolysed in the plates by adding fructanase (10 µl) and incubating it for 30 min at 40^{o}C. The reducing sugar levels were measured by treatment with working PAHBAH reagent (35 min incubation at 90^{o}C) and reading the absorbance (410 nm) against the reagent blank.

Fructan levels (mg ml^{-1}) in the samples were obtained based on the standard fructose concentrations and the mean absorbance of three replicated wells. Final fructan concentration

(mg g^{-1} dry weight (DW) or mg g^{-1} fresh weight (FW) in the samples were calculated based on fructose levels (mg ml^{-1}), extract volume, dilutions (1:1 or 1:2) and the actual mass of tissue sampled.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Negative control	Std 0	Std 0	Unknown 1	Unknown 1	Unknown 1	Unknown 9	Unknown 9	Unknown 9	Unknown 17	Unknown 17	Unknown 17
B	Std 0.125	Std 0.125	Std 0.125	Unknown 2	Unknown 2	Unknown 2	Unknown 10	Unknown 10	Unknown 10	Unknown 18	Unknown 18	Unknown 18
C	Std 0.250	Std 0.250	Std 0.250	Unknown 3	Unknown 3	Unknown 3	Unknown 11	Unknown 11	Unknown 11	Unknown 19	Unknown 19	Unknown 19
D	Std 0.375	Std 0.375	Std 0.375	Unknown 4	Unknown 4	Unknown 4	Unknown 12	Unknown 12	Unknown 12	Unknown 20	Unknown 20	Unknown 20
E	Std 0.500	Std 0.500	Std 0.500	Unknown 5	Unknown 5	Unknown 5	Unknown 13	Unknown 13	Unknown 13	Unknown 21	Unknown 21	Unknown 21
F	Std 0.825	Std 0.825	Std 0.825	Unknown 6	Unknown 6	Unknown 6	Unknown 14	Unknown 14	Unknown 14	Unknown 22	Unknown 22	Unknown 22
G	Std 0.750	Std 0.750	Std 0.750	Unknown 7	Unknown 7	Unknown 7	Unknown 15	Unknown 15	Unknown 15	Unknown 23	Unknown 23	Unknown 23
H	Fructan flour	Fructan flour	Fructan flour	Unknown 8	Unknown 8	Unknown 8	Unknown 16	Unknown 16	Unknown 16	Unknown 24	Unknown 24	Unknown 24

A1is the negative control comprising 10 µl water that has been taken through the $NaBH_4$ reduction and HOAc neutralization + 10 µl fructanase. The value of this well is subtracted from all the unknown well values.

Figure 2.2. Microplate map for fructan analyses.

2.5.3 NSC assay by HPLC-PAD method

NSC determination was performed by Helen Boldingh at the New Zealand Institute for Plant and Food Research, Ruakura using HPLC coupled with photodiode array detector (HPLC-PAD) method. Freeze-dried onion powder (10 mg) was redissolved in 1ml Milli-Q water. Aliquots (50 µl) were analysed on a DIONEX ICS-3000 system with a CarboPac PA1 column and pulsed amperometric detection with gold working electrode and Ag/AgCl reference. Eluents were A: 1 M NaOH, B: water, C: 1 M sodium acetate/100 mM NaOH. Column temperature was 30°C detector compartment 35°C and flow rate was 1 ml per minute. The gradient used for elution was A: 2.5%, B: 98.5% for 23 minutes ramping to A: 10% B: 90% over seven minutes then ramping to A: 0% B: 70% C: 30% over 36 minutes. The column was then washed with A: 10% b: 0% C: 90% for three minutes then equilibrating back to A: 2.5% B: 98.5% prior to subsequent injection. Fructo-oligosaccharides were

quantified relative to 1-kestose.

2.6 Enzyme activity assays

2.6.1 Enzyme extraction and total protein measurements

Protein extractions for enzyme activity assays were performed according to Dancer *et al*. (1990) , with some modifications. Ground plant tissue samples (500 mg) were homogenised with 0.5 ml of extraction buffer containing 50 mM HEPES-KOH pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.05% (w/v) Triton X-100. Extracted samples were centrifuged and desalted into 20 mM HEPES-KOH using BioRad spin columns. The eluted extract was used to analyse total protein levels, sucrose phosphate synthase (SPS), sucrose: sucrose 1-fructosyltransferase (1-SST), acid and neutral invertase activities. For each of the unknown eluted extract a blank sample with 0 min incubation time was maintained throughout the assay. The enzyme activity per milligram protein was calculated after deducting the absorbance value of each of the blank sample from the absorbance value of the incubated samples.

Total protein from eluted enzyme extracts was determined by the method of Bradford (1976). To 2 µl of enzyme extract, 8 µl of water and 300 µl of Bradford reagent (Quick Start Bradford 1x Dye Reagent # 500-0205, BIO- RAD) and the absorbance was measured at 595 nm.

2.6.2 Sucrose phosphate synthase activity

Sucrose phosphate synthase (SPS) activity was assayed under saturated (V_{max}) substrate conditions following the anthrone method described by Guo *et al*. (2002). Desalted enzyme extracts (10 µl) were incubated for 30 min at 30^{o}C with 10 µl of 10 mM UDP-glucose, 10 µl of 2.5 mM fructose-6-phosphate, 25 µl of 10 mM glucose-6-phosphate and 45 µl of 100 mM Hepes buffer (pH 7.5). Hepes buffer comprised 100 mM Hepes-KOH, 1M $MgCl_2$ and 0.5 M EDTA. The reactions were terminated by adding 100 µl 30% (w/v) KOH and heating it in a boiling water bath for 10 min. A control containing anthrone reagent (1 ml) was added to the cooled reaction mixture, which was incubated at 40^{o}C for 20 min, mixed and plated (250 µl) on to the microplates in triplicates. The absorbance was read at 620 nm. SPS activity

was measured against sucrose standard as µg sucrose formed per minute per milligram protein.

2.6.3 Acid invertase activity

Acid invertase (AI) activity was measured by the method of Zrenner *et al*. (1996) with minor modifications. The reaction mixture (100 µl) containing 10 µl of 20 mM sodium acetate (pH 4.7), 10 µl of 1M sucrose, 55 µl of water and 25 µl of desalted homogenates were incubated at 30^{o}C for 30 min. The reaction mixture was alkalinised by adding 25 µl of 1 M Tris-HCl, pH 8.0 just before stopping the reaction at 95^{o}C. The glucose and fructose were measured as per Section 2.5.1. As the crude enzyme extract exhibited 1-SST activity, AI activity was calculated based on the amount of fructose production. AI activity was measured as µg fructose formed per minute per milligram protein.

2.6.4 Neutral invertase activity

Neutral invertase (NI) activity was measured following a similar protocol to that of AI activity. The reaction mixture (100 µl), containing 10 µl of 100 mM Hepes buffer (pH 7.5), 10 µl of 1 M sucrose, 45 µl of water and 25 µl of desalted homogenates, was incubated at 30^{o}C for 30 min. After incubation the reaction was stopped by placing the tubes in boiling water for 4 min. Neutral invertase activity was measured as µg fructose formed per minute per milligram protein.

2.6.5 Sucrose: sucrose 1-fructosyltransferase activity

The activity of sucrose: sucrose 1-fructosyltransferase (1-SST) was measured by modifications of the method described by Puebla *et al*. (1999). The reaction mixture (50 µl) containing 10 µl of 200 mM sucrose, 25 µl of 100 mM acetate buffer (pH 5.2) and 15 µl of desalted enzyme extract was incubated at 30^{o}C for 120 min before stopping the reaction at 95^{o}C. 20 µl of the reaction mixture was then treated with 100 µl of maltase solution (Section 2.4.1) and incubated for about 16 h to completely hydrolyse the unreacted sucrose. To 50 µl of the hydrolyzed sucrose reaction mixture, 200 µl of 0.5 M NaoH was added and boiled at 100^{o}C. The fructosylsucrose formed was determined by adding 600 µl of working TBA-HCl reagent (Section 2.4.8) and developing the colour at 100^{o}C for 7 min. The absorbance of the

cooled reaction mixture (200 µl) was read at 432 nm. 1-SST activity was expressed as nanomoles of kestoses formed per milligram protein.

2.7 RNA extraction

Total RNA from leaf blade and leaf bases was isolated using Concert^{TM} Plant RNA Reagent (Catalog no. 12322-012, Invitrogen) following the manufacturer's protocol. Cold (+4^{o}C) Plant RNA Reagent (0.5 ml) was added to ground frozen plant tissues (0.1 g). The extract was thoroughly mixed by flicking the tubes and incubated for 5 min at room temperature. The solution was clarified by centrifugation (for 2 min) at 12, 000 x g in a microcentrifuge at room temperature. The supernatant was transferred to an RNase-free tube and was gently mixed with 0.1 ml of 5 M NaCl before mixing thoroughly with 0.3 ml of chloroform. The samples were centrifuged at +4^{o}C for 10 min at 12,000 x g to separate the phases. The aqueous phase was transferred to RNase-free tubes, mixed with an equal volume of isopropyl alcohol and incubated at room temperature for 10 min. The samples were centrifuged at +4^{o}C for 10 min at 12,000 x g to pellet the RNA and the supernatant was removed. 1 ml of 75% (v/v) ethanol was added to the pellet. This was centrifuged at room temperature for 1 min at 12,000 x g and supernatant was removed again. The pellet was dried and re-suspended in 50 µl of nuclease free water (Ambion® DEPC-treated water, Life Technologies).

2.8 RNA quantification

RNA concentration was determined by using the Quant-iT™ RNA Assay Kit with the Qubit™ fluorometer (Invitrogen, USA). 1 µl of RNA sample (diluted 1:10 in DEPC-treated water) was mixed by vortexing with 199 µl of Quant-iT™ RNA working solution (made by diluting- Quant-iT™ RNA reagent buffer in 1:200 and Quant-iT™). Standard #1(10 µl of 0 ng/µl *Escherichia. coli* rRNA + 190 µl of Quant-iT™ RNA working solution and standard #2 (10 µl of 10 ng/µl *E. coli* rRNA + 190 µl of Quant-iT™ RNA working solution) were simultaneously prepared. Standard and samples tubes were incubated for 2 min at room temperature. The Qubit™ fluorometer was calibrated using Standard #1 and Standard #2 before reading the sample absorbance. Final RNA concentration of the samples was calculated as follows:

$$\text{RNA concentration (ng/µl)} = \text{QF value} \times (200/x) \times \text{dilution factor}/1000$$

where:

QF value = the value given by the Qubit (ng/ml)

x = the volume of RNA (µl) added to the assay tube

2.9 RNA purification

RNA was treated with DNase to remove residual contaminating DNA with the TURBO DNA-free™ Kit from Ambion. 25 µl of TURBO DNase master mix (containing 0.1 volume of 10X TURBO DNase buffer, 1 µl of Turbo DNase, 1 µl of SUPERase-In™ RNase INHIBITOR) was added to 1 µg RNA in a volume of 20 µl RNA mixed gently and incubated at 37°C for 30 min. 0.1 volume DNase inactivation reagent was added, mixed well and incubated (with occasional mixing) for 2 min at room temperature. The sample was centrifuged for 1.5 min at 10,000 x g and the RNA was transferred to a fresh tube. 10 µl of purified RNA was transferred to new tubes and mixed with 90 µl nuclease free water to obtain a final concentration of 2 ng/µl of RNA. The samples were stored at -80°C.

2.10 Quantitative real-time RT-PCR (qRT-PCR)

Analysis of gene expression was performed using a StepOnePlus™ Real-Time PCR System, (Applied Biosystems, USA). Gene-specific primers along with TaqMan probes (Table 2.2) were designed using 'Primer3'-a primer design program (http://primer3.sourceforge.net/) (Untergasser A *et al.*, 2007).The primer and probes were screened against all known onion primer sequences using 'primersearch' an EMBOSS tool (http://helixweb.nih.gov/emboss/html/primersearch.html). Primers and probes showing significant homology with other onion sequences were not considered. Since the probe designed for acid invertase homologue ACE6067, showed only a few mismatches with other onion sequences, a locked nucleic acid (LNA) probe was designed for this gene (Table 2.2). The LNA probe along with the ACE6067 primers was checked using the Oligo Optimizer tool on the Exiqon website (www.exiqon.com). LNA probe labeled with 5′ FAM (reporter dye) and 3′ BHQ-1 (quencher dye) was supplied by Sigma-Aldrich, New Zealand. The primers were supplied by GeneWorks Pty Ltd, Australia and the probes by Gene Link, NY. Total acid invertase and 1-SST transcripts were quantified relative to the expression of the reference gene "18S rRNA". ACE6067 (accession number: AJ006067) primers with LNA

probe ACE6067-LNA and ACP041 (accession number: CF437610) primers with ACP041_ probe (Table 2.2) were used to quantify the expression of acid invertase genes. 1-SST (accession number: AJ006066) primers and 1-SST_probe (Table 2.2) were used to quantify 1-SST gene expression. C_T for each sample was determined by averaging the C_T's from three technical replicates. $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001) was used for comparative quantification of gene expression. The C_T's for genes of interest (GOI) in both the test and calibrator samples were adjusted to the reference gene C_T (18S). Relative gene expression was determined using the equation below.

Relative expression = $2^{-\Delta\Delta CT}$

where:

$-\Delta\Delta CT = \Delta C_{T\ sample} - \Delta C_{T\ calibrator}$

$\Delta C_{T\ sample} = C_{T\ (GOI^s)} - C_{T\ (normaliser^s)}$

$\Delta C_{T\ calibrator} = C_{T\ (GOI^c)} - C_{T\ (normaliser^s)}$

2.10.1 Thermocycler profile for one step qRT-PCR

Thermocycler profile for one step qRT-PCR included two stages: a holding stage and a cycling stage. The holding stage included two steps: Step 1 where the samples were incubated at 48^{o}C for 15 min for cDNA synthesis followed by step 2 during which the samples were incubated at 95^{o}C for 10 min. The holding stage was immediately followed by a 40 x cycling stage: Step 1 of the cycling stage included incubation at 95^{o}C for 15 s followed by step 2 during which the samples were incubated at 60^{o}C for 1 min. The data was collected and analysed using StepOnePlus^{TM} software.

2.10.2 Primer optimisation

A primer optimisation assay was conducted for acid invertase and 1-SST genes (Table 2.2) using four different concentrations (0.5 µM, 1.0 µM, 3.0 µM and 9 µM) of both forward and reverse primers to determine the combination that generated the lowest C_t value. The RT-PCR mix for primer optimisation per reaction consisted of 10 µl of TaqMan RT-PCR mix (2x), 1 µl probe (5 µM), 0.5 µl TaqMan RT enzyme mix (40x), 5 µl of 2 ng/µl of RNA and 1.5 µl of nuclease free water. The forward

(2 µl containing 0.5 µM, 1.0 µM, 3.0 µM or 9 µM primer concentration) and reverse primers (2 µl containing 0.5 µM, 1.0 µM, 3.0 µM or 9 µM primer concentration) were added last according to their concentrations. Analysed data showed 9 µM left and 9 µM right primers to be the best primer combinations for the transcript data studies of ACE6067, ACP041 and 1_SST markers. A representative primer optimisation plot is shown in Figure 2.3.

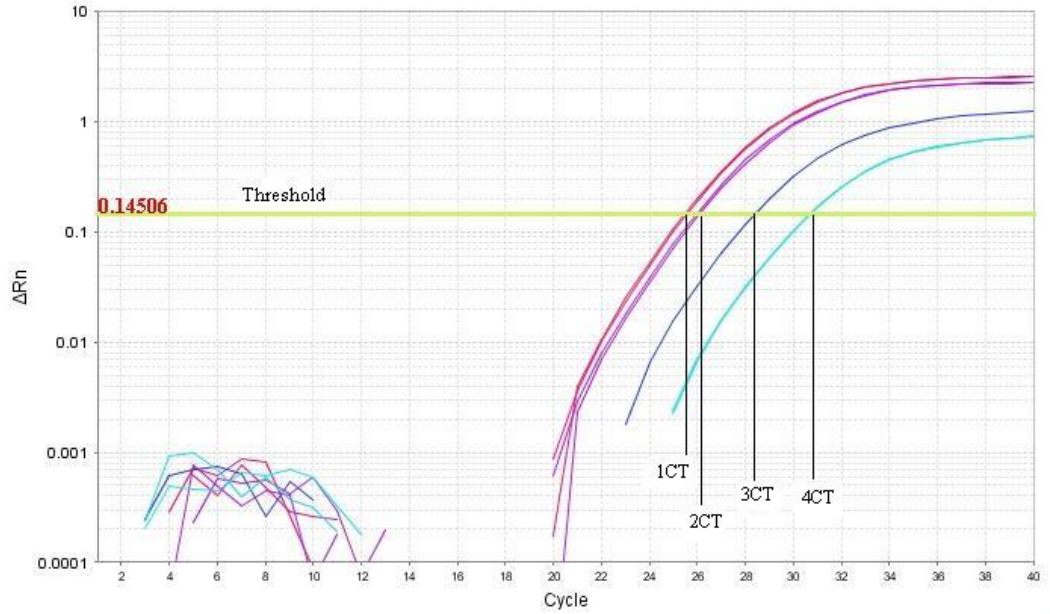

Figure 2.3. A representative primer optimisation plot. This plot shows cycle numbers $1C_T$ (9 µM left and 9 µM right primer), $2C_T$ (3 µM left and 3 µM right primer), $3C_T$ (1 µM left and 1 µM right primer) and $4C_T$ (0.5 µM left and 0.5 µM right primer) at which the fluorescent signal of the reaction crosses the threshold. ACE6067 marker primer is used here**.**

Table 2.2. Primers and probes (5′-3′) sequences for qRT-PCR experiments.

Gene	Marker name	Accession number	Oligo name	Primer Sequence	Probe Sequence
Acid invertase	ACP041	AJ006066	ACP041_RT_L_V2	GATCCGAATGGCCCAATGTA	[6-FAM]CCAATACAACCCAGAAGCAGCAGTATGGG [BHQ-1]
			ACP041_RT_R_V2	ACCCAGTTTAGAAGGTCTTTTGACA	
Acid Invertase	ACE6067	AJ006067	ACE6067_RT_L_V2	ACAGCGAAACTGCTGATATTGC	[6-FAM]AG[+A]A[+G]CCC[+A][+G]CCCTT[BHQ-1]
			ACE6067_RT_R_V2	GTCCTCGGAACACCCTGAAG	
1-SST	1-SST	CF437610	1-SST_RT_L_V2	AGGATGACCCTCCATCCG	[6-FAM]TGGGTATCATTTTCAACCCCCTAACCATTT[BHQ-1]
			1-SST_RT_R_V2	TTGCAGCATTGGGATCG	
Reference marker	18S		18S-F	CGTCCCTGCCCTTTGTACAC	[Hex]CCGCCCGTCGCTCCTACCG[BHQ-1]
			18S-R	CACTTCACCGGACCATTCAAT	

2.10.3 Probe optimisation

A probe optimisation assay was conducted for ACE6067-LNA, ACP041_ probe and 1-SST_probe (Table 2.2) using five different concentrations (50 nM, 100 nM, 150 nM, 200 nM and 250 nM) to determine the concentration that generated the lowest C_T value. RT-PCR mix for probe optimisation per reaction consisted of 10 µl of TaqMan RT-PCR mix (2x), 0.9 µl forward primer (20 µM), 0.9 µl reverse primer (20 µM), 0.5 µl TaqMan RT enzyme mix (40x), 5 µl of ng/µl of RNA and 0.7 µl of nuclease free water. The probe (2 µl) was added last according to their concentrations. The best probe concentration was 200 nM for ACE6067-LNA, 200 nM for ACP041_ probe and 250 nM for SST_probe. A representative probe optimisation plot is shown in Figure 2.4.

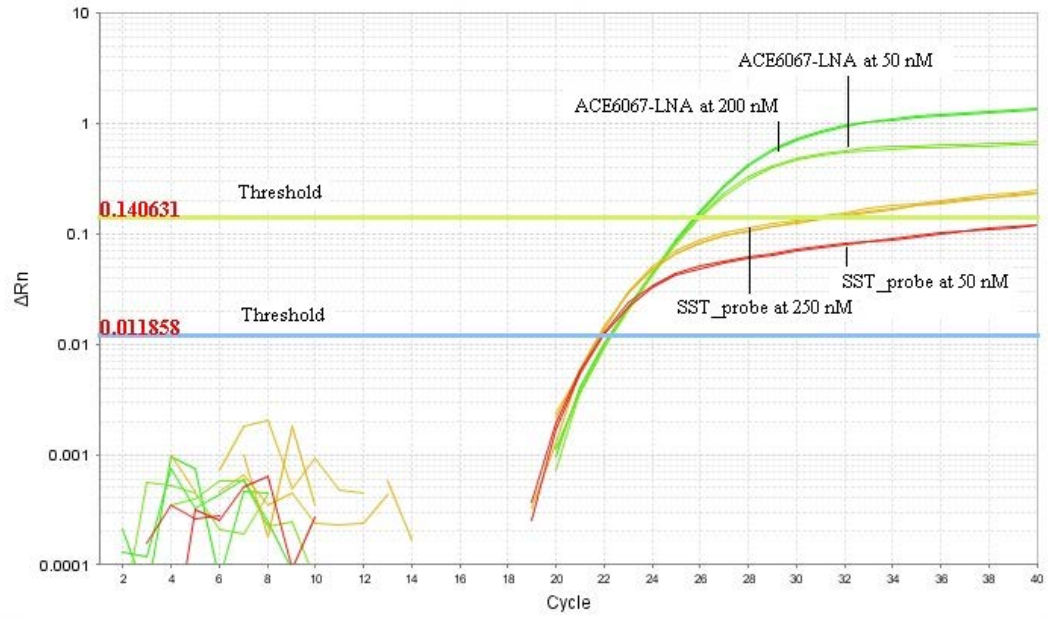

Figure 2.4. A representative probe optimisation plot. The plot shows ACE6067-LNA and 1-SST probe amplification at varying probe concentration.

2.10.4 Efficiency test

To assess the reaction efficiencies in a multiplex assay (target gene plus reference gene), a dilution (1:10) standard curve was obtained and their efficiencies calculated. The log of the

concentration of cDNA was plotted against the C_T values to generate a negative slope. The efficiency of the cDNA was then calculated using the equation: Efficiency = $10^{(-1/slope)}$. A slope of -3.32 gives a perfect efficiency of 2 (Pfaffl, 2001). The reaction efficiency for ACE6067 (with 18S) was 98% (Figure 2.5), ACP041 (with 18S) was 100% (Figure 2.6) and 1-SST (with 18S) was 100% (Figure 2.7).

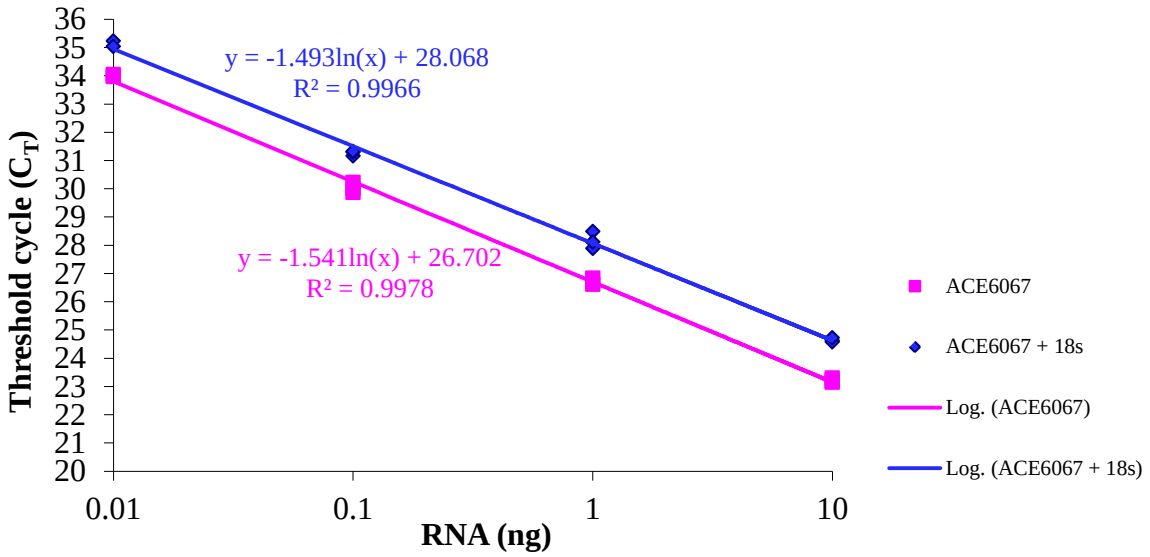

Figure 2.5. Standard curve of ACE6067. Serial dilutions of RNA from a representative sample were used to generate a standard curve representing reaction efficiencies of an acid invertase homolog, ACE6067, by itself and in a multiplex assay. Slope, Y-intercept, and R-squared values representing the performances of the reactions are indicated.

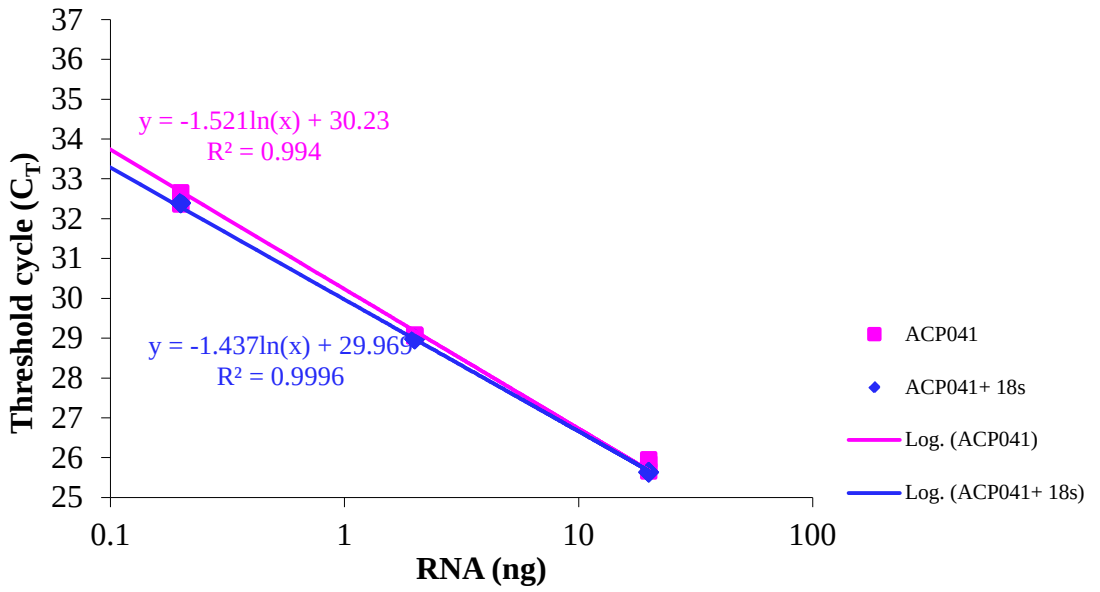

Figure 2.6. Standard curve of ACP041. Serial dilutions of RNA from a representative sample were used to generate a standard curve representing reaction efficiencies of an acid invertase homolog, ACP041, by itself and in a multiplex assay. Slope, Y-intercept, and R-squared values representing the performances of the reactions are indicated.

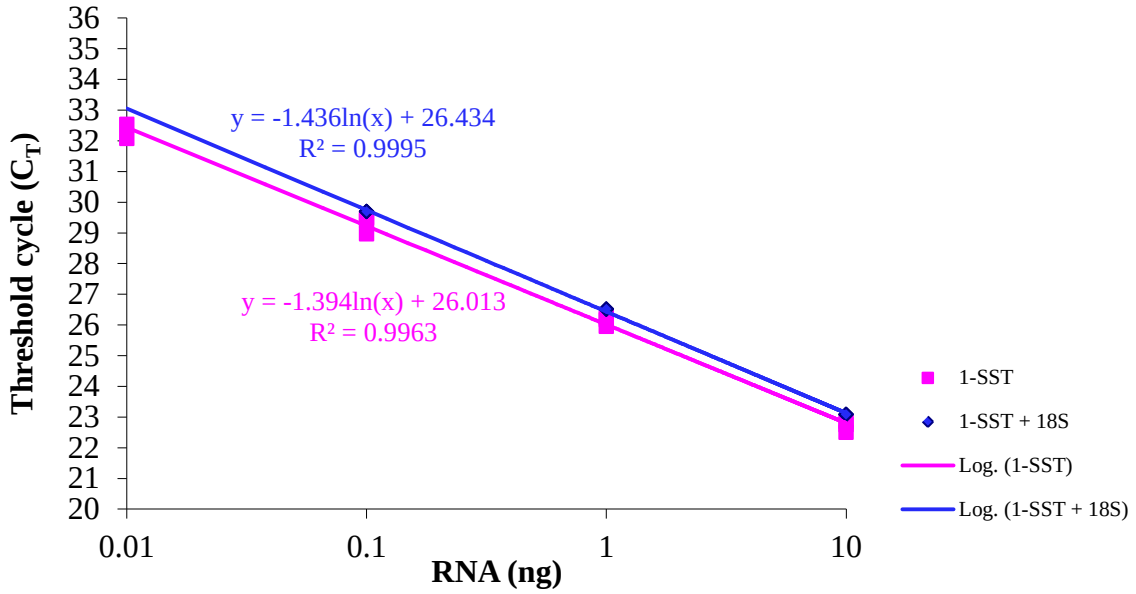

Figure 2.7. Standard curve of 1-SST. Serial dilutions of RNA from a representative sample were used to generate a standard curve representing reaction efficiencies of 1-SST and 1-SST in a multiplex assay. Slope, Y-intercept, and R-squared values representing the performances of the reactions are indicated.

2.11 DNA Extraction

DNA from onion tissue was isolated according to Lassner *et al*. (1989), with some modifications. Onion bulb samples (1-2 g, Section 2.2.1) collected in DNA bags were crushed using a wall paper seam roller. Warmed, modified CTAB buffer (3 ml, containing 200 mM Tris-HCL pH 8.0, 50 mM EDTA, 2.2 M NaCl, 2% CTAB (w/v) plus 0.5% sodium meta-bisulphite (w/v) added just before use) was added to the crushed tissues and rolled again to mix well with the plant material. The plant extract was then collected in a 15 ml centrifuge tube and 1ml 5% sarcosyl, 1 ml polyvinylpyrrolidone (PVP) and 1 ml 20% CTAB were added. The solution was mixed well by inversion and incubated at 65^{o}C for 1 h with occasional mixing during incubation. 5 ml of chloroform/ octan-2-ol (24:1) was added to the cooled mixture and mixed well before centrifugation it at 4000 rpm for 20 min. The supernatant was transferred to a new 15 ml tube, mixed with an equal volume of isopropanol followed by 1 ml of 5 M NaCl. The mixed tubes were incubated at -20^{o}C for at least 1 h. The tubes were centrifuged at 4000 rpm for 20 min, the supernatant discarded and the tubes were inverted to drain on a paper towel. 3 ml of ethanol/ sodium acetate solution (containing 190 ml 100% ethanol +25 ml 0.2 M sodium acetate solution + 35 ml sterile water) was added to the tubes and incubated at room temperature for 20 min. The ethanol/acetate solution was carefully removed leaving the pellet. The DNA pellet was air dried and resuspended in 200-400 µl of $T_{10}E_1$ buffer (prepared by adding 50 ml 1M Tris (pH 8), 20 ml 0.5 M EDTA (pH 8) to 930 ml of sterile water). DNA was quantified spectrophotometrically using Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen).

2.12 Oligonucleotides

Oligonucleotide primers (Table 2.3) were designed to sense (F) and antisense (R) strands of DNA and were commercially synthesised (Invitrogen, New Zealand).

For Cleaved Amplified Polymorphic markers (CAPS), the PCR products (5 µl) were digested in a 10 µl reaction using 3 U restriction enzyme (NEW ENGLAND BioLabs$_{inc}$.) with 1x buffer 4 (NEW ENGLAND BioLabs$_{inc}$.). The reactions were incubated for 4 h at 37^{o}C for digestion. The digested PCR products were separated on electrophoresis with a 4% (w/v) agarose gel (2% SeaKem® LE Agarose, Lonza, USA and 2% NuSieve® 3-1 Agarose, Lonza, USA) and visualised under UV after ethidium bromide staining.

Table 2.3. Primer sequences and restriction enzymes for PCR experiments.

Marker	Forward and reverse primer	Type	Restriction enzyme
ACP127	5′-AACCATTGCCAACACCAAAT-3′	CAPS	HaeIII
	5′-GCAATTCGTGACCTTGCTTT-3′		
ACI025	5′-CCGCCATAGTGAAATGTGAA-3′	INDEL	-
	5′-TGAAGCTAATGTGGGAGGACA-3′		
ACP273	5′-TTGCAACTGGAATTGACAACA-3′	CAPS	HinfI
	5′-GCAGACTTCAGAGGAAGCAGA-3′		
ACP720	5′-TGCTGGAAACAACACTAGTTGC-3′	CAPS	HaeIII
	5′-ACTCCTGACACAACCCCTTG-3′		

2.13 Polymerase chain reaction

Markers were amplified by polymerase chain reaction (PCR) using 15 µl reactions in an Eppendorf Mastercycler® ep Gradient 5341 (Eppendorf, Germany), using the following conditions for ThermoPrime Taq (ThermoFisher Scientific).

Step 1 Initial denaturation 94^{o}C, 2 min

Step 2 Denaturation 94^{o}C, 30 s

Step 3 Annealing 55^{o}C, 30 s

Step 4 Elongation 72^{o}C, 30 s

Repeat steps 2-4 for 40 cycles

Step 5 Final extension 72^{o}C, 7 min

The 15 µl reaction mixture contained 1x PCR buffer, 200 µM dNTP, 1.5 mM $MgCl_2$, 0.375 U Taq, 0.5 µM each of the primer pair and 1 µl of the template DNA.

2.14 Statistical analysis

Statistical analysis of the data was carried out using Genstat 14th edition (VSN International, Hertfordshire, UK). Correlations between the variates were calculated as Pearson correlation coefficients (r = +/- 0.70 or higher indicates very strong positive relationship, r = +/- 0.40 to +/- 0.69 indicates strong positive relationship, r = +/- 0.30 to +/- 0.39 indicates moderate positive relationship, r = +/- 0.20 to +/- 0.29 indicates weak positive relationship and r = +/- 0.01 to +/- 0.19 indicates no or negligible relationship).

Bland-Altman plots (Bland & Altman, 1986) were used to compare the concentrations of sugars obtained using different assay methods in Chapter 3. In Chapter 4, carbohydrate trait data on high- and low-fructan cultivars was tested by analysis of variance (ANOVA) on each variate at each of the sampling dates. Treatment and blocking structure for ANOVA analysis are as follows:

Treatment structure = Type / (cultivar high + cultivar low),

where :

Cultivar 'high' includes high-fructan cultivars Nasik Red, SWG-N96, 47BK and W202A and cultivar 'low' includes low-fructan cultivars 47AC, 47CB, 47P and Texas Grano 438.

Blocking structure = Replication

Relationships among the variates measured on 'Nasik Red x CUDH2150' F_2 populations in Chapter 5 were explored by principal component analysis (PCA) of trait correlation matrices and differences among populations were explored by canonical variates analysis (CVA).

3. MEASUREMENT OF NON-STRUCTURAL CARBOHYDRATE CONTENT IN ONION BY A HIGH-THROUGHPUT MICROPLATE ENZYMATIC ASSAY

3.1 Introduction

Non-structural carbohydrate (NSC: glucose, fructose, sucrose and fructan) composition and dry matter (DM) content in onion are fundamental quality parameters determining the market suitability for either fresh, storage or dehydration usage (Simon, 1995; Sinclair *et al.*, 1995a; Wall *et al.*, 1999; Vagen & Slimestad, 2008; Zdravkovic *et al.*, 2010; Mallor *et al.*, 2011). There is wide genetic variability in the dry matter content, fructan accumulation and the proportion of other NSCs (Jones & Bisson, 1934; Jones & Mann, 1963; Bajaj *et al.*, 1980; Sinclair *et al.*, 1995a; Sinclair *et al.*, 1995b; Wall *et al.*, 1999; Jaime *et al.*, 2001a; Kahane *et al.*, 2001) stored in onion bulbs, as discussed in Chapter 1 (Section 1.5). DM and soluble solid (SS) contents of high and low DM genotypes are linearly correlated and exhibit a strong positive correlation with fructan content and a negative correlation with reducing sugars (Sinclair *et al.*, 1995b; Vagen & Slimestad, 2008).

High DM onions (>150 g kg^{-1} DM) containing low hexose concentrations are required in the dehydrating industry as they have a direct impact on the energy required for drying and on the colour of dehydrated products. Refractometer (Mann & Hoyle, 1945) and near infrared (Birth *et al.*, 1985) spectrophotometer methods for measuring soluble solids (SS) have been widely used in dehydration onion breeding programmes to indirectly determine the percentage DM in onions (Sinclair *et al.*, 1995a). However, SS or percentage DM does not completely characterise the carbohydrate quality of onions, which governs the quality of the processed products. It is reported that accessions of some high and low DM cultivars exhibited a different NSC profile for a similar DM% (Kahane *et al.*, 2001). Studies on NSC composition in dehydration onions is important as reducing sugars contribute to the Maillard reaction, resulting in non-enzymatic browning of the dehydrated products (Yamaguchi *et al.*, 1975).

In contrast to the requirements of the dehydrating industry, the fresh market demands onion varieties that are rich in reducing sugars and typically sweeter than storage/dehydration onions. Selection for sweet onions is primarily based on their pungency as determined by pyruvic acid content (Schwimmer & Weston, 1961) and sweetness (sugars), along with other health-enhancing parameters such as FOS content, flavonols and antioxidant capacity (Vagen & Slimestad, 2008). Sweet onion cultivars are usually characterised by low pyruvic acid content, low FOS content and greater amounts of glucose and fructose.

Since NSC content and composition is such an important quality trait, breeding and selection for improved carbohydrate content requires analysis of NSC in a large number of onion samples. Various extraction methods and techniques have been described and used for the analysis of NSC in onion tissues. Chromatographic methods such as gas chromatography (Salama *et al.*, 1990) or high performance liquid chromatography (HPLC) using pulsed amperometric (Benkeblia *et al.*, 2004; Shiomi *et al.*, 2008), refractive index (Hansen, 1999; Jaime *et al.*, 2001a; Jaime *et al.*, 2001b; Gennaro *et al.*, 2002; Benkeblia & Varoquaux, 2003) or evaporative light scanning detection (Kahane *et al.*, 2001) are among the most frequently used methods to quantify NSC and fructan oligomers in onions. Though efficient, these methods are not always practical due to the requirement for expensive apparatus, columns and a considerable amount of time input per sample. Colorimetric methods reported for sugar analysis in onions are labour intensive, time consuming and are not suitable in a high-throughput context.

As an alternative for the above mentioned methods, enzymatic methods in combination with microplate screening can be used to measure various sugars. When compared with chromatographic or anthrone colorimetric methods, these methods are simple, rapid, cheap, amenable to automation and do not require aggressive chemicals or expensive apparatus to measure sugar content. Based on the enzymatic method for fructan analysis in food and food products (McCleary *et al.*, 2000), a simple, rapid and inexpensive microplate assay was adapted in this thesis for NSC analysis in onion. Microplate-format fructan assays in onions using Megazyme kits (McCleary & Blakeney, 1999) have been previously reported in onions (McCallum *et al.*, 2006; Yaguchi *et al.*, 2008). In research reported in this chapter, the results on validation and optimisation of these methods for comprehensive analysis of onion carbohydrate composition are reported.

3.2 Results

3.2.1 Comparison of yeast invertase with maltase activity

Table 3.1 presents summary statistics for sucrose and fructan, measured (as per Section 2.5.1 and 2.5.2) in population 4 of 'Nasik Red' x 'CUDH2150' F_2 (Table 3.1) making use of two different sucrose hydrolytic enzymes (Section 2.4.1 and 2.4.2). Mean sucrose level was 75.8 mg g^{-1} DW for maltase measured sucrose and 219.3 mg g^{-1} DW for yeast invertase (YI) measured sucrose. Mean fructan level was 391.6 mg g^{-1} DW for maltase measured fructan and 83.1 mg g^{-1} DW by YI measured fructan. The mean discrepancy (bias) between measurements using maltase and YI was very high: 143.5 mg g^{-1} DW for sucrose and -308.5 for fructans, indicating significant variation in the measurements between these two methods. There was a systematic overestimation of sucrose, with spread in the individual values, by using YI (Figure 3.1A). YI measured fructans were systematically underestimated and showed increased bias with an increase in the mean value of fructan (Figure 3.1B).

Table 3.1. Summary statistics for sucrose and fructan content in onions. Sucrose and fructan concentrations as determined by enzymatic analysis (maltase, invertase) in Population 4 of 'Nasik Red x CUDH2150' cross.

Sample	**'n'**	**Mean**	**Minimum**	**Maximum**	**Difference between means**	**SED**
Invertase Sucrose (mg g^{-1} DW)	189	219.3	113.5	324.2		
Maltase Sucrose (mg g^{-1} DW)	189	75.8	31.5	136.0	143.5	3.5
Invertase Fructans (mg g^{-1} DW)	189	83.1	21.4	198.7		
Maltase Fructans (mg g^{-1} DW)	189	391.6	153.7	571.4	-308.5	7.3

SED represents standard error of difference

n = number of lines

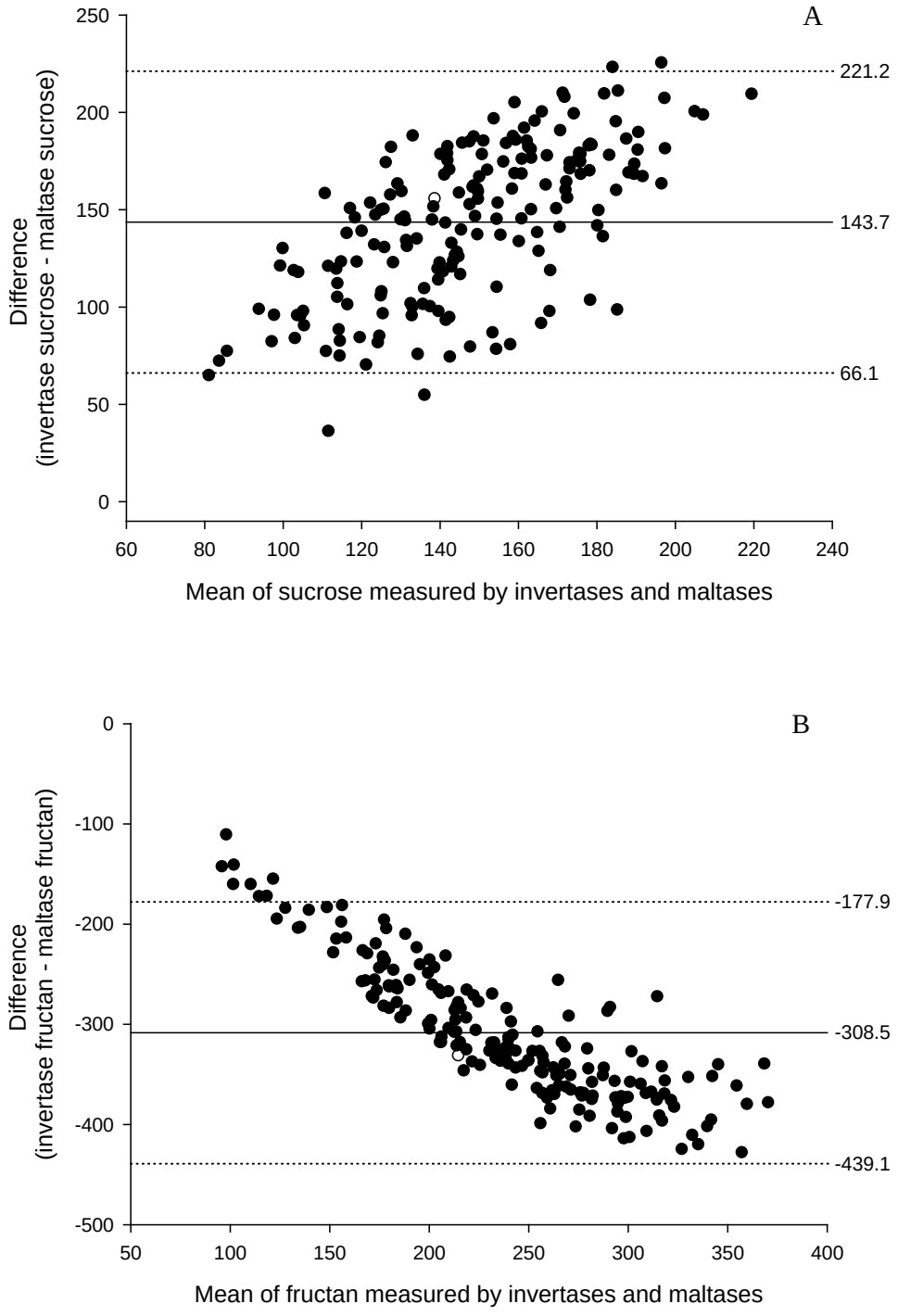

Figure 3.1. Bland-Altman plot for sucrose (A) and fructan (B) measured using two different sucrose hydrolytic enzymes. Bland-Altman plots of differences between sugar measurements by invertases and by maltases versus the mean with bias and upper and lower 95% levels of agreement, in population 4 of 'Nasik Red x CUDH2150' cross.

3.2.2 Standard addition analysis

Standard addition was used as confirmatory analyses for using maltase as a sucrose hydrolytic enzyme in the microplate enzymatic assay in onions. Recovery of pure sucrose at multiple concentrations showed a linear recovery response (R^2 = 0.9982, Figure 3.2, line A) with up to 99.21% recovery. Standard addition to a test onion sample (randomly selected from population 4 of 'Nasik Red x CUDH2150' cross), having a sucrose concentration of 0.137 mg ml^{-1}, showed a linear recovery response (R^2 = 0.9995, Figure 3.2, line B) with acceptable recoveries of 88-103%.

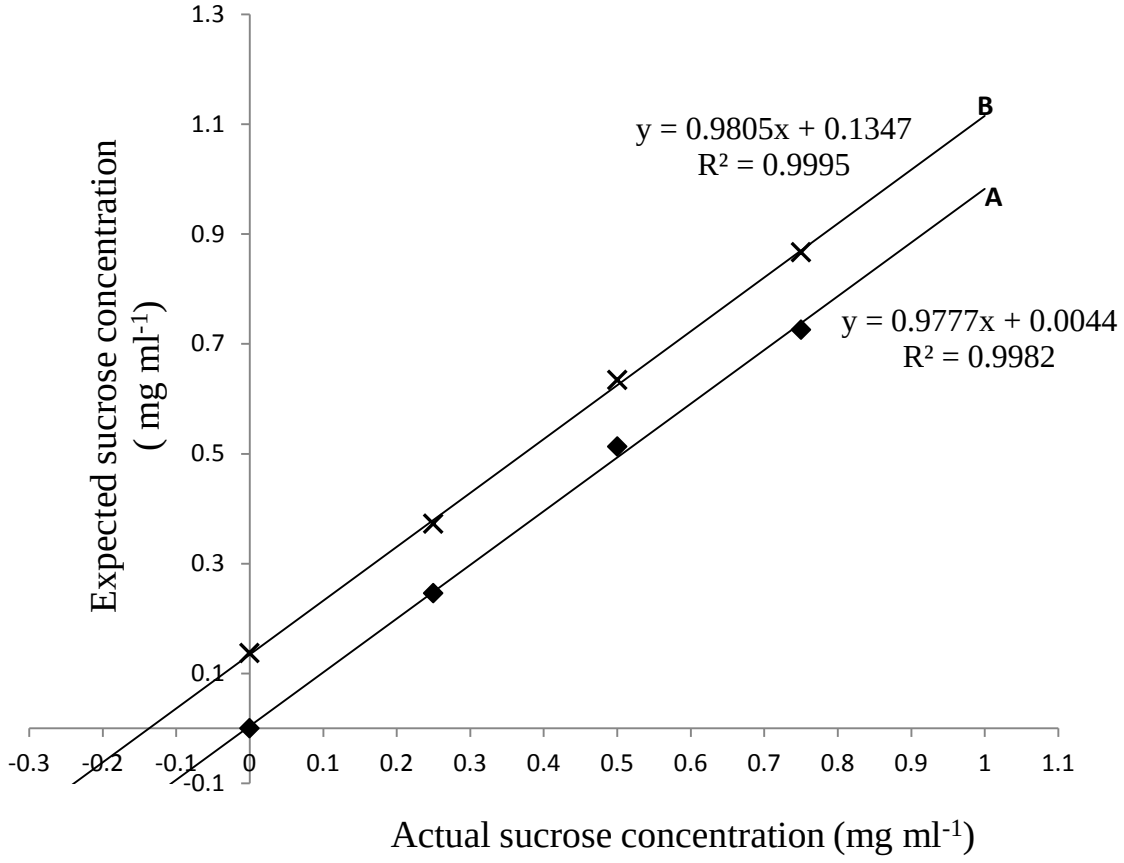

Figure 3.2. Standard addition plot for the determination of sucrose in an onion test sample. Line A**,** recovery response of pure sucrose at multiple sucrose concentration (◆). Line B**,** recovery response of sucrose in an onion test sample (0.137 mg ml^{-1} sucrose) with different concentration (0.250, 0.500 and 0.750 mg ml^{-1}) of sucrose standard (**x**).

3.2.3 Comparison of HPLC with enzymatic assays

The agreement between the microplate enzymatic method (Section 2.5.1 and 2.5.2) and HPLC-PAD (Section 2.5.3) was explored by correlation analysis and inspection of Bland-Altman plots (Bland & Altman, 1999) (Figure 3.3 and 3.4). A very strong correlation was found for measured sugars: fructose (r = 0.92), glucose (r = 0.97), sucrose (r = 0.76) and fructan (r = 0.85). The mean discrepancy between HPLC-PAD and enzymatic microplate measurements was very small for fructose (1.1 mg g^{-1} DW) and sucrose (1.2 mg g^{-1} DW). Individual differences for fructose content were close to zero and showed no systematic difference between the two methods at the lower end of the scale, while a systematic increase in individual differences was noticed at the higher end of the scale (Figure 3.3B). The variability of sucrose levels measured using the two assay methods was systematic (Figure 3.4A).

The bias for glucose content was -5.9 mg g^{-1} DW, suggesting underestimation of glucose by the HPLC-PAD method. There was no systematic bias (Figure 3.4A) in the relationship between the two methods for glucose content. The mean discrepancy between the two methods for fructans was very high (117.9 mg g^{-1} DW). The 95% limits of agreement (LOA) were very wide for fructans (216.4 mg g^{-1} DW, 19.3 mg g^{-1} DW) (Figure 3.4 B).

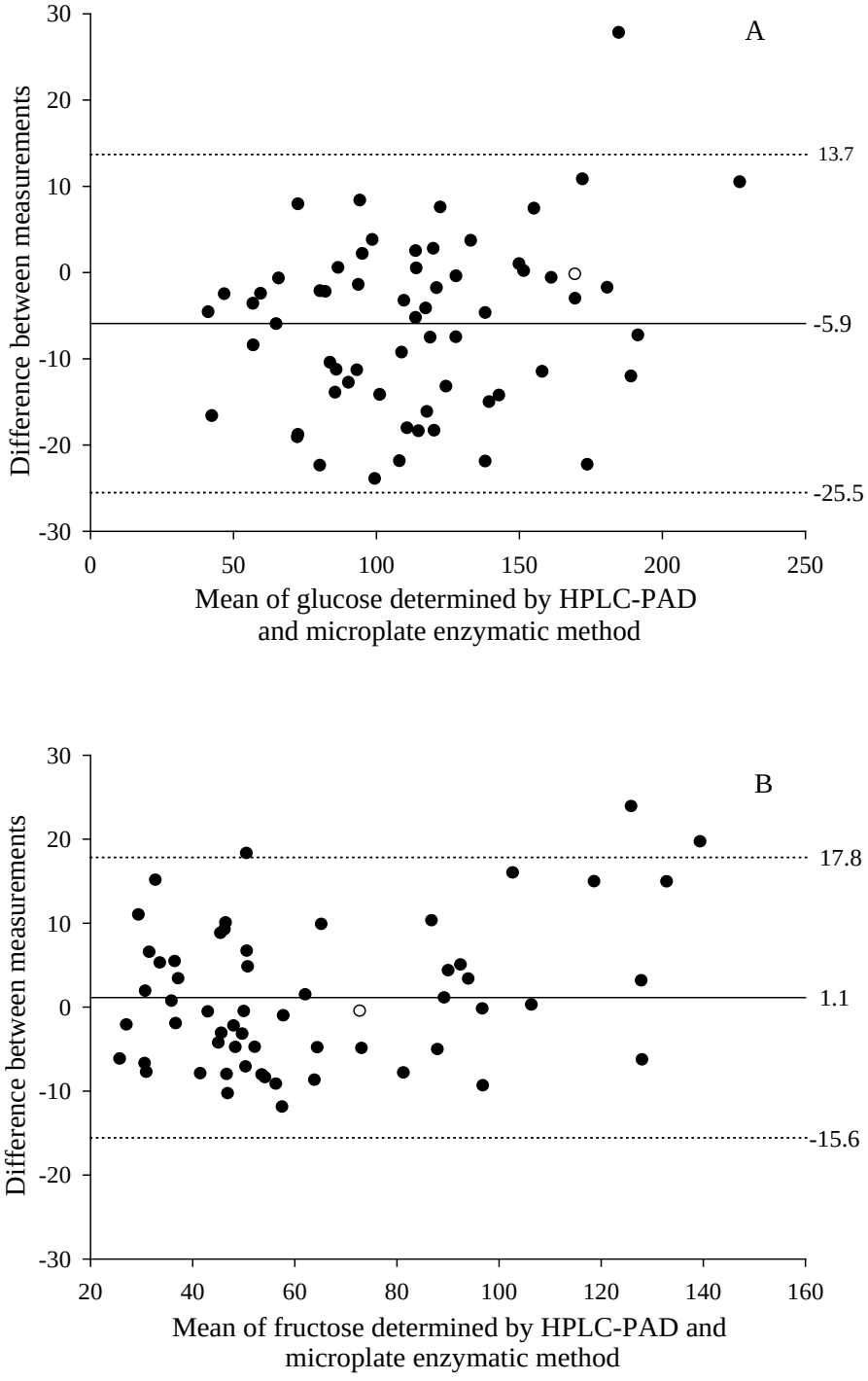

Figure 3.3. Bland-Altman plot for glucose (A) and fructose (B) measured using two different analysis methods. Bland-Altman plots of differences between sugar measurements by HPLC-PAD and by a microplate enzymatic method versus the mean with bias and upper and lower 95% levels of agreement, in 64 randomly selected population 4 of 'Nasik Red x CUDH2150' cross.

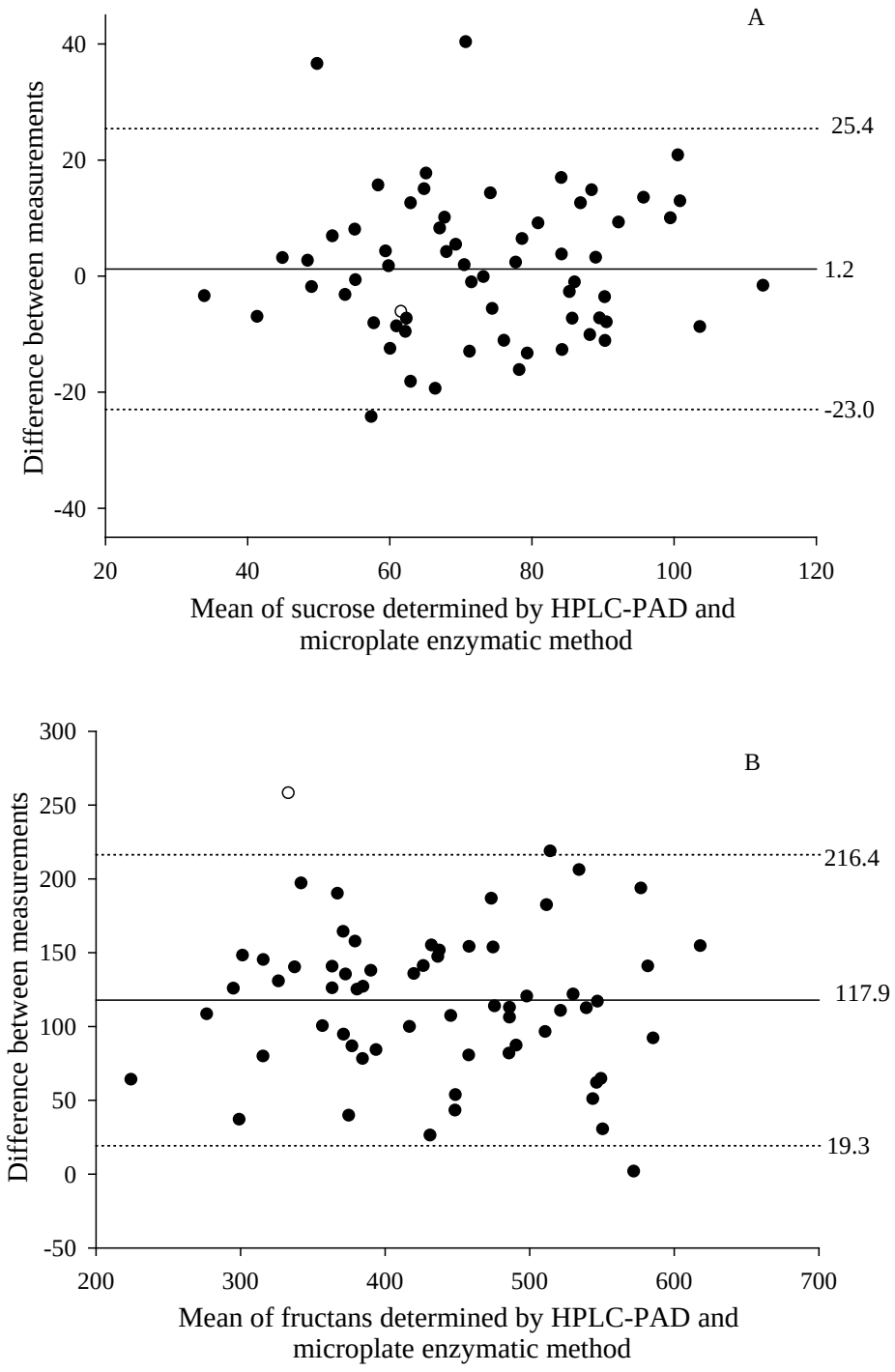

Figure 3.4. Bland-Altman plot for sucrose (A) and fructan (B) measured using two different analysis methods. Bland-Altman plots of differences between sugar measurements by HPLC-PAD and by a microplate enzymatic method versus the mean with bias and upper and lower 95% levels of agreement, in 64 randomly selected population 4 of 'Nasik Red x CUDH2150' cross.

3.3 Discussion

The use of more economical alternatives to the highly specific sucrase/maltase enzyme provided in the Megazyme fructan assay kits (McCleary *et al.*, 2000) which are widely used for fructan analysis in many plant species (Muir *et al.*, 2007), including onions (McCallum *et al.*, 2006), was explored in this chapter. Yeast invertase has been employed in enzymatic assays of sucrose in other species but its application in onions requires validation, as YI has been shown to hydrolyse members of the fructan series in some plant species (Megazyme, 2011). Since onions contains no maltose or starch, commercially available maltase can be used instead of expensive kits or YI for a reliable (Andersen & Sørensen, 1999) and cost effective way of analysing large numbers of onion samples.

The wide segregation for NSC in the 'Nasik Red x CUDH2150' cross provided a means to validate the use of alternative sucrose-hydrolysing enzymes in a microplate enzymatic method over a wide NSC range. From the results (Table 3.1) it is clear that there is lack of agreement between these two methods, suggesting variation in the substrate specificity between these two enzymes. YI hydrolyses fructooligosaccharides (Pontis, 1966; McCleary & Blakeney, 1999) in onions, as a result of which apparent mean sucrose increased while mean fructan concentration declined. As a consequence, it is recommend to use maltase, which is also in agreement with HPLC-PAD (Figure. 3.4A) to estimate the sucrose content in onions.

The individual differences for fructose and sucrose contents measured using the microplate enzymatic method and HPLC-PAD clustered around the mean (Figure. 3.3B and 3.4A), indicating agreement between the two methods. A wide 95% LoA for fructans (216.4 mg g^{-1} DW, 19.3 mg g^{-1} DW) suggests a considerable discrepancy between the microplate enzymatic and HPLC-PAD methods for fructan analysis. HPLC-PAD was successful in providing qualitative measurements of NSC, including FOS in onions. However, the method was limited in terms of resolving power and the accuracy of peak integration, resulting in inaccurate measurements of glucose and total fructan levels.

A standard addition plot (Figure 3.2) for enzymatic reactions showed no interfering matrix in our assays and confirmed that the microplate enzymatic method was highly specific, accurate and reproducible. The requirement of expensive apparatus, reference columns, and time

required per assay, also made the HPLC method less practical for high-throughput analyses. Each run took upward of 60 min to complete and about USD 6.18 were spent on consumables per sample. With only USD 2.65 per sample input cost, the microplate enzymatic method was an economical and practical way for large scale measurements of NSC in onions.

3.4 Conclusions

The microplate-enzymatic assay can be used as a reliable and practicable method for sugar analysis in onion. Maltase enzymes specifically hydrolyse sucrose in onions, providing an alternative tool in place of expensive sucrose and fructan assay kits. Microplate enzymatic methods were in agreement with HPLC measurements of fructose and sucrose measurements, and therefore can be used for large scale screening of onion samples, assisting in genetic analysis of carbohydrate trait in onions.

4. BIOCHEMICAL AND MOLECULAR CHARACTERISATION OF CARBOHYDRATE TURNOVER IN DEVELOPING LEAVES AND BULBS OF HIGH- AND LOW-FRUCTAN ONION CULTIVARS

4.1 Introduction

Fructans, along with other non-structural carbohydrates (NSCs; sucrose, glucose and fructose), form a substantial part of onion bulb dry matter, contributing up to 80% of the total dry weight (Darbyshire & Henry, 1978). Considerable genetic variation in the accumulation of these NSCs has been observed within and among *A. cepa* cultivars (Kahane *et al.*, 2001; Rodríguez Galdón *et al.*, 2009). Fructans, the main reserve carbon in onions, range from around 4% of the bulb dry matter in fresh market types to over 65% in dehydrator onions. It is observed that the cultivars accumulating high levels of fructans are those cultivars characterised by high bulb dry matter percentage, high degree of polymerization (DP) of fructan chains and low levels of reducing sugars. While cultivars with low fructan concentration have low dry matter content, low DP fructans and accumulate greater amounts of monosaccharides (Darbyshire & Henry, 1979; Sinclair *et al.*, 1995a), as described in Chapter 1 (Section 1.5).

Because fructan content contributes much towards the dry matter of the bulb, and this influences storage quality and the quality of the processed products, understanding the molecular mechanisms behind variable fructan content and the accumulation of other NSCs in onions is essential for the genetic improvement of the crop. Fructans in onions are synthesised by the activities of two fructosyltransferase enzymes, 1-SST and 6G-FFT (Vijn *et al.*, 1997; Vijn *et al.*, 1998; Ritsema *et al.*, 2003; Weyens *et al.*, 2004), as described in Section 1.3.4. Studies on NSC accumulation and enzyme activities during the growth of high- and low-solid onion cultivars have shown differences in their total sugar levels, sugar composition, and fructosyltransferase activity (Shiomi *et al.*, 1997). It was noticed that during the period of bulb expansion, high-solid onion cultivars exhibited high fructosyltransferase activity and accumulated greater amounts of FOS than low-solid onion cultivars. Fructan content, along with the expression and activity of fructosyltransferases in plants, has been

reported to be regulated by the level of metabolic sugars, mainly sucrose (Vijn *et al.*, 1997; Vijn *et al.*, 1998; Martinez-Noël *et al.*, 2009), developmental signals (Druart *et al.*, 2001) and environmental factors such as drought (De Roover *et al.*, 2000; Garcia *et al.*, 2011) and temperature stress (Hisano *et al.*, 2008).

In fructan-storing plants, sucrose not only acts as a substrate for fructan synthesis but, on reaching a certain threshold level, can also act as a signaling molecule in a signal transduction pathway resulting in fructosyltransferase gene expression (Müller *et al.*, 2000). Continuous illumination and feeding of 5% (w/v) sucrose to non-fructan storing onion leaves have resulted in accumulation of 1-SST and 6G-FFTs mRNA along with the synthesis of FOSs (Vijn *et al.*, 1998; Vijn *et al.*, 1999). Sucrose content has also shown positive correlations with fructan levels in *A. fistulosum*, alien monosomic addition lines (AALs), and in high- and low-fructan inbreds (Yaguchi *et al.*, 2008), suggesting the importance of sucrose metabolic pathways controlling the fructosylsucrose-synthesising activity.

Sucrose metabolism in plants is carried out by three major enzyme families: invertases, sucrose synthase (SuSy) and SPS, all belonging to the glycosyl hydrolase family 32 (GH32). Measurement of invertase (EC 3.2.1.26) activity in the bulbs of developing onion plants has previously shown that the low-fructan onion cultivars have higher invertase activity than high-fructan onion cultivars (Shiomi *et al.*, 1997). However, assignment of the SPS locus on the same chromosome (8) as that of the '*Frc*' locus (QTL conditioning major phenotypic difference between the fructan phenotypes) (McCallum *et al.*, 2006), along with an increase in sucrose levels and SPS enzyme activities in 'FF+8A' (AAL), has suggested a major role for SPS in regulating NSC composition in onions (Yaguchi *et al.*, 2008). Since no significant differences in the activity of SuSy were observed in leaf blades and bases of developing high- and low-fructan inbreds, variation in invertase/SPS was hypothesised to be a determinant of fructan phenotype in onions.

A preliminary study conducted on developing leaf blade and leaf bases of high- and low-fructan cultivars showed variation in NSC composition between the two fructan groups (Appendix 8.2, Figure 8.3 and 8.4). Based on these observations, an integrated study was performed to characterise carbohydrate turnover in developing leaf blade and leaf bases of high- and low-fructan onion cultivars, and to gain an understanding of variability of fructan metabolism in onions. Correlative relationships existing between transcript abundance, enzyme activity and NSC content in tissues of eight different onion cultivars exhibiting

differential fructan accumulation were investigated. The investigations in this chapter provide a basis for identifying major metabolic pathways controlling differential fructan accumulation in onions.

4.2 Results

4.2.1 Genetic variability in NSC composition in onion bulbs

Non-structural carbohydrates in onions were analysed enzymatically as per Section 2.5.1 and 2.5.2. Initial observations of the proportions of NSC in the bulbs of several commercial varieties and inbred lines (Section 2.1.1 to 2.1.6) showed wide genetic variability in the concentration of individual sugars (Table 4.1 and Figure 4.1). The concentrations of fructans in SWG-N96, Nasik Red and 47BK were high and were in the range of 234.4 – 490.9 mg g^{-1} DW. W202A (146.9 mg g^{-1} DW) and Texas Grano 438 (120.7 mg g^{-1} DW) had similar fructan levels, while 47P, 47AC and 47CB exhibited fructan concentrations in the lower range (92.1 to 110.7 mg g^{-1} DW). Based on the fructan accumulation studied over different years (Shiomi *et al.*, 1997; McCallum *et al.*, 2006; Yaguchi *et al.*, 2008) and from the present study, these onion cultivars were classified as 'high-fructan' (SWG-N96, Nasik Red, 47BK and W202A) and 'low-fructan' (Texas Grano 438, 47P and 47AC) types.

The sucrose concentration was very low in high-fructan onion cultivars – SWG-N96 (61.9 mg g^{-1} DW) and Nasik Red (90.1 mg g^{-1} DW). Hexose concentration in low-fructan cultivars ranged from 308.0 to 453.7 mg g^{-1} DW, while high-fructan cultivars exhibited a lower range (91.8 to 368.5 mg g^{-1} DW) of hexoses. DM% of high-fructan cultivars was higher than low-fructan onion cultivars (Table 4.1). High-fructan cultivars showed strong negative correlations between fructan content and the levels of sucrose (r = -0.96), glucose (r = -0.95) and fructose (r = -0.97) (Table 4.2). Fructan content in low-fructan cultivars showed weak correlations with sucrose and hexose sugars.

Table 4.1. Non-structural carbohydrate concentrations in bulbs of eight onion cultivars. The sugar levels are measured as mg g^{-1} DW, and dry matter as percentage dry matter. Values are the mean of three field replicates.

cultivar	Fructan	Sucrose	Glucose	Fructose	Hexose	Total	DM (%)
SWG-N96	490.9	61.9	67.6	24.1	91.8	644.6	18.7
Nasik Red	435.7	90.1	63.1	30.5	93.5	619.3	19.7
47BK	234.5	120.7	189.1	118.9	308.0	663.2	14.0
W202A	146.9	156.0	192.3	176.2	368.5	671.4	12.7
Texas Grano 438	120.7	164.4	229.9	204.6	434.5	719.7	11.6
47P	110.7	155.1	196.7	172.0	368.7	634.5	12.0
47AC	95.0	161.8	200.1	192.0	392.1	648.8	11.5
47CB	92.1	145.8	235.8	217.9	453.7	691.6	12.3

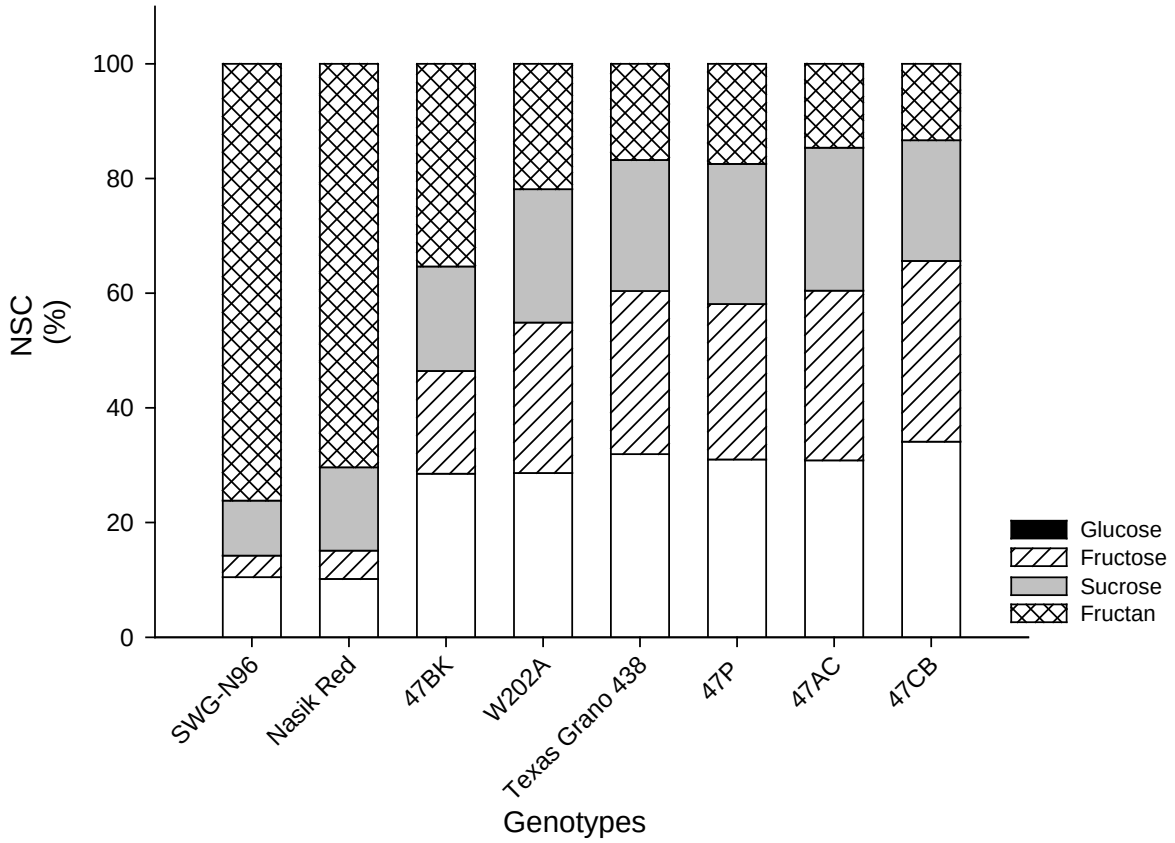

Figure 4.1. Non-structural carbohydrate concentrations as percentage total NSC in the bulb tissues of eight onion cultivars.

Table 4.2. Pearson's correlations, *r*, between the NSC components and DM% of onion cultivars. Correlation values in a) all eight cultivars, b) among high-fructan cultivars and c) among low-fructan cultivars.

		Total	DM	Fructan	Sucrose	Glucose	Fructose
a) All cultivars							
1	Total (mg g^{-1} DW)	1.00					
2	DM (%)	-0.46	1.00				
3	Fructan (mg g^{-1} DW)	-0.39	0.96	1.00			
4	Sucrose (mg g^{-1} DW)	0.43	-0.92	-0.96	1.00		
5	Glucose (mg g^{-1} DW)	0.58	-0.96	-0.96	0.90	1.00	
6	Fructose (mg g^{-1} DW)	0.56	-0.95	-0.97	0.94	0.97	1.00
b) High-fructan cultivars (SWG-N96, Nasik Red, W202A and 47BK)							
1	Total (mg g^{-1} DW)	1.00					
2	DM (%)	-0.63	1.00				
3	Fructan (mg g^{-1} DW)	-0.46	0.93	1.00			
4	Sucrose (mg g^{-1} DW)	0.40	-0.86	-0.96	1.00		
5	Glucose (mg g^{-1} DW)	0.62	-0.96	-0.95	0.85	1.00	
6	Fructose (mg g^{-1} DW)	0.61	-0.93	-0.97	0.93	0.94	1.00
c) Low-fructan cultivars (Texas Grano 438, 47AC, 47CB and 47P)							
1	Total (mg g^{-1} DW)	1.00					
2	DM (%)	0.13	1.00				
3	Fructan (mg g^{-1} DW)	0.45	-0.06	1.00			
4	Sucrose (mg g^{-1} DW)	0.24	0.02	0.07	1.00		
5	Glucose (mg g^{-1} DW)	0.86	0.15	0.16	-0.17	1.00	
6	Fructose (mg g^{-1} DW)	0.80	0.19	-0.11	0.01	0.88	1.00

4.2.2 Total NSC content in developing tissues

Leaf blade and leaf base total NSC were determined during the development of high-fructan (SWG-N96, Nasik Red, 47BK and W202A) and low-fructan (Texas Grano 438, 47AC, 47CB and 47P) onion cultivars (Figure 4.2). The total NSC concentration differed significantly between the leaf blades and the leaf bases, and throughout their developmental stage. Mean total NSC content in leaf blades of low-fructan type cultivars was low at all stages and showed moderately significant differences from mean total NSC of high-fructan cultivars at the first, fifth and sixth sampling stage (Figure 4.2A). Statistically significant differences in total NSC were observed in leaf bases between high- and low-fructan cultivars only at the first ($0.001 < P < 0.01$) and sixth harvests ($0.01 < P < 0.05$) (Figure 4.2B). Overall, the results showed no conspicuous difference in total NSC concentration between high- and low-fructan cultivars in the leaf bases. Mature bulb tissues (before conditioning) of high-fructan onion cultivars showed significantly higher amounts of total NSC than low-fructan onion cultivars.

4.2.3 Sugar concentrations in developing tissues

Analysis of sugar composition in developing onion tissues confirmed fructans as the major NSC component contributing to the variation between high- and low-fructan phenotypes (Figure 4.3). Leaf bases accumulated significantly greater amounts of fructans (17.52 to 80.08 mg g^{-1}DW) than leaf blades (0.39 to 35.84 mg g^{-1}DW). At all growth stages, mean fructan content in tissues of high-fructan cultivars remained higher than the mean fructan content of low-fructan cultivars (Figure 4.3A and 4.3B). Correlation analysis of sugars in leaf blades (Table 4.3) and leaf bases (Table 4.4) revealed strong negative correlation between fructans and fructose content (r = -0.42 and r = -0.57). Fructan levels in leaf blades and bases were very strongly correlated with sucrose content (r = 0.73 and r = 0.46).

Mean sucrose concentration in leaf blades and leaf bases of low-fructan cultivars was significantly lower than high-fructan cultivars at all sampling stages, except at the second sampling stage (where the plants experienced low temperature stress, Section 4.2.6) in both tissue types and at the fifth sampling stage in leaf bases (Figure 4.3B and 4.3C). Leaf blade sucrose was positively correlated with total NSC (r = 0.85) and fructans. Sucrose levels in

leaf bases showed a strong negative correlation with fructose content (r = -0.58) and was moderately associated with the levels of leaf base total NSC (r = 0.37) and fructans.

The general pattern of fructose accumulation in high-fructan onion cultivars was similar to that of low-fructan onion cultivars (Figure 4.3E and 4.3F). Generally, high-fructan onion cultivars accumulated significantly lower amounts of fructose than low-fructan onions. In both tissue types, fructose levels were very strongly correlated with glucose content (leaf blade, r = 0.91; leaf base, r = 0.70).

Significant differences in accumulation of glucose were noticed between the two fructan types (Figure 4.3G and 4.3F). Leaf blade and leaf bases of low-fructan onions accumulated significantly greater amounts of glucose than tissues of high-fructan onion cultivars. High-fructan cultivars showed significant variation among each other for glucose content, suggesting large genotypic differences in sucrose and fructan metabolism within these groups. Throughout the development stages, leaf bases of the high-fructan cultivars (W202A and 47BK) accumulated glucose levels similar to those of low-fructan cultivars and these results are in agreement with the bulb sugar data (Figure 4.1).

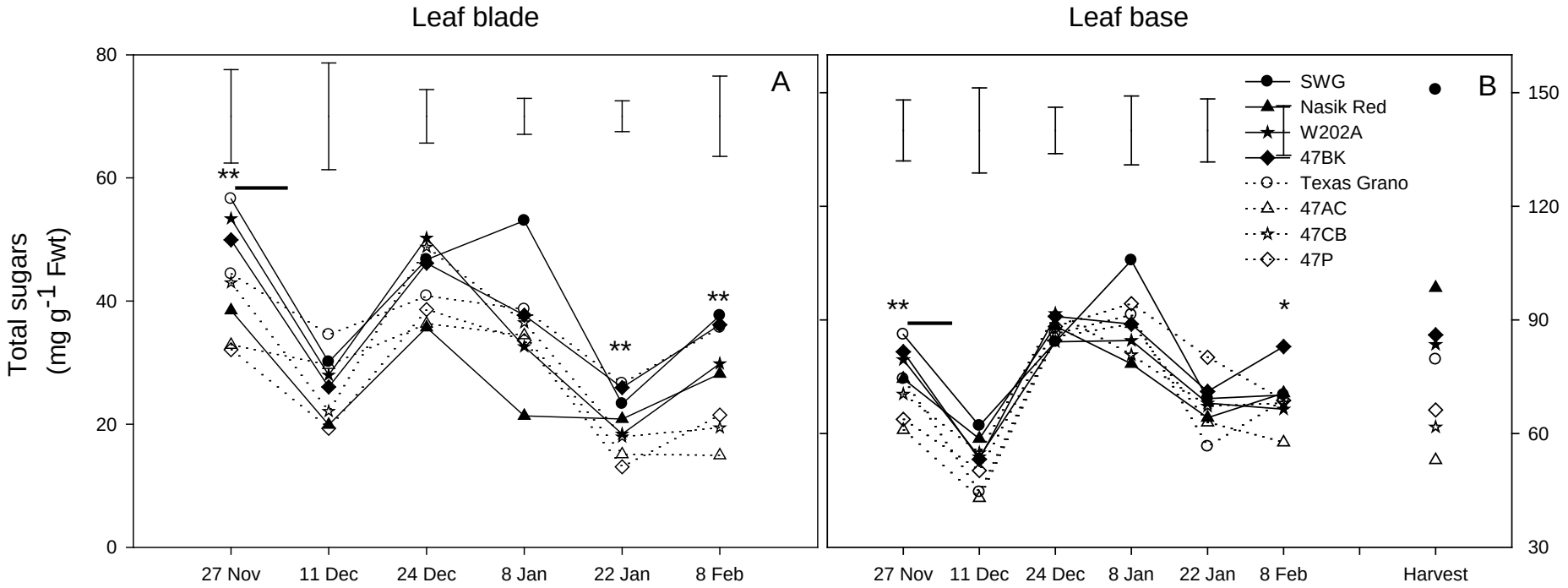

Figure 4.2. **Total NSC content of leaf blade and leaf bases excised from the youngest fully expanded leaf of developing high-fructan (solid symbols) and low-fructan (open symbols) onion cultivars**. The vertical bars represent least significant differences (LSD) at 5% level of probability. Horizontal bar represents the severe cold spell period (28/11/2009 to 4/12/2009) where the mean daily temperature was less than or close to 10^{o}C. Significance levels are represented as: *** ($0.001 < p < 0.01$), ** ($0.01 < p < 0.05$),* ($p > 0.05$).

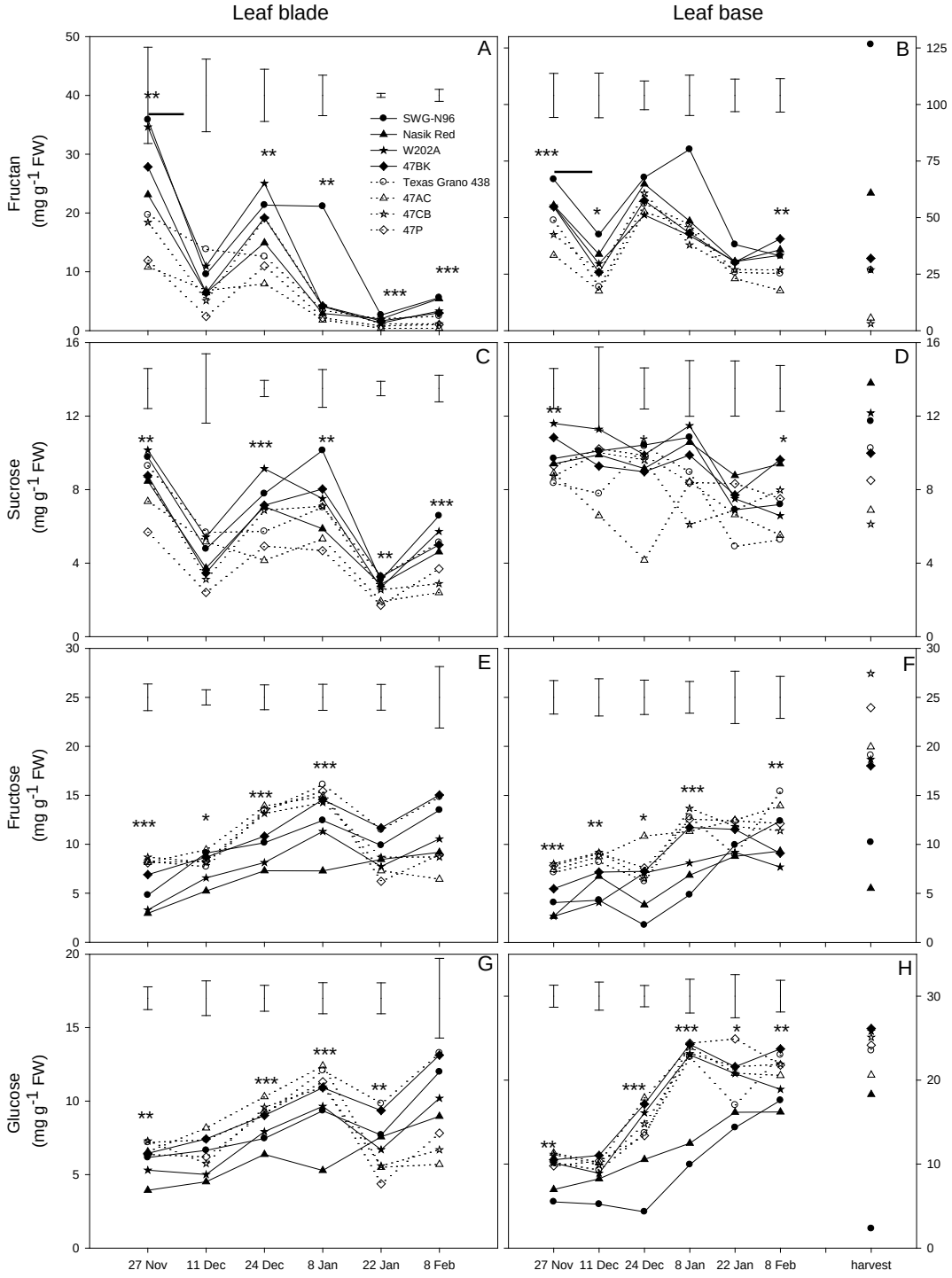

Figure 4.3. NSC concentrations in developing leaf blade and leaf bases of high-(solid symbols) and low-fructan (open symbols) onion cultivars. The vertical bars represent least significant differences (LSD) at 5% level of probability. Horizontal bar represents the severe cold spell period (28/11/2009 to 4/12/2009) where the mean daily temperature was less than or close to 10^{o}C. Significance levels are represented as: *** ($0.001 < p < 0.01$), ** ($0.01 < p < 0.05$),* ($p > 0.05$).

Table 4.3. Pearson's correlations, *r*, between NSC concentration, enzyme activity and transcript abundance in developing leaf blades of eight onion cultivars.

	Total sugars (mg g^{-1} FW)	Fructan (mg g^{-1} FW)	Sucrose (mg g^{-1} FW)	Glucose (mg g^{-1} FW)	Fructose (mg g^{-1} FW)	SPS activity	AI activity	NI activity	SST activity	ACE6067 RQ	ACP041 RQ
Fructan	0.82										
Sucrose	0.85	0.73									
Glucose	0.31	-0.26	0.11								
Fructose	0.15	-0.42	-0.05	0.91							
SPS activity	0.33	0.60	0.32	-0.43	-0.50						
AI activity	-0.05	-0.22	-0.29	0.31	0.43	-0.13					
NI activity	-0.03	-0.05	-0.20	0.12	0.27	-0.08	0.60				
SST activity	0.27	0.26	0.11	0.03	0.05	-0.10	0.30	0.26			
ACE6067 RQ	-0.12	-0.41	-0.05	0.54	0.48	-0.41	0.09	-0.20	-0.16		
ACP041 RQ	0.27	0.41	0.40	-0.30	-0.39	0.51	-0.39	-0.51	-0.15	-0.12	
1-SST RQ	0.37	0.32	0.27	0.10	-0.02	0.25	-0.03	-0.22	-0.02	0.30	0.29

Table 4.4. Pearson's correlations, *r*, between NSC concentration, enzyme activity and transcript abundance in developing leaf bases of eight onion cultivars.

	Total sugars (mg g-1 FW)	Fructan (mg g-1 FW)	Sucrose (mg g-1 FW)	Glucose (mg g-1 FW)	Fructose (mg g-1 FW)	SPS activity	AI activity	NI activity	SST activity	ACE6067 RQ	ACP041 RQ
Fructan	0.87										
Sucrose	0.37	0.46									
Glucose	0.29	-0.19	-0.29								
Fructose	-0.18	-0.57	-0.58	0.70							
SPS activity	0.28	0.31	0.09	-0.03	-0.17						
AI activity	-0.15	-0.34	-0.39	0.34	0.55	-0.18					
NI activity	-0.26	-0.26	-0.30	-0.02	0.22	0.24	0.41				
SST activity	0.34	0.17	-0.25	0.40	0.27	0.22	0.22	0.06			
ACE6067 RQ	0.40	0.09	-0.14	0.74	0.48	0.12	0.25	0.01	0.57		
ACP041 RQ	0.31	0.08	-0.15	0.56	0.35	0.12	0.17	0.06	0.33	0.73	
1-SST RQ	0.44	0.10	-0.19	0.78	0.57	0.18	0.33	-0.04	0.61	0.83	0.67

4.2.4 Sucrose metabolising enzyme activities in developing tissues

The activities of SPS, AI, NI and 1-SST were analysed (as per Section 2.6) to determine if their activities were related to the changes in NSC content and composition (Figure 4.4). Acid invertase activity measured on crude enzyme extracts also exhibited 1-SST activity in onions tissues (Appendix 8.3), because of which enzyme activity measured as μg fructose min^{-1} mg^{-1} protein were considered in the assays (see Section 5.2.4).

SPS did not show any significant difference in its activities between the high- and the low-fructan cultivars (Figure 4.4A and 4.4B). Leaf blades of both fructan types exhibited similar SPS activity at all sampling stages except at the sixth sampling stage, where a significant increase in SPS activity was observed in the low-fructan cultivars. SPS activity in leaf blades was strongly correlated with fructan content (r = 0.60) and hexose sugars (glucose, r = -0.43; fructose, r = -0.50). Sucrose levels, which usually are an indicator of SPS activity, were only moderately correlated with enzyme activity (r = 0.32) in leaf blades. In leaf bases, SPS activity varied among the eight cultivars but showed no significant differences between the two fructan types.

Enzyme activities of AI and NI revealed inverse correlations with sucrose levels in tissues of the eight onion cultivars (Table 4.3 and Table 4.4). AI activities in tissues of high-fructan cultivars were significantly lower than in low-fructan cultivars (Figure 4.4C and 4.4D) throughout the time course of the experiment. NI activities in leaf blades of the high-fructan group were generally lower than in the low-fructan group, but were not statistically different (Figure 4E and 4F). Although there were non-significant differences in leaf base AI activities at a given point in time, the overall experiment indicated significant and tissue-specific differences in AI activities between the two fructan groups. Variation in leaf base AI activity closely corresponded to the differences observed in fructose accumulation between the two fructan groups. A moderate negative correlation between AI/NI and sucrose (AI, r = -39; NI, r = -30) and a strong positive correlation with fructose sugars were observed in leaf bases (Table 4.4). AI and NI activities were positively correlated (leaf blade, r = 0.60; leaf base, r = 0.41).

Leaf blade and leaf bases of high- and low-fructan cultivars showed strong fluctuations in 1-SST activity throughout development. Low-fructan cultivars generally exhibited higher 1-

SST activity than high-fructan cultivars (Figure 4.4G and 4.4H). 1-SST activity in leaf blades of the low-fructan group differed significantly from the high-fructan group at the first and third harvest, while leaf base 1-SST activity varied significantly at the third, fourth and fifth harvests. Glucose level, which is indirectly indicative of 1-SST activity, was strongly correlated with the enzyme activity in leaf bases (r = 0.40). There was a strong correlation between 1-SST activity with glucose and AI activities at some of the sampling stages of each tissue type (prominent in leaf bases at 24/12/2009 and 08/01/2010) (Appendix 8.4).

4.2.5 Transcript analysis of AI and 1-SST genes

Since AI and 1-SST enzyme activities showed significant variation between the two fructan groups, the focus moved to quantifying their relative mRNA levels using quantitative real time (RT)-PCR (Section 2.10). The relative quantities (RQ) of AI homologs (ACE6067 and ACP041) and 1-SST transcripts showed variation between high- and low-fructan cultivars, but failed to statistically separate the two fructan groups at all sampling stages (Figure 4.5). ACE6067 transcript levels were low at the fourth leaf stage but were high during the bulb swelling stage (Figure 4.5A and 4.5B). ACP041 transcript levels were high at the first sampling stage (four leaf stage) in leaf blades of both high- and low-fructan cultivars but decreased at later stages (Figure 4.5C). During the development of leaf bases, ACE6067 and ACP041 transcript levels (Figure 4.4B and 4.5D) followed a similar pattern, but ACE6067 transcript levels were abundant. The RQs of 1-SST in leaf blades were low and fluctuated in their transcript abundance (Figure 4.5E and 4.5F). 1-SST transcript levels in leaf bases increased with developmental stage, exhibiting a similar pattern in both high- and low-fructan cultivars.

Correlation analysis in leaf blades and leaf bases (Table 4.3 and 4.4) showed that transcript abundance of the AI homologs was not correlated with their enzyme activity (leaf blade, r = 0.09; leaf base, r = 0.25) or fructose sugar concentrations, indicating post-transcriptional regulation of these genes. However, the 1-SST transcript level in leaf bases was strongly correlated with enzyme activity (r = 0.61) and sugar level (glucose), indicating transcriptional and tissue-specific regulation of the 1-SST gene. Transcript abundance of 1-SST in leaf bases was strongly correlated with transcript abundance of ACE6067 (r = 0.83) and ACP041 (r = 0.67).

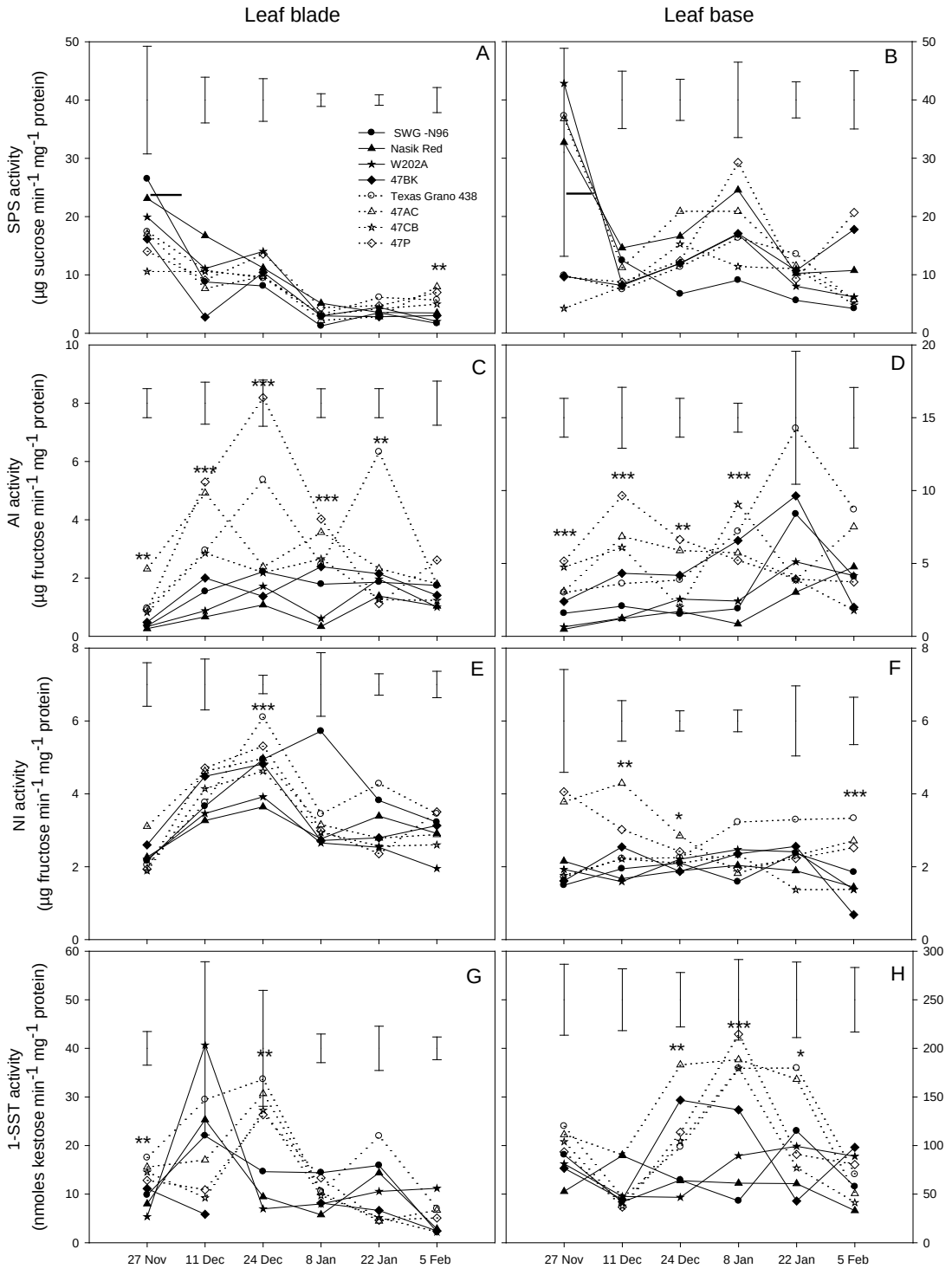

Figure 4.4. Enzyme activities in developing leaf blade and leaf bases of high- (solid symbols) and low-fructan (open symbols) onion cultivars. The vertical bars represent least significant differences (LSD) at 5% level of probability. Horizontal bar represents the severe cold spell period (28/11/2009 to 4/12/2009) where the mean daily temperature was less than or close to 10^{o}C. Significance levels are represented as: *** ($0.001 < p < 0.01$), ** ($0.01 < p < 0.05$),* ($p > 0.05$).

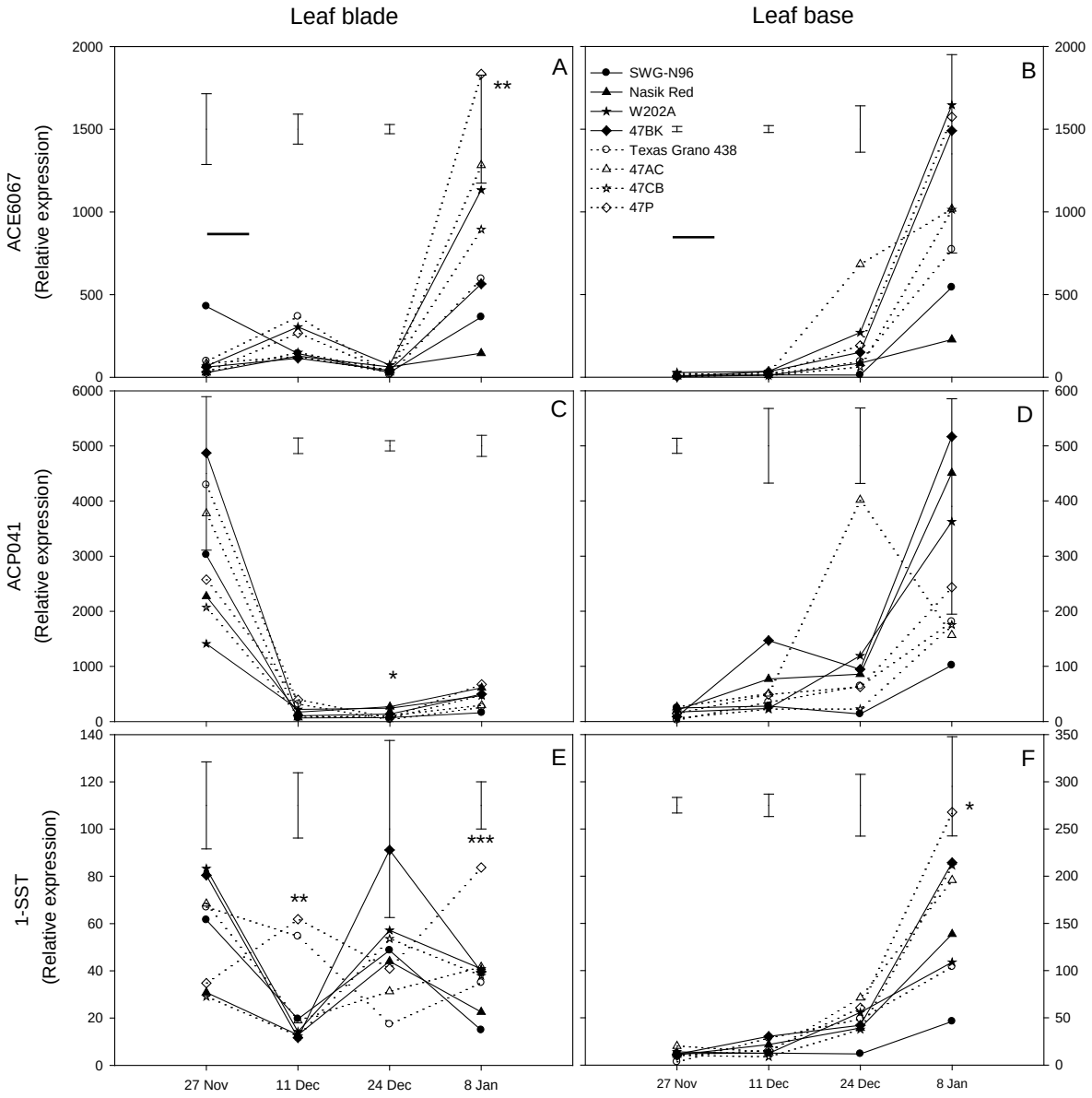

Figure 4.5. **Relative transcript levels of acid invertase and 1-SST genes in developing leaf blade and leaf bases of high- (solid symbols) and low-fructan (open symbols) onion cultivars.** The vertical bars represent least significant differences (LSD) at 5% level of probability. Horizontal bar represents the severe cold spell period (28/11/2009 to 4/12/2009) where the mean daily temperature was less than or close to 10^{o}C. Significance levels are represented as: *** ($0.001 < p < 0.01$), ** ($0.01 < p < 0.05$),* ($p > 0.05$).

4.2.6 Effect of low temperature on NSC traits

During the experiment, the plants experienced severe cold and frosty temperatures between 27/11/2009 and 05/12/2009, affecting the measured carbohydrate traits in developing tissues of high- and low-fructan cultivars. The mean daily temperature was seen to be less than or close to 10^{o}C during this period (Appendix 8.1). Irrespective of the fructan group, the total NSC concentrations in all eight onion cultivars declined markedly at the second sampling stage. The fructan content was low in both leaf blade and leaf bases (Figure 4.3A and 4.3B). Sucrose content in leaf blades of all eight cultivars dropped simultaneously at the second harvest, while leaf bases showed a varying pattern of sucrose accumulation between these eight onion cultivars at this sampling stage (Figure 4.3C and 4.3D).

Both high- and low-fructan cultivars followed a similar pattern in the activities of SPS, AI, and NI activities (Figure 4.4). The SPS activities in leaf blade and leaf bases declined markedly in both high- and low-fructan cultivars (Figure 4.4A and 4.4B), while AI showed an increase in its activity after experiencing the cold-stress period (Figure 4.4C and 4.4D). Generally, NI activities increased with low temperature stress in both tissue and fructan type in onions (Figure 4.4E and 4.4F). 1-SST activities in leaf bases of both high- and low-fructan cultivars (except Nasik Red) declined at second harvest, but 1-SST activities in leaf blades exhibited varying patterns between and within high- and low-fructan group (Figure 4.4G and 4.3H).

4.3 Discussion

A major drawback in breeding and selection for desired NSC content in onions is the lack of knowledge of the biochemistry and genetics of differential fructan accumulation in onions. The NSC content of onions is a multigenic trait and is under the influence of several QTLs associated with sucrose and fructan metabolism. *Frc* on chromosome 8 conditions most of the phenotypic effect on onion bulb composition (McCallum *et al.*, 2006), but the huge genome size (Arumuganathan & Earle, 1991; Ricroch *et al.*, 2005) and limited genetic resources complicate the dissection of this region. To assist genomic study of the *Frc* region, a combined biochemical and molecular approach was carried out in this chapter to identify the important carbohydrate metabolic genes/enzymes affecting the NSC composition in onions.

Since phenotypic differences in fructan accumulation and NSC composition has been previously reported in onions (Bajaj *et al.*, 1980; Galmarini *et al.*, 2001; Jaime *et al.*, 2001a; McCallum *et al.*, 2001; O'Donoghue *et al.*, 2004; McCallum *et al.*, 2006; Vagen & Slimestad, 2008), we analyzed the NSC content and DM% in bulbs of eight onion cultivars varying in fructan content. Fructan levels accounted for most of the variation among the eight onion cultivars (Figure 4.3A and 4.3B) and to ease the statistical analysis in characterisation studies they were grouped as high- or low-fructan types. Nasik Red, SWG-N96, 47BK and W202A were grouped in the high-fructan type, while 47AC, 47CB, 47P and Texas Grano 438 were grouped in the low-fructan type. W202A had similar levels of NSC composition as that of 'Texas Grano 438', but based on previous reports (McCallum *et al.*, 2006; Yaguchi *et al.*, 2008) it was classified as a high-fructan cultivar. Population studies has earlier revealed that W202A is not fixed at the *Frc* (McCallum *et al.*, 2007). It is assumed that the heterozygous nature of W202A at many loci and selections over years may have resulted in genetic drift of this cultivar towards a low-fructan phenotype. Total NSC content of mature bulbs did not show any significant variation among the two fructan groups, in contrast to previous reports (Darbyshire & Henry, 1979), where total NSC was reported to increase with fructan content. As shown by Sinclair *et al*. (1995a) fructans showed a strong positive correlation with bulb DM% and a negative correlation with others sugars, but the relationship was only limited to the high-fructan group (Table 4.2).

Fructosyltransferases (1-SST and 6G-FFT) make use of vacuolar sucrose to synthesize β (2-1) and β (2-6) linked inulin and inulin neo-series type of fructans in onion cells. Since sucrose is the principal form of translocated carbon and the main substrate for fructan synthesis, the level of sucrose loading from photosynthetic sources, and the activity of sucrose metabolic pathways (including degradation and re-synthesis) both affect fructan levels in onions. Variation in the significance and strength of correlations between the matrices of high- and low-fructan cultivars observed in the present study are likely due to the variation in these processes of carbohydrate metabolism (Kahane *et al.*, 2001).

4.3.1 During development, NSC composition and not total NSC varied among tissues of high- and low-fructan cultivars

During development, changes in total NSC content in tissues of high- and low-fructan cultivars overlapped each other and did not show any considerable difference between the

two fructan groups (Figure 4.2). However, the cultivars of the two fructan groups did vary in the levels of individual NSC components (Figure 4.3). In tissues of high fructan accumulating cultivars, high-fructan levels were accompanied by high sucrose content and lower levels of hexoses, confirming earlier reports (Kahane *et al.*, 2001; Vagen & Slimestad, 2008; Galdon *et al.*, 2009). High sucrose levels have previously been reported to induce fructosyltransferase gene expression and enzyme activity, enhancing fructan levels in onions (Vijn *et al.*, 1997; Vijn *et al.*, 1998). Strong positive correlations between sucrose and fructan levels in the leaf blades, along with significant differences in sucrose accumulation in tissues of both fructan phenotypes, clearly indicate that sucrose has a regulatory role in fructan metabolism.

4.3.2 Sucrose metabolism modulates fructan accumulation in onions

Sucrose is synthesised by the activity of SPS with sucrose phosphatase and also by the activity of sucrose synthase. Investigation of SPS activity in tissues of high- and low-fructan cultivars did not show any significant difference between the two groups (Figure 4.4A and 4.4B). No correlation was observed between SPS activity and sucrose levels in leaf blades and leaf bases of the eight fructan cultivars. These results are in contrast to a previous report where an FF+8A- alien monosomic addition line accumulating higher amounts of sucrose exhibited high SPS activity (Yaguchi *et al.*, 2008). High sucrose levels in inbred high-fructan *Frc_* onions also indicated SPS as the possible contributing factor for differential fructan accumulation. However, result for SPS activity and sucrose accumulation in this study enable clear rejection of this hypothesis. From this study it is inferred that the higher SPS activity in the FF+8A line than the control line FF is probably due to a dosage effect of having an extra chromosome - thus SPS activity is not a major determinant of fructan phenotype in onions.

Vacuolar sucrose available for fructan synthesis is controlled by the activity of vacuolar invertases. Soluble acid invertase activity was noticed to be significantly high in tissues of low-fructan cultivars than in the cultivars belonging to high-fructan group (Figure 4.4C and 4.4D). During development, cultivars in the high-fructan group had low (Shiomi *et al.*, 1997) and relatively constant acid invertase activity in leaf blades. The sugar data showed that throughout development, hexose levels were significantly high in low-fructan cultivars (Figure 4.3E-F) and were significantly correlated to AI activity. Relative transcript levels of acid invertase homologues ACE6067 and ACP041 did not vary between the two fructan groups and showed no linear relationship with acid invertase activities. Hence it is suggested

that post transcriptional regulation of AIs, and not transcript levels of AI genes, regulate variation in sucrose and hexose concentration in onions.

Sucrose-hydrolysing invertases are post-translationaly regulated by small proteins (<20 kDa) belonging to a large family of pectinmethylesterases-related proteins (PMEI-RPs) (Krausgrill S *et al.*, 1998; Rausch & Greiner, 2004; Jin *et al.*, 2009). Studies on the role of inhibitors from fructan (Kusch *et al.*, 2009b) and non fructan-accumulating plants (Greiner *et al.*, 1998; Greiner *et al.*, 1999; Link *et al.*, 2004) have shown that inhibitors of invertases are likely to regulate only AIs and not fructan active enzymes (1-SST, 1-FFT, 1-FEH I and 1-FEH IIa) (Kusch *et al.*, 2009b). A direct connection between transcript levels of potential C-DNA-encoding AI inhibitors and the inhibition of invertase activity has been reported in coffee genotypes varying in sucrose accumulation (Privat *et al.*, 2008). Expression of an acid invertase inhibitor (CiC/VIF) in fructan-storing *Cichorium intybus* has been related to reduced sucrose hydrolyses during the fructan accumulation phase of chicory taproots (Kusch *et al.*, 2009b). Study on silencing of the invertase inhibitor INVINH1 have revealed post-translational elevation of cell wall invertase activity resulting in increasing hexose levels in tomato (Jin *et al.*, 2009). Accumulation of high sucrose levels in tissues of high-fructan onion cultivars exhibiting low acid invertase activity throughout development suggests the possibility of such an inhibitor-regulated mechanism in onion and is worthy of further investigation.

Neutral invertase activities (reflecting cytoplasmic invertase activity) did not vary between the two fructan groups. Neutral invertase activities in leaf blades and leaf bases were similar in high- and low-fructan cultivars (Figure 4.4E and 4.4F). Mean neutral invertase activity was low and almost constant in leaf bases of the high-fructan group during development, as previously reported by Lercari (1982). Hexose sugars did not show strong a correlation with NI activities, suggesting a limited role of these enzymes in regulating differential sucrose levels between high- and low-fructan cultivars.

Sucrose levels are known to regulate fructan metabolism in plants. Apart from being the substrate for fructan synthesis, they are also known to play a major role in starting the signal transduction chain of events required for inducing fructosyltransferase gene expression. Several signaling molecules, such as protein kinases (PKs), protein phosphatases (PPs) - phosphatase type 2A, small GTPases and phosphatidyl inositol 3-kinase are reported to be involved in up-regulation of sucrose-mediated fructosyltransferase activity (mainly 6-SFT) in

fructan storing plants (Martinez-Noël *et al.*, 2006; Martinez-Noël *et al.*, 2007; Kusch *et al.*, 2009a; Martinez-Noël *et al.*, 2009; Ritsema *et al.*, 2009). It has been noticed that sucrose feeding to leaf blades of wheat and barley can induce the expression of 1-SST and 6-SFT (enzyme involved in catalyzing the formation and extension of (2-6)-linked fructan) by 3 and 20 times respectively (Müller *et al.*, 2000; Martinez-Noël *et al.*, 2001). Transcript levels of 1-SST in leaf bases of the high- and low-fructan onion cultivars strongly correlated with 1-SST activity, suggesting transcriptional regulation of the 1-SST gene. This finding is supported by reports in grasses (Luscher & Nelson, 1995; Luscher *et al.*, 2000; Lasseur *et al.*, 2006). However, a weak correlation between 1-SST activity and 1-SST transcript abundance with fructan levels, and higher 1-SST activity in low sucrose accumulating low-fructan onion cultivars suggest that the differential sucrose had a limited role in inducing 1-SST gene expression and activity in onions.

During the bulb swelling stage, 1-SST activity and its transcript levels showed a strong positive correlation with acid invertase transcript levels, its enzyme activity and hexose sugar content. These results are in agreement with the sugar data and northern blot analysis in developing chicory roots, where an increase in 1-SST expression and activity was accompanied by increased acid invertase expression and activity (Druart *et al.*, 2001). Sucrose levels did not seem to have a major affect on the 1-SST activity in chicory roots, while developmental signals accompanied with an increase in acid invertase activity just before tuber initiation induced 1-SST gene expression. It has been reported that fructosylsucrose synthase activity in wheat (*Triticum aestivum*) leaf blades can be induced by various sugars, including glucose and fructose (Martinez-Noël *et al.*, 2001). 1-SST and fructan: fructan 1-fructosyltransferase expression levels in hairy root cultures of chicory (*Cichorium intybus*) grown in high-carbon/low-nitrogen medium was reported to vary with the carbon source used in the medium (Kusch *et al.*, 2009a). Use of fructose, as a carbon source, induced 1-SST and 1-FFT expressions and the results were very much comparable to the expression levels as seen by using sucrose as a carbon source. These results indicate that hexose sugars derived from hydrolysed sucrose by invertase may possibly play a signaling role in inducing fructosyltransferases. Since high- and low- fructan onion cultivars showed significant variation in acid invertase enzyme activity, it is suggested that the hexose sugars may play a signaling role in inducing 1-SST gene expression in onions. Glucose levels, indicative of both invertase and 1-SST activity, were also significantly lower in Nasik Red and SWG-N96. Further experiments are needed to confirm these in onions.

Low-fructan cultivars exhibited high 1-SST activity but accumulated lower amounts of fructans than high fructan cultivars. Previous reports have suggested disaccharide mediated regulation of fructosyltransferases (6-SFT) in wheat and barley (Müller *et al.*, 2000; Martinez-Noël *et al.*, 2001), and the formation of sucrose induced products of 6G-FFT activity in onions and grasses (Vijn *et al.*, 1997; Vijn *et al.*, 1998; Lasseur *et al.*, 2006). Since 6-SFT and 6G-FFT both use 1-kestoses as substrates to form high DP fructans and 6G-FFTs are reported to be post-transcriptionally regulated (Lasseur *et al.*, 2006), we suggest the presence of such a disaccharide mediated signaling pathway in onions. Developing SWG-N96 onion bulbs accumulating high DP fructans have been reported to exhibit high 6-GFFT activity (Shiomi *et al.*, 1997). High sucrose content, accompanied by very low 1-SST activity in SWG-N96, supports our hypothesis of a sucrose-mediated induction of 6G-FFT in onions.

Apart from signaling molecules (PPs, PKs, GTPases etc), regulatory genes such as transcription factors are involved in fructan accumulation in temperate cereals. MYB13 genes (transcription factor genes) transactivated fructosyltransferase (1-SST and 6-SFT) genes in wheat. Sucrose along with stem developmental signals was reported to play a key role in upregulating the signal mediated MYB13 regulatory pathway of fructan synthesis in wheat (Xue *et al.*, 2011). Though the involvement of transcription factors in sucrose and fructan metabolism still remains undetermined in onions, the possibility of involvement of similar regulatory proteins controlling differential fructan accumulation is suggested.

4.3.3 Low temperature during growth affects carbohydrate traits in onions

Plants are frequently exposed to variable environmental stress conditions affecting plant growth, development and ultimately yield parameters. They have developed a series of mechanisms at physiological, biochemical, metabolic and molecular level to combat these environmental stresses (abiotic or biotic). Reduced growth, increased level of abscisic acid (Tuteja, 2007), changes in membrane lipid composition (Wang & Lin, 2006; Li *et al.*, 2008) and accumulation of compatible osmolytes (soluble sugars, sugar alcohols and low-molecular weight nitrogenous compounds) (Chu *et al.*, 1974; Chu *et al.*, 1978; Apostolova *et al.*, 2008; Guy *et al.*, 2008) are some of the adaptive mechanism seen in plants experiencing low temperature stress. During reprogramming of the metabolome at low temperatures, carbohydrate metabolism has been particularly affected in all cold stressed plants (Hannah *et*

al., 2006; Mo *et al.*, 2011). Onions exhibited similar variation in gene expression (Figure 4.5), and enzyme activities (Figure 4.4) affecting carbohydrate concentration (Figure 4.3) at the second harvest (Section 4.2.6) when the plants had gone through a period of low but above zero temperature stress (referred to as cold acclimation phase).

Formation of low DP fructooligosaccharides (from high DP fructans) along with an increase in hexose sugar concentration, has been previously associated with increasing freezing tolerance in plants (Suzuki & Nass, 1988). Low fructan concentrations accompanied by high fructose sugars in onion tissues (at second harvest) suggest adaptation of similar mechanism by onions to combat cold stress. A remarkable drop in fructan levels in onions is possibly due to the increased fructan exohydrolase activity at low temperatures, as previously reported in fructan storing plants (Van den Ende & Van Laere, 2002; Asega *et al.*, 2011; van Arkel *et al.*, 2012). High hexose sugars formed by sucrose or fructan hydrolysis during low temperature stress are known to protect plant tissues by reducing the freezing point of cell-sap by few degrees (Levitt, 1980) and/or by resisting plasmolysis by increasing osmotic pressure within cells.

At low temperature, the decline in sucrose levels in leaf blades of all eight onion cultivars (Figure 4.3C) is attributed to low SPS, high acid and neutral invertase activities (Figure 4.4C to 4.4F). These results are in contrast to the previous reports (Vargas et al., 2007; Usadel et al., 2008) where plants exposed to low temperature treatment exhibited high sucrose content with an increase in SPS activity. High acid invertase and neutral invertase activities noticed in leaf blades at second harvest were similar to the reports of Vargas *et al.* (2007) but were in contrast to Usadel *et al.* (2008) where invertases genes were repressed at low temperatures. Accumulation of 1-kestoses has been reported to increase with low temperature in timothy (*Phleum pratense*) (Thorsteinsson *et al.*, 2002). Transgenic tobacco expressing 1-SST gene accumulated 1-kestoses and showed significant reduction in electrolyte leakage than the wild type under low temperatures stress (-2^{o}C) (Li *et al.*, 2007). Increased 1-SST activity (Figure 4.4 G) and possibly high FEH activity (reducing the fructan levels) in leaf blades of onions may contribute towards accumulation of 1-kestoses and thus protecting plants from freezing damage.

Cold treatment (5^{o}C) of plants with intact leaves showed declined 1-SST activity in the rhizophores, while cold treatment to plants with excised leaves showed no 1-SST activity in the rhizophores, indicating the importance of temperature, photoassimilate production and

translocation of sugars to the sink organs in regulating fructan synthesis (Portes *et al.*, 2008). Reduced 1-SST activity in onion leaf bases (Figure 4.4H) may possibly due to reduced influx of sucrose to these sink tissues at low temperatures. Since the plants were sampled a few days after the severe cold stress (mean daily temperature increased, but night temperature was still below 10^{o}C), the plant response noticed in this study can also be an indicative of a cold stress recovery phase and not just a cold acclimation phase. Studies on plant responses to cold stress under controlled environmental conditions are required to gain a further insight into the variety of mechanisms employed by onion to evade low temperature stress.

4.4 Conclusion

The variation in fructan accumulation between high- and low-fructan cultivars reported in this chapter is mainly due to the variation in sucrose accumulation. SPS activity and gene expression did not vary between the two fructan groups and is excluded as a major control of sucrose levels in onions. Post-transcriptionally regulated AIs caused much of the variation in sucrose content. High sucrose level did not seem to induce 1-SST activity, and are therefore not regarded as the major fructosyltransferase affecting the fructan content. Since AI varied significantly between high- and low-fructan groups, it is hypothesised to be associated with *Frc* and thus further experiments were designed to check their association with *Frc* region in segregation onion progenies. These experiments are presented in the next Chapter 5. Low temperature during plant growth has been shown in this chapter to affect carbohydrate traits in onions.

5. BIOCHEMICAL AND GENETIC ANALYSIS OF NON-STRUCTURAL CARBOHYDRATE CONTENT IN ONION POPULATIONS

5.1 Introduction

Onion (*Allium cepa* L.) is a naturally out-crossing diploid (2n =16) species grown in many parts of the world. It is important for enhancing food flavour and to obtain health benefits (Section 1.2.2 and 1.3). The NSC content in onions forms a substantial part of onion DM (65-80%), as described in Chapter 1 (Section 1.4) and Chapter 4 (Section 4.2.1), and is an important quantitative trait to be considered in onion breeding programs. Genetic improvement of carbohydrate traits through conventional plant breeding can be difficult and slow. However, molecular marker-aided breeding can provide a rapid and versatile tool for genetic studies, assisting in selection and crop improvement of many quantitative traits including NSC.

Linkage mapping and quantitative trait locus (QTL) analysis in onions (Galmarini *et al.*, 2001; Havey *et al.*, 2004; McCallum *et al.*, 2006; Raines *et al.*, 2009) has identified genomic regions affecting carbohydrate traits in onions (Section 1.7.1 and 1.7.2). The genomic region on chromosome 8, named the*'Frc'* locus (McCallum *et al.*, 2006), has a very large effect on the relative levels of bulb fructans and hexoses. This *Frc* region, flanked by ACM033 and ACM235 markers (LOD >6) in 'Colossal x ELK P12' and *A. cepa* x *A. roylei* explained 93% of phenotypic variation for fructan content, while ACM033 and ACABE58 markers encompassing the *Frc* QTL in 'BYG15-123 x AC43' (ACM033 was monomorphic) explained 50.3% of the phenotypic variation for fructans (McCallum *et al.*, 2006). The *Frc* region is mapped in a 20 cM region and does not have tighly linked molecular markers that can be used for marker-aided breeding in any onion cross. High levels of residual heterozygosity in the genetic stocks used for development of these onion mapping populations further complicates sequence assembly, marker development and fine mapping of many QTLs, including the *Frc* locus. In order to overcome these problems for the development of new onion mapping families, making use of the doubled haploid line as one

of the parents is desirable in these open pollinated species. Highly fecund doubled haploid lines in onion (Alan *et al.*, 2003; Alan *et al.*, 2004) can be incorporated into mapping crosses, thus simplifying sequence assembly, marker development and genetic analysis of QTLs.

Phenotypic evaluation of high- and low-fructan onion crosses has previously shown bimodal, and in some cases overlapping bimodal, distribution for the fructan trait in onion bulbs. These studies were conducted on small sized (n = <60) onion families and involved inbred parents with residual heterozygosity. To study the genetic architecture of NSC or any other complex trait, studies on large mapping families from a wide onion cross are required. Phenotyic evaluation in large onion populations will help us understand the interacting loci that govern a given trait.

In this chapter, 'Nasik Red x CUDH2150' F_2 mapping familes previously developed (Section 2.1.7) were analysed for the population structure of fructan and other NSC content. The interaction of fructans with other sugars are reported here. The functionality of the *Frc* region was studied by single marker association studies with the identified factor, acid invertase, from Chapter 4 (Section 4.2.4) in one of the 'Nasik Red x CUDH2150' F_2 family.

5.2 Results

5.2.1 Phenotypic evaluation of NSC in onion populations

The validated enzymatic method (Chapter 3) was used to study the carbohydrate composition of the experimental onion populations. The summary statistics for the concentrations of NSCs along with DM% within the Nasik Red/CUDH2150 population are presented in Table 5.1. The distribution patterns of these carbohydrate components are presented in Figures 5.1 to 5.3.

The concentrations of fructans and other NSCs varied widely within Nasik Red/CUDH2150 populations (Figure 5.1 and Figure 5.2). Fructan concentration was high in the 'Nasik Red' parent, while the low fructan parent 'CUDH2150', accumulated less fructan (Figure 5.1A). The fructan levels in the segregating progenies were generally distributed normally and were seen to slightly skew towards the high fructan parent 'Nasik Red' in population 2, population 4 and population 5 (Figure 5.1A and Table 5.1). Some of the segregated onion progenies also

exhibited transgressive segregation patterns by accumulating greater fructan content than the parental line 'Nasik Red'(Figure 5.1A).

Fructose content in 'Nasik Red x CUDH2150' F_2 families strongly correlated with bulb fructan content (Table 5.2), and exhibited positive skewness towards the low fructan parent 'CUDH2150' (Figure 5.1B). Glucose levels strongly correlated with the bulb fructan content (Table 5.2), but unlike fructose (Figure 5.4A) the individual glucose levels plotted against fructans were more evenly spread (Figure 5.4B). Fructose levels were strongly correlated with glucose levels (Table 5.2). Individual values of glucose drawn against the fructose content was widely spread at the lower end scale (Figure 5.5B). Sucrose levels showed weak to moderate negetive correlation with frunctans (Table 5.2 and Figure 5.5A).

5.2.2 Principal component analysis

Principal component analysis (PCA) was performed to study the correlation structure among components affecting the carbohydrate composition within the 'Nasik Red x CUDH2150' F_2 populations. PCA indicated that the first two principal components accounted for 76–84% of the total trait variation in F_2 families of 'Nasik Red' and 'CUDH2150' (Table 5.3). The first principal component accounting for 57–67% of the total variance was largely represented by reducing sugars and fructans. In the second principal component, sucrose content exhibited the greatest weighting of all the components.

5.2.3 Canonical variate analysis

Differences between the mapping populations were explored by canonical variate analyses (CVA). From the CVA, it is noted that the first two canonical variates accounted for 85.6% of the among-population variance. Grouped scatter plots of the first two canonical variables overlapped but with distinct centers and showed a weak separation between the groups (Figure 5.6A and 5.6B). The first and second canonical discriminant functions were both dominated by loadings from DM content followed by fructose content (Figure 5.6B).

Table 5.1. Means, standard deviation (SD), ranges and skewness for carbohydrate traits in 'Nasik Red x CUDH2150' F_2 populations.

		Nasik Red x CUDH2150 F_2					
Population		population 1	population 2	population 3	population 4	population 4	population 5
Environment		WM 2007/08	WM 2007/08	WM 2008/09	Kimihia 2009/10	Kimihia 2009/10	Kimihia 2011/12
'n' plants/lines		115	112	503	189	197	95
Tissue		bulb	bulb	bulb	bulb	leaf	bulb
Sampling procedure		Freeze dried [b]	Freeze dried [b]	Freeze dried [b]	Freeze dried [b]	Fresh leaf extract [a]	Freeze dried [b]
Fructose	Mean	80.2	57.5	92.3	64.0	3.8	81.29
	SD	33.7	25.4	43.8	34.4	2.5	32.42
	Range	31.2 -159.3	19.9-136.0	21.8-225.6	7.6-206.4	0.01-18.8	33.3-172.9
	Skewness	0.88	1.09	0.86	0.97	1.34	0.96
Glucose	Mean	144.8	95.4	120.6	119.8	4.2	164.7
	SD	22.7	38.7	51.1	42.8	1.9	27.47
	Range	82.9-205.4	19.2-195.7	14.9-253.8	43.6-232.6	1.1-14.1	99.1-223.6
	Skewness	0.02	0.61	0.43	0.51	0.91	0.14
Sucrose	Mean	116.2	87.1	83.0	75.8	10.8	76.53
	SD	19.2	19.9	31.7	20.8	4.0	27.46
	Range	67.3-161.8	43.4-145.3	26.5-213.1	31.5-136.0	3.0-22.9	10.5-148.3
	Skewness	0.07	0.49	1.11	0.26	0.33	0.23
Fructan	Mean	271.9	321.3	343.8	391.6	2.5	318.6
	SD	78.9	83.2	109.9	92.0	1.4	56.4
	Range	106.3-514.2	117.5-532.9	79.6-619.1	153.7-571.4	0.3-8.7	173.4-453.2
	Skewness	0.02	-0.04	0.03	-0.31	1.47	-0.16
DM%	Mean	13.8	15.9	14.8	14.6	-	12.29
	SD	1.3	1.4	2.3	2.3	-	0.99
	Range	10.9-19.9	12.0-18.6	4.8-27.6	9.8-20.5	-	6.9-14.1

WM = West Melton, a= sugars in mg g^{-1} FW, b= sugars in mg g^{-1}D

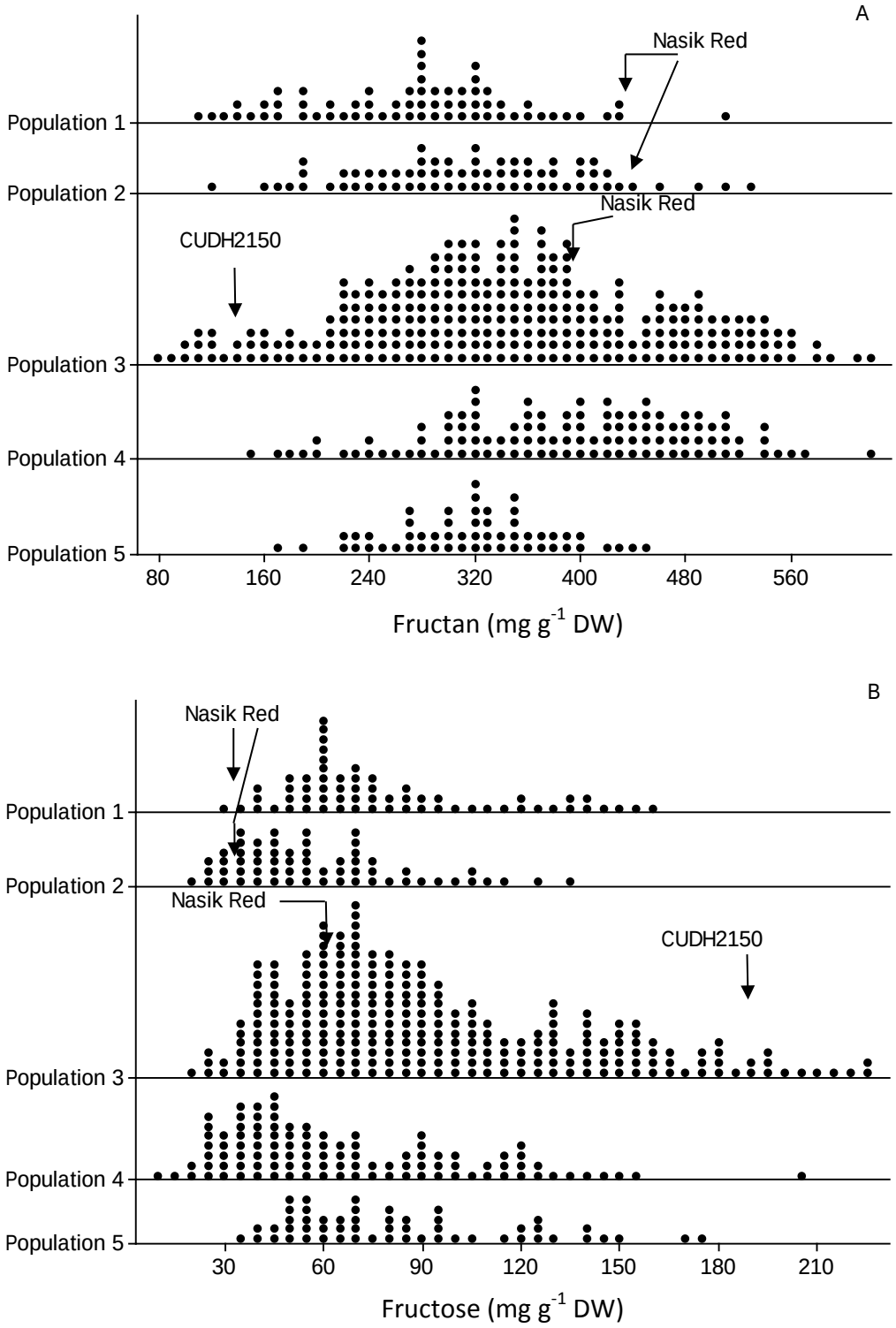

Figure 5.1. Distribution for fructan (A) and fructose (B) content in 'Nasik Red/CUDH2150' crosses. Parental means for bulb fructan and fructose content is indicated by arrows. Each dot represents 2 observations.

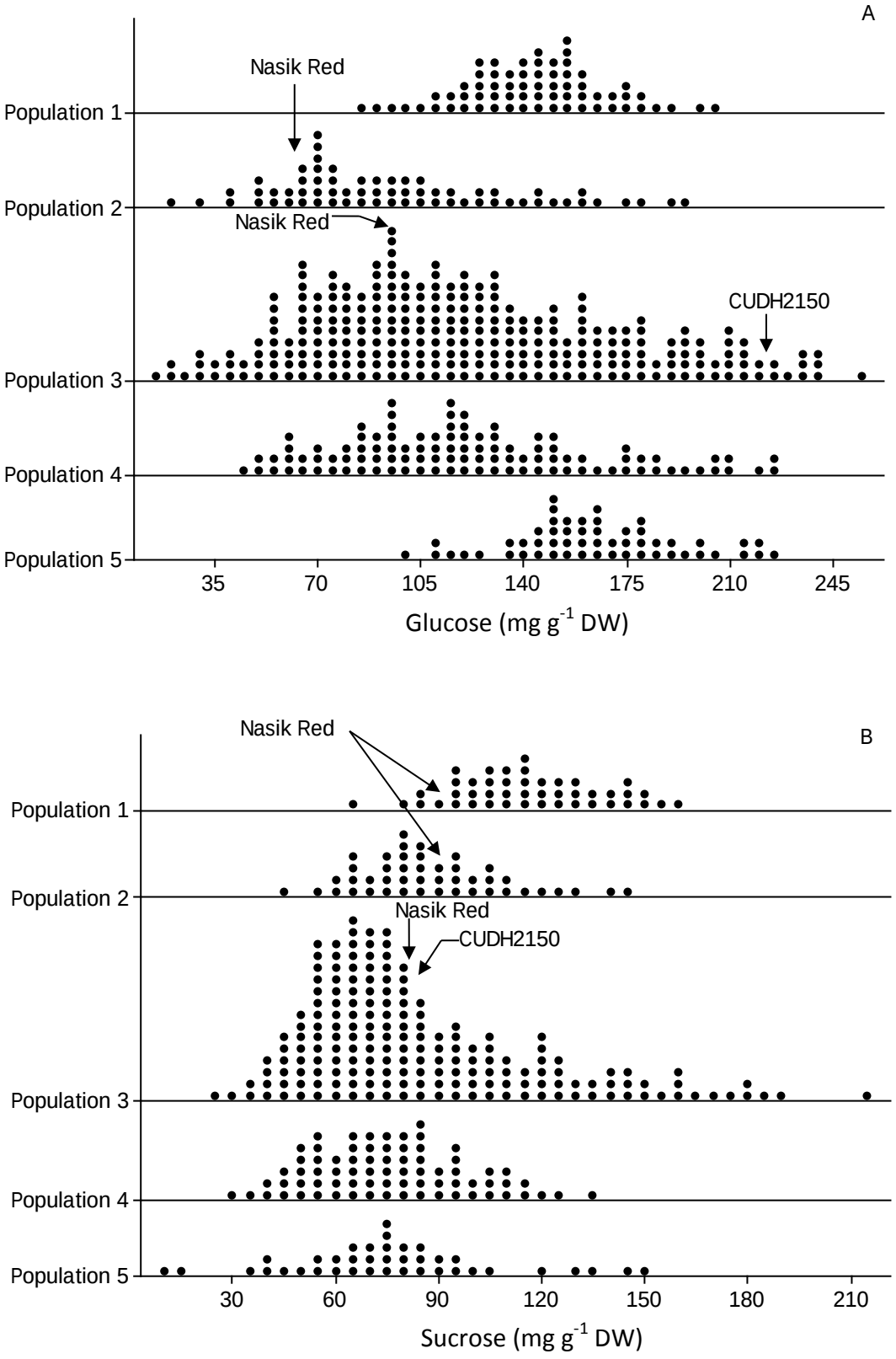

Figure 5.2. Distribution for glucose (A) and sucrose (B) in 'Nasik Red/CUDH2150' crosses. Parental means for bulb glucose and sucrose content are indicated by arrows. Each dot represents 2 and 3 observations, respectively.

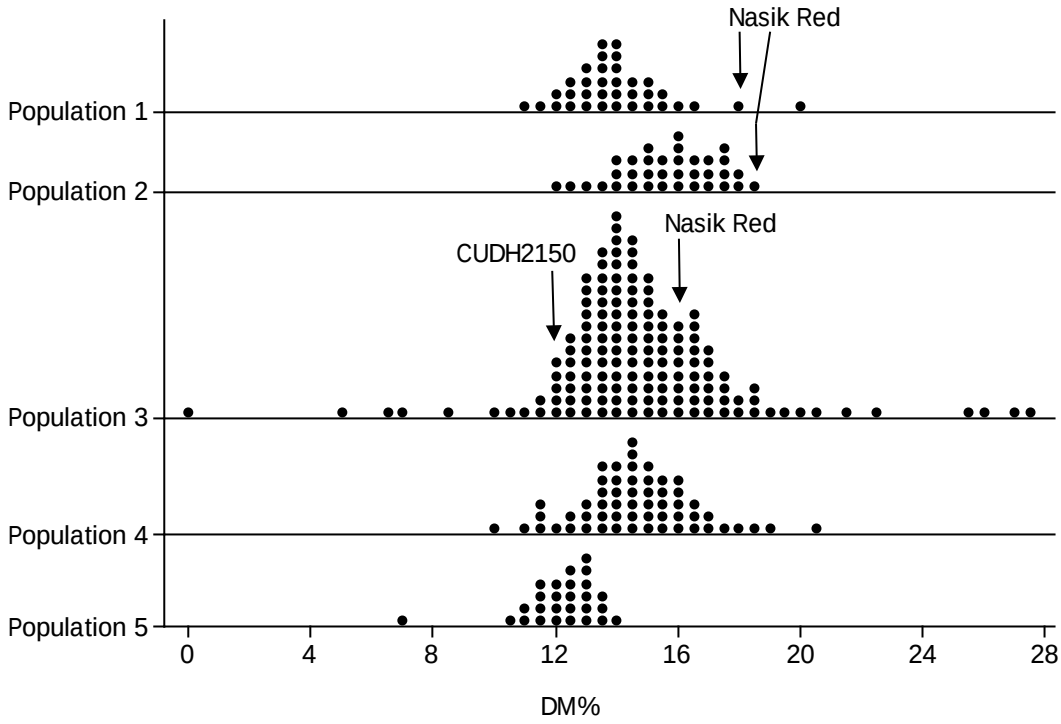

Figure 5.3. Distribution for DM% in 'Nasik Red/CUDH2150' crosses. Parental mean for bulb DM% are indicated by arrows. Each dot represents 4 observations.

Table 5.2. Pearson's correlation, *r*, between the NSC components and DM% in 'Nasik Red x CUDH2150' F_2 families.

		DM%	Fructose	Glucose	Sucrose
Fructose	Population 1	-0.54	-		
	Population 2	-0.47	-		
	Population 3	-0.38	-		
	Population 4	-0.61	-		
	Population 5	-0.40	-		
Glucose	Population 1	-0.37	0.74	-	
	Population 2	-0.63	0.54	-	
	Population 3	-0.43	0.59	-	
	Population 4	-0.61	0.58	-	
	Population 5	-0.55	0.56	-	
Sucrose	Population 1	-0.43	0.48	0.44	-
	Population 2	-0.58	0.68	0.70	-
	Population 3	-0.25	0.20	0.22	-
	Population 4	-0.52	0.21	0.25	-
	Population 5	-0.26	0.18	0.23	-
Fructan	Population 1	0.55	-0.83	-0.57	-0.35
	Population 2	0.54	-0.50	-0.53	-0.35
	Population 3	0.45	-0.76	-0.77	-0.25
	Population 4	0.80	-0.81	-0.79	-0.45
	Population 5	0.47	-0.70	-0.69	-0.34

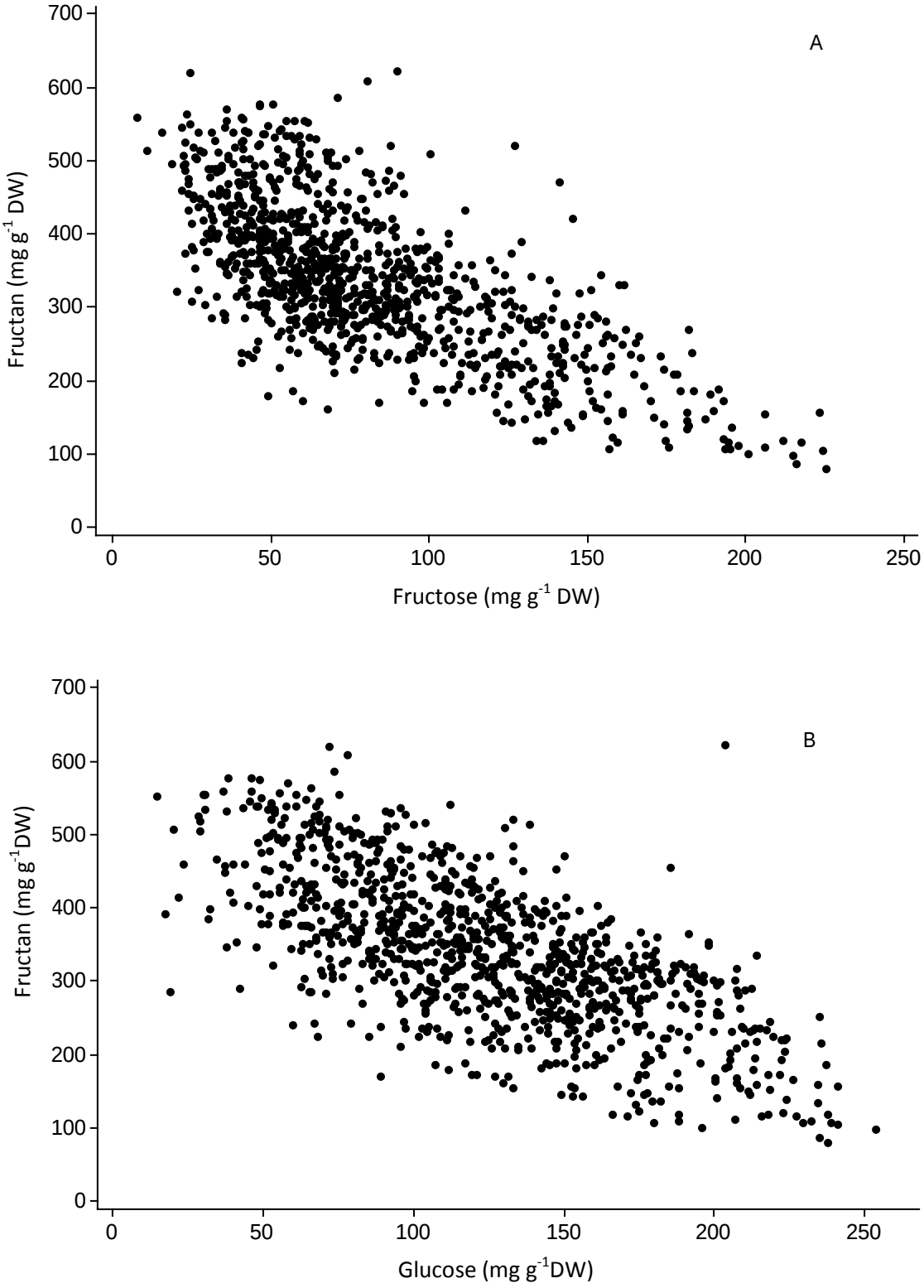

Figure 5.4 Correlation of bulb fructan with bulb fructose (A) and glucose (B) content in 'Nasik Red x CUDH2150' F_2 lines. Scatter plot shows all individual samples from population 1 to population 5.

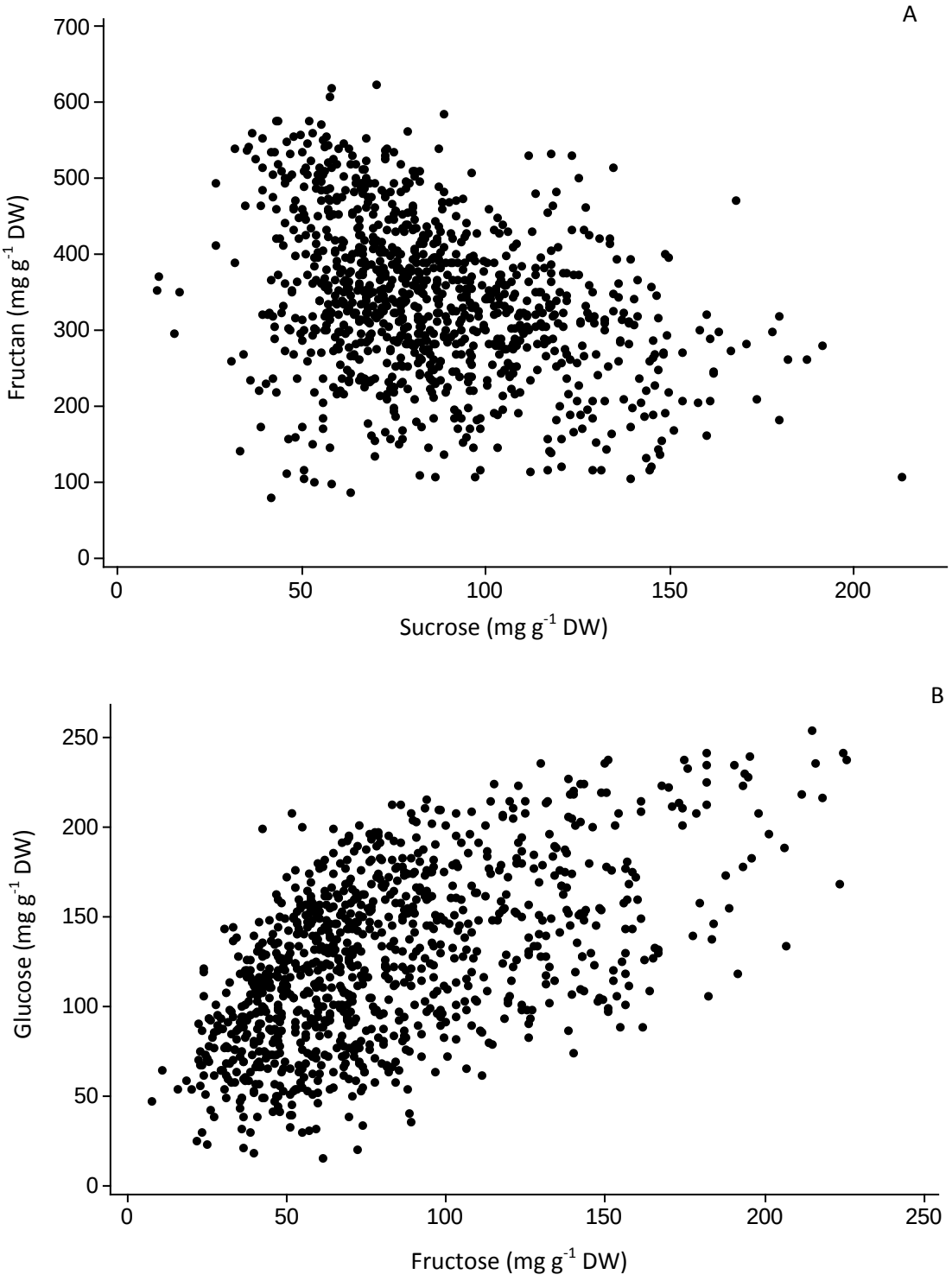

Figure 5.5 Correlation of bulb fructan with sucrose (A), and glucose with fructose (B) in 'Nasik Red x CUDH2150' F_2 lines. Scatter plot shows all individual samples from population 1 to population 5.

Table 5.3. Summary of first two principal components (PC) of bulb carbohydrate composition and DM%.

Population	Population 1		Population 2		Population 3		Population 4		Population 5	
Environment	West Melton 2007/08		West Melton 2007/08		West Melton 2008/09		Kimihia 2009/10		Kimihia 2011/12	
Percentage variation explained by PC1 & PC2	63.1	77.6	64.5	78.2	57.2	75.7	66.7	84.3	63.2	81.7
N plants/lines	115		112		504		189		92	
Weightings of PC1 & PC2	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
DM%	-0.40	0.30	-0.45	0.23	-0.40	-0.22	-0.48	-0.17	0.40	-0.29
Fructose	0.52	0.26	0.44	0.27	0.49	-0.23	0.45	-0.36	-0.53	0.02
Glucose	0.45	0.23	0.48	0.06	0.50	-0.18	0.45	-0.28	-0.51	-0.04
Sucrose	0.36	-0.81	0.47	0.54	0.24	0.91	0.30	0.87	0.23	0.93
Fructan	-0.48	-0.37	-0.40	0.76	-0.54	-0.19	-0.53	0.10	0.49	-0.22

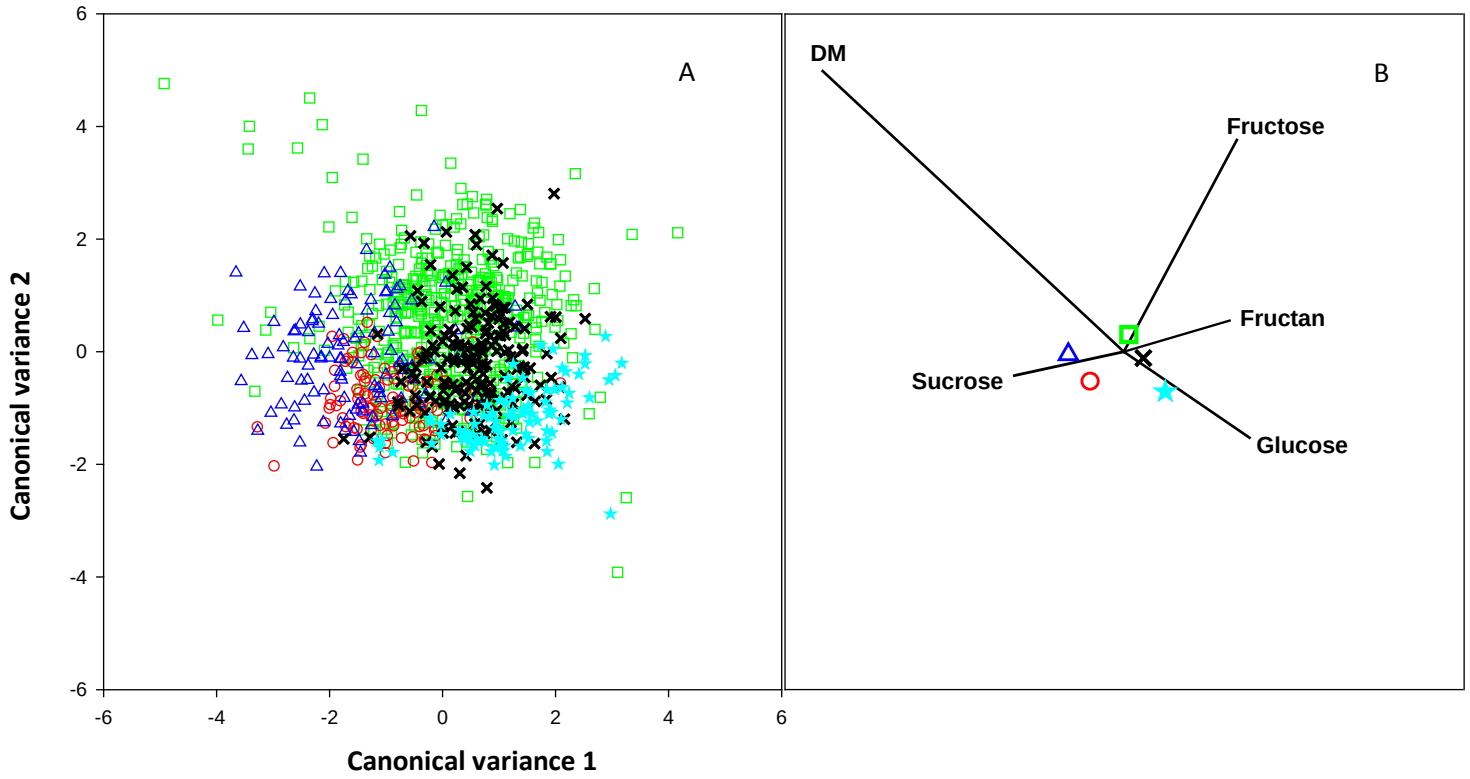

Figure 5.6. Canonical variance analysis scatter plot with 95% confidence region (A) and biplot of NSC and DM% with class means (B) in 'Nasik Red x CUDH2150' F_2 populations: Population 1 (○), population 2 (△), population 3 (□), population 4 (**x**) and population 5 (★).

5.2.4 Biochemical analysis in a 'Nasik Red x CUDH2150' F_2 family

Acid invertase was the major enzyme that differed significantly between high- and low-fructan cultivars of onion, as studied in Chapter 4 (Section 4.2.4). Biochemical analysis of 95 'Nasik Red x CUDH2150' F_2 lines of population 5 (Section 2.1.7 and Table 2.1) was performed to evaluate the role of these acid invertases on NSC accumulation and to determine their possible association with the *Frc* locus (reported in Section 5.2.5).

The distribution pattern and concentration range of NSC of population 5 are shown in Figure 5.1 and Figure 5.2, and Table 5.1. Fructan content across this population was normally distributed (Figure 5.1A) and ranged from 173.4 – 453.2 mg g^{-1} DW. Correlation studies showed that fructan content was highly correlated with hexose sugars (Table 5.2). PCA analysis showed that the first principal accounted for 63.2% of total trait variation and was dominated by loadings from fructan and hexose sugars, while the second principal component accounted for 18.5% of the total trait variation and was dominated by sucrose (Table 5.3).

Acid invertase activities (determined as µg fructose min^{-1} mg^{-1} protein) in bulbs of 'Nasik Red x CUDH2150' F_2 were low and undetectable in many lines. The crude enzyme extracts exhibited substantial 1-SST activity, because of which the enzyme activities measured as µg glucose min^{-1} mg^{-1} protein showed approximately 10 times more activity than that of enzyme activities measured as µg fructose min^{-1} mg^{-1} protein. Acid invertase activities ranged from 0 to 4.24 µg fructose min^{-1} mg^{-1} protein and showed negligible correlation with the amounts of glucose (r = 0.19) and fructose (r = 0.19).While the combined enzyme activity (CEA: acid invertase + 1-SST activity) varied widely ranging from 1.27 to 16.32 µg glucose min^{-1} mg^{-1} protein and showed strong correlation with the amount of fructose (r = 0.45). Weak correlation were notice between this CEA and other sugars (glucose, r = 0.27; sucrose, r = -0.29; and fructan, r = -0.24).

5.2.5 Genetic analysis of *Frc* in a 'Nasik Red x CUDH2150' F_2 family

Genetic markers ACP127, ACI025, ACP273 and ACP720 encompassing *Frc* on chromosome 8 (Baldwin *et al.*, 2012) were evaluated in the phenotyped population 5 of the Nasik

Red/CUDH2150 cross. Segregation analysis of these markers fitted a 1:2:1 ratio supporting phenotypic dominance for high fructans. Single factor ANOVAs revealed that fructose (Figure 5.8B) and CEA (measured as μg glucose min^{-1} mg^{-1} protein) differed significantly across all these markers (Figure 5.7B). ACI025 and ACP273 showed the most significance association with CEA ($P<0.001$) (Figure 5.7B), fructose ($P<0.001$) (Figure 5.8B), glucose (ACI025, $P<0.05$; ACP273, $P<0.001$) (Figure 5.8C) and fructan levels ($P<0.05$) (Figure 5.8A). Mean CEA activity in F_2 lines with homozygous CUDH2150 (low fructan line) alleles at *Frc* locus were higher than the lines with homozygous alleles from Nasik Red (high-fructan parent). This result is in line with our earlier results, where 1-SST activities in the developing leaf bases of low-fructan cultivars were higher than the enzyme activities of high-fructan cultivars (Section 4.3.2). Acid invertase activity (μg fructose min^{-1} mg^{-1} protein) did not show significant association with any of the *Frc* markers (Figure 5.7A). Mean sucrose concentrations in F_2 lines with homozygous Nasik red (XX) and heterozygous (H) alleles for *Frc* markers, were higher than those lines exhibiting CUDH2150 (YY) alleles (Figure 5.8D). Though sucrose levels were high in the high-fructan cultivars, the *Frc* region did not show any significant association with this trait (Figure 5.8D).

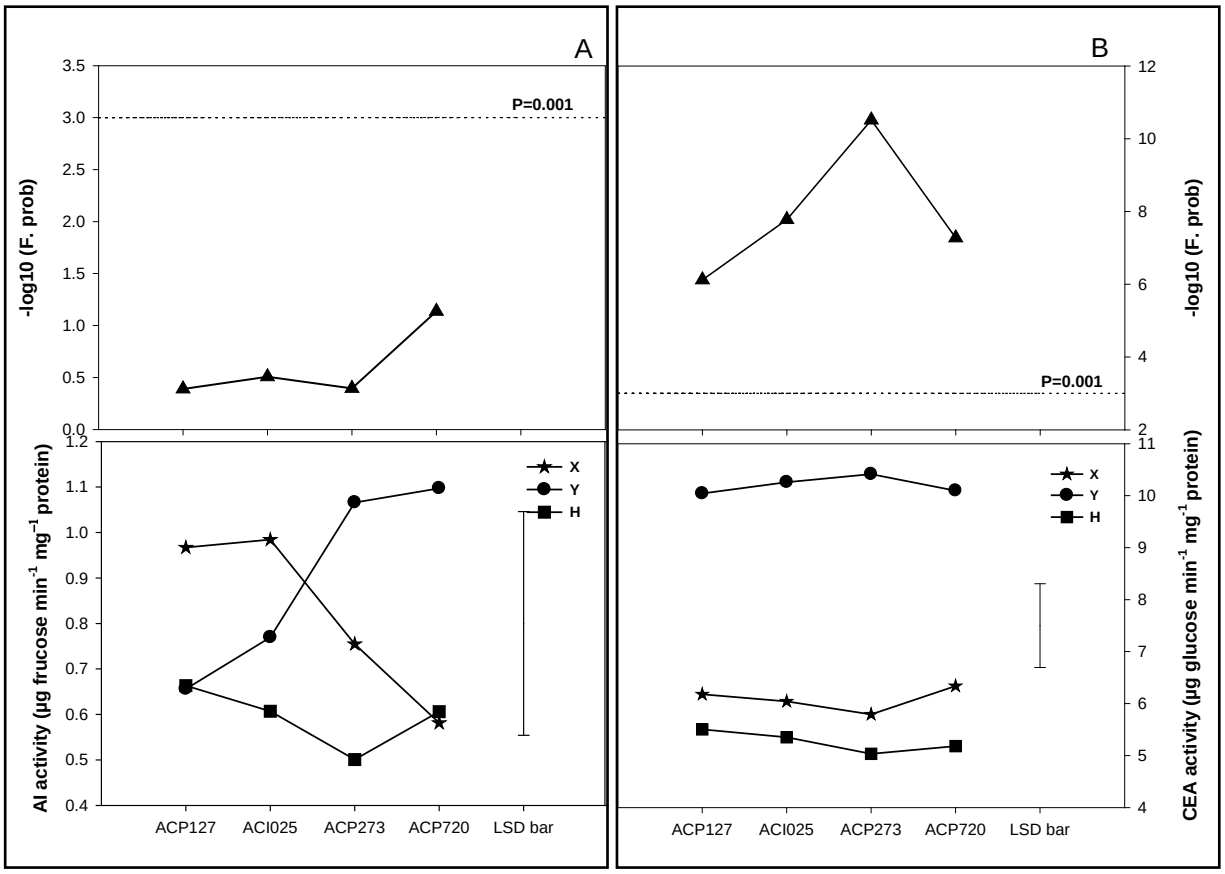

Figure 5.7. Mean enzyme activities for lines with X (Nasik Red), Y (CUDH2150) and H (Heterozygous) alleles for each marker in population 5 of Nasik Red/CUDH2150 cross. A) Acid invertase activity measured as µg fructose min^{-1} mg^{-1} protein B) combined enzyme activities (CEA: acid invertases and 1-SST) measured as µg glucose min^{-1} mg^{-1} protein. Bars are 95% confidence limits for the mean Least Significant difference between X, Yand H. These are the mean over all markers.

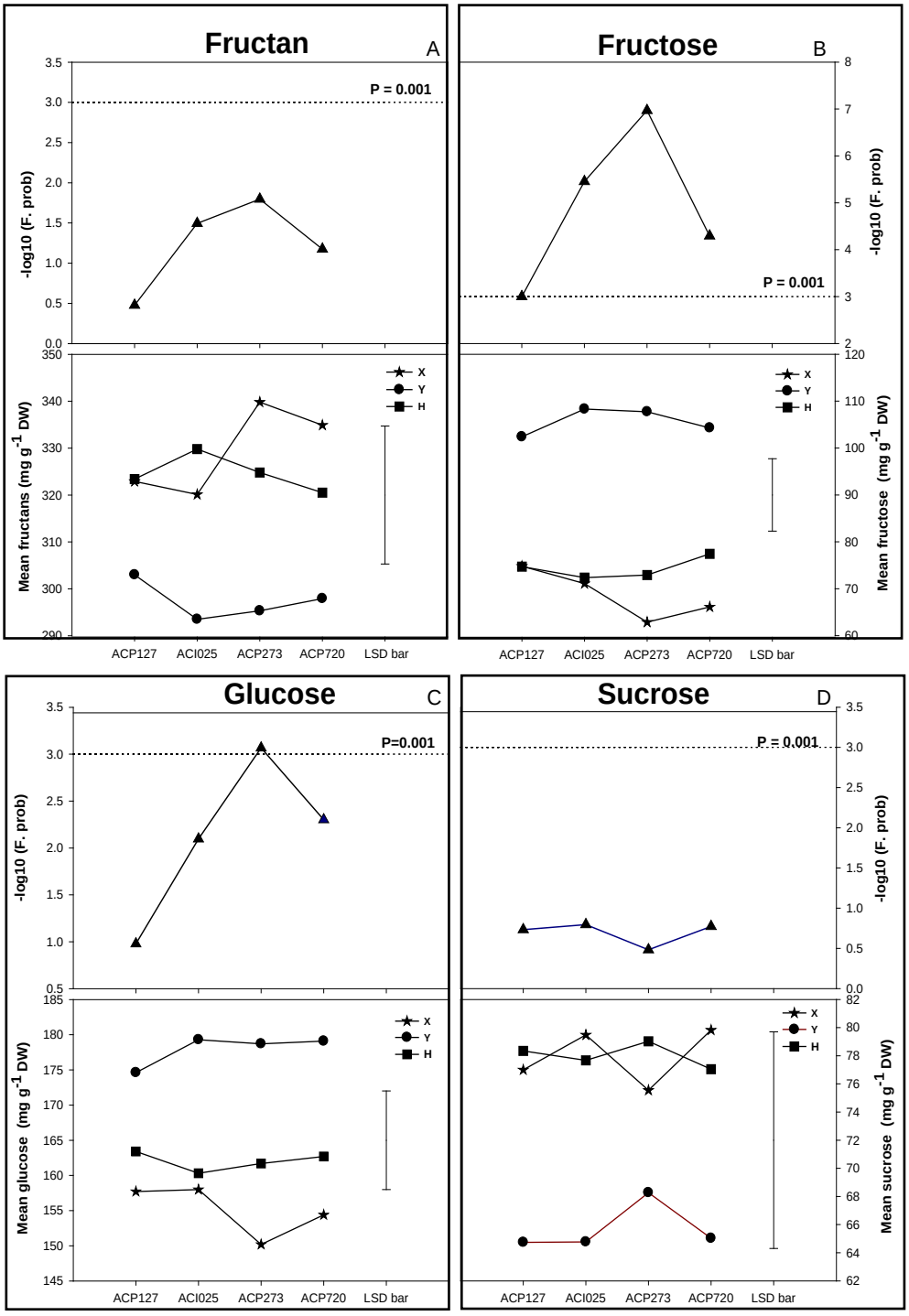

Figure 5.8. Mean sugars for lines with X (Nasik Red), Y (CUDH2150) and H (Heterozygous) alleles for each marker in population 5 of Nasik Red/CUDH2150 cross. (A) Mean fructan, (B) mean fructose, (C) mean glucose and (D) mean sucrose concentrations of X, Y and H alleles for each marker. Bars are 95% confidence limits for the mean Least Significant difference between X, Y and H. These are the mean over all markers.

5.2.6 Comparison of leaf blade sugars with bulb sugars in a 'Nasik Red x CUDH2150' F_2 family

The summary statistics for measured NSC variables in leaf blade and bulb tissues of population 4 of the 'Nasik Red' and 'CUDH2150' cross (Section 2.17 and Table 2.1) are presented in Table 5.1. Leaf analysis showed sucrose (accounting for up to 51% of total NSC) as the major NSC component accumulated during the early stages of the plant growth (Section 2.2.1). Although the fructans were seen to accumulate in leaves, their concentrations were significantly lower than the leaf glucose and fructose concentrations. Leaf glucose levels were seen to strongly correlate (r = 0.96) with leaf fructose levels. In bulb tissues, fructans were the major NSC, accounting for up to 60% of total sugars. Bulb fructan showed a very strong negative correlation with bulb glucose (r = -0.79) and fructose (r = -0.81), and a strong negative correlation with bulb sucrose (r = -0.45) (Table 5.2). Leaf and bulb sugar correlation data showed negligible to weak correlations between leaf and bulb NSC composition (Table 5.4). Studies of the distribution pattern of fructan content showed that leaf fructans were positively skewed, while bulb fructans were negatively skewed (Table 5.1 and Figure 5.1A).

Table 5.4. Pearson's correlations, *r*, between leaf blade and bulb NSC in segregating population 4 of 'Nasik Red x CUDH2150'-F_2 population.

Pearson's correlation (*r*)	Leaf glucose (mg g^{-1} FW)	Leaf fructose (mg g^{-1} FW)	Leaf sucroses (mg g^{-1} FW)	Leaf fructan (mg g^{-1} FW)
Bulb glucose (mg g^{-1} DW)	0.01	0.01	0.00	-0.10
Bulb fructose (mg g^{-1} DW)	0.17	0.18	0.00	-0.27
Bulb sucrose (mg g^{-1} DW)	-0.03	-0.01	-0.07	-0.05
Bulb fructan (mg g^{-1} DW)	-0.03	-0.04	0.05	0.23

5.3 Discussion

The F_2 families used for carbohydrate trait dissection studies in this chapter were developed from parental lines that exhibited contrasting carbohydrate traits. Nasik Red, a highly

heterozygous Indian landrace, was high in bulb fructan content and DM%, and accumulated fructose at levels lower than sucrose and fructan content. In contrast, CUDH2150, a homozygous doubled haploid line, accumulated low fructan content and DM%, and had fructose levels higher than sucrose and fructan content. Studies on families obtained from these crosses are much more useful than previously reported onion crosses, as the parental lines used in previous studies (Galmarini *et al.*, 2001; McCallum *et al.*, 2006; Yaguchi *et al.*, 2008) did not show wide phenotypic variation for carbohydrate traits and also exhibited residual heterozygosity (due to limited selection), thus hindering genomic studies. 'BYG15-23' and 'AC43' used in carbohydrate trait QTL studies (Galmarini *et al.*, 2001; Havey *et al.*, 2004) varied in their fructose, sucrose and FOS content, but unlike 'Nasik Red' and 'CUDH2150', both 'BYG15-23' and 'AC43' had glucose and fructose concentrations higher than sucrose and fructans, limiting the variability in segregating generation. Because 'Nasik Red' and 'CUDH2150' exhibit such high phenotypic variability, F_2 families of the 'Nasik Red x CUDH2150' cross are a very useful genetic resource and have been used successfully for carbohydrate trait dissection studies in this chapter.

5.3.1 Phenotypic evaluation of 'Nasik Red x CUDH2150' F_2 families provide evidence for epistatic gene action

Compared with previously reported segregating onion families (Havey *et al.*, 2004; McCallum *et al.*, 2006; Yaguchi *et al.*, 2009), microplate enzymatic evaluation of NSC in 'Nasik Red x CUDH2150' F_2 families showed wide segregation for fructan (79.6-619.1 mg g^{-1} DW) and other NSC carbohydrates (Table 5.1). Fructan content was normally distributed (with weak skewness towards the high fructan parent in some families), in contrast to the studies of McCallum (2006), which mainly indicated a bimodal segregation pattern for fructan. 'Nasik Red x CUDH2150' F_2 families showed transgressive segregation for NSC and DM%, in agreement with Galmarini *et al.* (2001). Transgressive segregation, along with differences in the distribution pattern of fructan content among F_2 families from the same cross, suggests epistatic gene action along with possible heterozygosity in the genetics of NSC composition within 'Nasik Red'. Fructan content was strongly correlated with fructose content and DM%, as previously reported in segregating onion populations (McCallum *et al.*, 2006; Yaguchi *et al.*, 2008). Concentrations of glucose and fructose correlated with each other, but were not as strongly correlated as seen in other crosses (Havey *et al.*, 2004; McCallum *et al.*, 2006; Raines *et al.*, 2009), suggesting wide variation in sucrose metabolism,

especially in the activity of fructosyltransferases in these 'Nasik Red x CUDH2150' F_2 families. Correlation between sucrose with glucose content varied (Table 5.2), but did not show negative to negligible positive correlation as seen in other studies (Havey *et al.*, 2004; McCallum *et al.*, 2006; Raines *et al.*, 2009), further suggesting variation in sucrose metabolism.

From the results on the loadings of original variables and the percentage variation explained by PCs (Table 5.3), it is clear that the emphasis of PC1 is on the measure of the balance between reducing sugars and fructan, similar to results seen the other mapping populations (McCallum *et al.*, 2006), while the emphasis of PC2 is on the measure of sucrose. The weighting of sucrose in PC1 was in opposite sign to fructans, which is in contrast to earlier reports (McCallum *et al.*, 2006). The first and second canonical discriminant functions were both dominated by loadings from DM% followed by fructose content, suggesting differences in the segregation for loci controlling DM and fructose in onions. Previously, QTL analyses of onion bulb composition have shown that a locus on chromosome 5 conditions DM in several crosses (Raines *et al.*, 2009), supporting these results. Since fructose content has such high weightings in PCA and CVA, and shows stronger correlations with fructan content than any NSC component (Table 5.2), it is suggested that the *Frc* conditioning of much of the phenotypic variation in bulb fructan content is associated with genes regulating sucrose hydrolysis, possibly acid invertases as reported in Chapter 4 (Section 4.5).

5.3.2 Biochemical and genetic analysis of the 'Nasik Red x CUDH2150' F_2

A major gene located to the *Frc* locus on chromosome 8, has previously been reported to affect bulb fructan content in onion (McCallum *et al.*, 2006). Since high- and low-fructan cultivars varied significantly in their acid invertase activities (Figure 4.4C and 4.4D), it was hypothesised (Section 4.9 and 5.3.1) that the *Frc* markers were associated with the altered expression of acid invertase enzyme activity and was investigated in a Nasik Red/CUDH2150 F_2 cross.

Biochemical and genetic analysis of a segregating 'Nasik Red x CUDH2150' F_2 family (Section 5.2.5 and 5.2.4) showed no significant association of acid invertase (as μg glucose min^{-1} mg^{-1} protein) with the *Frc* markers (Figure 5.7A) or correlation with the fructose

content. In contrast to these results, combined enzyme activity (CEA: acid invertase plus 1-SST, Figure 5.7B) showed significant association with the *Frc* markers and exhibited strong correlation with the fructose content. Acid invertases are highly homologous to fructosyltransferases at the gene level and have been reported to exhibit fructosyltransferase activity (Obenland *et al.*, 1993; Vijn *et al.*, 1998). *In vitro* studies using AcT1 (onion acid invertase protein) transformed protoplasts has previously shown to exhibit fructosyltransferase activity under high sucrose (100 mM) concentration (Vijn *et al.*, 1998). Since crude enzyme extracts and 100 mM sucrose was used in our assays, it is not clear what proportions of the enzyme activity measured as μg glucose min^{-1} mg^{-1} protein is represented by acid invertase or by 1-SST enzymes, respectively. If acid invertase has significantly contributed towards fructosyltransferase activity, it is possible that the enzyme activity measured as μg fructose min^{-1} mg^{-1} protein may not be the true representative of acid invertase activity in these studies. To overcome this problem, fractionation of GH32 enzyme activity is further required to resolve the particular enzyme activity associated with *Frc*.

Depending upon the predicted dominance of each of these enzyme activities in CEA we suggest the following two testable hypotheses:

In *situation one*, where CEA is dominated by acid invertases: Significant association of CEA (Figure 5.7B) and fructose (Figure 5.8B) with *Frc* markers suggests that the genes controlling variation in acid invertase activity may be controlled by *Frc.* Acid invertase genes have been previously assigned to chromosome 2 (Yaguchi *et al.*, 2008) and chromosome 3, thus suggesting the presence of acid invertase regulatory genes at the *Frc* locus. Post-translational regulation of acid invertases by small inhibitory proteins belonging to a large family of PMEI-RPs has been reported in plants (Greiner *et al.*, 1998; Greiner *et al.*, 1999; Link *et al.*, 2004; Privat *et al.*, 2008; Jin *et al.*, 2009; Kusch *et al.*, 2009b). These inhibitor proteins have been shown to regulate only acid invertases and not fructan active enzymes (1-SST, 1-FFT, 1-FEH AND 1-FEHIIa) (Kusch *et al.*, 2009b). Relative transcript level of acid invertases genes did not vary between high- and low- fructan cultivars but showed significant variation in the enzyme activity between the two fructan groups (Section 4.2.4 and 4.2.5), indicating post-transcriptional regulation of acid invertases in onions. Based on these results and the strong correlation noticed between CEA and fructose content in this chapter, we suggest the association of *Frc* with acid invertase regulatory genes and is worthy of further investigation.

In *situation two*, where CEA is dominated by 1-SST: Significant association of CEA with *Frc* (Figure 5.7B) markers suggests that the genes controlling variation in 1-SST activity may be controlled by *Frc.* Druart *et al.* (2001) earlier reported an increase in acid invertase and 1-SST enzyme expression /activities during the onset of tuber formation in chicory (*Cichorium intybus* L.). It was suggested that the developmental signals played a significant role in governing the transcriptional activities of these two enzymes in chicory. However, strong positive correlation of CEA activity with fructose in segregating F_2 progenies (harvested at bulb swelling, see Section 2.2.1) indicate that developmental signals along with fructose content (from sucrose hydrolysis) may have a signaling role in regulating 1-SST activity in onions. These results were further supported by strong positive correlations of 1-SST activity with acid invertase activity and fructose levels (Appendix 8.4: Table 8.3, 8.4, 8.7 and 8.8) during bulb swelling stages of high- and low-fructan cultivars, as reported in Chapter 4. Fructose has been previously shown to strongly induce 1-SST in plant tissues (Martinez-Noël *et al.*, 2001; Kusch *et al.*, 2009a), supporting these results. Sucrose mediated induction of enzymes (protein kinases, Ca^{2+} dependent protein kinases, protein phosphatases, small GTPases, and phosphatidyl inositol 3-kinase) involved in signal transduction chain of events resulting in enhanced fructosyltransferase (1-SST and 6-SFT) gene expression has been extensively studied in plants. Though fructose has been shown to induce 1-SST (Martinez-Noël *et al.*, 2001; Kusch *et al.*, 2009a), no studies have been so far conducted to address its role in the signaling process leading to fructosyltransferase gene expression and thus requires further investigation.

Assignment of 1-SST gene to chromosome 6 (Havey *et al.*, 2004), significant association of *Frc* markers to fructose, and high fructose concentration in F_2 lines with CUDH2150 (YY) alleles rather than homozygous Nasik red (XX) at each of the studied *Frc* markers, suggests that *Frc* may underlie either acid invertase regulatory genes or genes involved in fructose-mediated signaling of fructosyltransferase or both, as the carbohydrate metabolism is so closely interlinked in onions.

5.3.3 Leaf blade NSC cannot be used as a phenotypic marker for early selections of bulb NSC traits

Breeding and selection for bulb carbohydrate content in field experiments can be an expensive and tedious task. Any early and inexpensive selection tool in onion breeding

programmes could reduce the overall cost, labour and time spent on each sample. At the pre-bulbing stage, onion leaf blades and bases of high-fructan lines have been reported to accumulate significantly different sugar contents than low-fructan lines (Yaguchi *et al.*, 2008). Based on this report, it was hypothesised that the levels of NSC in developing plants could predict bulb NSC levels. However, there was no correlation between leaf and bulb sugar data that could be used in predicting sugar data at early stages of plant growth. Overall, the result suggests that factors other than *Frc*, conditions leaf fructan levels in onions. For early selections of NSC traits in onions, marker assisted selections may be the only appropriate tool and thus requires more genome-oriented research.

5.4 Conclusion

'Nasik Red x CUDH2150' F_2 families are a very useful genomic resource for mapping studies and physiology in onion. They segregate widely for fructan and other NSC traits and are thus very useful for carbohydrate trait dissection studies. Fructan content as studied in 'Nasik Red x CUDH2150' F_2 families is a transgressive trait and is controlled by the action of many genes including those associated with *Frc*. Genes regulating acid invertases or 1-SST or both has been suggested to underlie the *Frc* locus, and this requires further investigation.

6. GENERAL DISCUSSION AND CONCLUSIONS

Onion (*Allium cepa* L.) exhibits wide variation in fructan and other NSC accumulation (glucose, fructose and sucrose), affecting the quality of the fresh and processed onion produce. Research on onion fructans is becoming increasingly important, as onions are a very good source of dietary fructans which are known to benefit human health (Alexiou, 2010; Marteau *et al.*, 2011; Yen *et al.*, 2011). Fructans are positively associated with bulb DM and are an important quality trait to be considered in many dehydrator onion breeding programs. Fundamental research has provided significant gain in the understanding of fructan synthesis in onions (Vijn *et al.*, 1998; Ritsema *et al.*, 2003; Fujishima *et al.*, 2005), but there is still a large gap in the understanding of the biochemistry behind varying fructan accumulation. QTL analysis in onions has revealed many genomic regions affecting NSC traits in onion, the most notable one being '*Frc*', which has a very large effect on the relative levels of bulb fructan and hexoses (McCallum *et al.*, 2006). Limited genomic resources, especially in terms of the availability of suitable and reliable large mapping onion families, usable sequence information and availability of tightly linked polymorphic markers, have slowed down genomic research of many QTLs, including *Frc* in onions. This thesis describes biochemical, molecular and genetic approaches carried out to investigate variation in fructan and other NSC accumulation using high- and low-fructan cultivars, and segregating onion populations with a homozygous doubled haploid as one of the parents.

6.1 NSC measurements

An important requirement for physiological and genetic studies of NSC trait in onions is the availability of the corresponding phenotypic data. Measurements of NSCs in onions are either kit based or use chromatographic techniques that are usually not accessible in many resource-limited laboratories. These methods are also not readily adapted in a high-throughput phenotyping situation, as they are expensive to perform and require a considerable amount of time (60 min) per assay.

As an alternative to the use of highly specific sucrase enzyme provided in the Megazyme sucrose and fructan assay kits (McCleary *et al.*, 2000), commercially available maltases provided a cost effective way to analyse sucrose and fructan content in onions. Unlike yeast

invertases that hydrolysed both sucrose and fructans in onions, maltases hydrolysed only sucrose in the widely segregating 'Nasik Red x CUDH2150' F_2 cross, and were suitable to be employed in onion sugar assays.

Performing the enzymatic assays in 96 well microplates using maltases, as described in Chapter 2, provided a rapid, reliable and cheap NSC assay method for onions, which was also very much comparable to the HPLC-PAD sugar measurements. HPLC-PAD provided qualitative measurement of fructooligosaccharide in onions, but due to limitations in resolving power (between hexoses and higher oligomers) and inaccuracy in peak integration, fructan levels were overestimated in onions. Considering the research needs, the availability of resources (technical and financial) and reliability, the newly adapted high throughput-microplate enzymatic assay (as described in Chapter 2) was employed in place of expensive enzyme kits or chromatographic methods to obtain NSC data in this thesis (Chapter 4 and Chapter 5).

6.2 Biochemical and genetic analysis of NSC accumulation in onions

Fructans are significantly correlated with bulb DM, which is an important quality trait determining the market niche in onions. A comprehensive investigation was carried out in Chapter 4, to dissect the biochemical and molecular differences in fructan accumulation between high- and low-fructan onion cultivars. The study demonstrated that genotypic differences seen in fructan and other NSC accumulation in onions were determined by differential regulation of sucrose metabolic genes at transcriptional- and post-transcriptional levels. In addition, the study, identified key enzymes, acid invertases and 1-SST, that were not only correlated with variation in NSC accumulation, but their combined enzyme activity showed significant association with the *Frc* locus on chromosome 8, as described in Chapter 5.

Fructans accounted for most of variation in NSC composition between high- and low-fructan onion cultivars, and among 'Nasik Red x CUDH2150' F_2 progenies. They are synthesised in the vacuoles (Wagner *et al.*, 1983) and are stored as major carbon reserve in onions. Sucrose, the main substrate for fructan and hexose synthesis, played a central role in carbohydrate composition. Variation in sucrose metabolism through metabolic pathways governed by SPS,

fructosyltransferases and invertases has been reported in onions (Shiomi *et al.*, 1997; Yaguchi *et al.*, 2008). However these studies characterised a limited range of NSC related traits in a few onion cultivars or under *A. fistulosum* background (alien addition monosomic lines), providing little information on the differential regulation of fructan and sucrose metabolism in onion. In this thesis (Chapter 4), a range of cultivars and NSC traits were studied to identify the factors affecting the varying fructan accumulation in onion.

Sucrose phosphate synthase activity showed moderate correlation to sucrose levels in leaf blades of eight onion cultivars. However, the enzyme activities did not differ between high sucrose accumulating high-fructan and low sucrose accumulating low-fructan groups, clearly rejecting the hypothesis of Yaguchi *et al.*,(2009). SPS is located on chromosome 8, but the results on SPS activities and sucrose accumulation in this thesis (Chapter 4) have demonstrated that *Frc* is not associated with this enzyme activity.

Sucrose: sucrose 1-fructosyltransferase is the enzyme responsible for the synthesis of β-2,1-linked 1-kestoses in onions. The enzyme activities differed between the high- and low-fructan onion groups. In contrast to the report of Vijn *et al.* (1997), high sucrose levels did not seem to significantly increase fructan accumulation, 1-SST mRNA transcript level or 1-SST enzyme activity in high-fructan cultivars. Weak positive correlations between fructan levels and 1-SST enzyme activities and 1-SST transcript levels in the eight cultivars suggested a limited role of this enzyme in differential fructan accumulation. The transcript level of 1-SST showed no correlation with 1-SST enzyme activity in leaf blades (r = -0.02), but had a strong positive correlation (r = 60) in leaf bases. This suggests a tissue specific and transcriptional regulation of 1-SST in onions. Since sucrose meditated formation of 6G-FFT products has been noticed in onions and grasses (Vijn *et al.*, 1997; Vijn *et al.*, 1998; Lasseur *et al.*, 2006), it is hypothesised that the increased sucrose levels in high-fructan accumulating onion cultivars may have a signaling role in enhancing 6G-FFT gene expression and activity, and this requires further investigation. A very low glucose concentration in high-fructan accumulating cultivars, Nasik Red and SWG, supports this hypothesis.

Studies on invertase activities in developing tissues of high- and low-fructan cultivars demonstrated acid invertase as the major sucrose metabolic enzyme that differed significantly between the high- and the low-fructan groups. High-fructan accumulating cultivars showed lower AI activities and this was in agreement with the reports of Shiomi *et al.*,(1997). A strong positive correlation between AI activity and fructose, and a strong negative correlation

between fructose and fructans in both leaf blade and leaf bases suggested that AI is associated with the genotypic variation in fructan accumulation. Based on the correlation studies of NSC traits in high- and low-fructan cultivars, post-transcriptional regulation of AI is reported is suggested.

No significant differences in the activity of neutral invertases between high- and low-fructan groups were observed in this thesis. Neutral invertase activity in leaf bases was low and constant, and is in line with the report of Lercari (1982). A weak correlation between NI activity and hexose sugars and fructan levels indicates a limited role for this enzyme on varying sucrose and fructan accumulation in onions.

'Nasik Red x CUDH2150' F_2 families are a very useful genetic resource to help dissect carbohydrate traits in onions. Compared to previous studies (Galmarini *et al.*, 2001; Havey *et al.*, 2004; McCallum *et al.*, 2006; Yaguchi *et al.*, 2008), the 'Nasik Red x CUDH2150' F_2 families reported in this thesis are large in population size, segregate widely for NSC traits and show high repeatability among the observed traits. With a highly heterozygous cultivar (Nasik Red) as one parent and a homozygous doubled haploid line (CUDH2150) as the other, these F_2 families have been a very useful genetic resource to develop genetic map and enhance marker resources in onions (Baldwin *et al.*, 2012).

Phenotypic evaluation of fructan in 'Nasik Red x CUDH2150' F_2 families has showed normal distribution and transgressive segregation patterns, suggesting the action of additional genes and not just *Frc*, in conditioning the fructan trait in onions. PCA analysis has shown that the first principal component (accounting for 57-67% of total trait variation) weighted most heavily for reducing sugars and fructans, and was consistent with previous reports (McCallum *et al.*, 2006). This measure of balance between fructans and hexoses in the first PC, and domination of the sucrose trait in the second PC, has suggested major role of sucrose signaling and degradation pathways in conditioning fructan and hexose levels in onions. Sucrose synthase activity did not show any significant affect on the fructan phenotype in 'W202A x Texas Grano 438' mapping population (McCallum *et al.*, 2006). Also, assignment of sucrose synthase to chromosome 6 (Martin *et al.*, 2005; Yaguchi *et al.*, 2008), has earlier confirmed that it does do not determine the *Frc* phenotype, and thus assays of SuSy were not included in this project.

A 'Nasik Red x CUDH2150' F_2 family fitted a 1: 2: 1 ratio for *Frc* markers, supporting phenotypic dominance for high fructan content in onions (McCallum *et al.*, 2006). Single marker ANOVA was performed to study the association of varying enzyme activity with *Frc* markers. Combined enzyme activity (acid invertase plus 1-SST activity) measured as μg glucose min^{-1} mg^{-1} protein, showed wide variation (1.27 to 16.32 μg glucose min^{-1} mg^{-1} protein) in segregating F_2 lines, and were seen to significantly associate with the *Frc* markers. Since the combined enzyme activity is carried out on crude enzyme extracts, fractionation of the acid invertases from 1-SST is required to study the particular enzyme activity and their association with *Frc* locus. The *Frc* region showed significant marker associations with fructose, followed by glucose and fructans, indicating that this locus mainly conditions sucrose metabolism in onions. AI and 1-SST enzymes both use sucrose as a substrate to produce hexose sugars. Results obtained from characterising sugar traits in high- and low-fructan cultivars, segregating onion populations, and from previous knowledge on AI and 1-SST location (Havey *et al.*, 2004; Yaguchi *et al.*, 2008), suggest that AIs regulatory genes or genes controlling the variation in 1-SST or both may underlie *Frc*, and this requires further investigations in these areas.

6.3 Future directions for the study of regulation of fructan levels in onions

Following the investigations described in this thesis, additional experiments work could now be undertaken to study the regulation of variation in carbohydrate composition in onions.

6.3.1 Fractionation of enzymes belonging to GH32 family

Acid invertase activity measured as μg glucose min^{-1} mg^{-1} protein showed higher activity than the enzyme activity measured as μg fructose min^{-1} mg^{-1} protein (Figure 5.7 and Appendix 8.3), indicating the interference of 1-SST enzyme activity in the assays. These two enzymes are similar (deduced amino acid sequences show 61% identity to each other in onions) (Vijn *et al.*, 1998) and express their activity under similar assay conditions (L'Hocine *et al.*, 2000). Protein extracts of AcN1 (onion acid invertase)-transformed protoplasts when incubated with 20 mM sucrose exhibited only acid invertase activity, producing glucose and fructose, while incubation of AcN1 protein extract at 100 mM sucrose concentration exhibited both acid invertase activity and fructosyltransferase activity producing glucose, fructose, 1-kestose and

oligofructans. Analysis of sugar products generated by AcN2 (onion 1-SST) transformed protein extracts revealed only 1-SST activity, but at varying activity levels.

To untangle the contribution that each of these enzymes makes to the final levels of hexoses and other carbohydrates, physical fractionation of the crude protein extract from different cultivars by means such as ion exchange, gel permeation and/or affinity chromatographic techniques is desirable. Fractionation coupled with variable assay conditions is further required to enable discrimination of modes of enzyme activity at different substrate concentrations. The use of higher resolution techniques for enzymatic product identification, such as HPLC with pulsed amperometric detection or mass spectrometry would resolve the net contributions made by the enzyme present in each protein preparations.

6.3.2 Role of protein inhibitors in regulating AIs in onion

Fructose content, which strongly correlated negatively with fructan content in 'Nasik Red x CUDH2150' F_2 onion mapping families, had the highest weightings in PCA and CVA analyses, indicating variation in sucrose hydrolysis (possibly by acid invertases) in onions. Acid invertase activities were seen to vary significantly between high- and low-fructan cultivars. Since acid invertase activities in high-sucrose accumulating high-fructan and low-sucrose accumulating low-fructan onion cultivars were not affected by their transcript level, it is suggested that post-transcriptional regulation of acid invertases is important in onions. Small proteins (15-23 kD) belonging to a large family of pectinmethylesterase-related proteins (PMEI-RPs) have been reported to be involved in post-translational regulation of invertases in plants (Krausgrill S *et al.*, 1998; Hothorn M. *et al.*, 2004; Rausch & Greiner, 2004; Jin *et al.*, 2009; McKenzie *et al.*, 2012). These PMEI-RPs inhibitors bind irreversibly to invertases (forming an inactive complex) (Rausch & Greiner, 2004) affecting sucrose hydrolysis and sucrose composition in plants (Kusch *et al.*, 2009b). A direct connection has be noticed between the transcript levels of potential cDNA-encoding AI inhibitors and the inhibition of invertase activity in coffee genotypes varying in sucrose accumulation (Privat *et al.*, 2008). Also silencing of the invertase inhibitor, INVINH1, has revealed post-translational elevation of cell wall invertase activity, resulting in increased hexose levels in tomato (Jin *et al.*, 2009).

To enable studies on the post-translational regulation of acid invertase activity in onions, identification and molecular characterisation of the invertase inhibitor genes in onions is

required. The amino acid sequences of identified invertase inhibitors such as NtCIF and NtVIF from *N. tabacum* (Greiner *et al.*, 1998; Greiner *et al.*, 1999), and AtC/VIF 1 and 2 from *A. thaliana* can be used as query sequences for searching proteins encoded by putative onion invertase inhibitor genes in GenBank. Transcript level analysis of putative onion inhibitors and their correlation with acid invertase activity, sucrose or hexose sugar content would provide information on their functionality. Ectopic expression similar to the studies of Greiner *et al.* (1999) and Kusch *et al*. (2009b), or gene silencing studies (Jin *et al.*, 2009), would also assist in confirming the functionality of the putative invertase inhibitory protein.

6.3.3 Sucrose-mediated fructosyltransferase activity in onion

Sucrose-mediated induction of fructosyltransferases has determined in many plants including onions (Vijn *et al.*, 1998; Müller *et al.*, 2000). A weak correlation between 1-SST activity/1-SST transcript abundance and fructan levels, and higher 1-SST activity in the low sucrose accumulating low-fructan line, has indicated a limited role for sucrose in inducing 1-SST activity in onions. Differential induction of fructosyltransferases of 1-SST & 6-SFT (by 3 and 20 times) has been determined in sucrose-fed leaf blades of wheat and barley (Müller *et al.*, 2000; Martinez-Noël *et al.*, 2001). Similarly, it is suggested that sucrose might have a significant role in inducing 6G-FFT activity affecting fructan content in onions. Significantly low glucose concentration along with low 1-SST activity in high DP fructan-accumulating Nasik Red and SWG-N96 cultivars has indicated high 6G-FFT induction and activity by high sucrose.

To study the role of sucrose in inducing fructosyltransferases (1-SST and 6G-FFT) in onions, sucrose feeding experiments similar to previous reports (Müller *et al.*, 2000; Martinez-Noël *et al.*, 2001) are recommended. Transcript level and activities studies of 6G-FFT in high- and low-fructan cultivars, followed by their correlation with NSCs, will further elucidate their role in varying fructan accumulation in onions.

6.3.4 Fructose-mediated fructosyltransferase activity in onion

Variation in 1-SST activity between leaf bases of high- and low-fructan cultivars noticed during the bulb swelling stage is suggested to be due to developmental signals and variation in hexose sugar concentrations. During the bulb swelling stage, 1-SST activity and its transcript levels showed a strong positive correlation with acid invertase transcript levels, its

enzyme activity and hexose sugar content, and were similar to the studies of Druart *et al.* (2001). Sucrose levels did not seem to have a positive effect on the 1-SST activity in developing leaf bases. To study the role of hexose sugars in inducing 1-SST, leaf blades can be fed with various sugars similar to the experiments of Martinez-Noël *et al.* (2001), and their expression levels can be studied. Kusch *et al.* (2009a) have previously shown induced expression of fructosyltransferases (1-SST and 1-FFT) in hairy root cultures of chicory (*Cichorium intybus*) grown in high-carbon/low-nitrogen medium with sucrose or fructose as the main carbon source. Similar experiments with varying carbon source (in the growth media) to onions seedlings would be beneficial in understanding the role of sucrose, glucose and fructose in inducing fructosyltransferases in onions and this will further assist in elucidating the variation in 1-SST expression/activity level between high- and low-fructan cultivars.

6.3.5 Plant response to low temperature stress

Low temperature stress, which occurred during the field experiment in 2009 (Appendix 8.1) was shown to affect carbohydrate related traits in onions. Fructan and sucrose concentrations reduced markedly irrespective of the fructan type in leaf blades, while fructose content was seen to increase during the cold stress. Increased 1-SST activity in leaf blades during cold stress has been suggested to be an adaptive mechanism to combat cold stress in onions. High kestose concentration, seen as a result of increased 1-SST activity (Thorsteinsson *et al.*, 2002; Li *et al.*, 2007), has been reported to reduce electrolyte leakage in cold-stressed plants (Li *et al.*, 2007). Based on the reports of Portes *et al.* (2008) on cold-treated *Vernonia herbacea* rhizophores, reduced 1-SST activity in onion leaf bases is suggested to be due to reduced photoassimilate production and translocation to sink tissues at low temperatures. Since the data presented in this thesis was recorded a few days after the low temperature period, it is difficult to explain conclusively the mechanism employed by onions to overcome cold stress. To determine the effects of low temperatures in onion, a controlled experiment with varying temperatures is desirable.

6.3.6 'Nasik Red x CUDH2150' F_2 lines in genomic studies

'Nasik Red x CUDH2150' F_2 progenies, segregating widely for NSCs, are a very useful genetic resource to study genetic architecture of carbohydrate traits in onion. Use of the

doubled haploid as one of the parents has further facilitated genomic studies in onions. Transcriptome sequence information of Nasik Red and CUDH2150, obtained using 454^{TM} sequencing of normalized cDNA libraries of leaf and shoot, are now being used to develop PCR-based molecular markers in onions (Baldwin *et al.*, 2012). A framework genetic linkage map developed using a subset of one of the 'Nasik Red x CUDH2150' F_2 population is currently being populated with developed molecular markers. Since the 'Nasik Red x CUDH2150' intercross also exhibited variation in other phenotypic traits such as plant height, vigour, bolting, bulb colour, bulb size and centeredness, it can be used for genetic dissection of such segregating traits.

6.4 Conclusion/Significance

The aim of this thesis was to identify the factors that control variation in fructan and other NSC composition in high- and low-fructan cultivars. This is essential for understanding the biochemical and genetic basis of the *Frc* locus on chromosome 8 (which has been shown to have a major affect on the fructan trait in onions) in segregating onion progenies, and to develop closely linked molecular markers that can be useful in marker-aided breeding. Through biochemical and molecular analysis of high- and low-fructan cultivars, some of the factors affecting variable fructan and other NSC traits have been identified. In low-fructan cultivars there was generally high acid invertase activity affecting hexose and sucrose concentrations. Analysis of 'Nasik Red x CUDH2150' F_2 families showed wide variability for fructans and other NSCs, and transgressive segregation of fructan has indicated epistatic gene action in controlling the fructan trait in onions. A possible association of acid invertase activity with *Frc* was also investigated in a segregating 'Nasik Red x CUDH2150' F_2 family. Based on characterisation studies in high- and low-fructan cultivars, and association studies in a segregating F_2 family, acid invertase regulatory genes (inhibitory proteins) or genes controlling variation in 1-SST expression/activity have been suggested to underlie *Frc*. Elucidation of variation in fructan accumulation could have far reaching benefits, from crop manipulation for desirable sugar content and DM%, to increased health benefits of fructans in humans.

7. References

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8. APPENDIX

8.1 Temperature data

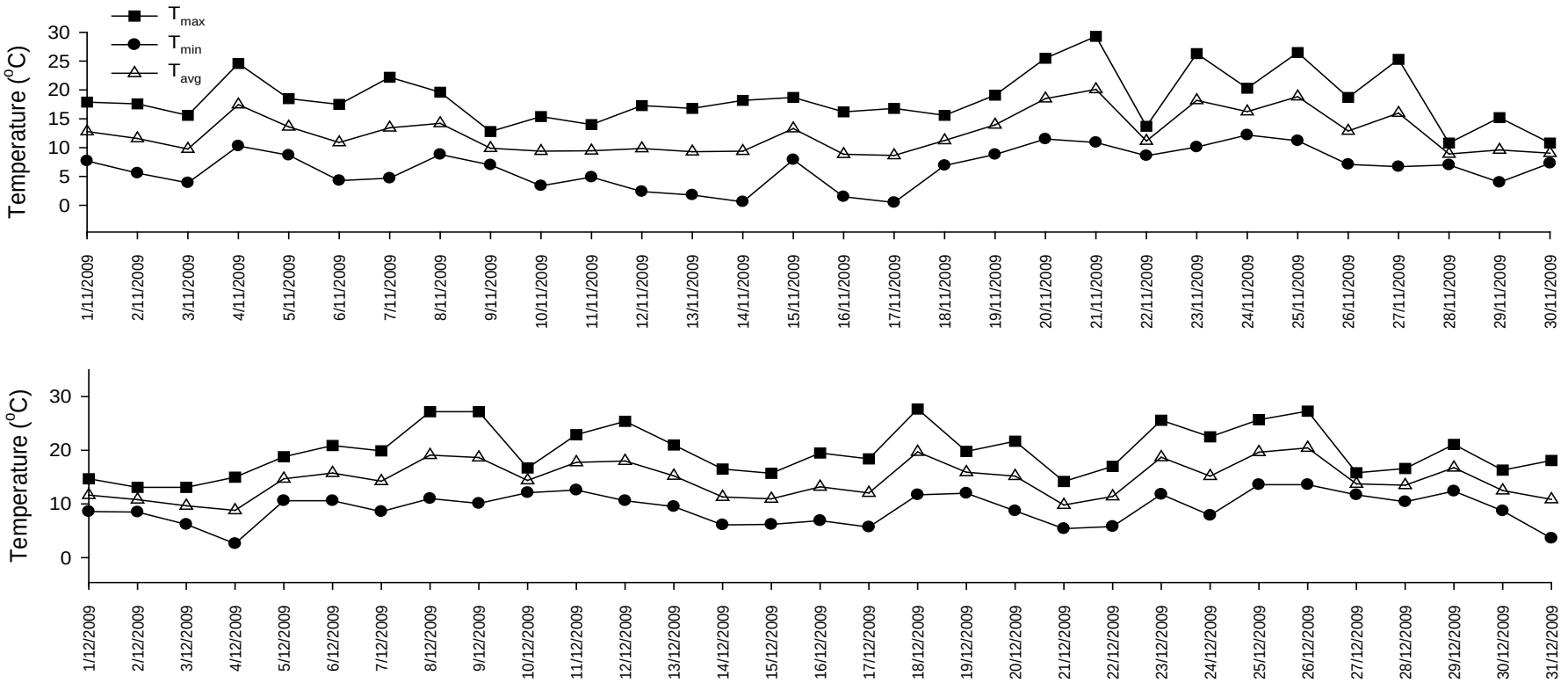

Figure 8. 1 Average daily temperature values for November and December during the NSC characterisation experiment (October 2009–March 2010). Maximum (T_{max}), minimum (T_{min}) and average (T_{avg}) temperature are indicated.

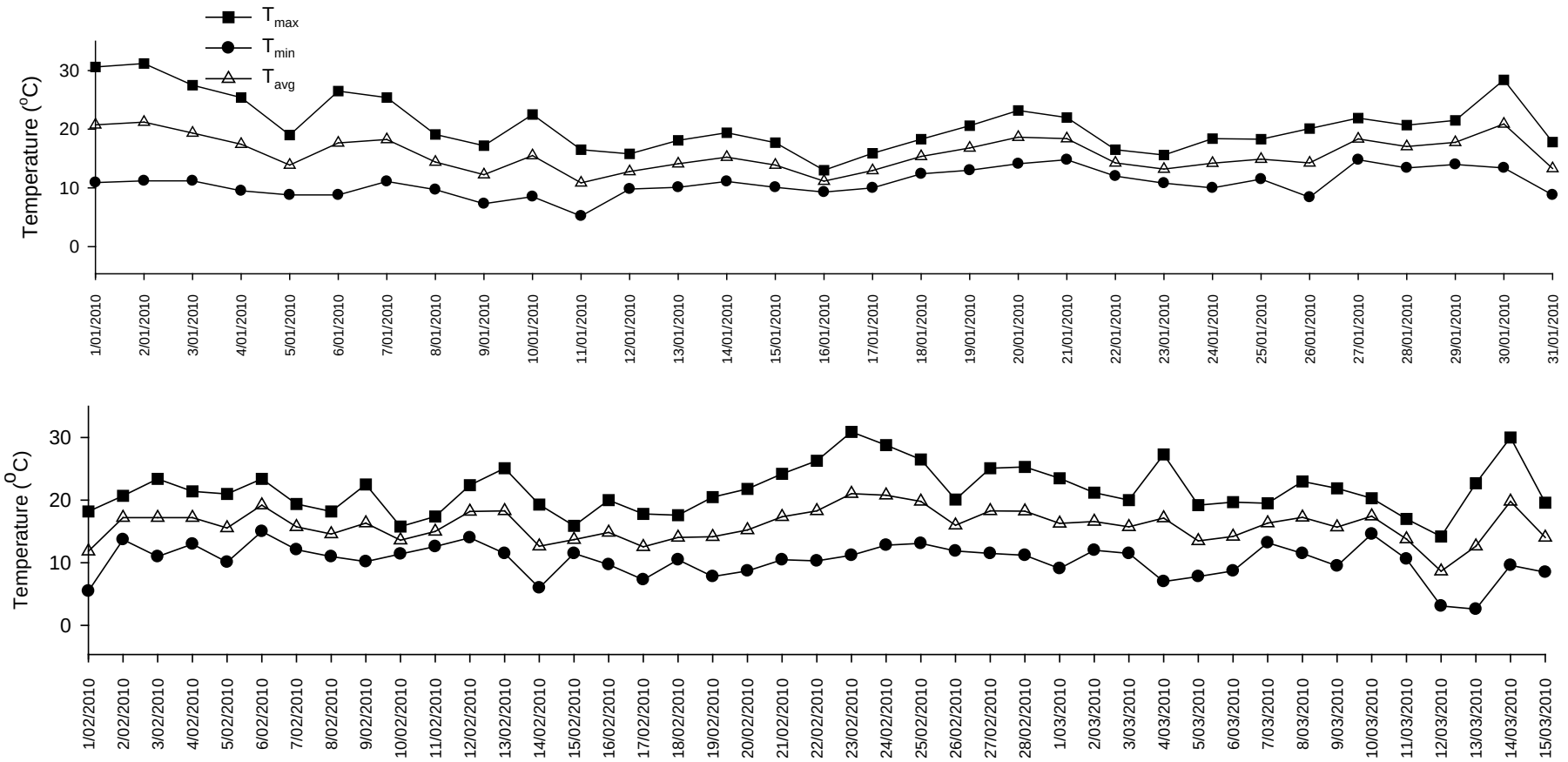

Figure 8. 2 Average daily temperature values for January to mid march during the NSC characterisation experiment (October 2009–March 2010). Maximum (T_{max}), minimum (T_{min}) and average (T_{avg}) temperature are indicated.

8.2 Non-structural carbohydrate concentrations in onion tissues (2008-2009)

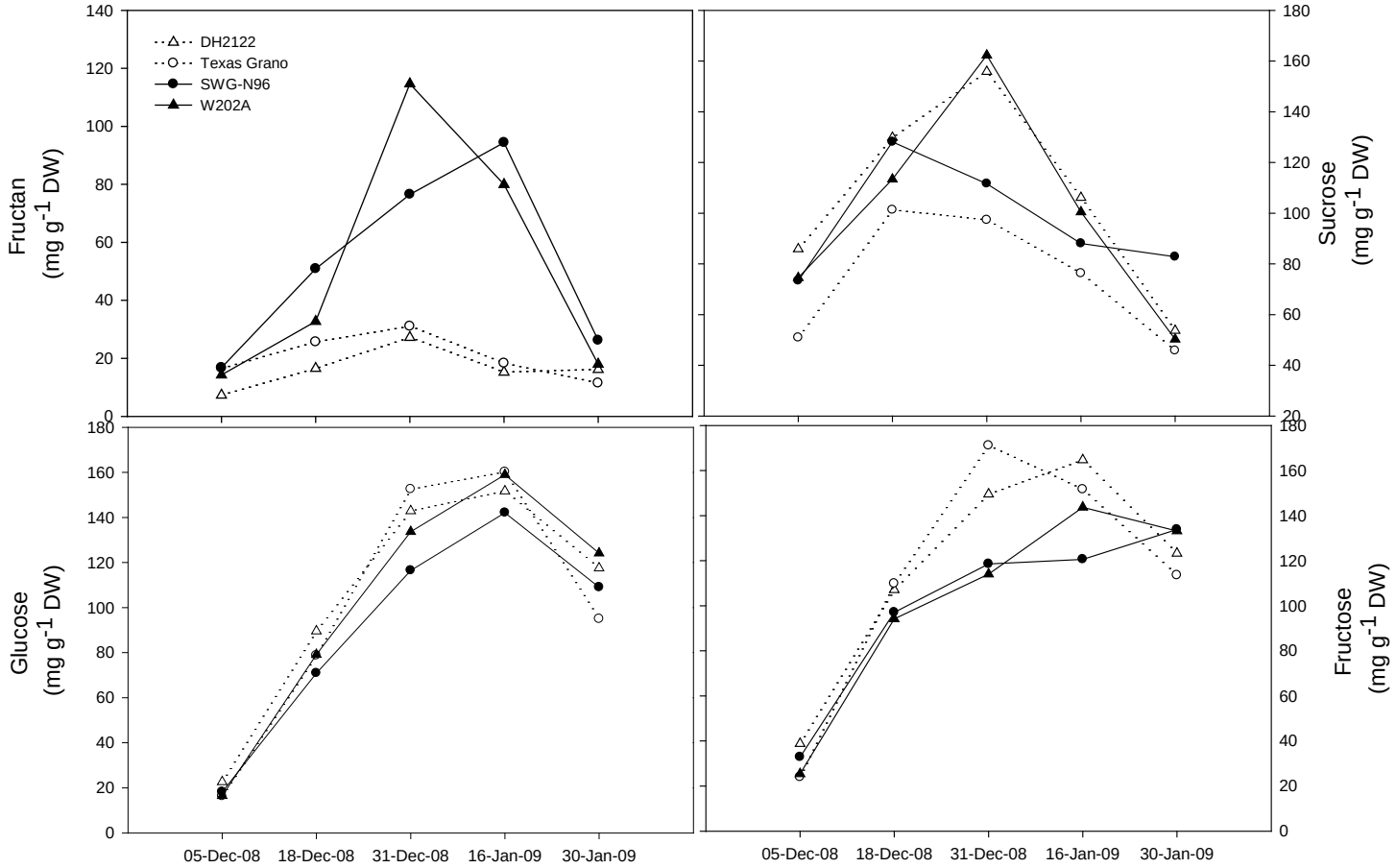

Figure 8.3. NSC concentrations in developing leaf blade of high-(solid symbols) and low-fructan (open symbols) onion cultivars.

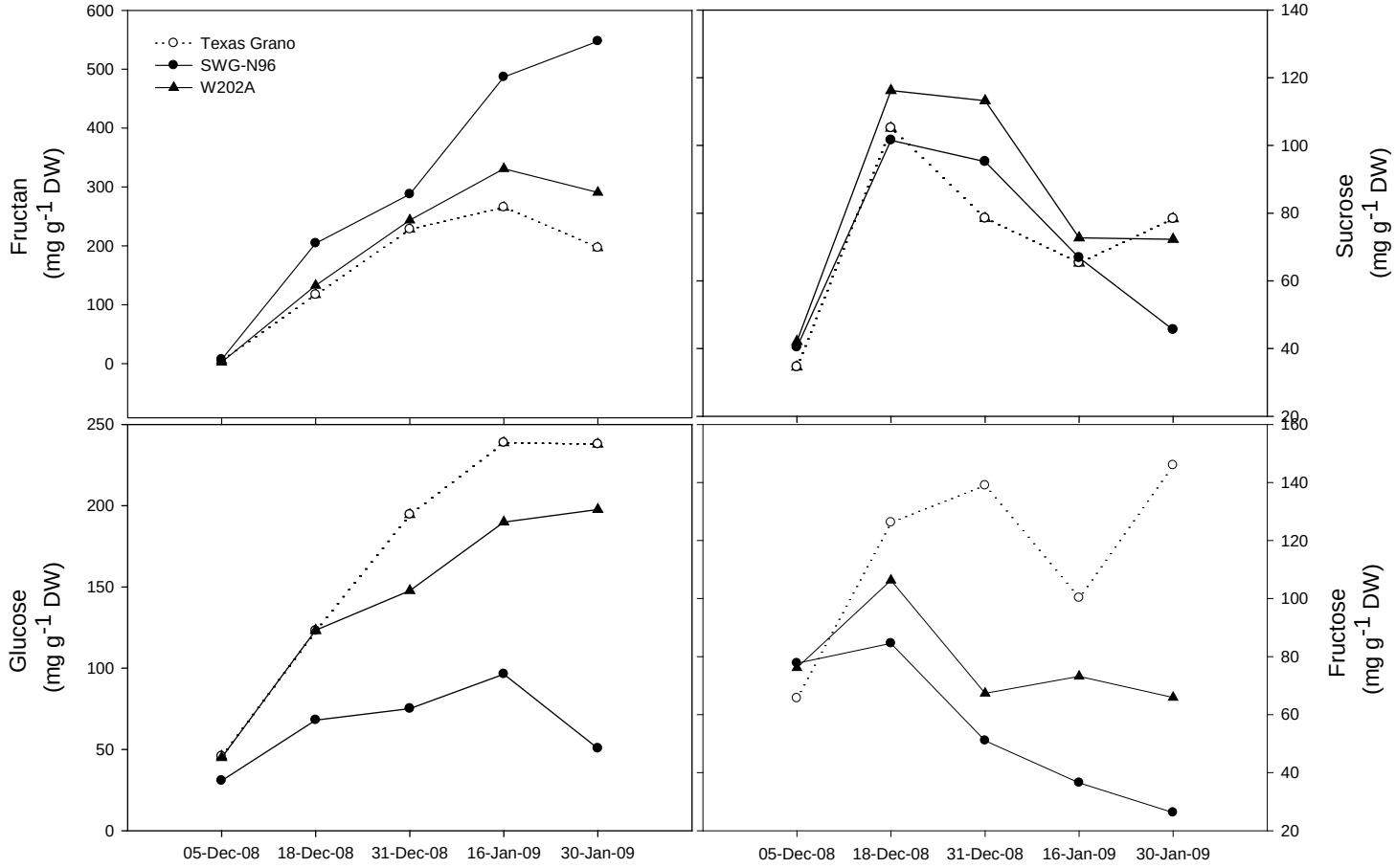

Figure 8.4. NSC concentrations in developing leaf bases of high-(solid symbols) and low-fructan (open symbols) onion cultivars.

8.3 Acid invertase activity in crude enzyme extracts

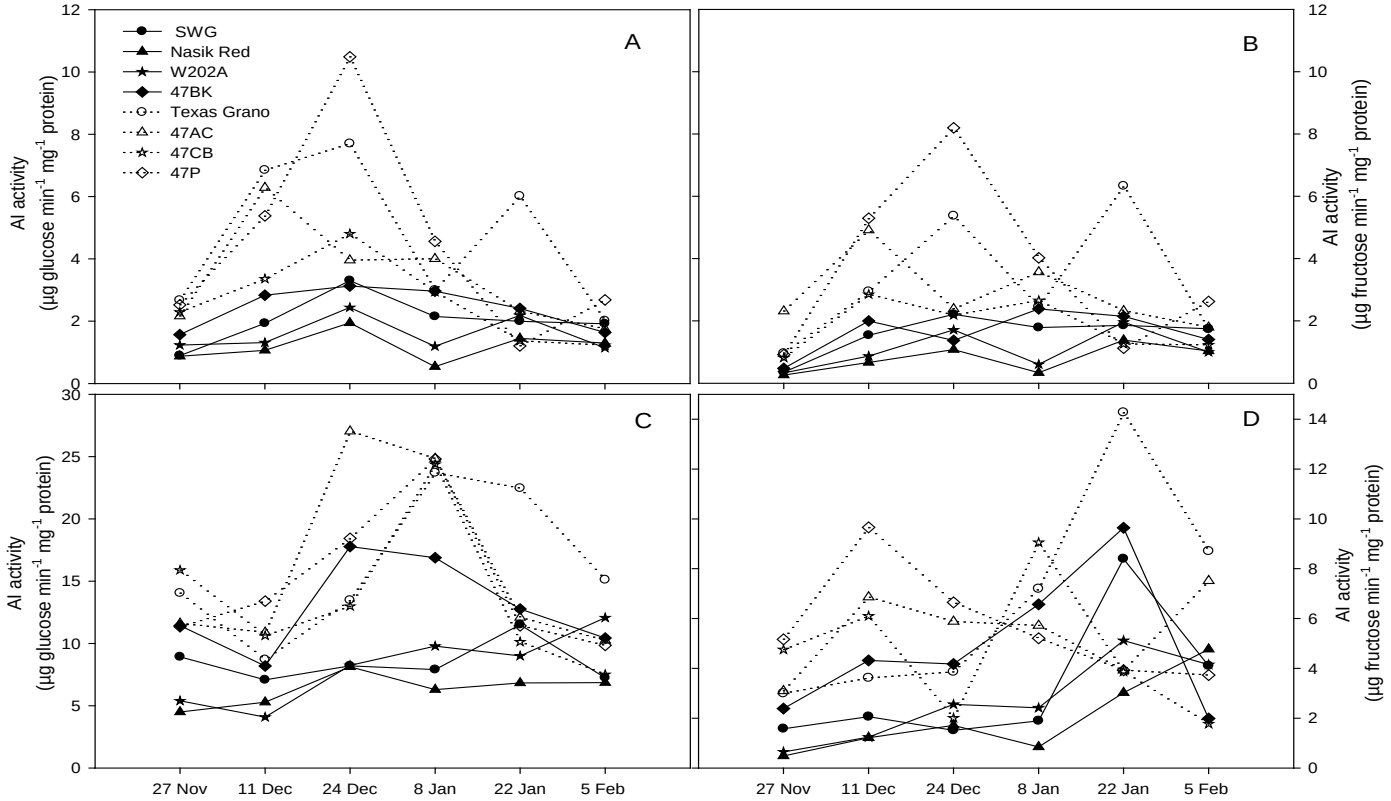

Figure 8.5. Acid invertase activities measured as µg glucose min^{-1} mg^{-1} protein and µg fructose min^{-1} mg^{-1} protein in developing leaf blade (A and B) and leaf bases (C and D) of high- (solid symbols) and low-fructan (open symbols) onion cultivars. Crude enzyme extracts exhibited 1-SST activity (releasing glucose), as a result of which the enzyme activity measured as µg glucose min^{-1} mg^{-1} protein (A and C) is higher than the enzyme activity measured as µg glucose min^{-1} mg^{-1} protein (B and D).

8.4 Correlation analysis

Table 8.1. Pearson's correlations, *r*, between NSC concentration, enzyme activity and transcript abundance in developing leaf blades of eight onion cultivars at first sampling (27/11/2009).

Harvest 1	Total	Fructan	Sucrose	Glucose	Fructose	SPS activity	AI activity	NI activity	SST activity	ACE6067 RQ	ACP041 RQ
Fructan	0.93										
Sucrose	0.72	0.64									
Glucose	-0.02	-0.37	-0.08								
Fructose	-0.34	-0.63	-0.40	0.90							
SPS activity	0.22	0.38	0.00	-0.41	-0.44						
AI activity	-0.36	-0.47	-0.46	0.42	0.57	-0.02					
NI activity	-0.34	-0.29	-0.18	-0.03	0.00	0.23	0.51				
SST activity	0.03	-0.17	-0.15	0.51	0.61	-0.11	0.39	-0.31			
ACE6067 RQ	0.06	0.00	0.29	0.17	-0.04	-0.12	-0.21	0.02	-0.13		
ACP041 RQ	-0.01	-0.04	0.03	0.03	0.06	0.23	0.09	0.23	0.19	-0.12	
1-SST RQ	0.52	0.47	0.50	0.00	-0.20	0.27	0.02	0.13	0.02	0.2 2	0.03

Table 8.2. Pearson's correlations, *r*, between NSC concentration, enzyme activity and transcript abundance in developing leaf blades of eight onion cultivars at second sampling (11/12/2009).

	Total	Fructan	Sucrose	Glucose	Fructose	SPS activity	AI activity	NI activity	SST activity	ACE6067 RQ	ACP041 RQ
Fructan	0.96										
Sucrose	0.93	0.89									
Glucose	0.78	0.58	0.68								
Fructose	0.14	-0.11	-0.06	0.52							
SPS activity	-0.16	0.02	-0.03	-0.50	-0.60						
AI activity	0.01	-0.15	-0.05	0.41	0.46	-0.21					
NI activity	-0.61	-0.72	-0.72	-0.13	0.47	-0.38	0.52				
SST activity	0.67	0.74	0.69	0.33	-0.27	-0.01	-0.15	-0.61			
ACE6067 RQ	-0.16	-0.18	-0.16	-0.08	0.09	0.06	0.23	0.16	-0.09		
ACP041 RQ	-0.32	-0.30	-0.33	-0.23	-0.04	0.22	0.28	0.21	-0.24	0.84	
1-SST RQ	0.08	0.06	0.01	0.15	0.11	0.00	0.37	0.10	0.03	0.64	0.76

Table 8.3. Pearson's correlations, *r*, between NSC concentration, enzyme activity and transcript abundance in developing leaf blades of eight onion cultivars at third sampling (24/12/2009).

	Total	**Fructan**	**Sucrose**	**Glucose**	**Fructose**	**SPS activity**	**AI activity**	**NI activity**	**SST activity**	**ACE6067 RQ**	**ACP041 RQ**
Fructan	0.88										
Sucrose	0.51	0.65									
Glucose	0.15	-0.29	-0.50								
Fructose	-0.12	-0.54	-0.68	0.83							
SPS activity	-0.01	0.06	0.17	-0.06	-0.24						
AI activity	-0.12	-0.31	-0.50	0.38	0.58	0.01					
NI activity	-0.03	-0.29	-0.54	0.52	0.69	-0.12	0.67				
SST activity	0.02	-0.18	-0.64	0.44	0.64	-0.28	0.42	0.60			
ACE6067 RQ	0.43	0.52	0.35	-0.18	-0.34	0.30	-0.30	-0.36	0.04		
ACP041 RQ	0.19	0.39	0.37	-0.34	-0.54	0.29	-0.42	-0.53	-0.12	0.87	
1-SST RQ	-0.11	0.17	0.32	-0.49	-0.62	0.28	-0.50	-0.66	-0.25	0.80	0.53

Table 8.4. Pearson's correlations, *r*, between NSC concentration, enzyme activity and transcript abundance in developing leaf blades of eight onion cultivars at fourth sampling (08/01/2010).

	Total	**Fructan**	**Sucrose**	**Glucose**	**Fructose**	**SPS activity**	**AI activity**	**NI activity**	**SST activity**	**ACE6067 RQ**	**ACP041 RQ**
Fructan	0.78										
Sucrose	0.63	0.56									
Glucose	0.33	-0.28	-0.15								
Fructose	0.34	-0.29	-0.10	0.94							
SPS activity	-0.56	-0.61	-0.38	-0.01	0.11						
AI activity	0.28	-0.05	-0.44	0.66	0.70	0.03					
NI activity	0.73	0.89	0.28	-0.10	-0.11	-0.60	0.21				
SST activity	0.51	0.29	0.19	0.37	0.36	0.03	0.38	0.35			
ACE6067 RQ	-0.23	-0.45	-0.55	0.40	0.45	0.47	0.53	-0.27	0.25		
ACP041 RQ	-0.18	-0.38	-0.46	0.43	0.38	0.32	0.46	-0.26	0.30	0.57	
1-SST RQ	-0.43	-0.43	-0.25	-0.06	0.02	0.53	-0.05	-0.48	-0.13	0.87	0.67

Table 8.5. Pearson's correlations, *r*, between NSC concentration, enzyme activity and transcript abundance in developing leaf bases of eight onion cultivars at first sampling (27/11/2009).

	Total	Fructan	Sucrose	Glucose	Fructose	SPS activity	AI activity	NI activity	SST activity	ACE6067 RQ	ACP041 RQ
Fructan	0.97										
Sucrose	0.24	0.23									
Glucose	-0.42	-0.62	-0.19								
Fructose	-0.57	-0.70	-0.58	0.68							
SPS activity	0.26	0.29	-0.14	-0.16	-0.22						
AI activity	-0.52	-0.62	-0.44	0.52	0.86	-0.36					
NI activity	-0.38	-0.40	-0.33	0.35	0.35	0.52	0.32				
SST activity	0.03	-0.01	-0.61	0.29	0.25	0.07	0.22	0.35			
ACE6067 RQ	0.25	0.24	-0.04	0.10	-0.22	0.32	-0.26	0.12	0.30		
ACP041 RQ	-0.04	-0.06	-0.26	0.10	0.17	-0.22	0.36	0.05	0.39	0.25	
1-SST RQ	0.11	0.19	-0.12	-0.18	-0.32	0.23	-0.30	-0.05	0.32	0.44	0.80

Table 8.6. Pearson's correlations, *r*, between NSC concentration, enzyme activity and transcript abundance in developing leaf bases of eight onion cultivars at second sampling (11/12/2009).

	Total	Fructan	Sucrose	Glucose	Fructose	SPS activity	AI activity	NI activity	SST activity	ACE6067 RQ	ACP041 RQ
Fructan	0.94										
Sucrose	0.82	0.70									
Glucose	0.22	-0.08	0.25								
Fructose	-0.47	-0.69	-0.45	0.41							
SPS activity	0.55	0.62	0.33	-0.10	-0.46						
AI activity	-0.19	-0.39	-0.12	0.45	0.65	-0.23					
NI activity	-0.63	-0.62	-0.68	0.02	0.45	-0.23	0.56				
SST activity	0.43	0.35	0.19	0.37	-0.04	0.53		-0.11			
ACE6067 RQ	-0.01	-0.09	-0.06	0.27	0.17	-0.24	-0.04	-0.14	0.10		
ACP041 RQ	-0.01	-0.07	0.09	0.28	-0.02	-0.26	0.01	-0.08	0.16	0.62	
1-SST RQ	-0.09	-0.19	-0.05	0.27	0.28	-0.18	0.05	0.01	0.09	0.63	0.78

Table 8.7. Pearson's correlations, *r*, between NSC concentration, enzyme activity and transcript abundance in developing leaf bases of eight onion cultivars at third sampling (24/12/2009).

	Total	Fructan	Sucrose	Glucose	Fructose	SPS activity	AI activity	NI activity	SST activity	ACE6067 RQ	ACP041 RQ
Fructan	0.76										
Sucrose	0.29	0.46									
Glucose	0.14	-0.52	-0.47								
Fructose	-0.13	-0.65	-0.73	0.80							
SPS activity	0.13	-0.15	-0.68	0.49	0.57						
AI activity	0.04	-0.34	-0.49	0.50	0.73	0.40					
NI activity	-0.11	-0.32	-0.64	0.33	0.68	0.42	0.68				
SST activity	0.14	-0.21	-0.63	0.54	0.67	0.46	0.55	0.46			
ACE6067 RQ	0.01	-0.37	-0.51	0.51	0.76	0.55	0.65	0.68	0.42		
ACP041 RQ	-0.13	-0.45	-0.77	0.56	0.78	0.59	0.57	0.82	0.62	0.95	
1-SST RQ	-0.19	-0.44	-0.80	0.51	0.70	0.60	0.50	0.72	0.57	0.73	0.62

Table 8.8. Pearson's correlations, *r*, between NSC concentration, enzyme activity and transcript abundance in developing leaf bases of eight onion cultivars at fourth sampling (08/01/2010).

	Total	Fructan	Sucrose	Glucose	Fructose	SPS activity	AI activity	NI activity	SST activity	ACE6067 RQ	ACP041 RQ
Fructan	0.85										
Sucrose	0.11	0.16									
Glucose	0.11	-0.41	-0.19								
Fructose	-0.30	-0.64	-0.59	0.60							
SPS activity	0.42	0.35	0.34	0.07	-0.29						
AI activity	0.06	-0.29	-0.56	0.60	0.76	-0.28					
NI activity	-0.07	-0.27	0.14	0.30	0.32	-0.19	0.41				
SST activity	0.02	-0.30	-0.54	0.58	0.68	0.12	0.66	0.06			
ACE6067 RQ	0.17	-0.08	-0.32	0.48	0.33	0.47	0.39	-0.23	0.67		
ACP041 RQ	0.23	-0.03	-0.07	0.52	0.14	0.16	0.18	0.00	0.50	0.53	
1-SST RQ	0.17	0.16	0.24	0.05	-0.29	0.23	-0.13	0.10	-0.05	0.61	0.34

8.5 Publications

Revanna R, Turnbull MH, Shaw ML, Wright KM, Butler RC, Jameson PE, McCallum J. (2013). Measurement of the distribution of non-structural carbohydrate composition in onion populations by a high-throughput microplate enzymatic assay. *Journal of the Science Food and Agriculture*. http://onlinelibrary.wiley.com/doi/10.1002/jsfa.6062/abstract

Revanna R, Shaw M, Pither-Joyce M, Butler RC, Turnbull MH, Wright KM, Baldwin S, Darryn Ward D, Jameson PE, McCallum J. Carbohydrate turnover in developing leaf blades and leaf bases of high- and low-fructan onion genotypes. *Under internal review*.

Baldwin S, Revanna R, Thomson S, Pither-Joyce M, Wright K, Crowhurst R, Fiers M, Chen L, MacKnight R & McCallum JA. (2012). A toolkit for bulk PCR- based marker design from nextgeneration sequence data: Application for development of a framework linkage map in bulb onion (*Allium cepa* L.). *BMC Genomics* **13**, 637.

Baldwin S, Revanna R, Pither-Joyce M, Wright K, Thomson S, , Moya L, Lee R, MacKnight R & McCallum JA. (2013). Genetic analysis of bolting in bulb onion (*Allium cepa* L.). *Theoretical and Applied Genetics*. [Epub ahead of print : http://www.ncbi.nlm.nih.gov/pubmed/24247236?report=abstract]