Cold-induced sweetening in potato (*Solanum tuberosum* L.): genetic analysis of the apoplastic invertase inhibitor gene

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by Sagar Datir

Potatoes are increasingly consumed in the form of processed foodstuffs such as French fries and crisps. After harvest, potatoes need to be kept at low temperature to prevent sprouting during storage for round-the-year processing. However, tubers accumulate reducing sugars at low temperatures, a phenomenon referred to as cold-induced sweetening (CIS). The processing of these high sugar potatoes into crisps or fries leads to a dark brown to black product that renders them unfit for human consumption and causes a great loss to the processing industry. To prevent sprouting and diseases, chemical treatments have been applied to tubers in storage. The recent withdrawal of these chemicals has increased the reliance on cold storage for potato tubers and highlighted the importance of CIS. Extensive research is required to produce a cultivar resistant to cold induced sweetening (CIS) along with good processing quality. The present work focused on increasing the understanding of the biological processes (physiological, biochemical and molecular) contributing to the initiation and/or controlling of CIS in potato tubers. The genetic basis of this trait was examined in progeny from a cross between potato cultivars with poor and excellent CIS response. This included molecular markers for candidate genes of known position on potato chromosomes to assess the role of different alleles involved in carbohydrate metabolism. Association studies between marker alleles and the phenotype of the progeny were performed. Among all the candidate genes, allele diversity for apoplastic invertase inhibitor gene was further studied from resistant and susceptible potato cultivars. In total five alleles were identified. Polymorphism was observed in both exon and intron regions. Three alleles had a unique substitution at the predicted junction of the signal peptide and mature protein. In order to identify the specific alleles that may play a role in resistance to CIS, transgenic potato plants have been produced with overexpression and antisense repression of apoplastic invertase inhibitor alleles. The results revealed that there were no consistent differences in CIS traits among the transgenic lines with the various

alleles. However, the results of the transcript analysis showed much higher and stable transcripts levels of the apoplastic invertase inhibitor in the 1021/1 derived transgenic lines. This greater accumulation or stability of the apoplastic invertase inhibitor transcripts in 1021/1 may be a key factor contributing to the CIS trait of this cultivar.

A key difference from previous studies involves the use of 1021/1, a potato cultivar known to have the very high resistance to CIS. This study provided to help identify key genes for the future genetic improvement of tuber properties with respect to long-term storage and processing characteristics. The development of a potato that does not sweeten in the cold will revolutionize the potato industry as this problem currently contributes 20 percent losses after crop harvest. Once identified, clones resistance to CIS could be used in potato breeding programmes for the development of cold-resistant processing cultivars. Eliminating the need for chemical application of sprout inhibitors will be helpful to develop sustainable approaches to benefit both mankind and potato industries. These biotechnological tools will be used to identify elite potatoes with improved properties of the tuber with respect to long-term storage and processing characteristics (cold-sweetening and after-cooking darkening).

Keywords: Potato, cold-induced sweetening, resistance, apoplastic invertase inhibitor, cultivar, crisps, processing, transgenic potato.

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Abbreviations

ADP	adenosine diphosphate
ANOVA	analysis of variance
AFLP	amplified fragment length polymorphism
AGPase	ADP-glucose pyrophosphorylase
AMY	amylase
ATP	adenosine triphosphate
bp	base pair
°C	degree Celsius
CaMV	Cauliflower mosaic virus
cm	centimetre
cDNA	Complementary DNA
CIS	cold-induced sweetening
CAPS	cleaved amplified fragment polymorphism
CO_2	Carbon dioxide
comm.	communication
CTAB	cetyldimethyl ethyl ammonium bromide
ddH_2O	double distilled water
kDa	kilodaltons
dNTP	deoxynucleoside-5'-triphosphate
DEPC	diethyl pyrocarbonate
df	degrees of freedom
DM	Dry matter
DNA	deoxy ribonucleic acid
EC	electrical conductivity
EDTA	ethylene diamine tetra acetic acid
EMBL	European Molecular Biology Laboratory
etc	et cetera
g	gram
gDNA	genomic DNA
GA	Gibberellic Acid
Gb	giga base pairs

Abbreviations

hr	Hours
i.e.	that is
indel	insertions/deletions
inh1	cell wall / apoplastic invertase inhibitor
inh2	vacuolar invertase inhibitor
IPTG	isopropyl-1-thio-β-D-galactoside
kb	kilobases
kDa	kilodaltons
l	litre
LB	Luria broth
LG	Linkage group
M	Molar
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog medium
Mw	Molecular weight
NAA	1-napthalene acetic acid
NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced form of NAD
nm	nanometer
ng	nanogram
nM	nanomoles
nptII	neomycin phosphotransferase II
Nt-inhh	*Nicotiana tabacum* putative vacuolar invertase inhibitor
Nt-VIF	*Nicotiana tabacum* vacuolar invertase inhibitor
OD	optical density
PCR	polymerase chain reaction
pers.	personal

Abbreviations

%	Percent
PGM	phosphoglucomutase
PHI	phosphohexoisomerase
pI	isoelectric point
PGSC	Potato Genome Sequencing Consortium
qRT PCR	quantitative real time polymerase chain reaction
QTL(s)	quantitative trait locus/loci
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolution per minute
RNAi	ribonucleic acid interference
SBE	starch branching enzyme
SCAR	sequence characterised amplified region
s	second
SNPs	single nucleotide polymorphisms
SP	starch phosphorylase
SPS	sucrose phosphate synthase
S6P	sucrose 6-phosphate phosphatase
SSRs	Simple Sequence Repeats
SS	starch synthase
Stinh	*Solanum tuberosum* vacuolar invertase inhibitor
$Stinh_{ap}$	*Solanum tuberosum* apoplastic invertase inhibitor
ST	Storage treatment
TAE	Tris-acetate-EDTA
TBE	Tris-boric acid/EDTA
U	Unit
UGPase	UDP-glucose pyrophosphorylase
µg	microgram
µm	micrometer
µl	microlitre

Abbreviations

µM	Micromolar
UK	United Kingdom
USA	United States of America
UTR	untranslated region
UV	Ultra violet
V	Volts
VI/VInv	vacuolar acid invertase
vs	versus
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside
χ^2	Chi square

Chapter 1

Literature review

Chapter 1 Literature review

1.1 Importance of potato crop

Potato (*Solanum tuberosum* L.) is globally one of the most important vegetable food crops of high nutritional value. Being a multi-facetted crop, it is used worldwide not only for human and animal consumption, but also in the food industry for starch and other industries for textile, paper and alcohol production. Tuber quality is one of the most important aspects of potato production which includes: various biological parameters such as proteins, carbohydrates and minerals; sensory traits which include flavour and texture; and processing traits such as tuber shape, cold sweetening and starch quality (Carputo et al. 2005). Genetic engineering and molecular marker technology offer enormous opportunities for improvement of all the above quality traits in potato. Among all the quality parameters, there is ample scope for the genetic improvement of cold-induced sweetening (CIS) in potato which causes a great loss to the processing industry.

1.2 Cold-Induced Sweetening (CIS)

After harvesting, potatoes need to be stored for long periods at a low temperature to prevent sprouting for round-the-year processing. The accumulation of free sugars by plants at low, non-freezing, temperatures is a widespread phenomenon, first observed by Müller-Thurgau (1882, as cited in Sowokinos 2001a), although, the type of sugars accumulated is species dependant (Guy et al. 1992). Storage organs, such as potato tubers, accumulate sucrose, and reducing sugars (glucose and fructose) when subjected to chilling temperatures, a phenomenon referred to as cold-induced sweetening (CIS) or low-temperature sweetening (Burton 1969; Dale and Bradshaw 2003). The accumulation of sugars at low temperatures causes diminution in starch content and ultimately affects the quality of the fried products. The increase in reducing sugars reduces the quality of fried potato products. Furthermore, these reducing sugars (glucose and fructose) reacts with amino acids such as asparagine in a Maillard type reaction (Shallenberger et al. 1959; Dale and Bradshaw 2003) during frying which forms dark brown to black colour and produces the harmful carcinogenic compound acrylamide (Mottram et al. 2002). Despite extensive research, the functional importance and the exact mechanism behind the cold accumulation of reducing sugars is not fully understood.

Currently CIS is considered as one of the most severe problems in potato production and processing with potatoes tolerant of CIS becoming a priority in a number of potato breeding programmes (Xiong et al. 2002; Hamernik et al. 2009). Cultivars resistant to cold-

induced sweetening would provide several economic benefits, such as less need for sprout inhibitors, reduced dry matter loss, easy maintenance of high relative humidity, less pathogen problems during storage and less chance of chilling injury during harvest, storage and transit (Burton 1969; Duplessis et al. 1996). There has been significant international breeding effort to produce germplasm with resistance to CIS, including the Plant & Food Research (PFR) potato breeding programme in New Zealand (Anderson et al. 2005). PFR potato line, 1021/1, has been shown to rival the best lines worldwide in terms of CIS resistance coupled with high agronomic performance (Anderson et al. 2005; McKenzie et al. 2005).

Chen et al. (2001) constructed a potato molecular genetic map mainly for candidate genes operating in carbohydrate metabolism and transport. The enzymes and transport proteins participating in these processes have been characterized biochemically and corresponding genes from potato or other plants have been cloned and characterised at the molecular level. Despite extensive research, there is no unequivocal evidence for genetic understanding of CIS and there are still major gaps in the knowledge of this trait.

1.3 Processing quality

Potatoes are consumed both in the natural state and as processed foods. French fries and crisps are key sectors of the processing industry and represent more than 70% of the potato processing industries. Potatoes are required year round, however, producing fresh potatoes throughout the year is impracticable and long term storage is mandatory (Sonnewald 2001). Usually 8-10°C is the recommended storage temperature for potatoes to prevent the accumulation of reducing sugars and to control the development of various diseases, weight loss and sprouting. With the removal of chemicals used to inhibit sprouting, the storage of potatoes at 4°C becomes an important management strategy (Perlasca 1956; Sowokinos and Preston 1988; Sowokinos 2001a). However, this causes a large accumulation of sugars and ultimately degrades starch (Hajirezaei et al. 2003) and diminishes the tuber quality. The level of reducing sugars increases as they are stored below 8°C. The aldehyde groups of reducing sugars such as glucose and fructose interact with amino acids (free amino groups) at high frying temperatures (the Maillard reaction) and form unsightly and unpalatable products (Shallenberger et al. 1959; Dale and Bradshaw 2003).

To overcome the deterioration in chip (crisp) colour, cold-stored potatoes can be reconditioned at warmer storage temperatures (i.e. > 10 °C) prior to processing, which results in a decrease in reducing sugar content as some of these sugars are converted back

into starch (Isherwood 1973; Samotus et al. 1974). However, Samotus et al. (1974) reported that reconditioning does not always lower the concentration of reducing sugars to acceptable levels and long term CIS is considered to be irreversible. Cheng et al. (2004) studied the effect of variation of different enzymes on reducing sugar content and processing quality of potato tubers and observed a linear relationship between reducing sugar content and chip colour index of potato tubers.

In recent years, interest has been developed in CIS because of the discovery of acrylamide in fried potatoes (Pedreschi and Zuniga 2009). The two major substrates for acrylamide formation in processed potato products are reducing sugars and asparagine (Göekmen and Palazoğlu 2008). Acrylamide is a potent neurotoxin (Tilsen and Cabe 1979) and group 2A potential carcinogen (Tareke et al. 2002), which is formed from asparagine and reducing sugars via an N-glycoside intermediate in a side reaction of the Maillard reaction (Mottram et al. 2002; Stadler et al. 2002). A positive correlation between the amount of acrylamide and the reducing sugar content of the tubers was observed in fried potato products, which is further exaggerated by prior storage of the potatoes at low temperature (Olsson et al. 2004; De Wilde et al. 2005; Williams 2005). Chie et al. (2006) studied the effects of storage temperature on the content of sugars and free amino acids in tubers from different potato cultivars and acrylamide in chips and concluded that reducing sugar content was the limiting factor for acrylamide formation. Controlling the concentration of reducing sugars remains a promising strategy for the manipulation of cold-induced sweetening. CIS is documented as one of the most persistent and severe problems of the processing industry (Bhaskar et al. 2010; Brummell et al. 2010).

1.4 Carbohydrate metabolism

Carbohydrate metabolism in potato tuber involved several enzymes and pathways that include starch synthesis, starch breakdown i.e. starch to sugar conversion, glycolysis, mitochondrial respiration etc. The enzymes involved in these processes are well known and have been extensively studied (Isherwood 1973; Isherwood 1976; Amir et al. 1977; Preiss 1982; Sowokinos 2001a). Figure 1.1 shows the simplified version of carbohydrate metabolism in potato tubers adapted from Sowokinos (2001a). The figure shows only few enzymes involved in the CIS process and only key enzymes are explained in detail.

1.4.1 Biochemical mechanism of cold-induced sweetening (CIS)

Sowokinos (2001a) postulated that the control of sugar accumulation in potatoes is a

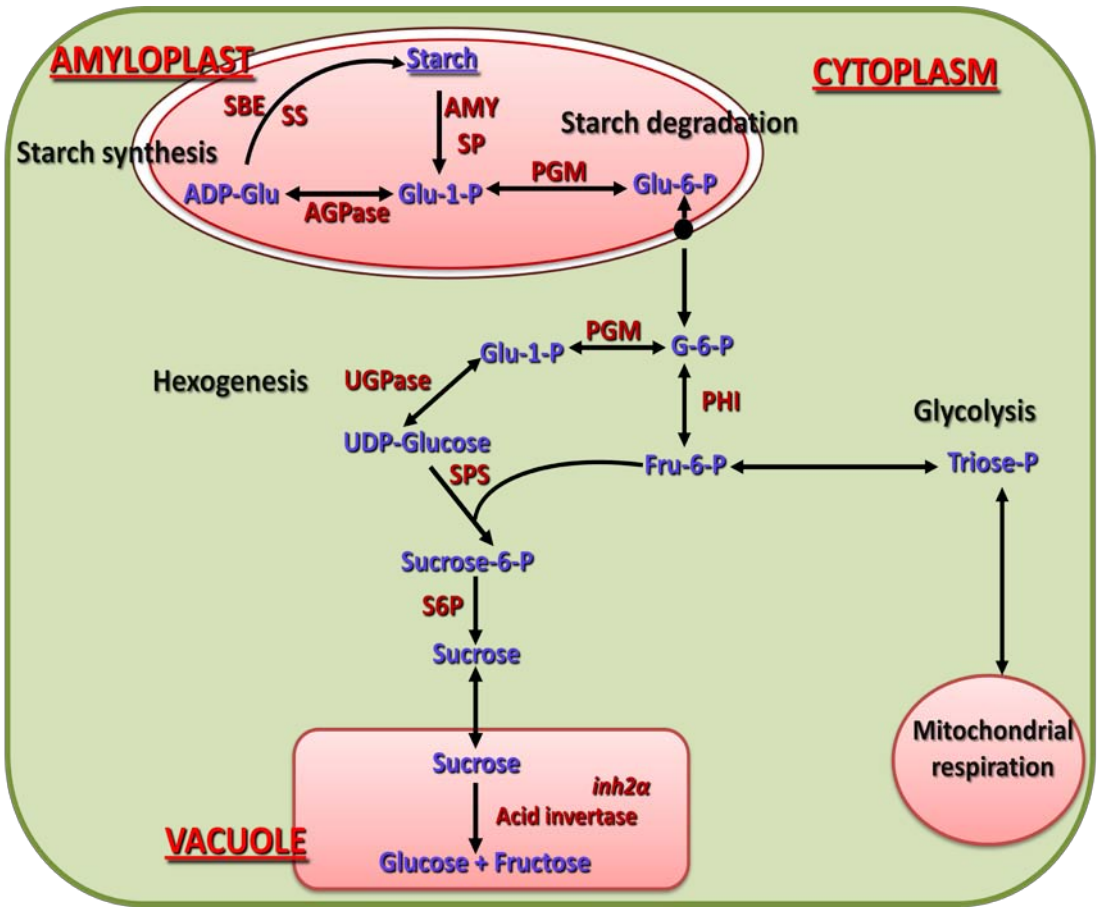


Figure 1.1 Simplified schematic overview of starch and sugar metabolism in potato tubers. The figure is a modified version taken from a paper published by Sowokinos (2001a). The figure explains the simplified pathway of starch and sugar metabolism in potato tubers. Only the key enzymes involved in the pathway are explained in the text. Starch synthesis involves ADP-glucose pyrophosphorylase (AGPase), Starch synthase (SS) and Starch branching enzyme (SBE). AGPase is the key enzyme involved in the synthesis of starch in amyloplasts. This process involved more than one SS and SBE (not shown in figure). Further it is degraded into Glucose-1-Phosphate (Glu-1-P) by amylase (AMY) and starch phosphorylase (SP) which forms Glucose-6-Phosphate (Glu-6-P) via phosphoglucomutase (PGM). Fructose-6-Phosphate (Fru-6-P) is formed from Glc-6-P via phosphohexoisomerase (PHI) and enters into glycolysis and respiration. In the hexogenesis pathway, sucrose is formed in the cytoplasm from UDP-Glucose. UDP-glucose pyrophosphorylase (UGPase), sucrose phosphate synthase (SPS) and sucrose 6-phosphate phosphatase (S6P) are involved in the process. Sucrose is transported into the vacuole and converted by acid invertase leading to the formation of reducing sugars glucose and fructose. Acid invertase is inhibited by vacuolar invertase inhibitor (*inh2α* as per Brummell et al. 2011 and *Stinh* as per Baldwin et al. 2011) in the vacuole.

complex metabolic process and it is affected by several levels of cellular control. For example, the regulation of cold-induced sweetening, has been reported as a relatively slow sweetening process (Ewing 1981), which may initially act through hormone alterations (Doucette and Pritchard 1993; Isherwood 1973, 1976). As a consequence, membrane permeability and composition is affected (Isherwood 1976; Knowles and Knowles 1989; Spychalla and Desbotough 1990; O'Donoghue et al. 1994). Furthermore changes in membrane structure and function can result in cellular adjustments in the compartmentalisation of key ions, substrates, and enzyme effector molecules (Isherwood and Kennedy 1975; Sowokinos 1990a). In addition, sweetening also involves the transport of metabolites across the amyloplasts membrane. Ultimately, modulation of post-translational activity of key enzymes that influence the flux of carbon toward sugar formation occurs because of the altered gene expression resulting from the sweetening process (Sowokinos 1990b). Although the pathway of carbohydrate metabolism is available, the exact mechanism involved behind the cold regulation of various enzymes in carbohydrate metabolism still remains to be elucidated.

The tuber is a storage organ where diverse biochemical events occur. The main carbohydrates found in potato tuber are starch, sugars (i.e. sucrose, glucose and fructose) and non-starch polysaccharides (cell wall components, often termed dietary fibre) (Lister and Munro 2000). Burton et al. (1992, as cited in Song 2004) reported that 60-80% of the dry matter (DM) consists of starch; there is a major correlation between DM content and the starch content of the tuber (Khayatnezhad et al. 2011). Potato starch is composed of 20 to 30% amylose and 70 to 80% amylopectin (Shannon and Garwood 1984, as cited in Visser et al. 1997; Schwall et al. 2000; Hoover 2001). Both starch and sugars play an important role during tuber formation (biosynthesis of starch) and during storage of potatoes (breakdown of starch) (Bánfalvi et al. 1999). Although the carbohydrate metabolism in both developing and stored potato tubers is well documented, the initial stimuli and mechanisms regulating the process of cold sweetening are still poorly understood (ap Rees and Morrell 1990). Sowokinos (2001a) postulated that CIS involved the interaction of a number of pathways of carbohydrate metabolism (Figure 1.1), including: starch synthesis, starch breakdown, glycolysis, hexogenesis (i.e. formation of glucose and fructose), and mitochondrial respiration.

1.4.2 Starch synthesis

Starch synthesis takes place in the plastids (amyloplasts) of potato cells. ADP-glucose pyrophosphorylase (AGPase EC 2.7.7.27), starch synthase (SS EC 2.4.1.21) and starch branching enzyme (SBE EC 2.1.4.18) are involved in the process of starch synthesis while amylases and starch phosphorylase (SP EC 2.4.1.1) are responsible for the breakdown (Preiss 1982) (Figure 1.1). It has been well documented that storage tissues contain one or more soluble starch synthases (Abel et al. 1996) as well as granule-bound starch synthase (Khoshnoodi et al. 1996; Larsson et al. 1998). ADP-glucose pyrophosphorylase (AGPase) is the chief enzyme responsible for the synthesis of starch in amyloplasts (Preiss 1982; Rathore et al. 2009). Okita et al. (1990) suggested that AGPase consisted of two regulatory subunits and two slightly smaller catalytic subunits. Further evidence from Iglesias et al. (1993) and Ballicora et al. (1995) suggests that the function of the large subunit of AGPase is to modulate the regulatory properties of the small subunit (sAGP) and the function of sAGP (small subunit of ADP-glucose pyrophosphorylase) is primarily catalysis. Starch synthase (SS) and starch branching enzyme (SBE) form α-1, 4-glucan macromolecules. ADP-glucose is used as the substrate by starch synthase enzymes, which add glucose units to the end of a growing polymer chain to build up a starch molecule (releasing the ADP in the process) (Preiss 1982).

As a step forward, Lorberth et al. (1998) cloned a gene involved in starch metabolism that encoded a protein (R1, protein associated with starch granules) capable of introducing phosphate into starch-like glucans. In order to reduce the activity of the R1 protein an antisense technology was used. Using this technology they were able to produce a reduced-phosphate starch in transformed potatoes that were resistant to degradation when compared to starch from wild type potatoes. As a result, upon cold storage the genetically modified potatoes accumulated less hexose. Greiner et al. (1999) claimed that genetic approaches may be successful in reducing sugar concentration of free sugars in cold stored potatoes, but they would be of limited commercial value if either the quality or the quantity of starch was altered. However, research is in progress to increase both quantity and quality of starch by manipulating the enzyme AGPase by molecular and genetic approaches (Song Batao et al. 2007, pers. comm., Huazhong Agricultural University, Wuhan, China).

1.4.3 Starch degradation

It has been reported that starch can be degraded either hydrolytically or phosphorolytically. The hydrolytic pathway involves enzymes like α-amylase (EC 3.1.2.1) and

β-amylase (EC 3.2.1.2) while the phosphorolytic pathway involves starch phosphorylase (Preiss 1992; Solomos and Mattoo 2005) (Figure 1.1). Increase in starch phosphorylase (SP, Figure 1.1) activity acts as a triggering event in the sweetening of potato tubers during cold storage (Claassen et al. 1993). Cottrell et al. (1993) showed that the activities of starch-degrading enzymes (i.e., α-amylase & β-amylase) increase during cold sweetening of potatoes. As shown in Figure 1.1, starch breaks down either hydrolytically or phosphorolytically and the products are exported from the amyloplasts as hexose phosphates (hexose-P) via the glucose phosphate-phosphate translocator (Weber 2004; Smith et al. 2005). Morrell and ap Rees (1986a) reported that the process of starch degradation during cold sweetening is predominantly phosphorlytic rather than hydrolytic in nature. However, other studies have reported increased activities of α-amylase, β-amylase (Cochrane et al. 1991, Cottrell et al. 1993, Nielsen et al. 1997) and phosphorylases (Sowokinos et al. 1985; Claassen et al. 1993) in some cultivars when tubers are stored at 4°C, and that these changes are absent or involve other activities in other cultivars (Davies 1990; Hill et al. 1996). The role of starch degrading enzymes in potato tubers is not entirely clear, but they might remain a potential target for manipulation of cold-induced sweetening. Sonnewald et al. (1995) reported that there are two starch phosphorylase isozymes, types L and H, in potato and they are believed to be responsible for the complete breakdown of starch where type L is found in amyloplasts, while type H is cytosolic in location.

1.4.4 Glycolysis

Glycolysis (Figure 1.1) is also thought to be involved in cold storage of potato tubers. Dixon and ap Rees (1980a) suggested that sweetening occurs because the entry of hexose-P into glycolysis is restricted at low temperature and thereby diverting the products of starch breakdown into the pathway of sucrose synthesis. Several enzymes (fructose-1, 6-bisphosphatase EC 3.1.3.11, adenosine triphosphate linked 6-phosphofructokinase EC 2.7.1.11, pyrophosphate linked 6-phosphofructokinase EC 2.7.1.90 and pyruvate kinase EC 2.7.1.40; enzymes involved in glycolysis pathway are not shown in Figure 1.1) have been investigated during cold sweetening and various possibilities have been proposed (Sowokinos 2001a). ap Rees et al. (1981) explained that the manner in which potato tubers, and other plant tissues, metabolize [^{14}C] glucose suggests that the conversion of fructose 6-phosphate to fructose 1, 6-bisphosphate is the first committed step of glycolysis, is restricted in the cold. The experiments conducted by ap Rees et al. (1981) showed that by lowering the temperature from 25°C to 4°C the proportion of metabolised glucose that enters the

respiratory pathway is decreased. This results in an accumulation of hexose-P and, subsequently sucrose (Pollock and ap Rees 1975b; Dixon and ap Rees 1980a). The entry of hexose-P into glycolysis is restricted at low temperature, which is thought to be due to the cold inactivation of the enzymes catalysing the synthesis of fructose 1, 6-bisphosphate from fructose-6-phosphate.

1.4.5 Hexogenesis

The hexogenesis pathway (Figure 1.1) is also considered highly important during cold storage. UDP-glucose pyrophosphorylase (UGPase EC 2.7.7.9), sucrose phosphate synthase (SPS EC 2.4.1.21) and sucrose 6-phosphate phosphatase (S6P EC 3.1.3.24) are involved in the CIS process (Figure 1.1) (Sowokinos 2001a) only a few are explained here. Sucrose is formed via UDP-glucose pyrophosphorylase and sucrose phosphate synthase (Pollock and ap Rees 1975a; Sowokinos 2001b; Hill et al. 1996). It is believed that the formation of UDP-Glucose (UDP-Glc) via enzyme UGPase (UDP-glucose pyrophosphorylase) is the first step to the cold-induced sweetening process in potato tubers (Sowokinos et. al. 1997). Post harvest studies and storage at low temperature revealed the relationship between UGPase and sucrose contents in potato tubers. Sowokinos et al. (1997) observed a relationship between allelic polymorphism of UGPase from potato clones and their ability to accumulate sugars in cold storage. Furthermore they concluded that the differences in kinetic properties of cold-resistant UGPase-isozymes may provide a biochemical basis for explaining the variation in sugar accumulation between potato cultivars.

Sucrose phosphate synthase is thought to play a major role in sucrose biosynthesis in both photosynthetic and non-photosynthetic tissues (Huber and Huber 1996). Reimholz et al. (1997) reported that potato plants contain several forms of SPS that have different functions in growing and mature tissues, in flower parts, and multiple forms in cold-stored tubers. Deiting et al. (1998) reported that the time course and temperature dependence of the appearance of the novel form of SPS is correlated with sugar accumulation. Antisense technology was also used to suppress SPS in transgenic potato plants to analyse the contribution of this enzyme in the cold-induced sweetening process (Krause et al. 1998) and a decrease of 10 to 40% of soluble sugars in cold stored tubers was observed. It is concluded that the increased sucrose production at low temperature is due to changes in the kinetic properties of SPS, rather than an increase in the catalytic capacity (Krause et al. 1998).

Sucrose synthase (EC 2.4.1.13) (not shown in Figure 1.1) and acid invertase (EC 3.2.1.26) (Figure 1.1) are also important hydrolytic enzymes promoting sweetening ability of

potato tubers. Sucrose is hydrolysed into reducing sugars i.e. glucose and fructose by soluble acid invertases (Pressey 1969; Richardson et al. 1990; Zrenner et al. 1996; Greiner et al. 1999), although the extent of the sugar accumulation is cultivar dependant (Hammond et al. 1990; Richardson et al. 1990; Zrenner et al. 1996). Studies were conducted by Zrenner et al. (1996) in cold-stored tubers of 24 potato cultivars differing in their cold-induced accumulation of reducing sugars. They observed no correlation between the total amount of invertase activity and the accumulation of reducing sugars. However, a striking correlation between the hexose/sucrose ratio and the extractable soluble invertase activity was noticed.

Pressey (1967) reported the presence of an inhibitor for acid invertase in potato tubers (Figure 1.1). Pressey (1969) and Sasaki et al. (1971) reported that acid invertase activity increases several fold during low temperature storage leading to a high concentration of reducing sugars. The lack of correlation between sugar levels and invertase activity following three months storage at low temperature was due to the presence of an invertase inhibitor (Pressey 1969). Acid invertase and its inhibitor are reviewed in more detail below (Section 1.5).

1.4.6 Mitochondrial respiration

Isherwood (1973) and Solomos and Laties (1975) indicated that a sharp increase in respiration rate in cold-stored potato tubers is associated with a concomitant rise in the ATP concentration, and a subsequent increase in sugars. These results therefore suggest that the increase in respiration is responsible for generating ATP, which is then utilized in sucrose synthesis. Amir et al. (1977) also observed an increase in respiration rate, ATP levels and sugar content in tubers stored at 4°C. However, they mentioned that the rapid increase in respiration preceded the increase in sugars, but occurred later than the increase in ATP.

In addition to carbohydrate metabolic pathways, electrolyte leakage and membrane lipid peroxidation have also been implicated in CIS (Workman et al. 1979; Spychalla and Desborough 1990; Wismer et al. 1998).

It has been well documented that activities of several enzymes and their isoforms are interlinked and closely related to CIS. Much research addressed the enzymatic changes that occur in potato tubers of single cultivars when they are stored at cold temperatures. There are however fewer examples of comparisons between cultivars known to have varying degrees of CIS resistance (Richardson et al. 1990, Cottrell et al. 1993; Zrenner et al. 1996; Sowokinos 2001b). Isherwood (1976) suggested that CIS requires longer term changes since there is often a delay of several days following transfer to low temperature before sugars

begin to accumulate. This delay may be associated with changes in gene expression that form part of the process of cold acclimation (Van Buskirk and Thomashow 2006; Yamaguchi-Shinozaki and Shinozaki 2006). Despite extensive research and numerous proposed mechanisms, there is still very little conformity with respect to the exact mechanism of CIS in potato tubers at the biochemical and molecular level (Blenkinsop et al. 2002).

1.5 Key enzymes involved in cold-induced sweetening

The CIS process in potato tubers involves a network of genes and the final content of sugar in a potato tuber, at any given point in time, is influenced by several genes (Sowokinos 2001a). Three key enzymes are described in the following sections.

1.5.1 Acid invertase (AcInv: β-fructofuranosidases, EC 3.2.1.26)

Acid invertase is the key enzyme involved in cold sweetening of potato tubers (Figure 1.1). The presence of invertase in potatoes was first reported over 100 years ago (Kastle and Clark 1903, as cited in Bracho and Whitaker 1990b). During low temperature, starch is degraded primarily into sucrose and then subsequently hydrolyses to reducing sugars (glucose and fructose) via acid invertase. According to its biochemical properties and sub-cellular locations, invertases (β-fructofuranosidases EC 3.2.1.26) are classified into 1) vacuolar, 2) cell wall bound (apoplastic) and 3) neutral invertases (Sturm 1999). Apoplastic invertases have a low pH-optimum (pH 3.5 to 5.0), vacuolar invertase have an acidic pH-optimum (pH 5.0-5.5), while neutral invertase, located in the cytosol with a pH optimal for sucrose cleavage in the neutral or slightly alkaline range (pH 6.8-8.0) (Roitsch and González 2004). Numerous reports demonstrate the relationship between acid invertase activity and hexose accumulation in potato tubers during low temperature and it is well known that the concentration of glucose and fructose is higher than sucrose during cold storage. Chie et al. (2006) studied changes in sugar content and activity of vacuolar acid invertase during low-temperature storage of potato tubers from six Japanese cultivars. On the basis of sugar accumulation capacity, they divided cultivars into three types:

Type 1, high sugar accumulating, cold-sensitive cultivars,

Type 2, low sugar accumulating, cold-tolerant cultivars,

Type 3, increased sugar, but not reducing sugars.

Furthermore, they suggested that the patterns of sugar changes during low temperature storage are classified into the above three types and are closely related to the activities of vacuolar acid invertase.

McKenzie et al. (2005) investigated the role of acid invertase in potato clones with varying resistance to cold-induced sweetening. Basal acid invertase activity showed a positive correlation to the glcose:sucrose ratio across the clones that were significant before and after cold temperature storage. They suggested that acid invertase plays a dominant role in the hexogenic pathway by regulating the hexose:sucrose ratio. Cheng et al. (2004) studied the effect of invertase activity on reducing sugar accumulation and the processing quality of potato tubers at 4°C. They found that when tubers were stored at 4°C, the activity of acid invertase showed a significant linear correlation with reducing sugar content. Furthermore, they suggested that acid invertase could play a major role in the cold-sweetening of potato tubers through regulating starch-sugar metabolism and may provide a new strategy and approaches in improving potato processing quality. This can be achieved by either overexpressing the invertase inhibitor or by knock down expression of invertase.

Low temperature regulated gene expression in cold stored potato tubers has been studied extensively by van Berkel et al. (1994). They suggested that the characterisation and the study of cold-inducible gene expression in potato tubers and their promoters is a useful part of a strategy to suppress cold temperature sweetening by manipulating the expression of certain genes at low temperature. Bournay et al. (1996) indicated that the potato acid invertase gene contains 6 exons, including an extremely small exon (exon 2), that codes only for the core tripeptide (DPN) of the highly conserved motif NDPNG (among invertases of plants, yeast and bacteria, Sturm and Chrispeels 1990). Bournay et al. (1996) and Simpson et al. (2000) have shown that low levels of exon skipping of the mini exon 2 of potato invertase pre-mRNA occurs under cold-stress conditions. Whether this low-level exon skipping reflects regulation of splicing or is an effect of low temperature on the integrity of the spliceosome is unknown.

1.5.2 Invertase Inhibitor

It has been well documented that invertase activity is also regulated by a proteinaceous inhibitor (Pressey 1967). Schwimmer et al. (1961) first reported evidence for an endogenous proteinaceous inhibitor of invertase in potatoes. Pressey (1966, 1967) purified the inhibitor to homogeneity and partially characterised it as a protein with a molecular weight of about 17,000 Da. It binds slowly and irreversibly to potato tuber invertase (Ewing and McAdoo 1971). Invertase inhibitors have also been found in red beet, sugar beet and sweet potato (Pressey 1968; Matsushita and Uritani 1976), as well as in maize endosperm (Jaynes and Nelson 1971). Pressey (1966) claimed that tuber storage

temperature also affects the inhibitor activity and further suggested that the inhibitor plays a physiological role in intact tubers. He postulated that the lack of correlation between sugar levels and invertase activity following three months storage at low temperature was due to the presence of an invertase inhibitor (Pressey 1969). Richardson et al. (1990) noted that total acid invertase activity (i.e., assayed after destroying the endogenous inhibitor) generally reflected sugar changes more closely than did basal activity (i.e., assayed with the inhibitor present).

Liu et al. (2010) cloned and characterised four invertase inhibitor genes from potato. Furthermore, they also studied the transcript levels from cold-susceptible and resistant genotypes from cold stored potato tubers. In addition, they investigated the interaction between invertase and invertase inhibitor which significantly influenced the reducing sugar accumulation in cold stored potato tubers. Similarly, Brummell et al. (2011) cloned and sequenced apoplastic (*inh1*) and vacuolar invertase inhibitor (*inh2α*) genes from potato and studied their expression patterns from cold-stored potato tubers from cold-susceptible and resistant cultivars. In addition to *inh2* which shows developmentally regulated alternative splicing, two hybrid mRNAs (*inh2β*A* and *inh2β*B*) resulting from mRNA splicing of an upstream region of *inh2* to a downstream region of *inh1*, were also hypothesised to contribute to the cold induced sweetening process in potato tubers. These hybrid mRNAs encode deduced vacuolar invertase inhibitors which possess divergent C- termini. Their investigation leads to the hypothesis that during cold storage, *inh2α* and the hybrid *inh2β* mRNAs accumulate to higher abundance in cultivars resistant to cold-induced sweetening than in susceptible cultivars.

1.5.3 ADP-glucose-pyrophosphorylase (AGPase: EC 2.7.7.23)

Adenosine diphosphate glucose pyrophosphorylase (AGPase) catalyses an important regulatory step in starch biosynthesis (Müller-Röber et al. 1992; Stark et al. 1992; Fu et al. 1998). In the presence of ATP and glucose-1-P (Glu-1-P), AGPase produces ADP-glucose and releases PPi. The ADP-glucose is then used as a substrate by starch synthase enzymes (*SS*; EC 2.4.1.21), which add glucose units to the end of a growing polymer chain to build up a starch molecule, releasing the ADP in the process (Müller-Röber et al. 1992). The potato tuber AGPase consists of two different subunits of 51 and 50 kDa. The major function of the larger subunit is to modify regulatory properties of the smaller subunit, which is the subunit primarily involved in catalysis (Ballicora et al. 1995). The structure and function of AGPase

has been extensively studied and cDNA encoding AGPase have been characterized from potato tubers (Ballicora et al. 1995).

Müller-Röber et al. (1992) confirmed that the enzyme AGPase is a key enzyme of starch biosynthesis by transforming potatoes with a chimeric gene containing the coding region of one of the subunits of the AGPase linked in an antisense orientation to the CaMV 35S promoter. They observed almost complete inhibition of the AGPase in tubers. Furthermore, there was abolition of starch formation in tubers, thus proving that AGPase has a unique role in starch biosynthesis in plants. Furthermore, the activity of AGPase is subject to allosteric regulation by 3-P-glycerate (activation) and inorganic phosphate (inhibition) (Sowokinos and Preiss 1982).

Stark et al. (1992) suggested an increasing starch synthesis rate and reduced accumulation of hexose in transgenic plants overexpressing a mutated AGPase gene (*glgC16*) under CaMV (cauliflower mosaic virus) 35S promoter from *E. coli*. However, the same increase in starch synthesis was not detected when overexpressing the same gene under a patatin promoter in potato in spite of a four to five fold increase of the activity of AGPase being noticed (Sweetlove et al. 1996a).

1.6 Use of transgenic potatoes to investigate cold-induced sweetening

1.6.1 Genetic manipulation of acid invertase

Genetic modification of acid invertase activity in potatoes has successfully resulted in decreased concentrations of reducing sugars by antisense repression of invertase (Zrenner et al. 1996). Zrenner et al. (1996) transformed potato with a cold-inducible vacuolar invertase (VI) isoform in an antisense configuration. The cDNA of this transcript was expressed in the antisense orientation under control of the CaMV 35S promoter. Analysis of the harvested and cold-stored transgenic tubers showed that the soluble acid invertase activity was reduced. As a result decreased hexose and increased sucrose content were noted compared with controls. There was no correlation between the total acid invertase activity and the accumulation of reducing sugars in transgenic potato tubers, but there was a correlation between the hexose/sucrose ratio and the extractable soluble acid invertase activity. Furthermore, after storage at 4°C, hexose/sucrose ratios were significantly reduced in transgenic tubers.

Recently the use of antisense technology or gene silencing techniques has been employed to reduce the hexose accumulation in potato tubers and also to modify starch quantity and quality by suppressing the activity of acid invertase. This approach is

anticipated to reduce hexose production and minimise cold sweetening which ultimately results in improved processing quality of potato tubers. Using antisense technology, Liu et al. (2011) showed that suppression of *StvacINV1* (vacuolar invertase gene) in transformed potato lines resulted into lower *StvacINV1* transcripts, lower reducing sugars and lighter chip colour in cold-stored transgenic lines. Similarly Wu et al. (2011) silenced the potato vacuolar acid invertase gene (*VInv*) and observed ~93% reduction in reducing sugars in cold-stored transgenic lines.

In order to reduce the cold accumulation of reducing sugars in potato tubers, Cheng et al. (2007) introduced *Nt-VIF* (*Nicotiana tabacum* vacuolar inhibitor of β-fructosidase, a vacuolar invertase inhibitor) gene regulated by potato tuber specific promoter (patatin class I). The results showed that there were no significant differences in reducing sugar content of transgenic and control tubers stored at 20°C. However, reducing sugar content of transgenic lines was reduced at 4°C compared to the control as a result of decreased activity of vacuolar invertase. Furthermore, a positive correlation between low vacuolar invertase activity and low reducing sugar content was observed which suggests that the quality improvement in potato could be achieved by increasing cold sweetening resistance using this strategy.

Bhaskar et al. (2010) demonstrated that both processing quality and acrylamide problems in potato can be controlled effectively by silencing the potato vacuolar acid invertase gene *VInv*. RNA interference (RNAi) suppression of *VInv* has been used to decrease *VInv* gene expression by up to 78% (Zhang et al. 2008). However, the experiment failed to control CIS.

Therefore, transgenic plants with altered invertase activity or reducing sugar content offer opportunities for the potato processing industry to increase the quality of potato tubers.

1.6.2 Genetic manipulation of invertase inhibitor

To overcome cold-induced hexose accumulation, vacuolar invertase inhibitors were overexpressed in transgenic potato plants and prevented cold induced sweetening of potato tubers (Greiner et al. 1999; Cheng et al. 2007). Important studies for the biotechnological use of *Nt-inhh*, a putative vacuolar invertase inhibitor from tobacco in potato transformations was observed by Greiner et al. (1999). Co-localising with the invertase in the cell wall (apoplastic), the inhibitor is presumed to play an important physiological role in the regulation of invertase activity (Greiner et al. 1999). A vacuolar invertase inhibitor from

tobacco was overexpressed in potato and prevented cold induced sweetening of potato tubers. They investigated the possibility of repressing the action of cold-induced vacuolar invertase (VI) by expression of a putative vacuolar invertase inhibitor from tobacco, called *Nt-inhh* in potato plants under the control of the CaMV 35S promoter. They found 75% reduction in hexose accumulation without affecting tuber yield. Transgenic tubers had the same quantity and quality of starch observed as seen in the control tubers.

To slow down the accumulation of reducing sugar in potato tubers exposed to low temperature storage, *Nt-VIF*, a vacuolar invertase inhibitor from tobacco regulated by a potato tuber-specific promoter (patatin class I), was transformed into potato (Cheng et al. 2007). The results showed that there were no significant differences in reducing sugars content between transgenic and untransformed (control) tubers stored at 20°C. However, reducing sugar content of transgenic lines was reduced at 4°C compared to the control, implying that vacuolar invertase activity was inhibited by *Nt-VIF* expression resulting in reduced content of reducing sugars. Furthermore, a positive linear relationship was observed between vacuolar invertase (VI) activity and reducing sugar content leading to the conclusion that the transgenic lines met the requirements of potato chips in terms of their low reducing sugar content after cold storage.

1.6.3 Genetic manipulation of ADP-glucose pyrophosphorylase

Manipulation of potato cold sweetening by regulating AGPase activity may have effects on the transition between sucrose and starch. The mechanism of co-regulation of this enzyme and its modification by using the antisense technology for enhanced starch synthesis is being investigated as a possible approach to improve potato cold sweetening. Song Batao et al. (2007, pers. comm., Huazhong Agricultural University, Wuhan, China) transformed the *sAGP* gene which encodes a small subunit of ADP-glucose pyrophosphorylase in both sense and antisense orientation in potato. They found that upon cold storage the reducing sugar content in the transformed lines decreased significantly and chip colour index declined with an increase in *sAGP* expression. Their results further lead to the conclusion that the *sAGP* gene may play a crucial role in CIS of potato tubers.

1.7 Molecular genetic studies on cold-induced sweetening in potatoes

Tuber starch and sugar contents are important quantitative as well qualitative parameters in processing of potato tubers. Carbohydrate metabolism and the network of genes involved in sugar to starch conversion are now well characterised and have been cloned. Various types of molecular markers, such as restriction fragment length

polymorphism (RFLP), amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD), sequence characterised amplified region (SCAR), single nucleotide polymorphism (SNP), microsatellite or simple sequence repeat (SSR), inter-simple sequence repeat (ISSR), cleaved amplified polymorphic sequence (CAPS) and transposon tagging are available to study the genetic variations.

RFLP markers were the first molecular markers used to produce linkage maps in tomato and potato. Initially a molecular marker map was produced for diploid potato using RFLP markers from tomato and for nearly every tomato genomic or cDNA sequence tested there was a homologous sequence in potato (Bonierbale et al. 1988). A second RFLP map was produced by Gebhardt et al. (1989) using gDNA and cDNA RFLP probes of potato. The twelve linkage groups were assigned and common markers from the two maps were used to number certain linkage groups (Gebhardt et al. 1991). Tanksley et al. (1992) further increased the number of mapped RFLP markers for tomato and potato to over 1400 which allowed the further identification of inversions on chromosomes XI and XII in potato, compared to tomato. Jacobs et al. (1995) constructed a genetic map of potato based on integrating molecular markers, including transposons and classical markers using a backcross population of 67 diploid potato plants. The RFLP technique is expensive, laborious and time-consuming compared to the easier PCR-based analyses used nowadays.

Randomly Amplified Polymorphic DNA (RAPD) markers (Williams et al. 1990) are dominant and have been used for detecting marker trait associations in potato. RAPD analysis is a PCR based technique that produces polymorphism using one nonspecific random 10-mer primer. However, low reproducibility of RAPDs is a major disadvantage (Song 2004). RAPD analysis combined with Bulked Segregant Analysis (BSA) (Michelmore et al. 1991) has been used to identify DNA sequences linked to traits of interest in potatoes (Jacobs et al. 1996; Hosaka et al. 2001; Bryan et al. 2002).

Amplified fragment-length polymorphism (AFLP) markers are dominant and were developed by Vos et al. (1995) and have been used in potato mapping studies (van Eck et al. 1995). In potato, 6 primer combinations of *Eco*RI/*Mse*I primer set and a diploid mapping population, van Eck et al. (1995) constructed an AFLP map. This map was consisted of 264 AFLP markers and the length of the map was 1170 cM. van Os et al. (2006) constructed an untlradense genetic linkage map consisted of > 10,000 AFLP loci in diploid potato mapping population comprising 136 individuals. The population was analysed with a total of 381 AFLP

primer combinations derived from three enzyme combinations. This potato map is the densest map of potato.

Simple sequence repeats (SSRs), also called microsatellites are short tandemly repeated, highly polymorphic sequences. SSR have been used in the identification of cultivars, fingerprinting and potato genome mapping projects. SSR marker systems have many advantages over other markers, such as ease of analysis, high polymorphism rate, high reliability, co-dominance and transferability among related species (Yildirim et al. 2009). SSR technology has been developed for potato using DNA from modern cultivars. The relative abundance of SSRs in the EMBL and the GenBank databases of potato and related sequences were first reported by Provan et al. (1996). Milbourne et al. (1998) developed 112 SSR markers and 98 markers had a high level of polymorphism. Using 65 of these markers, investigators mapped 89 loci in two potato mapping populations. Ghislain et al. (2004) developed 48 SSR markers and used them to identify 931 potato germplasm accessions. In addition, they mapped 31 of these markers using mapping populations. Feingold et al. (2005) developed 94 SSR markers and used 61 of them for mapping and 30 for genetic characterisation of 30 potato cultivars. Hamilton et al. (2011) identified a large number of Single Nucleotide Polymorphisms (SNPs) in three potato cultivars. Their results revealed that based on 2Gb of sequence for each cultivar generated 575,340 SNPs were identified within these three cultivars along with 2,358 SNPs from three additional cultivars. A total of 69,011 high confidence SNPs were identified using six cultivars. Furthermore they have identified 2,117,754 raw SNP calls, of which, 147,525 SNPs remained after filtering with the Bowtie/SAMTools pipeline using a separate computational pipeline that mapped sequence reads directly to the DM reference genome. With these results they concluded that the SNPs will enable high-throughput genotyping of germplasm and populations, which in turn will enable more efficient marker-assisted breeding efforts in potato.

Collard et al. (2005) mentioned that many agriculturally important traits such as yield, quality and disease resistance are controlled by several genes and are known as quantitative traits. The regions within genomes that contain genes associated with a particular quantitative trait are known as quantitative trait loci (QTLs) (Geldermann 1975; Collard et al. 2005). Three widely-used methods for detecting QTLs are single-marker analysis, simple interval mapping and composite interval mapping (Tanksley 1993). Weller (1987) reported that in order to avoid the complexities of tetrasomic inheritance, most of QTL mapping in potatoes initially proceeded in diploids. Some valuable monogenic inherited

agronomic traits have already been mapped: Yellow flesh colour (Y) on chromosome III (Bonierbale et al. 1988), purple skin colour (PSC) on chromosome X (Gebhardt et al. 1989), flower colour loci for red (D) and blue (P) anthocyanins on chromosome II and XI (van Eck et al. 1994a) and tuber shape (Ro) on chromosome X (van Eck et al. 1994b). Resistance genes for several diseases and pathogens have also been mapped (Gebhardt and Valkonen 2001; Naess et al. 2000; Jacobs et al. 1996; Ballvora et al. 1995; Ritter et al. 1991). Schäfer-Pregl et al. (1998) identified 17 QTLs in potato for tuber starch content and demonstrated that they were distributed on all the 12 linkage groups in two diploid populations.

Association mapping has been defined as the method of choice for identifying loci involved in the inheritance of complex traits (Gupta et al. 2005; Yu et al. 2006). It provides a statistical association between genotypes at a marker locus and the phenotype. Association or linkage disequilibrium mapping using DNA-based markers is a novel tool in plant genetics for the analysis of complex traits (Gupta et al. 2005; Holland 2007). Luo et al. (2001) stated that association genetics is particularly useful in cultivated potato, a tetraploid, non-inbred crop species, where linkage analysis in experimental, tetraploid populations is complicated by tetrasomic inheritance.

A major breakthrough in the characterisation and identification of quantitative traits for sugar and starch in potato were based on the studies conducted by Chen et al. (2001) and Menéndez et al. (2002). The natural variation at candidate gene loci has been shown to be responsible for quantitative trait variations in potato (Menéndez et al. 2002; Li et al. 2008, Baldwin et al. 2011). So far most of the CIS research in potato involved phenotypic evaluations, molecular markers, identification of QTLs for sugar and starch and association based studies (Menéndez et al. 2002; Li et al. 2008; Baldwin et al. 2011). To identify genetic factors that contribute to chip colour in potato, Douches and Freyre (1994) constructed a genetic map with a combination of 10 isozymes, 44 restriction fragment length polymorphisms (RFLPs) and 63 randomly amplified polymorphic DNA (RAPD) markers. Their studies showed that 13 genetic markers identified six QTLs which contribute to chip colour variation. These QTLs explained 50% of the chip colour variation in the population. With these results they concluded that these QTLs can be introgressed via marker assisted selection into the cultivated potato germplasm to broaden the genetic base for chip processing ability or low reducing sugar levels.

Chen et al. (2001) created a molecular function map for carbohydrate metabolism and transport comprising 85 loci from 69 genes involved in starch synthesis and degradation,

sucrose metabolism, membrane transport, the Calvin cycle and photorespiration, glycolysis and the oxidative pentose phosphate pathways, and the TCA (tricarboxylic acid) cycle. They concluded that the candidate gene approach is a powerful method which correlates a phenotype with its underlying biochemical or physiological basis by demonstrating that candidate genes are tightly linked to the genetic locus of interest.

Association studies have been conducted in order to identify the molecular markers and candidate genes associated with the cold-induced sweetening process in populations of tetraploid potato individuals. Studies conducted by Li et al. (2005) showed the first association between chip colour and natural DNA variation at the invertase locus *invGE/GF* on potato chromosome IX which co-localises with a QTL for tuber sugar content (Menéndez et al. 2002). Invertase converts sucrose in the reducing sugars glucose and fructose therefore genes encoding invertase are obvious functional candidates. Studies have suggested that there are QTLs for tuber starch and sugar content on each of the 12 chromosomes and that one of them has candidate *invGE/GF* locus (Schäfer-Pregl et al. 1998; Menéndez et al. 2002; Gebhardt et al. 2005). Menéndez et al. (2002) concluded that genetic variation in cold sweetening in potato is the net result of allelic variants of enzymes operating in carbohydrate metabolic pathways. Associations between alleles of the carbohydrate metabolism genes invertase (Li et al. 2005) and UDP-glucose pyrophosphorylase (Sowokinos 2001b) with cold-induced sweetening have been reported (Baldwin et al. 2011). QTL for fry colour have been identified in a tetraploid mapping population on chromosomes 1, 6 and 11 and one unanchored linkage group from tubers stored at 4°C or 10°C (Bradshaw et al. 2008). D'hoop et al. (2008) identified associations between AFLP markers localised on chromosomes 1, 2, 4, 7 and 10 and tuber quality traits such as fry colour and chip colour after cold storage. Li et al. (2008) studied markers for 36 loci including 23 candidate gene loci and detected marker-trait associations with 15 candidate genes explaining 1.8-12.6% of the variation with chip colour (before or after cold storage). Recently, Baldwin et al. (2011) conducted an association mapping approach to identify alleles of candidate genes associated with cold-induced sweetening of potato using a collection of 161 lines. This includes markers designed for β-amylase, citrate synthase, apoplastic invertase inhibitor, acid invertase, sucrose synthase, UDP-glucose pyrophosphorylase, apoplastic invertase and vacuolar invertase inhibitor. Two candidate genes i.e. UDP-glucose pyrophosphorylase and apoplastic invertase were identified as being associated with cold-induced sweetening (Baldwin et al.

2011). In addition they have identified an interaction between alleles of the apoplastic invertase and the apoplastic invertase inhibitor.

Extensive studies have been conducted on a number of agronomically important quantitative traits such as potato tuberisation, tuber shape, tuber dormancy (Van den Berg et al. 1996a, b; Simko et al. 1997) and specific gravity (Freyre and Douches 1994). Thill and Peloquin (1994) evaluated a population of *S. tuberosum* dihaploid x wild species hybrid clones for potato chip colour. They proposed a three key loci hypothesis for the inheritance of chip colour at the diploid level and suggested that the simplest model for good chipping involves at least one dominant allele at each of the three loci. Furthermore, they concluded that regardless of the allelic constitution of the remaining two loci, poor chipping quality occurred when at least one locus had two recessive alleles. These studies suggest that there is ample scope to study the allelic variations of candidate genes operating in carbohydrate metabolism and their association with CIS of potato tubers.

1.8 Context of this thesis

Extensive research is required to produce a cultivar resistant to cold-induced sweetening (CIS) along with good processing quality and agronomic performance. The tools of genomics and genetic engineering can contribute to resolving this persistent problem in potato tubers. However, to achieve this, a sound understanding of the molecular genetic basis of CIS is needed for the germplasm being used by potato breeders. The Plant & Food Research (PFR) potato breeding programme in New Zealand has developed an elite potato line, 1021/1, that has become recognised as one of the best lines worldwide in terms of CIS resistance coupled with high agronomic performance (Anderson et al. 2005; McKenzie et al. 2005). The overall aim of the research in this thesis was to gain a better understanding of the genes controlling tolerance to CIS in 1021/1.

To achieve this aim, four objectives were established:

Objective 1: Phenotypic assessment for pigmentation, physiological and cold-induced sweetening traits in a tetraploid mapping population of potato (Chapter 2);

Objective 2: Association-based studies on tuber quality traits in potato (*Solanum tuberosum* L.) using single marker allele ANOVA analysis (Chapter 3);

Objective 3: Allele diversity of the apoplastic invertase inhibitor gene in potato (Chapter 4);

Objective 4: Overexpression and antisense suppression of the apoplastic invertase inhibitor gene in transgenic potatoes (Chapter 5).

In order to accomplish the aims of the present thesis, the progeny from a cross between potato cultivars with poor and excellent CIS response was established. Subsequently, the further studies pursue to establish phenotypic and genotypic characterisation of the mapping population for cold-induced sweetening and relevant traits. Association-based studies were carried out between marker alleles and the phenotype of the progeny. Based on previous studies on genetic transformation (Greiner et al. 1999; Cheng et al. 2007), QTL detection (Menéndez et al. 2002) for potato tuber starch and sugar contents and association analysis between allelic variations of important candidate genes (Baldwin et al. 2011) and cold-induced sweetening in potato tubers, apoplastic invertase inhibitor was found to meet the criteria of being a functional candidate. Additional studies led to the identification and characterisation of allelic variation of the apoplastic invertase inhibitor gene in potato genotypes showing varying response to CIS. In order to identify the specific alleles that may play a role in resistance to CIS, transgenic plants were produced with overexpression and antisense repression of apoplastic invertase inhibitor alleles. Analysis of the resulting plants provided additional evidence to verify the identity of key genes for the future genetic improvement of tuber properties with respect to long-term storage and processing characteristics.

Chapter 2

Phenotypic assessment for pigmentation, physiological and cold-induced sweetening traits in a tetraploid mapping population of potato

Chapter 2

Phenotypic assessment for pigmentation, physiological and cold-induced sweetening traits in a tetraploid mapping population of potato

2.1 Introduction

Potato (*Solanum tuberosum*) is an important nutritional vegetable food crop and is of particular importance to the processing industry. Potato breeding aims at the enhancement and alteration of various qualitative and quantitative traits. Among various quality parameters, cold-induced sweetening (CIS) in potato is considered the most widespread and undesirable characteristics which causes a great loss to the processing industry. Potato breeding programmes aims at selecting genotypes tolerant to CIS (Xiong et al. 2002; Anderson et al. 2005; Hamernik et al. 2009). Since quality demands of consumers and potato processing industry have become increasingly stringent, breeding efforts need to focus on quality traits in potato (D'hoop et al. 2008).

Quality of the processed French fries and crisp are largely depend on dry matter (DM) and sugar content (Pritchard and Scanlon 1997; Marwaha 1999). The frying colour is an important criterion for potatoes destined for the French fries and crisps industries. The colour of the fried products is determined to a large extent by the reducing sugar (glucose and fructose). The higher the content of reducing sugars the darker the frying colour (Chaudhry et al. 1987; Coffin et al. 1989) which is a result of non-enzymatic Maillard reaction between amino acids and reducing sugars at high temperatures (Shallenberger et al. 1959; Dale and Bradshaw 2003). Reconditioning (controlled tuber warming) after cold storage decreases levels of reducing sugars and is a common practice when potatoes are stored, especially below 10°C (Habib and Broal 1957; Accatino 1973; Harvey et al. 1998; Kyriacou et al. 2009). Although reconditioning decreases the reducing sugar levels, not all genotypes respond similarly to reconditioning and some do not respond at all (Stevenson and Cunningham 1961; Thill and Pleoquin 1994, 1995; Hamernik et al. 2009). Nevertheless, chipping cultivars resistant to cold sweetening directly from cold storage or after a minimal reconditioning time is the main interest of potato breeders (MacKay et al. 1990; Periera et al. 1994; Hayes and Thill 2002c; Xiong et al. 2002).

As the weight of the processed product depends directly on the amount of DM present per quantitative unit of fresh potatoes, the dry matter (DM) content is considered as a very important factor for the quality of both boiled and fried potato products (Burton et al.

1992 as cited in Song 2004). A major or strong correlation between DM content and the starch content (Burton et al. 1992 as cited in Song 2004; Khayatnezhad et al. 2011) of the tuber exists as 60-80% of the DM consists of starch (Burton et al. 1992 as cited in Song 2004). Therefore selection of potato genotypes for high dry matter content can be considered as one of the most important potato breeding objectives.

The autotetraploid status (2n = 4x = 48) of virtually all commercial potato cultivars presents one of the major difficulties associated with potato breeding and genetics. The resulting tetrasomic inheritance, in conjunction with the associated high heterozygosity, adds considerable complexity to potato genetics (Howard 1970), making genetic analysis and mapping more difficult (Milbourne et al. 2007). Van Eck et al. (1994b) mentioned that the complex segregation ratios and the difficulties involved in establishing linkage relationships are the major drawbacks of the tetrasomic inheritance. The expected segregation ratios for tetraploid potatoes are 1:1, 3:1, 5:1, 11:1, 35:1 and 1:0 for simplex-nulliplex, simplex-simplex, duplex-nulliplex, duplex-simplex, duplex-duplex and triplex/quadruplex- simplex or triplex/quadruplex-duplex, respectively. Coupled with the high heterozygosity in tetraploid potato is the potential for a genetic load of lethal and sub-lethal alleles that contribute to severe inbreeding depression and distorted segregation (Howard 1970; Milbourne et al. 2007). Therefore the use of diploid wild species and especially the development of techniques to obtain dihaploid clones of *S. tuberosum* have simplified inheritance studies and made the study of potato genetics more feasible (Milbourne et al. 2007).

So far studies have been conducted to examine the tuber composition and inter-relationships of chip colour and tuber sugar concentration in diverse *Solanum* species (McCann et al. 2010), segregation pattern and correlations between chip colour and reducing sugars after cold storage in dihaploid and tetraploid potato populations (Song 2004) and chip processing potential of potato cultivars in response to long term cold storage and reconditioning (Samotus et al. 1974; Sasaki et al. 2004; Kyriacou et al. 2009). A key difference from previous studies involves the use of 1021/1, a potato breeding line known to have the best resistance to CIS (Anderson et al. 2005; McKenzie et al. 2005; Anderson et al. 2006). Progress in the development of cultivars with desired characters requires the creation and identification of appropriate parents. Therefore the present studies were aimed to study the phenomenon of cold-induced sweetening in potato. For this purpose tetraploid mapping populations were established from a cross made from the parents with contrasting pigmentation, physiological and tuber quality (crisp colour and sugar contents) characters.

The extent of genetic variability and segregation pattern among progeny lines for various pigmentation (hypocotyls, stem, flower and tuber colour), physiological (emergence and flowering time in field) and tuber quality parameters were assessed. The phenotypic variation of pigmentation and physiological parameters will be helpful in studying their inheritance pattern. In order to determine the response of the individual progeny, assessment of phenotypic components of CIS were made at harvest and after cold storage (after one and four months) at 6°C with or without reconditioning (storage at 18°C for 10 days) for crisp colour, sugars and dry matter contents. Furthermore the correlations between crisp colour and sugar contents were determined. The studies would then improve the knowledge of biochemical events of CIS and the results could be useful in association based studies to understand the genetic basis of this complex trait.

2.2 Materials and methods

2.2.1 Plant material

Mapping populations

Two tetraploid populations were used to study the phenomenon of cold-induced sweetening in potato. The first population (K) is the F_1 resulting from a cross between Summer Delight and 1021/1, whereas the second population (S) is the F_1 resulting from a cross between Karaka and 1021/1 (Figure 2.1). 1021/1 is an advanced breeding line derived from a cross between the cultivar Fianna and the breeding line L115-1. It is used for processing purposes and is known to be highly resistant to cold-induced sweetening. Both Karaka and Summer Delight are non processing cultivars susceptible to cold-induced sweetening and share a common parent, V394.

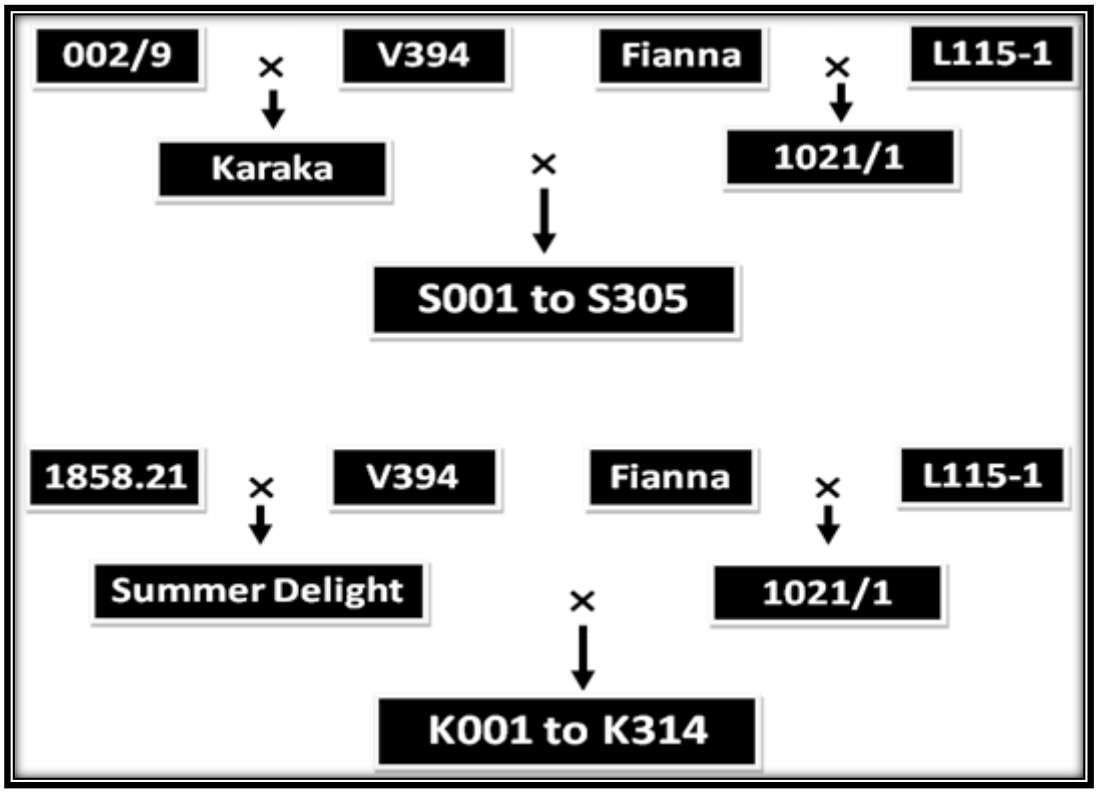


Figure 2.1 Origin of the two mapping populations

Establishment of mapping populations

Mapping populations were established for Summer Delight x 1021/1 or Karaka x 1021/1 at Plant & Food Research in 2007 using true seeds obtained from the Plant & Food Research potato breeding programme. Three hundred and fifty (350) dried seeds for each

population were soaked in 100 µg ml^{-1} GA_3 for 24-36 hr to ensure immediate and uniform germination. They were surface sterilised using the procedure described by Tynan et al. (1990) by washing in 1% sodium hypochlorite (NaClO) for 10 min, followed by three rinses in sterile distilled water, then sown in pre-sterilised plastic pottles (85 mm diameter X 35 mm high) containing potato multiplication media (Conner et al. 1991). The agar was added after pH was adjusted to 5.8 with 0.1 M KOH, then the medium was autoclaved at 121°C for 15 min. Aliquots of 50 ml were dispensed into (80 mm diameter x 50 mm high) pre-sterilised plastic containers (Vertex Plastics, Hamilton, New Zealand). At the germination step each seed was numbered and germination percentage was recorded. At this stage the colour of the hypocotyls was recorded when the cotyledons were just emerging.

Out of 350, 343 seeds of the S population germinated while 344 seeds for K population germinated. In order to obtain healthy plants, surviving seedlings were cultured onto potato multiplication media (Appendix A). Each seedling was then clonally propagated into six plants in tissue culture and upon rooting transferred (337 for S and 340 for K) to the greenhouse in October 2007 in small black 6-cell punnets containing potting mix (potting mix - 60% shredded pine bark, 20% crusher dust, 20% soil supplemented with lime and slow release fertiliser) as previously described (Conner et al. 1994). After two to three weeks individual plants were transplanted to a black PB5 polythene bag (15cm × 15cm × 15cm), along with the mapping population parents established from tubers obtained from the Plant & Food Research potato breeding programme. Plants were grown in a greenhouse (Figure 2.2, heating was provided at below 15°C and ventilation above 21°C) and watered daily. The watering was reduced once the plants started to senescence. Tubers were harvested in April/May 2008 after natural senescence which resulted in sufficient tubers from 307 progeny from the S population (Karaka x 1021/1) and 314 progeny for the K population (Summer Delight x 1021/1) to establish a field trial in spring 2008. The populations were also maintained in tissue culture on potato multiplication media (Appendix A) in a growth room at 20°C - 24°C with a 16 hr light/ 8 hr dark photoperiod. The stem colour was recorded from greenhouse grown plants of both the populations.

2.2.2 Field trials

A field trial was planted on 3rd November 2008 for both populations at Lincoln University Farm, Christchurch, New Zealand (Figure 2.3). As number of tubers was limited, one five

Figure 2.2 Greenhouse multiplication, December 2007

Figure 2.3 Field trial, January 2009

plant plot of each progeny line was randomised in an unreplicated design. Total number of progeny lines planted for the S population was 305 while it was 304 in case of K population. Plots of the parental clones were planted which were distributed randomly throughout the trial to allow any field variation to be assessed. Several plots of check lines (10 plots) selected from the Plant & Food Research potato breeding programme were also distributed randomly throughout the trial. These check lines were used as an internal standard selected to check their performance for CIS. The distance between plots within rows was 1 m and spacing between rows was 75 cm. The distance between plants within a plot was 30 cm. The field trial was surrounded by a guard row of potato plants (cultivar Red Rascal). Standard cultural practices i.e. irrigation, weeding etc were carried out at regular intervals consistent with the usual operations for the Plant & Food Research potato breeding programme.

2.2.3 Pigmentation and physiological traits

Various pigmentation traits were recorded from tissue culture, greenhouse and field grown S & K populations along with the parents and grandparents. Hypocotyl colour was recorded at seed germination stage from tissue culture grown seedlings while stem colour was noted from greenhouse grown plants. Tuber and flower colour were assessed from field grown S and K populations lines. In addition two physiological traits i.e. emergence in the field and flowering time were noted as early and late in "S" population only. The observation for emergence was taken from 18th to 25th November 2008. Flowering time was recorded from the same field growing plants when the flower buds observed on the plant. Early flowering was considered when the first flower bud was observed on the plant (24th December 2008). After seven days (31st December 2008) another observation for flowering time was recorded and this observation was considered as late flowering. Observed and expected frequencies for all the pigmentation and physiological traits were calculated. To test goodness-of-fit for segregation of these traits, a Chi-square test (with one degree of freedom) was used to test whether the observed data differ significantly from expected values.

2.2.4 Cold storage

Tubers from both populations were manually harvested in March 2009 (S population) and April 2009 (K population). Twenty five tubers of each line were transferred to a paper bag for the quality assessment. The remainder of the harvested tubers from each line were used for propagation/maintenance purposes. The quality assessment was performed at five sampling dates or storage treatments (a, b, c, d & e). Of the twenty five tubers, five tubers

were immediately processed for the fresh analysis (a) and the rest (20 tubers) were stored in the dark at 6°C (with humidity around 70%) in paper bags. These twenty tubers were divided into four samples consisting of five tubers each for sampling at four storage intervals, i.e. after one month of cold storage at 6°C (b); after one month of cold storage at 6°C, plus 10 days of reconditioning at 18°C (c); after four months of cold storage 6°C (d); and after four months of cold storage 6°C, plus 10 days of reconditioning 18°C (e).

2.2.5 Assessment of phenotypic components of cold-induced sweetening from parents, grandparents and progeny

Field harvested potato tubers were analysed for biochemical parameters such as dry matter and sugar content along with crisp quality assessment at the five storage treatments/sampling dates (a, b, c, d & e) described in section 2.2.4. The tubers of the parents and grandparents were analysed along with the mapping population. The results of obtained from the several plots of parents and grandparents were averaged.

2.2.5.1 Sampling

Five tubers were taken out from the cold storage at different storage treatments (b, c, d & e) for quality assessment. Tubers were washed, dried on paper towels and evaluated for tuber colour. Each tuber was then cored at about one third of the total tuber length from the stolon end using a six mm cork borer. The cores of five tubers from one progeny line were transferred to a sample bag (4 OZ/118mL capacity sample bags, Nasco Whirl Pack). The fresh weight of the five cores was recorded and the samples immediately stored at -80°C. These stored samples were freeze dried in a machine fitted with a Javac rotary vane high vacuum pump, with the condenser set at -25°C. Dry weight was noted from these freeze dried potato samples. Dry matter content was calculated from fresh and dry weights using the following formula: Dry matter % = 100[(Dry weight – bag weight) / (Fresh weight – bag weight)].

2.2.5.2 Crisp quality

Crisp quality was evaluated at the five storage treatments (a, b, c, d & e) from the five cored tubers on the same day. For frying the cored tuber half at the stolon end was removed and the other half was used for preparing crisps. Two mm slices were prepared from each tuber using a vegetable slicer. The third or fourth slice was taken for frying. Five slices per genotype (one per tuber) were deep fried in canola oil for two min at 190°C. The fried crisps were drained and the excess oil removed by placement on paper towels. Frying colour was determined by visually scoring five slices per genotype using an Agtron colour

scale (24 to 56; based on pictures) where a lower number corresponds to dark crisps/ bad crisp quality while a higher value corresponds to light (yellow) crisp colour /good crisp quality (Harvey et al. 1998). Crisps were stored in the darkness at 18°C in plastic zip lock bags.

2.2.5.3 Sugar analysis

Extraction of sugars

The freeze dried potato samples were ground to a fine powder and stored at room temperature in darkness for later sugar analysis. The extraction and determination of sucrose, D-glucose and D-fructose were carried out by Microplate assay measured by a sequential enzymatic assay (Boehringer Mannheim, Mannheim, Germany) with modifications for freeze dried potato samples. Sugars were extracted from 10 mg freeze dried powder in two ml Eppendorf tubes using 500 µl ethanol (80% v/v) in a water bath at 60°C for five minutes. Samples were centrifuged at 14,269 × g (Eppendorf centrifuge 5415C, Hamburg, Germany) for 4 min. The supernatants were transferred to 1.5 ml Eppendorf tubes. The ethanol extraction step (500 µl 80% ethanol in a water bath at 60°C for five minutes) was repeated to ensure that all the simple sugars have been removed from the sample, and the extract added into the first supernatant. The combined supernatants were dried down in the Automatic environmental SpeedVac® (Thermo Savant, AES2000, NY, USA) overnight to completely remove the ethanol. For sample preparation 200 µl deionised water was added to the SpeedVac® dried samples. After vortexing, samples were centrifuged for two minutes at 14,269 × g. The supernatant was then transferred into 96 well plates. Each plate was assigned with a number and to identify which samples came from which plate a plate layout sheet containing sample names was prepared for each batch. All plates were stored at -20°C until required. These stored samples were thawed at room temperature and 10 µl per well of each sample was taken for sugar analysis. Standards were prepared from a stock solution containing both glucose and fructose at 10 mg ml^{-1}. The standards prepared were 0 to 1.00 mg ml^{-1}. The analysis of the sucrose, glucose and fructose was performed in the same microtitre plate using standard plate layout along with 10 µl of sample, standards and quality controls dispensed by a Biomek Robotic Pipettor (Beckman Coulter, CA, USA). Samples were laid out in two replicate blocks of triplicates. Plates were covered with film and stored at -20°C until required. See Appendix A for the details of buffers, assay reagents and enzymes.

Assay

When required, microtitre plates were thawed, then 10 µl of β-fructosidase (Invertase enzyme, 0.942 U) solution was added to the first replicate block and plates were incubated at 40°C in an incubator (Gallenkamp, LOUGHB, UK) for 30 minutes. Sucrose is hydrolysed by the enzyme β fructosidase to D-glucose and D-fructose. After incubation, 250 µl of Solution 1 (Appendix A) and 10 µl of hexokinase/glucose-6-phosphate dehydrogenase (0.850 and 0.425 U of each enzyme respectively) were added to all wells. Hexokinase catalyses the phosphorylation of D-Glucose to Glucose-6-phosphate (G-6-P) and D-fructose to fructose-6-phosphate while converting ATP to ADP. Glucose-6-Phosphate is converted in the presence of Glucose-6-phosphate dehydrogenase (G6P-DH) to gluconate-6-phosphate with the reduction of NAD to NADH. As D-Glucose exists both as a raw component and as part of sucrose molecules found in potato tissue it is necessary to obtain the amount of free glucose as well as the total amount of glucose in the sample in order to be able to later determine the concentration of sucrose. So the first replicate block containing G-6-P derived from both hydrolysed sucrose and endogenous glucose, while the second replicate block will only have G-6-P from endogenous glucose. The difference in values after reading @ A340 nm between the same duplicate samples is then multiplied by 1.9 to give the concentration of sucrose in mg ml^{-1}. The absolute reading from the same sample in the second replicate block is due to free glucose present in the sample. Plates were then incubated at 25°C (Orbital mixer incubator, Ratek, Biolabs Scientific Limited, VIC, Australia) for 20 minutes to allow the reaction to reach its end point. On a plate reader (Victor 1420, Multilabel Counter, Perkin Elmer, CA, USA), the absorbance of NADH of reacted glucose from original sucrose and glucose was measured at 340 nm. The level of glucose/fructose and sucrose was estimated using a regression equation from a calibration curve established using glucose/fructose standards assayed as above, and the calibration regression equation was generated in the plate reader. To measure the fructose content, 10 µl, 1.75 U of phosphoglucose isomerase (PGI) was added into the wells of the second replicate block. The conversion of fructose-6-phosphate to Glucose-6-phosphate by Phosphoglucose Isomerase allows the reaction to continue. The resulting conversion of this Glucose-6-Phosphate molecule to Gluconate-6-phosphate means that the concentration of D- fructose can then be measured by the absorbance of NADH after subtracting the original absorbance due to endogenous glucose content. Plates were incubated at 25°C for 20 minutes to allow the reaction to reach its end

point. The fructose content was measured n a plate reader at 340 nm. The amounts of glucose/fructose/sucrose mg g^{-1} dry weight were calculated.

2.2.6. Statistical analysis of the data

Graphical representations, statistical parameters, Pearson correlation analysis, phenotypic variance (R^2) and regression analysis were performed in Microsoft office excel 2003, Minitab 15, SigmaPlot 10.0 and GenStat 12^{th} edition. A Chi-square goodness-of-fit test was used to test whether the observed segregation of individual pigmentation traits differed significantly from expected values. The expected segregation ratios were 1:1, 3:1, 5:1, 11:1, 35:1 and 1:0 for simplex-nulliplex, simplex-simplex, duplex-nulliplex, duplex-simplex, duplex-duplex and triplex/quadruplex-simplex or triplex/quadruplex-duplex, respectively.

2.3 Results

2.3.1 Mapping populations and field trial

The objective of this study was to establish the mapping populations to study the phenomenon of cold-induced sweetening in potato tubers. Both the mapping populations Summer Delight x 1021/1 or Karaka x 1021/1 were referred to as “K” and “S”, respectively. Initially the populations were grown in greenhouse (October 2007) and each produced more than 300 progeny. These greenhouse harvested tubers were stored in cold (6°C) and five tubers of each line were planted in field (November 2008) in order to produce sufficent tuber material for phenotypic analysis. Various pigmentation traits and physiological traits were recorded from tissue culture, greenhouse and field grown plants of both the mapping populations. Only one population (“S”) was investigated in detail for cold-induced sweetening traits. The progeny of “S” population was analysed using SSRs (microsatellites) in Chapter 3 and based on these microsatellite marker data, non-hybrids were eliminated from all data sets.

2.3.2 Pigmentation and physiological traits

Evaluation of pigmentation traits from parents and progeny

The pigmentation traits such as hypocotyl, stem, flower and tuber colour provide valuable classical genetic markers to determine the suitability of a mapping population for further genetic characterisation. Parents of both mapping populations i.e. Karaka, Summer Delight and 1021/1 differed for stem, flower and tuber colour. However, parental phenotype for hypocotyl colour was not available as it can only be assessed on germinating seed. A χ^2 test was used to determine the most likely segregation ratio of the phenotypes (Tables 2.1, 2.2) evaluated from greenhouse grown and field grown progeny.

Hypocotyl colour: Hypocotyl colour was observed in tissue culture grown seedlings for purple and green colours at the stage when the cotyledons were just emerging. The purple pigmentation visible in the hypocotyl is controlled monogenically by the *P* gene required for the production of blue anthocyanins (van Eck et al. 1993). The segregation was significantly different (P-value <0.001) from 1:1 ratio in both mapping populations (Tables 2.1, 2.2).

Stem colour: The stem colour was observed on greenhouse grown adult plants of both the mapping populations. Karaka and Summer Delight have green stems while 1021/1 has purple stems. Chi-square tests indicate segregation distortion to a 1:1 ratio in both mapping populations (Tables 2.1, 2.2).

Table 2.1: Segregation ratios of pigmentation and physiological traits in S population

Trait	Parental phenotype		Observed frequencies		Expected ratio	χ^2	*P value
	Female	Male					
Hypocotyl			170 (Purple)	117 (Green)	1:1	9.79	0.00
Stem	Green	Purple	162 (Green)	125 (Purple)	1:1	4.77	0.04
Emergence	Early	Early	222 (Early)	65 (Late)	3:1	0.84	0.35
Flowering	Very early	Early	221 (Early)	66 (Late)	3:1	0.61	0.43
Flower	White	Blue	159 (White)	128 (Blue)	1:1	3.35	0.06
Tuber skin	Colourless	Coloured	153 (Colourless)	134 (Coloured)	1:1	1.26	0.26
Tuber skin	Colourless	Red/Purple	73 (Red)	61 (Purple)	1:1	1.07	0.30

Note: - * P value was assigned for the calculated χ^2 for 1 degree of freedom (d.f)

Table 2.2: Segregation ratios of pigmentation and physiological in K population

Trait	Parental phenotype		Observed frequencies		Expected ratio	χ^2	*P value
	Female	Male					
Hypocotyl			145 (Purple)	199 (Green)	1:1	8.48	0.00
Stem	Green	Purple	181 (Green)	141 (Purple)	1:1	4.97	0.03
Flower	Blue	Blue	210 (Blue)	82 (Blue)	3:1	1.47	0.22
Tuber skin	Colourless	Coloured	169 (Colourless)	133 (Coloured)	1:1	4.29	0.04
Tuber skin	Colourless	Red	108 (Red)	25 (Purple)	5:1	0.43	0.51
			108 (Red)	25 (Purple)	3:1	2.72	0.09

Note: - * P value was assigned for the calculated χ^2 for 1 degree of freedom (d.f)

Flower colour: The flower colour was scored as blue and white from field grown plants. Few progeny failed to produce flowers. Karaka produced white coloured flowers while 1021/1 produced blue coloured flowers. A χ^2 test indicates a good fit to a 1:1 (simplex:nulliplex, *Pppp* X *pppp*) ratio in case of "S" population (Table 2.1). Summer Delight produced blue flowers and Chi-square test indicated a good fit to a 3:1 (simplex:simplex, *Pppp* X *Pppp*) in case of "K" population (Table 2.2).

Tuber skin colour: Tuber skin colour was noted after harvesting of field grown plants from both mapping populations. The segregation pattern for tuber skin colour of "S" population is illustrated in Figure 2.4. The tuber skin colour was classified as colourless (white) and coloured (red/purple). The "S" mapping populations showed a good fit to a 1:1 (simplex:nulliplex, *Pppp* X *pppp*) ratio (Table 2.1) while the "K" population showed segregation distortion to a 1:1 ratio (Table 2.2). The coloured category was further assessed for red or purple. The segregation for this category showed best fit to 1:1 ratio for goodness by Chi-square method in case of "S" population (Table 2.1). However, in case of "K" population the Chi-square test showed fit to both 5:1 (duplex:nulliplex, *PPpp* X *pppp*) and 3:1 (simplex:simplex, *Pppp* X *Pppp*) ratios (Table 3.2).

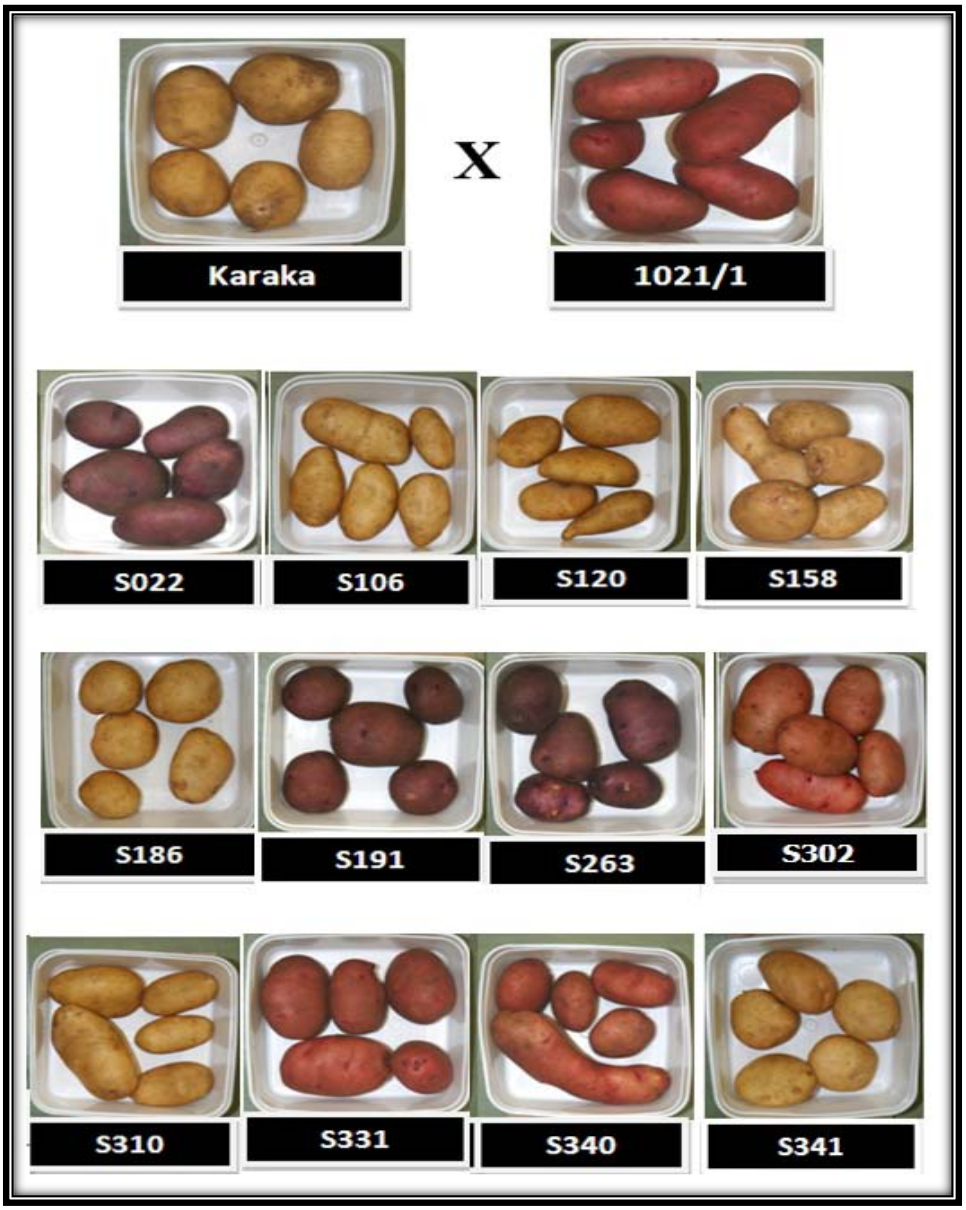


Figure 2.4 Representative tuber colour segregation in progeny of a cross between Karaka X 1021/1

Evaluation of physiological traits from parents & progeny

Two physiological traits such as emergence and flowering time were noted at two time intervals described in Materials and Methods (Section 2.2.3) from field grown progeny of the "S" population.

Emergence: The emergence in the field was considered to be early when occurring within three weeks of planting and plants emerging after three weeks were considered as late. Segregation for this trait fitted a 3:1 expected ratio consistent with monogenic nature of this character (Table 2.1).

Flowering: Flowering time was observed till development of the flower buds on the plant. The flowering time denoted as early or late from field grown progeny which showed best fit to 3:1 ratio (Table 2.1).

2.3.3 Assessment of phenotypic components of cold-induced sweetening from parents, grandparents and progeny

The detailed CIS studies were conducted from only one (S) population, the parents/ grandparents of K population were not included in this analysis. Based on the segregation studies on pigmentation traits, the "K" population shows greater distorted segregation, therefore "S" population was chosen for the CIS studies.

Parents & grandparents of the "S" mapping population

In order to evaluate the genetic variability in response to cold storage, the parents and grandparents of the "S" mapping population were assessed for crisp colour, sugar (glucose, fructose & sucrose) and dry matter at five storage treatments described in Materials and Methods (Section 2.2.4). The graphical representation of (A) crisp colour, (B) glucose, (C) fructose, (D) sucrose and (E) dry matter content is depicted in Figure 2.5.

Crisp colour: At harvest (storage treatment a) crisp colour score was greater than 45 in all parents and grandparents [Figure 2.5 (A)]. However, it varied markedly between these genotypes after cold storage (storage treatment b & d). Tubers of the cold-tolerant breeding line 1021/1 maintained lighter crisp colour (indicated by higher Agtron values) compared to the tubers of cold-susceptible line Karaka, irrespective of cold storage duration. Compared to 1021/1, the crisp colour declined in Fianna, L115-1, 002/9 and V394 after cold storage [Figure 2.5 (A)]. This resulted in unacceptable crisp colour as indicated by low Agtron scores, and acceptable crisp colour was not recovered after reconditioning (storage treatments c & e) at 18°C.

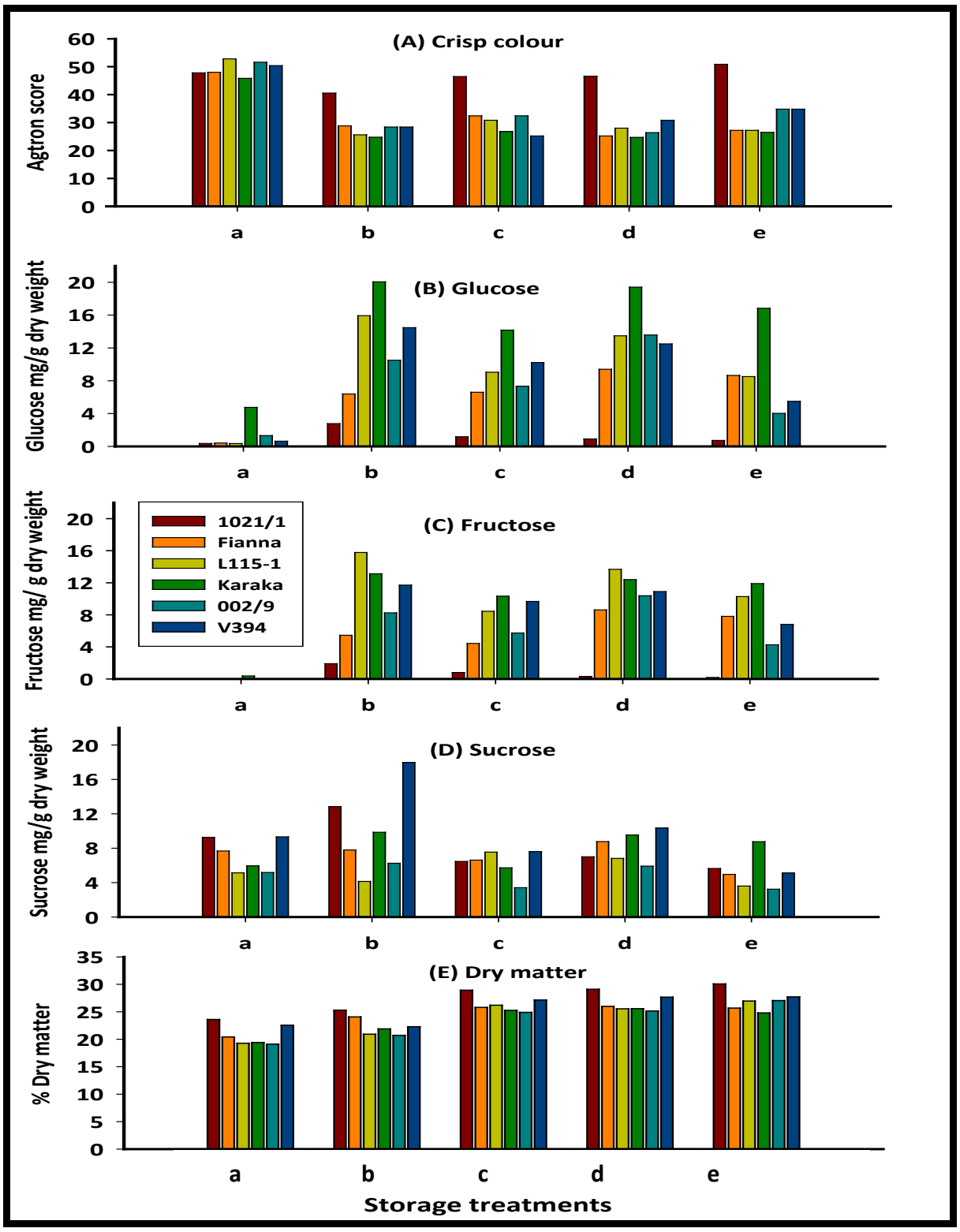


Figure 2.5 Assessment of phenotypic components from parents and grandparents. Axis X shows storage treatments (a, b, c, d and e) and Y axes show values for the measured parameters [crisp colour (A), glucose (B), fructose (C), sucrose (D) & dry matter (E)]. Crisp colour values are the "Agtron" scores, which measures the colour of the crisps. The higher value means better crisp quality, with a value greater than or equal to 40 being the acceptable industry standard (Genet et al. 1995).

Sugar analysis: Tuber sugar contents (sucrose, glucose and fructose) varied greatly among parents and grandparents. At harvest (storage treatment a), glucose [Figure 2.5 (B)] and fructose [Figure 2.5 (C)] contents were almost negligible (except glucose content in Karaka). After one month cold storage (storage treatment b) it increased rapidly, while reconditioning (storage treatment c) resulted in decreased in glucose [Figure 2.5 (B)] and fructose [Figure 2.5 (C)] content, except in Fianna. Longer cold storage (storage treatment d) increased the sugar levels. Reconditioning at storage treatment e again helped to reduce the levels of glucose [Figure 2.5 (B)] and fructose [Figure 2.5 (C)], except in Fianna where reconditioning had little effect. In addition fructose [Figure 2.5 (C)] content in Karaka marginally fluctuated at storage treatments d and e.

In general glucose and fructose levels were high in grandparents after cold storage and did not decrease greatly after reconditioning, resulting in unacceptable crisp colour. However, it is important to note that glucose [Figure 2.5 (B)] and fructose [Figure 2.5 (C)] levels remained low in breeding line 1021/1 after cold storage (storage treatments b and d) and changed marginally after reconditioning (storage treatments c and e). In contrast to 1021/1, the concentration of glucose [Figure 2.5 (B)] and fructose [Figure 2.5 (C)] in Karaka remained very high after cold storage and did not decrease to a great extent after reconditioning.

Changes in sucrose [Figure 2.5 (D)] levels exhibited a different pattern to glucose and fructose. Freshly harvested tubers (storage treatment a) had high levels of sucrose [Figure 2.5 (D)] in all parents and grandparents. After one month of cold storage (storage treatment b) it increased in 1021/1, Karaka and V394, however it was relatively stable in Fianna, L115-1 and 002/9. Reconditioning at 18°C for 10 days (storage treatment c) caused a reduction in sucrose levels, except in L115-1. Small fluctuations were observed in sucrose levels upon longer cold storage (storage treatment d) and slightly decreased at storage treatment e [Figure 2.5 (D)].

L115-1, one of the parents of 1021/1 had higher levels of glucose [storage treatments b, c & d, Figure 2.5 (B)] and fructose [storage treatments b to e, Figure 2.5 (C)] compared to Fianna although sucrose levels were higher in Fianna than L115-1 at both cold storage periods (storage treatments b & c). On the other hand, V394, one of the parents of Karaka had higher levels of glucose (storage treatments b, c & e), fructose (storage treatments b to e) and sucrose (storage treatments b to e) compared to 002/9. In conclusion breeding line 1021/1 accumulated lowest levels of glucose and fructose with lightest crisp colour, while

higher levels of glucose and fructose resulted in dark and unacceptable crisp colour in Karaka.

Dry matter content: Dry matter [Figure 2.5 (E)] content remained always high in 1021/1 at all the storage treatments compared to all the other genotypes.

2.3.4 Segregation among progeny of the mapping population

The objective of this study was to evaluate the segregation for crisp colour, sugar (glucose, fructose & sucrose) and dry matter in the "S" mapping population at five storage treatments. The statistical parameters are depicted in Table 2.3 while Figures 2.6 and 2.7 represents the segregation pattern for crisp colour (A), glucose (B), fructose (C), sucrose (D) and dry matter (E) content. The progeny (287) data was analysed for the above traits after eliminating all non hybrids based on the molecular markers analysis conducted in Chapter 3.

Crisp colour (Agtron score) segregation

Twenty five tubers of each progeny line were evaluated for crisp colour over five storage treatments (five tubers at each storage treatment). Figure 2.6 shows the crisp colour of representative progeny immediately after harvest, then the tubers of the same progeny were stored for one and four months of cold storage at 6°C (with and without ten days of reconditioning at 18°C). An Agtron score of 40 or more is considered a commercially acceptable light colour (Genet et al. 1995).

The segregation of values among the 287 progeny is illustrated in Figure 2.7 (A) and exhibits wide variability in crisp colour over the five storage treatments. Table 2.3 shows the average values obtained for crisp colour at five storage treatments which showed that in general the average crisp colour was declined upon cold storage (storage treatments b & d) while it was improved after reconditioning (storage treatments c & e) at 18°C. Figure 2.7 (A) indicated that crisp colour of some progeny was superior to that of 1021/1, while some progeny were inferior to that of Karaka, establishing transgressive segregation for this trait. At harvest (storage treatment a), except for a few progeny [2%, Figure 2.7 (A)], the crisp colour was within the acceptable industry standard (≥ 40, Genet et al. 1995). After cold storage at 6°C for one and four months [storage treatments b and d respectively, Figure 2.7 (A)] tubers from many progeny showed the effects of low temperature and dark crisps were obtained after frying as indicated by low Agtron scores. However, no specific trend was observed in crisp colour quality with respect to cold storage duration. Some progeny consistently produced black crisp colour (Figure 2.6) and were unacceptable at both cold

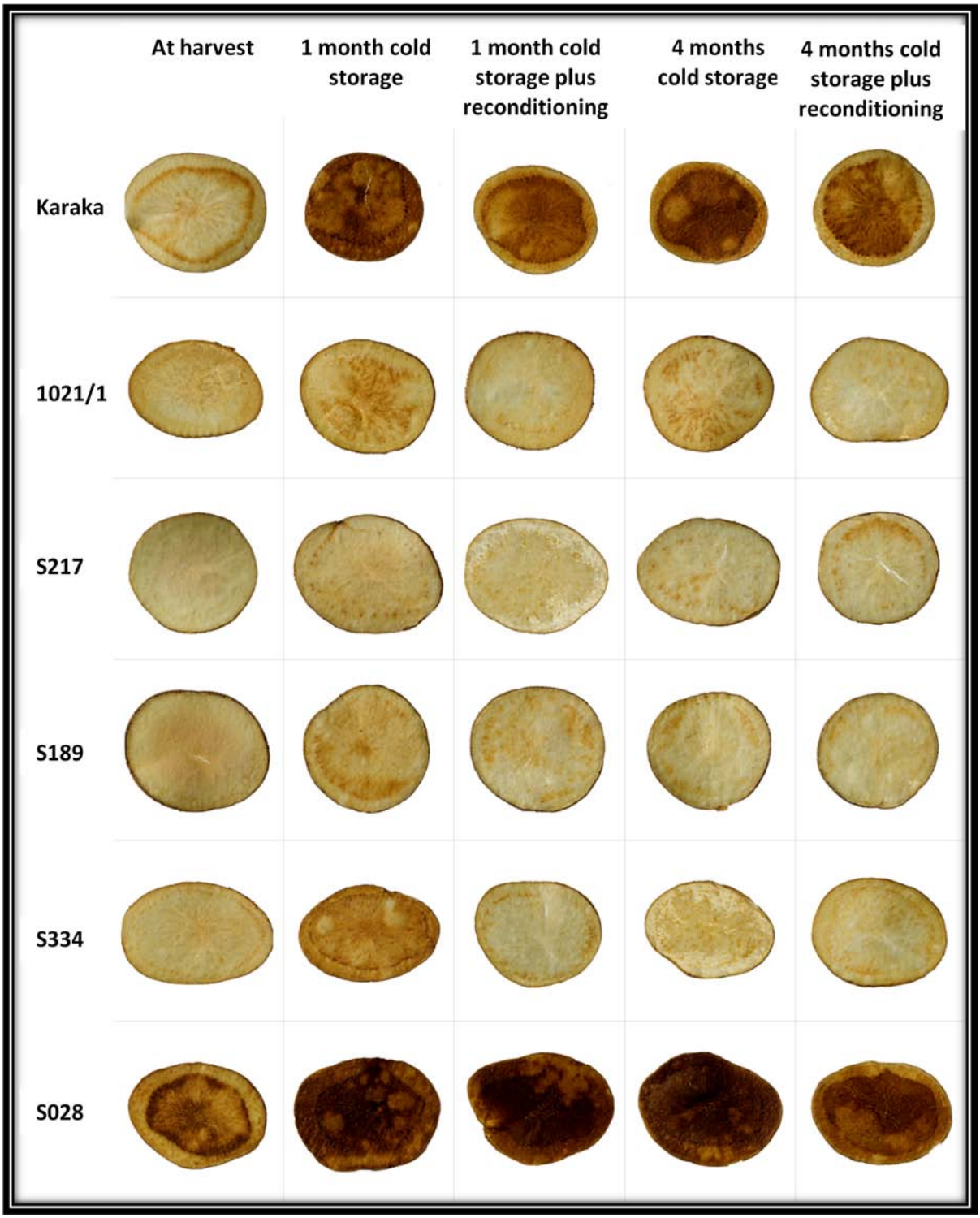


Figure 2.6: Crisp colour of the parents and representative progeny. Figure represents crisp colour of the parents (Karaka x 1021/1) and representative progeny (S217, S189, S334 and S028) at five storage treatments (at harvest and following one and four months of cold storage at 6°C (with and without ten days of reconditioning at 18°C).

storage durations [storage treatments b and d, Figure 2.7 (A)]. On the other hand, crisp colour in some genotypes deteriorated only after four months of cold storage. Some progeny lines produced black crisp colour after only one month of cold storage but crisp colour was found to be improved in these lines after four months of cold storage. Agtron scores of few genotypes were exceptionally high and consistently produced light crisp colour irrespective of storage duration [Figure 2.6, 2.7 (A)]. Overall, 10% and 16% of the progeny produced crisp colour ≥ 40 at storage treatments b and d respectively.

After cold storage treatment, tubers were subjected to reconditioning at 18°C for 10 days, a standard industry practice to improve crisp colour. Different tendencies were observed in reconditioning behaviour. Reconditioning clearly improved the crisp colour of some progeny, while some other progeny consistently produced bad crisp colour even after reconditioning [Figures 2.6, 2.7 (A)]. Overall, 44% progeny produced crisp colour≥ 40 after reconditioning (storage treatments c and e).

Tuber glucose and fructose contents (mg g^{-1} dry weight)

Tuber glucose and fructose contents were estimated from the same five tubers that were analysed for crisp colour at each storage treatment. The average glucose [Figures 2.7 (B)] and fructose [Figures 2.7 (C)] contents at five storage treatments are presented in Table 2.3. The average values of glucose and fructose contents were increased after cold storage periods (storage treatments b & d) while reconditioning (storage treatments c & e) helped to reduce the levels (Table 2.2). The content of glucose and fructose varied substantially among the progeny [Figures 2.7 (B) & (C)]. In general, the concentration of glucose was much higher than that of fructose however, there were few exceptions where some progeny exhibited the opposite (Appendix B). At harvest (storage treatment a) glucose contents ranged from 0 to 11.52 [mg g^{-1} dry weight, Figure 2.7 (B)]. In contrast, fructose [mg g^{-1} dry weight, Figure 2.7 (C)] contents were almost negligible or zero at harvest in most of the progeny. Upon cold storage (storage treatment b and d) glucose and fructose levels increased rapidly. The contents of glucose and fructose were exceptionally high in some progeny relative to Karaka and were remarkably low relative to 1021/1 upon cold storage [Figures 2.7 (B) & (C)]. Like crisp colour, in few instances no specific trend was observed in reducing sugars (glucose & fructose) accumulation profiles with respect to cold storage duration. For example, a few genotypes exhibited high glucose and fructose levels after one month of cold storage, while it decreased after four months of cold storage in these genotypes [Figures 2.7 (B) & (C), Appendix B]. An increase in the glucose and fructose contents was more pronounced in a

few progeny after four months of cold storage as compared to one month cold storage [Figures 2.7 (B) & (C)].

Table 2.3 Statistical parameters of crisp colour, glucose, fructose, sucrose and dry matter in "S" population (sample size 287)

Parameter	Storage treatment	Mean	Median
Crisp colour (Agtron score)	**a**	49.04	49.20
	b	31.49	30.80
	c	37.88	38.40
	d	33.47	32.40
	e	38.38	38.00
Glucose (mg g^{-1} dry weight)	**a**	1.34	0.63
	b	9.02	8.19
	c	4.54	3.06
	d	8.24	7.09
	e	4.18	2.92
Fructose (mg g^{-1} dry weight)	**a**	0.06	0.00
	b	6.53	5.78
	c	3.36	2.50
	d	5.87	5.55
	e	2.91	1.90
Sucrose (mg g^{-1} dry weight)	**a**	7.37	6.96
	b	10.06	9.65
	c	6.29	6.01
	d	8.36	8.32
	e	5.10	4.96
Dry matter (%)	**a**	22.68	22.58
	b	23.81	23.81
	c	29.01	28.89
	d	29.77	29.59
	e	30.06	30.02

Reconditioning at both storage treatments (c and e) lowered the contents of glucose and fructose in most progeny while no response or rather increase was observed in rest of the progeny [Figures 2.7 (B) & (C)].

Sucrose (mg g^{-1} dry weight)

Sucrose content was estimated from the same five tubers that were analysed for crisp colour, glucose and fructose contents at each storage treatment. The average sucrose content at five storage treatments is presented in Table 2.3. A wide variation was observed in sucrose accumulation profiles among the progeny [Figure 2.7 (D)]. In contrast to glucose and fructose, sucrose content were ranged from 0 to 23.17 (mg g^{-1} dry weight) in freshly harvested tubers [Figure 2.7 (D)]. Cold storage at 6° for one month (storage treatment b) generally resulted in an increase in sucrose levels. However, further cold storage (after four months, storage treatment d) resulted in a decline in sucrose levels, although it remained unchanged in few progeny (4%). A few progeny exhibited transgressive variation in sucrose relative to those observed in Karaka and 1021/1 [Figure 2.7 (D)].

Following reconditioning, levels of sucrose declined in most progeny while they remained relatively stable with only subtle fluctuations in a few cases. In general average sucrose content increased after cold storage and decreased after reconditioning (Table 2.2).

Dry matter content (%)

Average percent dry matter at five storage treatments is presented in Table 2.2. The pattern of dry matter accumulation in progeny is depicted in Figure 2.7 (E). In general the average dry matter content were increased after both cold storage periods (storage treatments b & d) and reconditioning periods (storage treatments c & e) further enhanced the levels (Table 2.3). Percent dry matter varied widely among the progeny. At harvest it ranged from 17 to 29%. Upon one month cold storage (storage treatment b) it did not differ much but slightly increased after four months of cold storage (storage treatment d) [Figure 2.7 (E)].

After reconditioning at storage treatment c dry matter contents were ranged from 22 to 36%. The average dry matter content was ranged from 20 to 45% after reconditioning at storage treatment e. Only two progeny lines showed exceptionally high dry matter content (41 and 45%) at storage treatment e [Figure 2.7 (E)]. In conclusion % dry matter content remained relatively stable at storage treatments c, d and e.

Figure 2.7 (A) Individual value plot of crisp colour

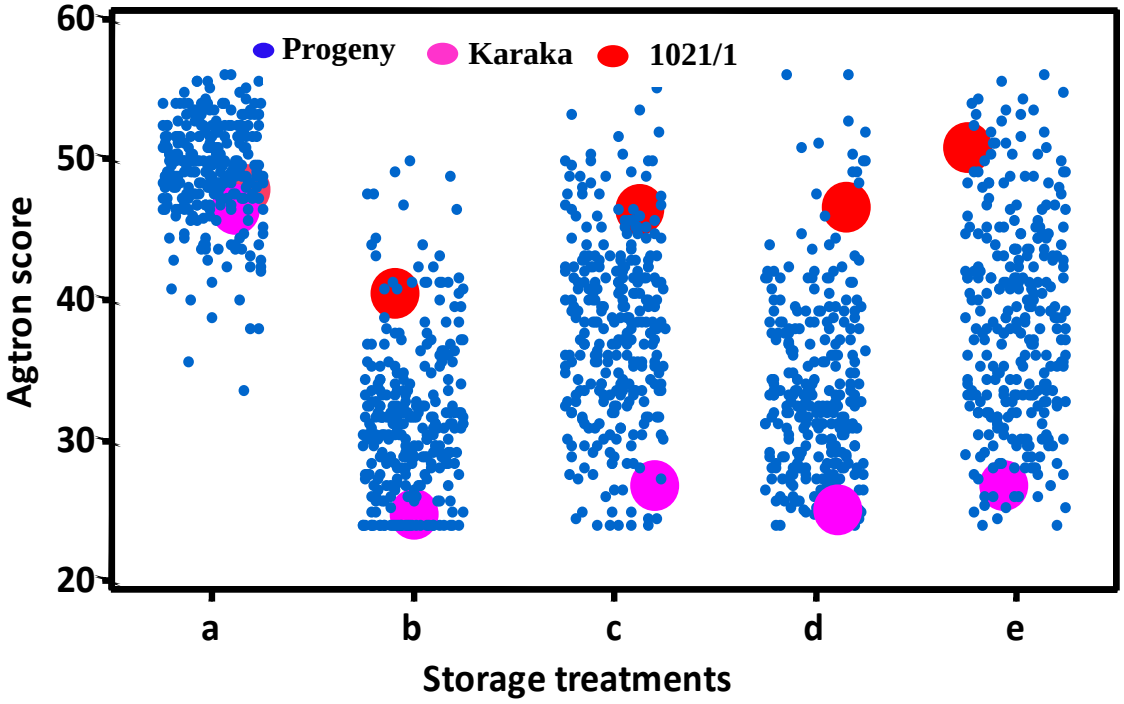


Figure 2.7 (B) Individual value plot of glucose

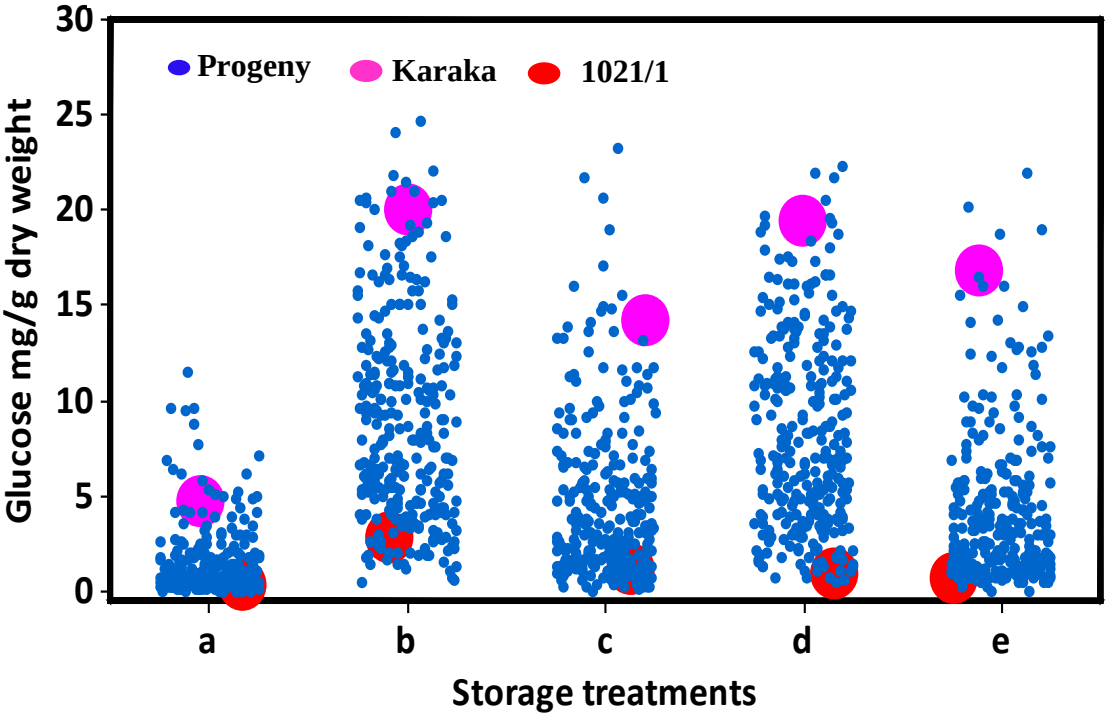


Figure 2.7 (C) Individual value plot of fructose

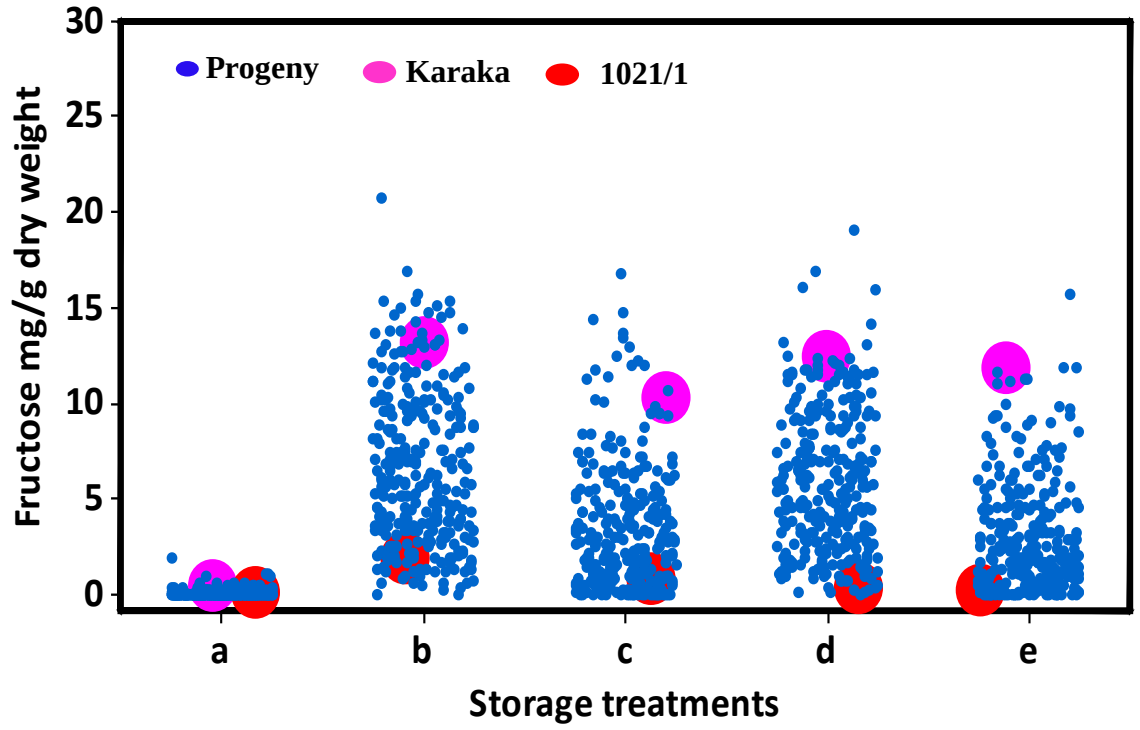
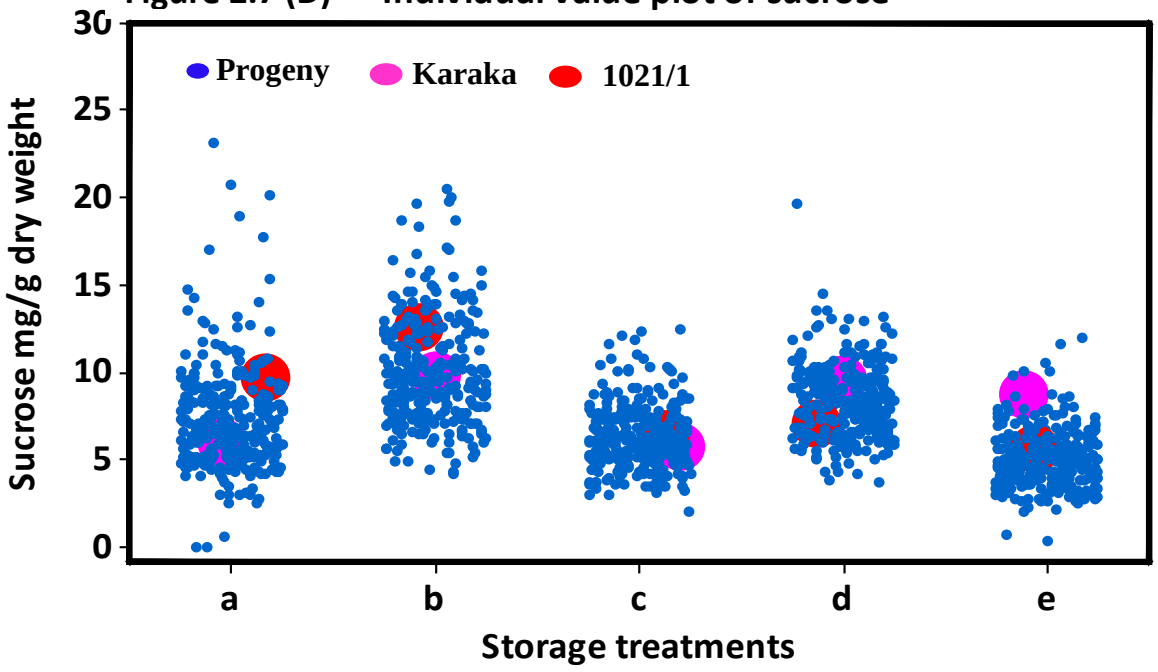


Figure 2.7 (D) Individual value plot of sucrose



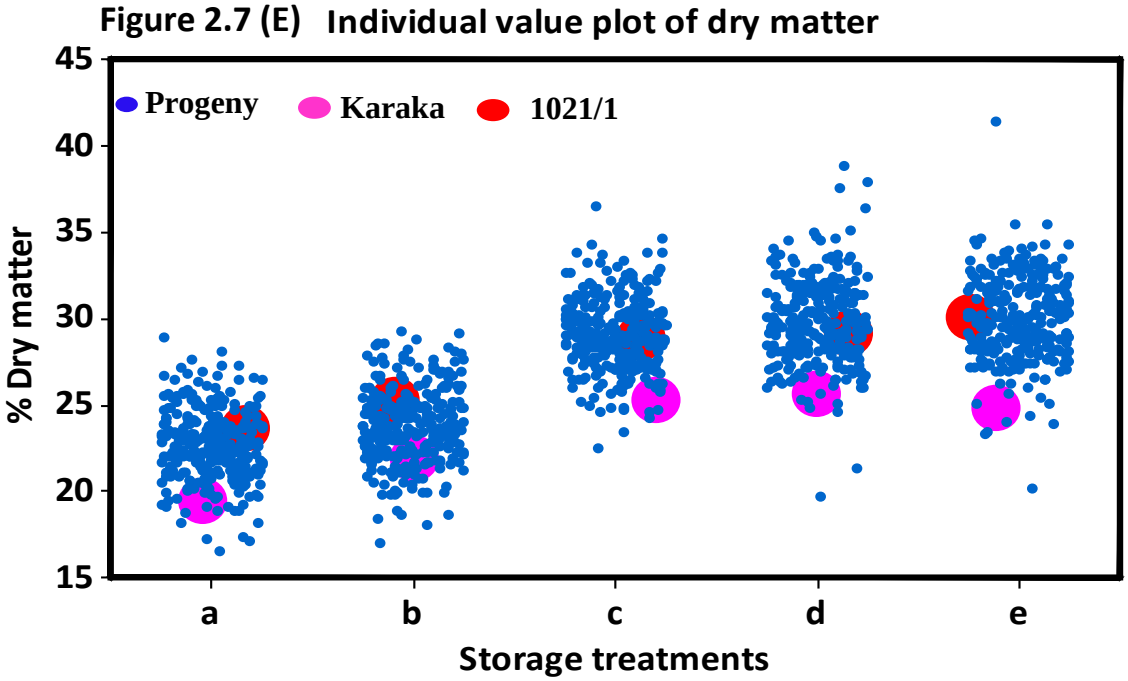


Figure 2.7 Assessment of phenotypic components from progeny. X axis represents storage treatments (a, b, c, d and e) while Y axis represents parameter (Crisp colour, glucose, fructose, sucrose and dry matter). Where (A) Individual plot of crisp colour, (B) Individual value plot of glucose, (C) Individual value plot of fructose, (D) Individual value plot of sucrose and E) Individual value plot of dry matter. The values of parental plots represent the average of several plots. Please note the scale difference.

2.3.5 Correlation between crisp colour and content of specific sugars

The purpose of this study was to interpret the relationship between crisp colour with that of glucose, fructose, sucrose and dry matter at five storage treatments (Table 2.4). The analysis of Pearson correlation coefficient (r) was carried out between crisp colour and specific sugars with a significance level of $p<0.0001$. The Pearson correlation coefficient (r) measures the strength of the association between two variables showed that crisp colour is negatively correlated with glucose and fructose contents (Table 2.4). Only the correlation between fructose and crisp colour is depicted in scatter plots [Figure 2.8 (A) to 2.8 (E)] at the five storage treatments. The reason to consider correlation between crisp colour and fructose mainly because the phenotypic variance R^2 between glucose, fructose contents and crisp colour at storage treatment b was (0.54 and 0.57 respectively) while it was 0.52 and 0.61 respectively at storage treatment d which indicates that fructose contents has slightly more influence on crisp colour than glucose content (Table 2.4). In contrast, only a very

weak correlation was apparent between sucrose and crisp colour. Dry matter content showed weak but a positive correlation with crisp colour. The consistently higher negative r values across all storage treatments show that the higher the reducing sugar content (glucose and fructose) in the tuber, the darker the chip colour (low Agtron values).

Table 2.4 Correlations between crisp colour and glucose, fructose & sucrose in population at various storage treatments

Parameter	Glucose		Fructose		Sucrose	Dry matter
Storage treatment	r	R^2	r	R^2	r	r
a	-0.49	0.24	-0.28	0.08	-0.31	0.20
b	-0.73	0.54	-0.76	0.57	-0.22	0.25
c	-0.69	0.48	-0.76	0.58	-0.23	0.33
d	-0.72	0.52	-0.78	0.61	-0.29	0.36
e	-0.67	0.45	-0.77	0.59	-0.33	0.38

Note: Correlations were determined by Pearson Correlation coefficient (r) analysis with a significance level of $p<0.0001$. Pearson Correlation coefficient (r) indicates the strength of a relationship between two variables.

Figure 2.8 (A)

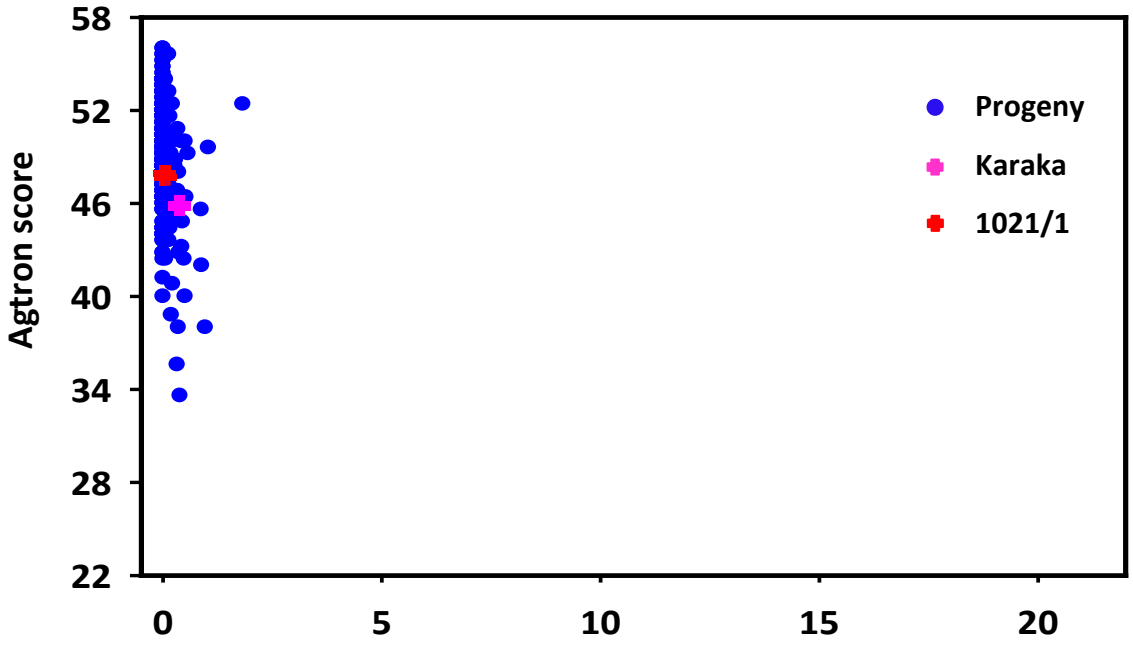


Figure 2.8 (B)

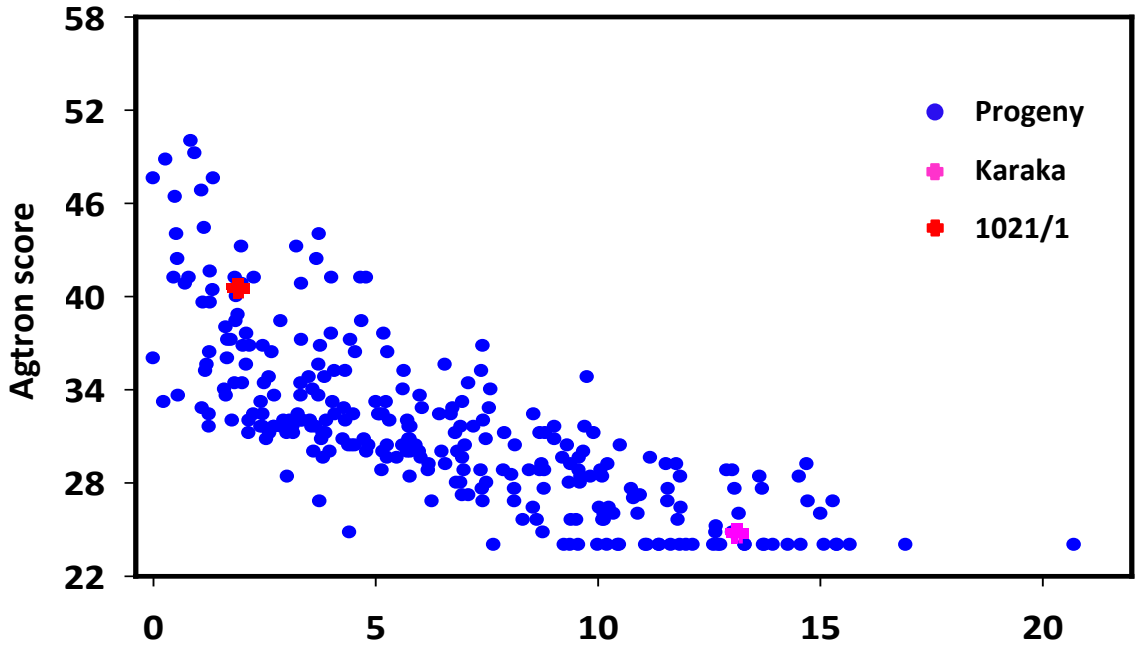
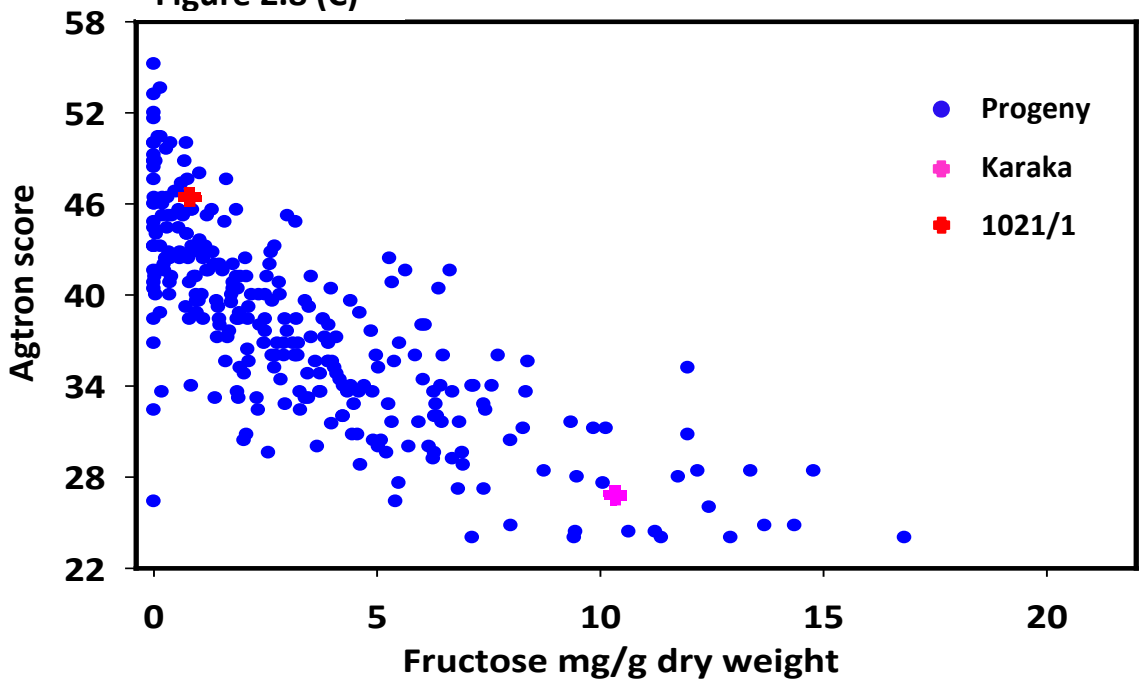


Figure 2.8 (C)



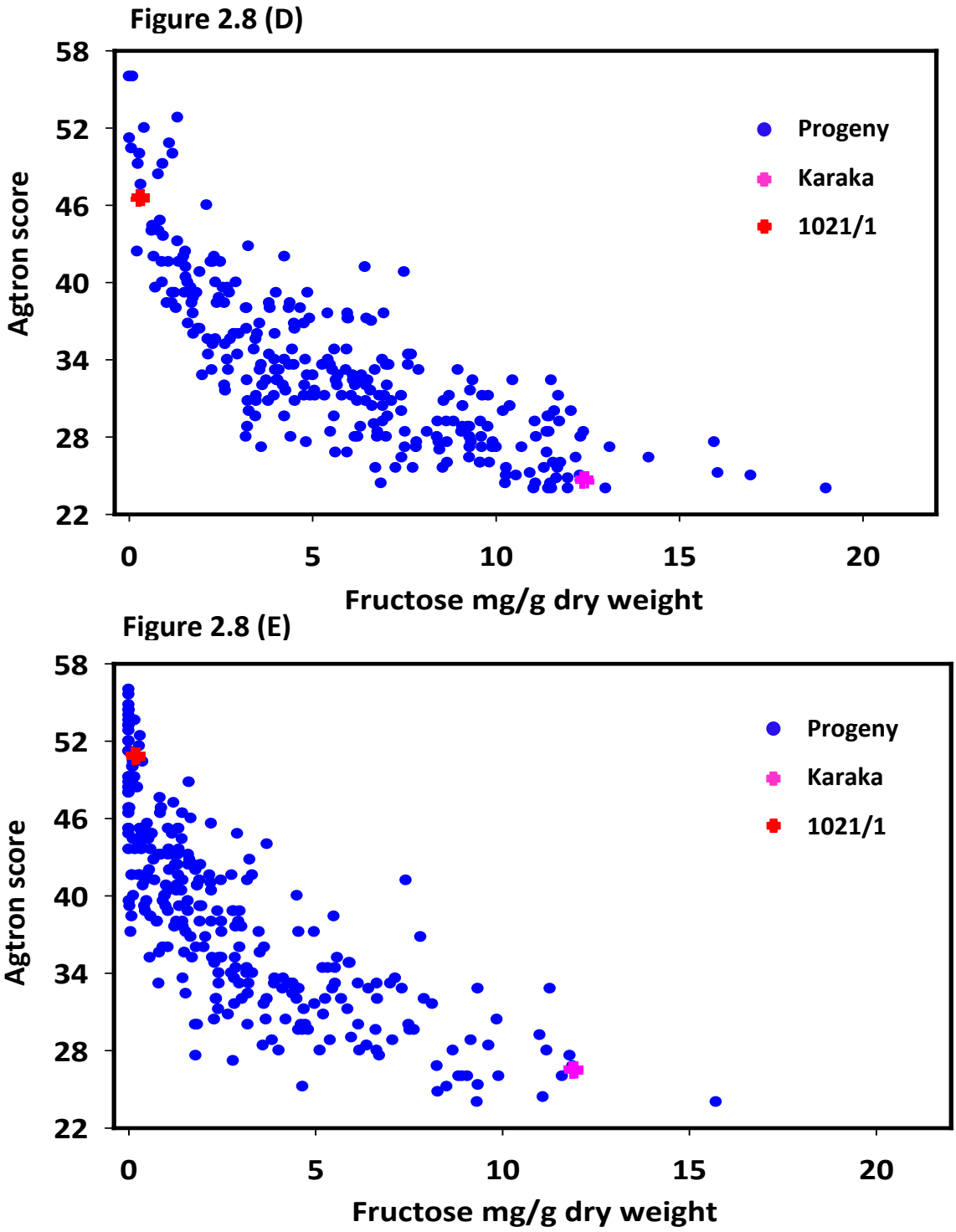


Figure 2.8: Scatter plots of crisp colour vs. fructose. Figures represents scatter plots showing relationship between crisp colour and fructose among segregating progeny of the cross between Karaka X 1021/1 for five storage treatments. A) Storage treatment a, B) Storage treatment b, C) Storage treatment c, D) Fructose at Storage treatment d, E) Fructose at Storage treatment e.

2.4 Discussion

The overall aim of this study was to understand the phenomenon of cold-induced sweetening in potato tubers. Extensive germplasm evaluations (McCann et al. 2010), dihaploid and tetraploid mapping populations (Song 2004; Menéndez et al. 2002) focused on understanding of this complex trait. The very key difference from the previous studies is the use of 1021/1, a potato cultivar known to have the best resistance to CIS. This study included very comprehensive analysis of pigmentation, physiological and cold-sweetening traits from a tetraploid mapping population at various storage treatments. The population established from the parents with diverse genetic background provided the extent of genetic variability and segregation patterns for pigmentation, physiological and cold-sweetening traits.

Mapping populations

Two mapping populations (Figure 2.1) were successfully established from parents with contrasting characters. In particular, both the mapping populations provided a unique resource to study pigmentation traits and phenomenon of CIS in potato tubers although the studies were continued with only one mapping population. Both the populations provided more than 300 progeny which is larger than that of similar studies that have been carried out in potato (Song 2004; McCann et al. 2010).

Pigmentation and physiological traits

Although all these pigmentation and physiological traits may not be relevant to CIS studies, the data was utilised for the estimation of genetic contribution of each parent in segregating populations. This provided confidence that the mapping populations exhibited appropriate segregation of phenotypic markers.

Evaluation of pigmentation traits from parents and progeny

One of the objectives of this study was to investigate the genetic basis of the pigmentation and physiological traits segregating in both mapping populations such as variation for hypocotyls, stem, flower and tuber skin colour (Tables 2.1 and 2.2). Knowledge of inheritance pattern and linkage between different morphological traits is of major interest of plant breeders and geneticists because it facilitates the efficient utilization of marker genes in crop improvement programmes (Khosravi et al. 2010). All these traits evaluated in the progeny are considered as useful morphological markers. However, to date, gene mapping in potato has resulted in the localisation of only a few other morphological traits (Jacobs et al. 1995). In case of both the populations most of the observed segregation for pigmentation traits was in agreement with that of Jacobs et al. (1995) displaying 1:1 ratio

(Tables 2.1, 2.2) (except for flower colour & tuber skin coloured category in case of K population, Table 2.2). The population was scored for the purple colour for hypocotyl and stem, which is controlled monogenically by the *P* gene required for the production of blue anthocyanins (van Eck et al. 1993). Genetic studies aiming at the identification of loci controlling the inheritance of colour characteristics in potato have been reviewed by several authors (Dodds and Long 1956; van Eck et al. 1993, 1994a, b; Gebhardt et al. 1989). For tuber skin colour four different names *R*, *E*, *I* and *PSC* have been used for a locus on chromosome 10 (van Eck et al. 1994b) whereas the inheritance of flower colour in diploid potato (2n = 2x = 24), was found to be controlled by three unlinked loci *D*, *F* and *P* (van Eck et al. 1993). Locus *D*, involved in the biosynthesis of red anthocyanins, was mapped on chromosome 2, while locus *P*, involved in the production of blue anthocyanins, was mapped on chromosome 11. Locus *F*, involved in the flower-specific expression of gene(s) accommodated by the *D* and *P* loci, was mapped on chromosome 10 (van Eck et al. 1993). Loci *E* and *R* explain the two different tuber skin phenotypes in tetraploids (van Eck et al. 1994). Segregation distortion observed in both the mapping populations (Tables 2.1, 2.2) for the pigmentation traits located on chromosomes 2 and 11. Jacobs et al. (1995) also observed the segragation distortion for pigmentation traits on chromosmes 2 and 11. Segregation distortion further attributed to the high genetic load and marked inbreeding depression of potato (Howard 1970).

In case of the “S” population the tuber skin colour (red/purple) category fitted well to 1:1 ratio while in case of “K” population it fitted to both 3:1 and 5:1 ratios (Tables 2.1, 2.2).

Evaluation of physiological traits from parents & progeny

Only one mapping population was evaluated for two physiological traits i.e. emergence and flowering time which revealed monogenic segregation in a 3:1 ratio (Table 2.1). Abdalla and Hermsen (1971) studied the inheritance of flowering time in wild *Solanum* species. They expressed the flowering time as the number of days from sowing till opening of the first flower on the plant. Their results indicated that flowering time showed best fit to 3:1 ratio in some populations while 9:7 in others. The present results confirm the 3:1 ratio for the flowering time although the parental phenotypes did not differ for flowering time (Table 2.1). According to the parental genotypes the progeny should be early however the progeny is segregating in a 3:1 ratio which confirms the expectation. More studies may be necessary to confirm this hypothesis.

Assessment of phenotypic components of cold-induced sweetening from parents, grandparents and progeny

Parents & grandparents of the mapping population

The purpose of this study was to investigate the genetic variability for processing quality and biochemical parameters (glucose, fructose, sucrose and dry matter contents) in parents and grandparents of "S" population.

Crisp colour and sugars in parents & grandparents

Colour is one of the most important characters which determine the crisp quality in potato (Stevenson et al. 1964). "Yellowish brown" (Burton et al. 1992 as cited in Asmamaw et al. 2010), "uniform light golden" (Stevenson et al. 1964), and "lighter coloured" (Cunningham and Stevenson 1963) crisps are accepted by consumers. However, dark brown crisps may have an undesirable flavour and generally are unattractive to the consumers (Stevenson et al. 1964). Crisp colour and sugar contents (glucose, fructose and sucrose) were highly variable in parents and grandparents of the mapping population irrespective of cold storage duration. Breeding line 1021/1 always maintained better crisp colour [Figure 2.5 (A)] and high dry matter content [Figure 2.5 (E)] with very low glucose [Figure 2.5 (B)] and fructose [Figure 2.5 (C)] levels compared to Karaka and other grandparents studied over the duration of storage. Anderson et al (2005) tested crisping performance of eight potato lines after cold storage at 5.5°C up to 29 weeks and reported that 1021/1 had consistently light fry colour across cold storage. Furthermore the reconditioning treatment after removal from cool storage had little effect on crisp colour. Crisp colour of Karaka was below Agtron score 30 (storage treatments b, c, d and e) making Karaka a relatively uniform breeding line with dark crisp colour. All the other grandparents of the progeny had average crisp colour scores lower than 40 (storage treatments b, c, d and e; Figure 2.5). These studies clearly indicate that crisp colour is predominantly determined by the amount of glucose and fructose present in the tuber tissue. The variability observed in CIS traits depends on factors such as genetic background of cultivar, temperature, handling of the tubers, storage temperature and storage duration (Habib and Brown 1956; Linnemann et al. 1985; Meena et al. 2009; Asmamaw et al. 2010). A wide range of variability observed in the parents of "S" mapping population (1021/1 and Karaka) for CIS traits reflect that such variability or segregation can be expected in the progeny. Overall results raise question that what makes 1021/1 resistant to CIS? What are the factors/ mechanism that control the process? As a step forward an

attempt has been made to understand the biochemical events involved in CIS in further research.

Segregation for CIS traits among progeny of the mapping population

The segregation pattern of various tuber quality traits were studied detailed in "S" population.

Crisp colour and sugars after cold storage

The evaluation of mapping population for crisp colour and sugars at various storage treatments exhibited a wide range of variability. A crisp colour score ≥ 40 is an acceptable industry standard (Genet et al. 1995). Progeny that showed good crisp quality [Figure 2.7 (A)] with acceptable colour with low glucose [Figure 2.7 (B)] and fructose [Figure 2.7 (C)] levels at harvest would probably due to low levels of invertase. Therefore light coloured crisps can be produced from freshly harvested potato. Similar results were recorded by Stevenson et al. (1964) and Asmamaw et al. (2010).

In response to cold (storage treatments b & d) the increase in glucose [Figure 2.7 (B)], fructose [Figure 2.7 (C)] and sucrose [Figure 2.7 (D)] with concomitant decrease in crisp colour was noticed [Figure 2.7 (A)] could be due to the genetic background of the parents (Figure 2.5). Genotypic variation in crisp colour and reducing sugar concentrations from cold stored potato cultivars has been reported (Matsuura-Endo et al. 2004; McCann et al. 2010). These results revealed that crisp colour is predominantly determined by the amount of reducing sugars (glucose + fructose) present in potato tubers (Gould 1979) as sucrose do not contribute to darkening of processed potato products (Leszkowiat et al. 1990; Pritchard and Adam 1994; Zrenner et al. 1996). Also starch is converted into sucrose which is furthermore broken down into glucose and fructose by invertase. It has been well documented that the activity of soluble acid invertase increases in cold-stored tubers (Pressey 1969; Richardson et al. 1990) implying that invertase is the key enzyme involved in CIS process of potato tubers.

Variations in the accumulation of glucose and fructose levels were also noted in several progeny lines after cold storage durations. For example, at low temperature although glucose was distinctly the dominant sugar, higher fructose than glucose or unequal amount of both has been observed [Figures 2.7 (B) & (C), Appendix B]. Such variations were also noted by McCann et al. (2010). Davies and Viola (1994) reported that glucose concentrations are frequently higher than fructose concentrations in stored potato tubers. Samotus et al. (1974) observed fructose as a dominant sugar in cold stored potato tubers. Based on these results several possibilities can be emerged. For example higher

concentrations of fructose than glucose could occur if glucose produced from invertase mediated sucrose cleavage can be cycled into tuber respiration (McCann et al. (2010). It has also been possible that the unequal amounts of fructose and glucose could occur if invertase is suppressed by its invertase inhibitor and sucrose would be cleaved by sucrose synthase rather than invertase. As a result more fructose and UDP-glucose would accumulate after cold storage. Presence of invertase inhibitor in potato tubers have been reported (Pressey 1969) that controls the activity of invertase at post-translation level (Rausch and Greiner 2004). Apart from this, genotypic differences during low temperature storage in sugar accumulation profiles have been reported by Burton (1965), Samotus et al. (1974), Verma et al. (1974) and Coffin et al. (1987). The reducing sugar content of the potato is affected by several factors, including variety, growing conditions, maturity at harvest, post harvest handling stress and storage environment (Uppal and Verma 1990).

Progeny that consistently produced light crisp colour [Figures 2.6, 2.7 (A)] with low reducing sugars (glucose + fructose) after cold storage [Figure 2.7 (B), (C)] it can be hypothesised that invertase may be inhibited by its inhibitor during cold storage. Therefore the post-translational interactions between invertase and invertase inhibitor may play a critical role in controlling cold-induced sweetening in potato tubers. The hypothesis can be supported by the results presented by Greiner et al. (1999), Liu et al. (2010) and Brummell et al. (2010). It can also be proposed that as the parents of the mapping population are from a diverse genetic background the natural variation of the invertase and invertase inhibitor genes may control the cold-induced sweetening in potato tubers. Few genotypes produced bad crisp colour at all storage treatments [Figures 2.6, Figure 2.7 (A)] which might be due to very high invertase activity.

Transgressive segregation was observed for crisp colour [Figure 2.7 (A)], sugars [Figure 2.7 (B) to (D)] and dry matter [Figure 2.7 (E)] where genotypes with crisp colour, glucose, fructose, sucrose and dry matter contents were equal to or lower than those of 1021/1 or higher than the Karaka were found. Transgressive segregation for reducing sugars from potato progenies were also reported by Pereira et al. (1993). These results suggest that the transgressive segregation might have originated from a combination of the genetic components from both parents.

Dry matter

The dry matter concentration of tubers is an important measure of quality to assess suitability for processing purposes (Stevenson et al. 1964; Asmamaw et al. 2010). High dry

matter is desirable in chipping potatoes because raw product with high dry matter content absorbs less oil during frying than product with lower dry matter content (McCann et al, 2010). Results revealed that compared to harvest (storage treatment a) the average dry matter content (Table 2.3) were increased at all the storage treatments (b to e). The more pronounced in average dry matter content at storage treatments d & e compared to storage treatments b & c is probably a result of tuber drying out and loosing water in storage. The increase in dry matter observed after storage was mainly caused by moisture loss through evaporation (Verma et al. 1971, 1972). Genotypes that showed high dry matter content can be further utilised for the processing purpose. Positive relationship between dry matter content and starch has been previously noted (Akeley and Stevenson 1944). Potatoes with dry matter concentrations 20 - 25% are ideal for preparing crisps (Bewell 1937; Akeley and Stevenson 1944; Asmamaw et al. 2010). High specific gravity or high dry matter content has been reported by several authors to be desirable because of less sugar accumulation during storage (Watada and Kunkel 1955; Iritani and Weller 1976a, b). Therefore it could be worth to study the allelic variations of these starch synthesising genes investigate the possibility of allelic variations that may play a critical role in controlling dry matter content in potato tubers.

Crisp colour and sugars after reconditioning

High level of sugar in potato tubers after cold storage required reconditioning (Samotus et al. 1974) at warmer storage temperatures (i.e., > 10°C) prior to processing, which results in a decrease in reducing sugar content as some of these sugars are converted back into starch (Isherwood 1973). In general, the reconditioning periods (storage treatments c & e) resulted into decrease in average glucose, fructose and sucrose levels with improved crisp colour (Table 2.3). Similar results reported by Sasaki et al. (2004) when tubers stored at 6°C for three months. Therefore it can be hypothesised that upon reconditioning these sugars might have converted back into to starch probably due the conversion of sugars to starch upon reconditioning suppress the activities of enzymes involved in reducing sugar accumulation.

Different tendencies in reconditioning behaviour for crisp colour [Figure 2.7 (A)], glucose [Figure 2.7 (B)], fructose [Figure 2.7 (C)] and sucrose [Figure 2.7 (D)] content were observed in the present studies. Although reconditioning was effective in improving chip colour, it was not effective in decreasing glucose and fructose contents in few genotypes and vice versa. Coffin et al. (1987) reported that, reconditioning does not always lower the

concentration of reducing sugars to acceptable levels and long term CIS is considered to be irreversible. Therefore in such genotypes the sugars might have not converted back into starch upon reconditioning and activities of enzymes those convert starch to sucrose may have induced.

Correlation between crisp colour and content of specific sugars

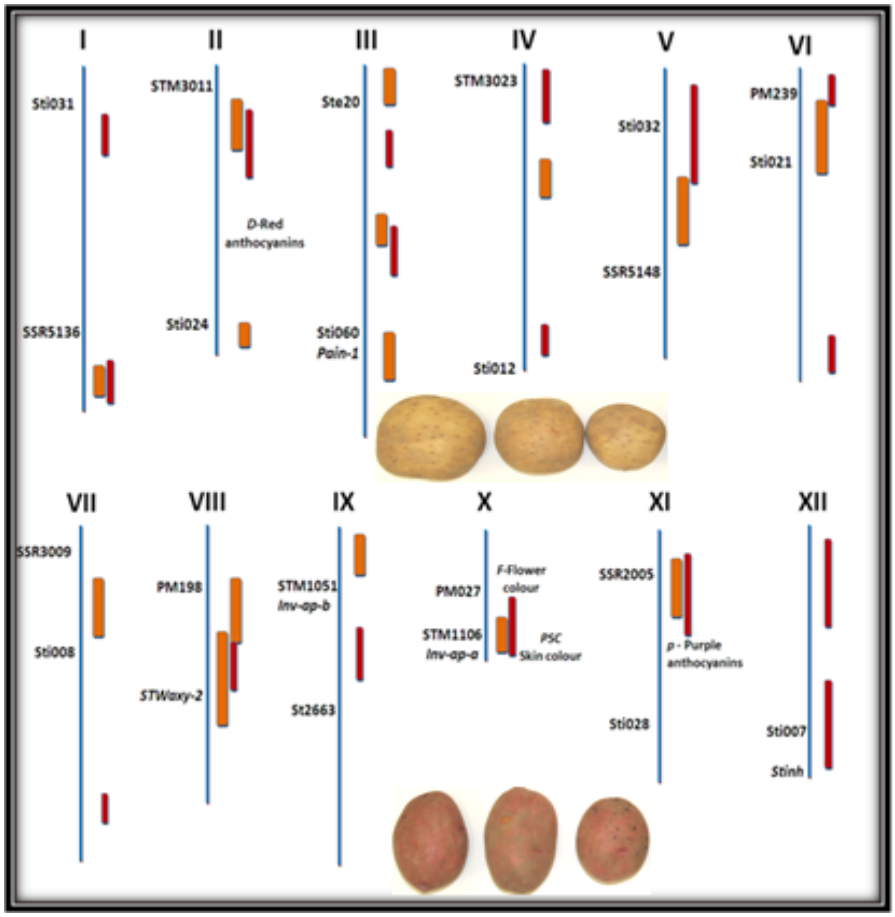
In order to determine which sugar is predominantly contributing to crisp colour, the relationship between sugar concentrations and crisp colour was also examined by Pearson correlation coefficient analysis (Table 2.4). Although all the three sugars (glucose, fructose and sucrose) found to be negatively correlated with crisp colour, the correlation coefficient (r) was smaller in case of sucrose. These results clearly showed that sucrose has very little or no influence on crisp colour. Secondly among glucose and fructose, the correlation coefficient (r) for fructose was greater than that of glucose. In addition phenotypic variance (R^2) between crisp colour and fructose was slightly greater than crisp colour and glucose which indicates that fructose has more influence on crisp colour than glucose. Similar results were noted by Song (2004) and Olsson et al. (2004). The observed negative correlation (low glucose content and high Agtron score) between crisp colour and glucose and fructose indicates that high Agtron score is associated with low sugars. Similar observations are noted by Mazza (1983). Because reducing sugars are negatively correlated with chip colour, positively correlated responses to selection for chip quality would be expected in selecting for reduced sugar content (Pereira et al. 1994).

Concluding remarks

This work illustrates valuable biochemical information that can be used to identify the genetic components responsible for the phenotypic differences. By combining biochemistry data with genomic tools may help in investigating the genetic regulation of CIS in potato tubers. The variability observed in tuber quality traits suggest that further research required to study the allelic diversity of genes or the genomic regions that may contribute to these phenotypic variations associated with CIS. The identification of molecular markers associated with phenotypic variation can provide information on further understanding of cold-induced sweetening in potato tubers. Based on the results obtained in the present studies, the further research will now elucidate the genetic dissection of this trait by molecular markers.

Chapter 3

Association based studies on tuber quality traits in potato (*Solanum tuberosum* L.) using single marker allele ANOVA analysis



Chapter 3

Association based studies on tuber quality traits in potato (*Solanum tuberosum* L.) using single marker allele ANOVA analysis

3.1 Introduction

Potato breeding aims at improvement of tuber traits and selection of novel genetic variants. One of the storage related problems is cold-induced sweetening (CIS) in potato tubers caused by accumulation of reducing sugars i.e. glucose and fructose at low temperatures that affect the processing quality of fried potato products (Shallenberger et al. 1959; Burton 1969; Dale and Bradshaw 2003). The quality improvement in potato by increasing CIS resistance is important in potato breeding. The breeding strategies aiming to improve cold-induced sweetening resistance have gained momentum in recent years by the use of molecular marker technologies (Menéndez et al 2002; Li et al. 2008). In order to determine the level of genetic variability molecular markers such as simple sequence repeat (SSR) markers have proven to be extremely useful. SSRs or microsatellites are a marker of choice in many genetic studies such as linkage mapping (Powell et al. 1996; Milbourne et al. 1998; Zhang et al. 2007, 2009), comparative mapping (Bérubé et al. 2006; Albrecht and Chetelat 2009), association mapping (Gebhardt et al. 2004; Malosetti et al. 2007; D'hoop et al. 2008; Baldwin et al. 2011) and QTL mapping (Menendez et al. 2002; Bradshaw et al. 2004; Bryan et al. 2004; Costanzo et al. 2005; Simko et al. 2006a; Sørensen et al. 2006). The popularity of SSR markers is due to their availability, distribution across the genome, co-dominant behaviour, multi-allelism, high level of polymorphism, locus specificity, relative abundance and reproducibility (Powell et al. 1996; Jones et al. 1997, Milbourne et al. 1998; Mc Gregor et al. 2000; Varshney et al. 2005).

As a fundamental aim of genetics is to connect genotype to phenotype (Botstein and Risch 2003), association mapping seeks to identify specific functional variants (i.e., loci, alleles) linked to phenotypic differences in a trait, to facilitate detection of trait causing DNA sequence polymorphisms and/or selection of genotypes that closely resemble the phenotype (Oraguzie and Wilcox 2007). In simple terms association mapping seeks the significant association of a molecular marker with a phenotypic trait (Gupta et al. 2005). Variations in the DNA sequence of genes and their regulatory regions underlie most of the phenotypic variation that has been exploited in modern crops (Bryan et al. 2000; Masouleh et al. 2009). It has been well documented that the phenotypic variation of many complex

traits of agricultural importance is influenced by multiple quantitative trait loci (QTLs), their interaction, the environment, and the interaction between QTL and environment (Zhu et al. 2008). For dissecting complex traits, therefore association mapping is the most commonly used tool (Zhu et al. 2008).

CIS is one of the most complex tuber quality traits. The association mapping approach has been used in potato to identify markers and loci associated with various tuber quality traits including CIS (Li et al. 2008; D'hoop et al. 2008; Baldwin et al. 2011; Urbany et al. 2011). Li et al. (2005) found that the invertase *invGE/GF* locus on potato chromosome IX co-localises with cold-sweetening QTL *Sug9* and showed that invertase alleles are associated with chip quality and tuber starch content. Li et al. (2008) evaluated tetraploid potato cultivars and breeding clones for various tuber traits (chip quality, tuber starch content, yield and starch yield) before and after cold storage, as well as genes coding for enzymes that function in carbohydrate metabolism or transport (candidate loci). They concluded that the natural DNA variation at candidate loci is associated with potato chip colour, tuber starch content, yield and starch yield. Baldwin et al. (2011), using an association mapping approach, concluded that allelic variation in UDP-glucose pyrophosphorylase and apoplastic invertase genes were significantly associated with cold-induced sweetening in potato tubers. They also identified a possible interaction between apoplastic invertase and apoplastic invertase inhibitor as being potentially important.

This Chapter describes results on association mapping conducted using single marker allele ANOVA (e.g. Kreike et al. 1994) analysis for a tetraploid mapping population established in Chapter 2. The allelic variation for 24 SSRs markers with known position on the potato genetic map (one marker per chromosome arm), including candidate genes associated with carbohydrate metabolism, were examined in 305 progeny ("S" population). Furthermore the marker-trait associations for pigmentation traits, physiological traits and for crisp quality, glucose, fructose, sucrose and dry matter contents were conducted before and after cold storage at various storage treatments described in Chapter 2 (Section 2.2.4).

3.2 Materials and methods

3.2.1 Plant material

Described in Section 2.2.1

3.2.2 Genomic DNA extraction

Healthy, young potato leaves were collected from all the progeny and parents of the mapping populations (1021/1, Karaka and Summer Delight) into 1.5 ml microfuge tubes (approximately three leaves per tube) from greenhouse-grown plants. The tubes were snap frozen in liquid nitrogen and stored at - 80°C until use. Total genomic DNA was isolated using a nuclear lysis method (Bernatzsky and Tanksley 1986) with slight modifications. DNA was extracted from stored leaf material by grinding to a fine powder using a pestle under liquid nitrogen. One ml of extraction buffer (Appendix A) was added to the ground tissue and the tube was inverted to mix. The mixture was centrifuged at 4°C for 20 min at 7428 x g (Eppendorf, 5810R, Hamburg, Germany) and the supernatant was carefully decanted. The pellet was resuspended in nuclear lysis buffer (Appendix A) (200 µl or one volume) and 80 µl (0.4 volume) 5% (w/v) sodium-N-lauroylsarcosine were added and mixed. The solution was incubated at 65°C for five min, then inverted 10 times and incubated for a further 10 min. Chloroform/isoamyl alcohol (24:1) solution (500 µl or one volume) was added and the sample was inverted 40 times. The sample was centrifuged at 4°C for five min at 14,857 x g. The top aqueous layer was transferred to a sterile 1.5 ml tube and re-extracted with an equal volume chloroform/isoamyl alcohol (24:1) solution. The DNA was precipitated using 250 µl (2/3 volume) ice cold (-20°C) isopropanol. The tube was inverted 15 times and the precipitated DNA was hooked out and transferred to a sterile 1.5 ml tube. The DNA was washed using 250 µl of 70% ethanol. The samples were centrifuged briefly and the ethanol drained off. The DNA was air dried at room temperature and resuspended in 250 µl of TE^{-4} (Appendix A).

3.2.3 PCR conditions for simple sequence repeats (SSRs)

Twenty four simple sequence repeat markers (SSRs) were selected using the following criteria 1) map location with at least one SSR marker for each chromosome arm and 2) single locus marker. All the SSR markers and their primer sequences are given in Table 3.1. The amplification of SSRs was performed either with two or three primers. In the case of two primers one of the primers was directly labelled with a fluorescent dye, either 6-carboxyfluorescein (6-FAM), hexachloro-6-carboxyfluorescein (HEX) or 7', 8'- benzo-5' fluoro-2', 4, 7 trichloro-5-carboxyfluorescein (NED). In the case of three primers, the forward

primer from each pair was tailed with the sequence 5'-GACGTTGTAAAACGACGGCC-3' (Xin et al. 2004) and a universal, fluorescently labelled, M13 primer with the same tail sequence containing a fluorescent dye (FAM, HEX or NED) was used. Microsatellites were amplified by PCR in a 10 µl reaction volume containing 1X reaction buffer [750mM Tris-HCl, pH 8.8, 200mM $(NH_4)SO_4$, 0.1 % v/v Tween®20], 1.5 mM $MgCl_2$, 0.2mM each of dATP, dCTP, dGTP, dTTP, 0.1 µl Red Hot *Taq* DNA polymerase (5.0 U μl^{-1}, Thermo Scientific, UK), 0.2 µM of each primer at, 1.0 µl of genomic DNA (10-50 ng). PCR was performed on a Master cycler (Eppendorf, Hamburg, Germany) and BioRad (BIO-RAD, C1000™ Thermal Cycler, CA, USA). Two different amplification protocols were used:

1) A regular PCR with four min initial denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at annealing temperature, 30 s at 72°C and a final extension of 10 min at 72°C.

2) A "touchdown PCR" with four min of initial denaturation at 94°C, followed by 4 touchdown cycles of 30 s at 94°C, 30 s annealing temperature, -2°C each cycle and extension at 72°C for 30 s. This was followed by 35 cycles of 30 s at 94°C, 30 s at annealing temperature and 30 s at 72°C. The programme was ended with a final extension for 10 min at 72°C. Amplified products were separated by electrophoresis in a 2% agarose gel (0.5X TBE buffer at 12 V cm^{-1} for 20 min) and visualised under UV light after staining with ethidium bromide (1 mg l^{-1}) for 30 mins.

3.2.4 Simple sequence repeat (SSR) analysis

Three differently labelled PCR products (FAM, HEX and NED) from one individual were pooled into one precipitation reaction. The PCR product was diluted by adding 10 µl of milli-Q water. Four µl of each microsatellite (total 12 µl) was pipetted into a 96 well plate (Axygen) and 18 µl of precipitation mix (Appendix A) was added to each well. The plate was covered with aluminium foil. The precipitation reaction was mixed by inverting the plates and centrifuged at 4°C for 30 min at 3,200 x g (Centrifuge 5810R, Eppendorf, Hamburg, Germany). After centrifugation the plates were inverted on to a tissue paper and re-centrifuged for 200 x g for 30 s to remove supernatant. The plates were then incubated in an incubator (INFORS, AGCH-4103, Bottmingen, Switzerland) at 37°C for 10 min to dry. The precipitated DNA was then re-constituted in 10 µl of ABI3100 loading buffer containing 0.075 µl ROX400 size ladder and denatured at 95°C for five min. After denaturation the plates were put on ice.

The fluorescently labelled products were separated on an ABI3100 Genetic Analyser (Applied Biosystems Pty Ltd, Australia & New Zealand). Electropherograms were analysed

using GeneMapper v3.7 (Applied Biosystems Pty Ltd, Australia & New Zealand) or GeneMarker v1.4 (SoftGenetics, LLC State College, PA, USA).

3.2.5 Statistical analysis

The size of the SSR fragments was visually checked for each individual. DNA fragments of all markers were recorded in each individual as 1 for presence, 0 for absence, or as missing value in unclear cases. A Chi-square goodness-of-fit test (with one degree of freedom) was used to test whether the observed segregation of individual marker alleles differed significantly from expected values.

Polymorphic SSR fragments which were scored as presence or absence of the fragment were tested for association by analysis of variance using GenStat (12^{th} edition, VSN International Ltd, Oxford, UK). Data of individual genotypes for each individual marker allele (0/1) was then associated with their respective phenotypic values (crisp colour, glucose, fructose, sucrose and dry matter contents before and after cold storage). Unclear SSR fragments in genotypes were declared as missing values and therefore not considered in the analysis. The SSR fragment was considered to be associated with the trait at $P < 0.05$.

Table 3.1 Characteristics of 24 SSRs isolated from potato

Marker	Description	F/R	Primer sequence 5' – 3'	Annealing Temp. (°C)[1]	LG	Fluorescent label[2]	Reference
SSR5136	Unknown	F R	GGGAAAAGGAAAAGCTCAA ATATGAACCACCTCAGGCAC	50	I	TAIL	Reid et al. 2009
Sti031	DAG protein	F R	AGGCGCACTTTAACTTCCAC CGGAACAAATTGCTCTGATG	TD 60-54	I	TAIL	Feingold et al. 2005
STM3011	cation-chloride co-transporter	F R	GTGTGGTTGATTGATTGATTTAGC TTAGGCAGTTCTTGGGG	60	II	TAIL	Milbourne et al. 1998
Sti024	Nucleoporin	F R	CGCCATTCTCTCAGATCACTC GCTGCAGCAGTTGTTGTTGT	TD 60-54	II	TAIL	Feingold et al. 2005
Sti060	Invertase *Pain-1*	F R	ACTTCTGCATCTGGTGAAGC GGTCTGGATTCCCAGGTTG	TD 60-54	III	TAIL	Feingold et al. 2005
Ste20	Auxin-responsive protein IAA27	F R	TTTGTGCTCAACTTTCCATGTC GCCAAAAAGAGAAACCCCAT	58	III	NED	Tang et al. 2008
STM3023	TIR-NBS-LRR resistance protein	F R	AAGCTGTTACTTGATTGCTGCA GTTCTGGCATTTCCATCTAGAGA	58	IV	FAM	Reid et al. 2009
Sti012	Dehydrin	F R	GAAGCGACTTCCAAAATCAGA AAAGGGAGGAATAGAAACCAAAA	TD 58-52	IV	TAIL	Feingold et al. 2005
SSR5148	Nuclear transport factor 2	F R	TCTTCTTGATGACAGCTTCG ACCTCAGATAGTTGCCATGTCA	50	V	TAIL	Reid et al. 2009
Sti032	Zinc finger protein	F R	TGGGAAGAATCCTGAAATGG TGCTCTACCAATTAACGGCA	TD 64-60	V	TAIL	Feingold et al. 2005
PM239	Unknown	F R	TCATGTGAAATACTTTGAAGTTAGAGGG CATGCTGCAGAGTTGGCTATAATC	TD 64-58	VI	TAIL	Potato Genome Sequencing Consortium 2011
Sti021	HSF30	F R	TCATCAAGTCGTCGTCATCAA TCGAATGATCCAAAGCTTCC	TD 60-54	VI	TAIL	Feingold et al. 2005
Sti008	Arabinogalactan protein	F R	CATCTCCTTCACCTGCTCCT CGACAAAGGAGGAAATCCAA	TD 58-52	VII	TAIL	Feingold et al. 2005

Table 3.1 Characteristics of 24 SSRs isolated from potato

Marker	Description	F/R	Primer sequence 5' – 3'	Annealing Temp. (°C)[1]	LG	Fluorescent label[2]	Reference
STM3009	Unknown	F R	TCAGCTGAACGACCACTGTTC GATTTCACCAAGCATGGAAGTC	58	VII	NED	Milbourne et al. 1998
PM198	Unknown	F R	GATTGGTAAATGGGATCACTCTCG TCTTTCACGACTTGGGGTGTTAAT	60	VIII	TAIL	Potato Genome Sequencing Consortium 2011
STWaxy2	Granule bound starch synthase	F R	CCCATAATACTGTCGATGAGCA GAATGTAGGGAAACATGCATGA	TD 60 - 54	VIII	NED	Milbourne et al. 1998
St2663	Unknown	F R	CCTCCTCCTCATCATGCACT ACACCCTCCTCTCCCAAAAA	58	IX	NED	Tang et al. 2008
STM1051	Invertase GE/GF Inv_{ap}-b	F R	TCCCCTTGGCATTTTCTTCTCC TTTAGGGTGGGGTGAGGTTGG	60	IX	FAM	Milbourne et al. 1998
STM1106	Invertase Inv_{ap}-a	F R	TCCAGCTGATTGGTTAGGTTG ATGCGAATCTACTCGTCATGG	61	X	HEX	Milbourne et al. 1998
PM027	Unknown	F R	CAAAGTAATGAAAATTCCCTCTGTGAA CACTTCGTTCTTTCCTTCTCTTCG	TD 64-58	X	TAIL	Potato Genome Sequencing Consortium 2011
Sti028	Unknown	F R	ATACCCTCCAATGGGTCCTT CTTGGAGATTTGCAAGAAGAA	60	XI	TAIL	Feingold et al. 2005
SSR2005	UPA15, hom with arab mannan synthase/ transferase	F R	TTTAAGTTCTCAGTTCTGCAGGG GTCATAACCTTTACCATTGCTGGG	56	XI	NED	Milbourne et al. 1998
Sti007	Glucosyl transferase	F R	TATGTTCCACGCCATTTCAG ACGGAAACTCATCGTGCATT	TD 60-54	XII	TAIL	Feingold et al. 2005
inh2α	Vacuolar invertase inhibitor (FJ810209, FJ810208, FJ810207, FJ810206)	F R	AAAGTTGAATTCAAATGAGAAATTTATTC ATGGGGTCTCCCTACACGTT	59	XII	HEX	Baldwin et al. 2011

Note: - [1] TD - Touchdown in which the range of annealing temperatures denotes a touchdown profile with -2°C per cycle, until lower annealing temperature was reached. [2] Sequence of labelled forward Tail primer - 5'-GACGTTGTAAAACGACGGCC-3'.

3.3 Results

3.3.1 Microsatellite polymorphisms

The results obtained from the 24 microsatellite polymorphisms observed from 305 individuals is summarised in Table 3.2. Alleles at the microsatellite loci were scored based on the parental bands. The progeny that consistently produced unexpected size bands not present in the parents were considered as non-hybrids and were eliminated from the progeny set. All the 24 markers were found to be polymorphic (Table 3.2) in the progeny. A total of 84 alleles were produced from 24 microsatellite markers. Forty-nine alleles were specific to one parent; 24 alleles (49%) were specific to 1021/1 while 25 alleles (51%) were specific to Karaka. The remaining 35 alleles were shared by both parents and the number of shared alleles varied from one to three alleles per marker. Of the 35 shared alleles, 31 alleles (89%) were segregating and four alleles (11%) were not segregating in the progeny (Table 3.2). The number of different bands produced by each SSR marker varied from two to six bands (average 3.5) (Table 3.2). Of the 24 markers tested five were candidate genes operating in carbohydrate metabolism in potato tubers (Table 3.1, Figure 3.1). The five candidate gene markers produced 17 alleles, of which six were specific to 1021/1 and four were specific to Karaka. The remaining seven alleles were shared by both parents (Table 3.2). The highest number of alleles (six) was produced by marker Sti028, while markers SSR5136 (LG I), Sti012 (LG IV), SSR5148 (V) and *STWaxy2* (LG VIII) each produced 5 bands (Table 3.2).

3.3.2 Segregation ratios and Chi square statistics

The segregation ratios and Chi square statistics are summarised in Table 3.2. Chi-square tests were performed on segregation ratios of 84 marker alleles by comparing observed genotype frequencies with the expected ratios. Several segregation ratios were expected depending on the presence or absence of a microsatellite marker allele in the parents. When a marker allele was present in only one parent, segregation ratios of 1:1 or 5:1 (presence versus absence) were expected for simplex and duplex markers respectively. When a marker allele was present in both parents, segregation of 3:1, 11:1, 35:1 and 1:0 (presence versus absence) were expected for simplex-simplex, simplex-duplex, duplex-duplex and simplex-triplex/quadruplex or duplex-triplex/quadruplex respectively. The results showed that in total 80 marker alleles (95%) out of 84 were segregating (Table 3.2). The remaining four (5%) were non-segregating alleles belonging to STM3023 (LG IV), Sti008 (LG VII), PM198 (LG VIII) & *inh2*α (LG XII) (Table 3.2). Out of 80 segregating alleles, a good fit to

Table 3.2:- Segregation ratios and Chi square statistics

Marker	Allele	Linkage group	Karaka	1021/1	No of present	No of absent	Segregation ratio	χ_1^2	P - value
SSR5136	240	I	0	1	130	156	1:1	2.36^{ns}	0.12
	249	I	1	1	216	70	3:1	0.04^{ns}	0.84
	252	I	1	0	152	134	1:1	1.13^{ns}	0.29
	269	I	1	1	213	73	3:1	0.04^{ns}	0.84
	272	I	1	0	139	147	1:1	0.22^{ns}	0.64
Sti031	**149**	**I**	**1**	**1**	**250**	**34**	**11:1**	**4.92***	**0.03**
	161	I	1	1	263	21	11:1	0.33^{ns}	0.57
	164	I	1	1	206	78	3:1	0.92^{ns}	0.34
STM3011	122	II	1	0	229	57	5:1	2.19^{ns}	0.14
	144	II	1	1	258	28	11:1	0.79^{ns}	0.37
	148	II	0	1	235	51	5:1	0.28^{ns}	0.60
	150	II	0	1	146	140	1:1	0.13^{ns}	0.72
Sti024	157	II	1	1	215	61	3:1	1.24^{ns}	0.27
	166	II	0	1	128	148	1:1	1.45^{ns}	0.23
	182	**II**	**1**	**1**	**270**	**6**	**11:1**	**13.71*** **	**0.00**
	187	II	0	1	142	134	1:1	0.23^{ns}	0.63
Sti060	179	III	1	1	209	73	3:1	0.12^{ns}	0.73
	182	III	0	1	128	154	1:1	2.40^{ns}	0.12
	185	III	1	0	135	147	1:1	0.51^{ns}	0.47
	194	III	1	1	270	12	35:1	2.28^{ns}	0.13
Ste20	190	III	1	1	283	4	35:1	2.04^{ns}	0.15
	193	III	0	1	144	143	1:1	0.00^{ns}	0.95
STM3023	176	IV	1	1	282	0	36:0	--	--
	196	IV	0	1	233	49	5:1	0.10^{ns}	0.75
Sti012	189	IV	1	0	132	149	1:1	1.03^{ns}	0.31
	192	**IV**	**1**	**1**	**196**	**85**	**3:1**	**4.13***	**0.04**
	195	IV	1	1	263	18	11:1	1.37^{ns}	0.24
	201	IV	0	1	125	156	1:1	3.42^{ns}	0.06
	208	IV	1	0	145	136	1:1	0.29^{ns}	0.59
SSR5148	439	V	0	1	145	137	1:1	0.23^{ns}	0.63
	451	**V**	**1**	**0**	**162**	**120**	**1:1**	**6.26***	**0.01**
	468	**V**	**1**	**0**	**122**	**160**	**1:1**	**5.12***	**0.02**
	473	V	1	1	259	23	11:1	0.01^{ns}	0.91
	493	V	1	1	221	61	3:1	1.71^{ns}	0.19
Sti032	138	V	1	0	156	130	1:1	2.36^{ns}	0.12
	145	V	1	0	145	141	1:1	0.06^{ns}	0.81
	148	V	1	1	266	20	11:1	0.67^{ns}	0.41
	151	V	1	1	202	84	3:1	2.91^{ns}	0.09
PM239	**242**	**VI**	**1**	**1**	**286**	**1**	**35:1**	**6.27***	**0.01**
	248	VI	1	0	236	51	5:1	0.25^{ns}	0.62
Sti021	122	VI	0	1	149	135	1:1	0.69^{ns}	0.41
	125	VI	1	0	151	133	1:1	1.14^{ns}	0.29
	129	**VI**	**1**	**1**	**282**	**2**	**35:1**	**4.52***	**0.03**
	132	**VI**	**0**	**1**	**164**	**120**	**1:1**	**6.82****	**0.01**
SSR3009	150	VII	0	1	150	136	1:1	0.69^{ns}	0.41
	164	**VII**	**1**	**1**	**284**	**2**	**35:1**	**4.58***	**0.03**
Sti008	163	VII	1	1	287	0	36:0	--	--
	169	VII	1	0	140	147	1:1	0.17^{ns}	0.679
	175	VII	1	0	237	50	5:1	0.12^{ns}	0.731
PM198	186	VIII	1	1	287	0	36:0	--	--
	195	VIII	1	0	152	135	1:1	1.01^{ns}	0.32

Table 3.2:- Segregation ratios and Chi square statistics

Marker	Allele	Linkage group	Karaka	1021/1	No of present	No of absent	Segregation ratio	χ_1^2	P - value
STWaxy2	**219**	**VIII**	**1**	**0**	**122**	**163**	**1:1**	**5.90***	**0.02**
	222	VIII	0	1	130	155	1:1	2.19 ns	0.14
	223	VIII	0	1	144	141	1:1	0.03 ns	0.86
	226	**VIII**	**1**	**0**	**278**	**7**	**5:1**	**41.4*****	**0.00**
	233	VIII	0	1	148	137	1:1	0.42 ns	0.51
STM1051	183	IX	1	0	145	140	1:1	0.09 ns	0.77
	190	IX	0	1	158	127	1:1	3.37 ns	0.07
	225	IX	1	1	220	65	3:1	0.73 ns	0.39
	250	**IX**	**1**	**1**	**197**	**88**	**3:1**	**5.25***	**0.02**
St2663	176	IX	0	1	146	139	1:1	0.17 ns	0.68
	190	IX	1	0	245	40	5:1	1.42 ns	0.23
	192	IX	1	1	277	8	35:1	0.00 ns	0.98
	195	IX	0	1	138	147	1:1	0.28 ns	0.59
STM1106	150	X	0	1	132	148	1:1	0.91 ns	0.34
	156	X	1	1	262	18	11:1	1.33 ns	0.25
PM027	148	X	1	1	212	75	3:1	0.20 ns	0.66
	150	**X**	**1**	**1**	**285**	**2**	**35:1**	**4.60***	**0.03**
Sti028	194	XI	0	1	137	146	1:1	0.29 ns	0.59
	197	XI	0	1	140	143	1:1	0.03 ns	0.86
	200	XI	1	0	243	40	5:1	1.31 ns	0.25
	203	XI	1	0	138	145	1:1	0.17 ns	0.68
	211	XI	0	1	238	45	5:1	0.12 ns	0.73
	217	XI	1	0	135	148	1:1	0.60 ns	0.44
SSR2005	**151**	**XI**	**1**	**0**	**255**	**29**	**5:1**	**8.52****	**0.00**
	171	XI	1	1	255	29	11:1	1.31 ns	0.25
	183	XI	1	0	149	135	1:1	0.69 ns	0.41
Sti007	**141**	**XII**	**1**	**1**	**244**	**41**	**3:1**	**17.12*****	**0.00**
	144	**XII**	**1**	**1**	**199**	**86**	**3:1**	**4.07***	**0.04**
	147	**XII**	**1**	**1**	**258**	**27**	**3:1**	**36.6*****	**0.00**
	150	XII	1	0	127	158	1:1	3.37 ns	0.066
	157	**XII**	**0**	**1**	**116**	**169**	**1:1**	**9.86****	**0.002**
inh2α	130	XII	1	1	257	26	11:1	0.27 ns	0.60
	139	XII	1	1	283	0	36:0	--	--

Note: - No segregation denoted by --. Bold highlight show segregation distortion. Note: $\chi 2_{0.05,1}=3.84$; $\chi 2_{0.01,1}=6.63$; $\chi 2_{0.001,1}=10.83$, *, ** and *** indicate significance at 0.05, 0.01 and 0.001 probability level, respectively.

the expected segregation ratios (1:1, 3:1, 5:1, 11:1 and 35:1, $P < 0.05$) was observed in 62 marker alleles (77.5%). The remaining 18 marker alleles (22.5%) significantly deviated ($P < 0.05$) from the expected segregation ratios and four out of 18 marker alleles (22%) were highly ($P < 0.01$, $P < 0.001$) distorted (Table 3.2). The distorted marker alleles belong to Sti031 (LG I), Sti024 (LG II), Sti012 (LG IV), SSR5148 (LG V), PM239 & Sti021 (LG VI), SSR3009 (LG VII), *STWaxy2* (LG VIII), STM1051 (LG IX), PM027, (LG X), SSR2005 (LG XI) and Sti007 (LG XII) (Table 3.2).

Sixteen segregating alleles were produced by candidate gene markers (Table 3.2). Thirteen (81%) showed a good fit to 1:1, 3:1, 11:1 and 35:1 ratios ($P < 0.05$).

3.3.3 Associations between microsatellites and pigmentation & physiological traits

Single marker allele ANOVA was used to conduct the association analysis and to identify the microsatellites associated with the pigmentation and physiological traits in the population. A total of six traits and their association statistics are described in Table 3.3. The tuber skin colour was categorised into two different columns: colour and colourless, and the colour category separated into red and purple. Table 3.3 also describes the effect of the presence of a marker allele on a trait and the direction of the effect, indicated by upward (↑) and downward (↓) arrows calculated based on the mean scores in the population.

Out of 24 marker loci, 18 markers (75%), which accounted for 27 alleles, showed significant associations ($P < 0.05$) with at least one of the measured pigmentation and physiological traits (Table 3.3). Six marker alleles were specific to 1021/1; nine were specific to Karaka while 12 were shared by both parents. For these 27 marker alleles, 54 marker-trait associations were observed at the 0.05 significance level, 21 of which showed highly significant ($P < 0.001$) associations with hypocotyl colour (five alleles), stem colour (five alleles), flower colour (three alleles) and tuber skin colour (three alleles for colour/colourless & five alleles for red/purple). The associated markers were located on linkage groups X (STM1106 & PM027) and XI (Sti028 & SSR2005) (Table 3.3). Only two markers showed significant association ($P < 0.05$) with flowering time (SSR5136 & Sti031, LG I) while nine markers were associated with emergence in the field.

1021/1 specific marker alleles accounted for nine significant associations ($P < 0.05$). In particular in the presence of 1021/1 specific allele STM1106 allele 150 (LG X) showed highly significant association ($P < 0.001$) with the stem, flower and tuber skin colour (Table 3.3). Karaka specific alleles accounted for 21 significant associations ($P < 0.05$). In particular five Karaka specific alleles from two markers (Sti028 & SSR2005, located on LG XI) showed highly significant ($P < 0.001$) marker-trait associations with hypocotyls, stem and tuber skin colour. Twelve alleles shared by both parents accounted for 24 significant associations ($P < 0.05$). In particular the presence of shared allele, 156 (STM1106 LG X) may not be required for determination of flower and tuber skin colour, while presence of PM027 shared allele 148 (LG X) may require to determine these traits (Table 3.3).

Table 3.3: Marker-trait associations in Karaka x 1021/1 population using ANOVA

Marker	Allele	Parent	LG	Hypocotyl colour	Stem colour	Flowering Time	Flower colour	Emergence in field	Tuber skin colour Colour/colourless	Tuber skin colour Red/Purple n = 129
SSR5136	240	P2	I	**0.036↓**	ns	**0.002↑**	ns	ns	Ns	ns
	249	K/ P2	I	ns	ns	ns	**0.024↓**	ns	**0.024↓**	ns
Sti031	149	K/ P2	I	ns	**0.023↑**	**0.002↑**	**0.015↑**	**0.021↑**	**0.025↑**	ns
STM3011	144	K/ P2	II	ns	**0.041↑**	ns	ns	ns	Ns	**0.020↓**
Ste20	190	K/ P2	III	**0.014↑**	ns	ns	ns	ns	Ns	ns
STM3023	196	P2	IV	ns	ns	ns	ns	**0.006↑**	Ns	ns
Sti012	208	K	IV	ns	ns	ns	**0.007↓**	ns	Ns	ns
SSR5148	493	K/ P2	V	**0.044↓**	ns	ns	ns	ns	Ns	ns
PM239	248	K	VI	ns	ns	ns	ns	**0.018↓**	Ns	ns
Sti008	169	K	VII	ns	**0.005↑**	ns	ns	ns	Ns	ns
STWaxy2	223	P2	VIII	ns	ns	ns	**0.009↑**	ns	Ns	ns
	226	K	VIII	**0.014↑**	**0.022↓**	ns	ns	ns	Ns	ns
	233	P2	VIII	ns	ns	ns	ns	**0.005↓**	Ns	ns
St2663	176	P2	IX	ns	ns	ns	ns	ns	Ns	**0.003↓**
STM1051	225	K/ P2	IX	ns	ns	ns	**0.015↓**	ns	**0.014↓**	ns
	250	K/ P2	IX	ns	ns	ns	ns	**0.013↓**	Ns	ns
STM1106	150	P2	X	ns	**<0.001↑**	ns	**<0.001↑**	ns	**<0.001↑**	ns
	156	K/ P2	X	ns	ns	ns	**<0.001↓**	ns	**<0.001↓**	ns
PM027	148	K/ P2	X	ns	ns	ns	**<0.001↑**	**0.019↑**	**<0.001↑**	ns
Sti028	200	K	XI	**<0.001↑**	**<0.001↓**	ns	ns	ns	Ns	**<0.001↑**
	203	K	XI	**<0.001↓**	**<0.001↑**	ns	ns	ns	**0.012↑**	**<0.001↓**
	217	K	XI	**<0.001↑**	ns	ns	ns	**0.035↓**	Ns	**<0.001↑**
SSR2005	151	K	XI	**<0.001↑**	**<0.001↓**	ns	ns	ns	Ns	**0.001↑**
	171	K/ P2	XI	**0.004↑**	ns	ns	ns	**0.007↓**	Ns	**0.022↑**
	183	K	XI	**<0.001↓**	**<0.001↑**	ns	ns	ns	Ns	**<0.001↓**
Sti007	147	K/ P2	XII	**0.015↓**	ns	ns	ns	ns	Ns	ns
inh2α	130	K/ P2	XII	ns	ns	ns	ns	**0.009↓**	Ns	ns

Note: K & P2 denote Karaka and 1021/1 respectively. ns- not significant. The numbers in the table represent the F-values obtained from ANOVA. Tuber skin colour Red/Purple is derived from colour category where n = 129. Arrows indicate the direction of the effect of the presence of a marker allele on a trait calculated based on mean scores in the population. ↑ indicates effect of the presence of an allele having colour while ↓ indicates effect of the presence of an allele not having colour. The direction of the effect cannot be determined in case of tuber coloured category (Red/Purple). ↑ & ↓ indicate quicker and delayed days for emergence and flowering respectively. LG- Linkage group.

3.3.4 Associations between microsatellites & phenotypic components of CIS

Association analysis was carried out between five phenotypic components of CIS (crisp colour, glucose, fructose, sucrose and dry matter content) evaluated at five storage treatments (a, b, c, d & e described in Chapter 2, section 2.2.4) and microsatellites using single marker allele ANOVA. The summary of the statistics is described in Table 3.4 along with the effect on a trait in the presence of the marker allele indicated by upward (↑) and downward (↓) arrows calculated based on the mean scores in the population. Decrease in glucose and fructose is indicated by ↓ and considered as a positive effect. Better crisp colour is indicated by ↑. Similarly effect on sucrose is indicated by ↑ ↓. For the dry matter content, ↑ indicates increased dry matter as a positive effect of the presence of allele.

Of 24 markers tested, 22 (92%) which accounted for a total of 60 alleles showed significant associations ($P < 0.05$) with at least one of the phenotypic components of CIS and ten of these alleles involved those of candidate genes (Table 3.4). For these 60 alleles, a total of 199 significant marker-trait associations ($P < 0.05$) were found. Seventeen alleles were specific to 1021/1, 22 were specific to Karaka while 21 were shared by both parents (Table 3.4). All these significant associations were found to be spread all over the 12 potato chromosomes. Twelve highly robust ($P < 0.001$) associations were recorded. In general 21 markers showed associations with more than one trait at least one storage treatment, except marker Sti008 - LG VII (Table 3.4). Sti008 showed significant association ($P < 0.05$) with sucrose at storage treatments a, d and e (Table 3.4).

Twenty six marker alleles were significantly associated ($P < 0.05$) with crisp colour; 22 alleles were associated with glucose and fructose contents, 31 with sucrose content and 21 with dry matter content (Table 3.4). Most frequent associations were with crisp colour, sucrose and dry matter content. The majority of the associations were recorded after cold storage (b & d) and reconditioning periods (c & e). Alleles specific to 1021/1 showed both positive and negative effects on the traits. For example four marker alleles (Sti060 - LG III, SSR5148 - LG V, Sti021 - LG Vi & SSR3009 - LG VII) specific to 1021/1 showed significant association ($P < 0.05$) with crisp colour at storage treatment b and presence of these alleles showed a positive effect on crisp colour indicated by ↑. In the presence of these four alleles decreased glucose, fructose or sucrose contents were observed at least one of the storage treatments indicated by ↓ (Table 3.4). Allele 196 (STM3023 - LG IV) showed significant association ($P < 0.05$) and increased dry matter content (↑) at storage treatments a, b, c & e as an effect of presence of a 1021/1 specific allele with concomitant decreased in glucose,

fructose (storage treatments c & a) and sucrose (storage treatment b) respectively (Table 3.4). On the other hand the presence of 1021/1 specific Sti012 allele 201 (LG IV) increased glucose and fructose contents (↑) and concomitantly decreased (↓) dry matter content was observed at storage treatments b & c (Table 3.4).

Similar effects were noted in the presence of alleles that were specific to Karaka. For example SSR2005 allele 183 (LG XI) showed significant association ($P < 0.05$) and increased glucose and fructose (↑) contents at storage treatments b, d & e, while Sti028 allele 217 (LG XI) showed significant association ($P < 0.05$) and increased crisp colour (↑) with concomitant decreased glucose, fructose and sucrose contents at storage treatment b (Table 3.4) indicated by ↓.

Among all the markers associations, those specific to SSR5136 (LG XII) were notable. For example, Karaka specific allele (SSR5136 allele 252) significantly associated with crisp colour, glucose and fructose ($P < 0.05$, $P < 0.001$) at all storage treatments except a (Table 3.4). In the presence of this allele, a negative effect (↓) on crisp colour and an increase in glucose and fructose (↑) contents were observed. In addition, sucrose contents were also found to be increased at storage treatments a & c. Similarly SSR5136 allele 272 (shared by both parents) showed a significant ($P < 0.05$) and negative association (↓) with crisp colour (storage treatment b) and dry matter content (storage treatments a, b, c &e). Interestingly SSR5136 shared allele 269 showed significant association ($P < 0.05$) and increased crisp colour (storage treatments b & e) and dry matter contents especially at storage treatments a, c, d & e.

Notably SSR3009 allele 164 (LG VII) shared by both parents was negatively associated with crisp colour at all the five storage treatments (Table 3.4). It is also worth mentioning the effects of Karaka specific alleles (Sti032 - LG V) on sucrose and dry matter contents. For example allele 138 exhibited positive, while allele 145 showed negative effect on sucrose and dry matter contents at various storage treatments (Table 3.4)

Candidate gene markers Sti060 (LG III), *STWaxy2* (LG VIII), STM1051 (LG IX), STM1106 (LG X) & *inh2*α (LG XII) showed significant associations (ranging from $P < 0.05$ to $P < 0.001$) with either positive or negative effect on phenotypic components of CIS (Table 3.4, Figure 3.1). The effects of markers Sti060 & *STWaxy2* were notable. For example Sti060 (soluble acid invertase) had three alleles. In the presence of Sti060 allele 182 (LG III, specific to 1021/1) showed significant association ($P < 0.05$) and better crisp quality (↑) at storage treatment b and decreased (↓) fructose at storage treatment c. In the presence of allele 185

(specific to Karaka), glucose content decreased (⇓) at both cold storage treatment periods (storage treatments b & c) and similarly in the presence of shared allele (194) crisp colour was found to decrease (↓) at storage treatments e (Table 3.4). In the case of *STWaxy2* (LG VIII) only one of the three alleles was specific to 1021/1. A negative effect (↓) on dry matter content (storage treatments a & b) was observed in the presence of 1021/1 specific allele (233) while a positive influence (↑) on crisp colour (storage treatments b, c, d & e) was noted in the presence of Karaka specific allele (219) with concomitant decreased in the glucose & fructose at one of the storage treatments (Table 3.4).

Table 3.4:- Marker-trait (phenotypic components) associations in Karaka x 1021/1 population using ANOVA

	Marker	SSR5136					Sti031	STM3011		Sti024				Ste20	
	Allele	240	249	252	269	272	149	122	148	157	166	182	187	190	193
Trait	Parent	P2	K/ P2	K	K/ P2	K	K/ P2	K	P2	K/ P2	P2	K/ P2	P2	K/ P2	P2
	ST/LG	I	I	I	I	I	I	II	II	II	II	II	II	III	III
Crisp colour	a	ns	ns	ns	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	b	ns	ns	**0.027↓**	ns	**0.013↓**	ns	ns	ns	ns	ns	ns	ns	**0.046↑**	ns
	c	ns	ns	**0.009↓**	**0.028↑**	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	d	ns	ns	**0.016↓**	ns	Ns	ns	ns	ns	ns	ns	**0.007↓**	ns	**0.046↑**	ns
	e	ns	ns	**0.032↓**	**0.006↑**	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Glucose	a	**0.041↓**	ns	ns	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	b	ns	ns	**0.033↑**	ns	**0.034↑**	ns	ns	ns	ns	ns	**0.039↑**	ns	ns	ns
	c	ns	ns	**0.009↑**	ns	Ns	**0.001↓**	ns	ns	ns	ns	ns	ns	ns	ns
	d	ns	ns	**0.030↑**	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	e	ns	**0.034↓**	**0.046↑**	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	**0.016↑**
Fructose	a	ns	ns	ns	ns	Ns	ns	ns	ns	ns	ns	ns	**0.037↓**	ns	**0.047↑**
	b	ns	ns	**0.019↑**	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	c	ns	ns	**<0.001↑**	**0.041↓**	Ns	**0.024↓**	ns	ns	ns	ns	ns	ns	ns	ns
	d	ns	ns	**0.012↑**	ns	Ns	ns	ns	ns	ns	ns	**0.039↑**	ns	ns	ns
	e	ns	ns	**0.027↑**	ns	**0.046↑**	**0.041↓**	ns	**0.025↑**	ns↑	ns	ns	ns	ns	ns
Sucrose	a	ns	ns	**<0.001↑**	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	b	ns	ns	ns	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	c	ns	ns	**0.041↑**	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	d	ns	ns	ns	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	e	ns	ns	ns	ns	Ns	ns	**0.004↑**	ns	ns	ns	ns	ns	ns	ns
Dry matter	a	ns	ns	ns	**0.040↑**	**0.034↓**	ns	ns	ns	**0.047↑**	**0.029↓**	ns	ns	ns	ns
	b	ns	ns	ns	ns	**0.005↓**	ns	ns	ns	**0.003↑**	ns	ns	ns	ns	ns
	c	ns	ns	ns	**0.016↑**	**0.019↓**	ns	ns	ns	ns	**0.047↓**	ns	ns	ns	**0.019↓**
	d	ns	ns	ns	**0.004↑**	Ns	ns	ns	ns	**0.005↑**	ns	ns	ns	ns	ns
	e	ns	ns	ns	**0.036↑**	**0.008↓**	**0.016↑**	**0.040↑**	ns	**<0.001↑**	ns	ns	ns	ns	**<0.001↓**

Table 3.4:- continue…..

Trait	Marker	Sti060			STM3023	Sti012				Sti032			
	Allele	182	185	194	196	192	195	201	208	138	145	148	151
	Parent	P2	K	K/ P2	P2	K/ P2	K/ P2	P2	K	K	K	K/ P2	K/ P2
	↓ ST/LG →	III	III	III	IV	IV	IV	IV	IV	V	V	V	V
Crisp colour	a	ns	ns	ns	ns	ns	ns	ns	ns	**<0.001↓**	**0.011↑**	**0.048↑**	ns
	b	**0.028↑**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	c	ns	ns	ns	ns	ns	ns	ns	**0.029↑**	ns	ns	ns	**0.034↑**
	d	ns	ns	ns	ns	ns	ns	ns	**0.022↑**	ns	ns	ns	ns
	e	ns	ns	**0.046↓**	ns	ns	ns	ns	ns	ns	ns	ns	**0.007↑**
Glucose	a	ns	ns	ns	ns	ns	ns	ns	ns	**0.030↑**	ns	ns	ns
	b	ns	**0.016↓**	ns	ns	ns	ns	**0.030↑**	ns	ns	ns	ns	ns
	c	ns	ns	ns	**0.008↓**	ns	ns	**0.023↑**	ns	ns	ns	ns	ns
	d	ns	**0.011↓**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	e	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Fructose	a	ns	ns	ns	**0.033↓**	ns	ns	ns	ns	ns	ns	ns	ns
	b	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	c	**0.038↓**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	d	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	e	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**0.034↓**
Sucrose	a	ns	ns	ns	ns	ns	ns	ns	**0.035↓**	**0.008↑**	**0.007↓**	ns	ns
	b	ns	ns	ns	**0.037↓**	ns	ns	ns	ns	ns	ns	**<0.001↑**	ns
	c	ns	ns	ns	ns	ns	ns	ns	ns	**0.021↑**	**0.026↓**	ns	ns
	d	ns	ns	ns	ns	**0.044↑**	ns	ns	ns	**0.043↑**	**0.005↓**	ns	ns
	e	ns	ns	ns	**0.046↓**	ns	ns	ns	ns	ns	**<0.001↓**	ns	ns
Dry matter	a	ns	ns	ns	**0.040↑**	ns	ns	ns	ns	ns	ns	ns	ns
	b	ns	ns	ns	**0.031↑**	ns	ns	**0.010↓**	ns	**0.019↑**	**0.013↓**	ns	ns
	c	ns	ns	ns	**0.023↑**	ns	ns	**0.005↓**	ns	**0.024↑**	**0.031↓**	ns	ns
	d	ns	ns	ns	ns	ns	ns	ns	ns	ns	**0.010↓**	ns	ns
	e	ns	ns	ns	**0.009↑**	ns	ns	ns	ns	**0.049↑**	ns	ns	ns

Table 3.4:- continue…..

	Marker	SSR5148					Sti021			Sti008	SSR3009		PM198
	Allele	439	451	468	473	493	122	125	132	175	150	164	195
Trait	Parent	P2	K	K	K/ P2	K/ P2	P2	K	P2	K	P2	K/ P2	K
	↓ST/LG→	V	V	V	V	V	VI	VI	VI	VII	VII	VII	VIII
Crisp colour	a	ns	**0.004↓**	**0.030↑**	Ns	ns	ns	ns	**0.018↑**	ns	ns	**0.036↓**	ns
	b	**0.022↑**	ns	ns	Ns	ns	ns	ns	**0.015↑**	ns	**0.013↑**	**<0.001↓**	ns
	c	**0.015↑**	ns	ns	Ns	ns	ns	ns	ns	ns	ns	**0.015↓**	ns
	d	ns	ns	ns	Ns	ns	ns	ns	ns	ns	ns	**<0.001↓**	ns
	e	ns	ns	ns	Ns	**0.045↓**	ns	ns	ns	ns	ns	**0.005↓**	**0.024↑**
Glucose	a	ns	**0.029↑**	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns
	b	ns	ns	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns
	c	ns	ns	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns
	d	ns	ns	ns	Ns	ns	ns	ns	ns	ns	ns	**0.043↑**	**0.026↓**
	e	ns	ns	ns	Ns	ns	ns	**0.040↓**	ns	ns	ns	ns	ns
Fructose	a	ns	ns	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns
	b	**0.007↓**	ns	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns
	c	ns	ns	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns
	d	**0.012↓**	ns	ns	Ns	ns	ns	ns	ns	ns	ns	**0.047↑**	ns
	e	ns	ns	ns	Ns	ns	ns	**0.019↓**	ns	ns	ns	ns	ns
Sucrose	a	ns	**0.004↑**	ns	Ns	ns	ns	ns	**0.032↓**	**0.016↓**	ns	ns	ns
	b	ns	**<0.001↑**	**0.032↓**	Ns	ns	ns	ns	ns	ns	ns	ns	ns
	c	ns	ns	ns	**0.005↓**	ns	ns	ns	ns	ns	ns	ns	ns
	d	ns	**0.004↑**	ns	Ns	ns	ns	**0.033↓**	ns	**0.014↓**	ns	ns	ns
	e	ns	ns	ns	Ns	ns	ns	ns	ns	**0.015↓**	ns	ns	ns
Dry matter	a	ns	**0.045↑**	ns	Ns	ns	**0.012↑**	ns	ns	ns	ns	ns	**0.044↑**
	b	ns	**0.033↑**	**0.007↓**	Ns	ns	ns	ns	ns	ns	ns	ns	ns
	c	**0.040↑**	ns	ns	Ns	ns	**0.014↑**	ns	**0.046↓**	ns	ns	ns	ns
	d	ns	**0.031↑**	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns
	e	ns	ns	ns	Ns	ns	**0.049↑**	ns	ns	ns	ns	ns	**0.030↑**

Table 3.4:- continue…..

	Marker	*STWaxy2*			St2663		STM1051				STM1106	
	Allele	219	226	233	190	192	183	190	225	250	150	156
Trait	Parent	K	K	P2	K	K/ P2	K	P2	K/ P2	K/ P2	P2	K/ P2
	↓ ST/LG →	VIII	VIII	VIII	IX	IX	IX	IX	IX	IX	X	X
Crisp colour	a	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	b	**0.042↑**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	c	**0.022↑**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	d	**0.005↑**	ns	ns	**0.021↑**	ns	ns	ns	ns	ns	ns	ns
	e	**0.029↑**	ns	ns	**0.015↑**	ns	ns	ns	ns	ns	ns	**0.035↓**
Glucose	a	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	b	ns	ns	ns	**0.017↓**	ns	ns	ns	ns	ns	ns	ns
	c	**0.023↓**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	d	**0.008↓**	ns	ns	**0.005↓**	ns	ns	ns	ns	ns	ns	ns
	e	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**0.048↑**
Fructose	a	**0.008↓**	ns	ns	ns	ns	ns	ns	**0.041↓**	ns	ns	ns
	b	ns	ns	ns	**0.011↓**	ns	ns	ns	ns	ns	ns	ns
	c	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	d	**0.048↓**	ns	ns	**0.005↓**	ns	ns	ns	ns	ns	ns	ns
	e	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Sucrose	a	ns	**0.008↓**	ns	ns	ns	ns	**0.039↑**	ns	ns	**0.028↑**	ns
	b	ns	ns	**0.028↓**	ns	ns	**0.041↓**	ns	ns	ns	ns	ns
	c	ns	**<0.001↓**	ns	ns	ns	**0.008↓**	ns	ns	ns	ns	ns
	d	ns	ns	ns	**0.013↓**	**0.007↑**	ns	ns	ns	ns	ns	ns
	e	ns	ns	ns	ns	ns	ns	**0.026↑**	ns	**0.005↓**	ns	**0.037↑**
Dry matter	a	ns	ns	**0.010↓**	ns	ns	ns	ns	ns	ns	ns	ns
	b	ns	ns	**0.011↓**	ns	ns	ns	ns	ns	**0.041↓**	ns	ns
	c	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	d	**0.043↑**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	e	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table 3.4:- continue…..

	Marker	Sti028				SSR2005			Sti007			*inh2α*
	Allele	197	200	203	217	151	171	183	147	150	157	130
Trait	Parent	P2	K	K	K	K	K/ P2	K	K/ P2	K	P2	K/ P2
	ST/LG	XI	XI	XI	XI	XI	XI	XI	XII	XII	XII	XII
Crisp colour	a	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**<0.001↑**
	b	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	c	ns	ns	ns	**0.017↑**	ns	ns	ns	ns	ns	ns	ns
	d	ns	ns	ns	ns	ns	ns	ns	ns	**0.048↑**	ns	ns
	e	ns	ns	ns	ns	ns	ns	ns	ns	**0.049↑**	ns	ns
Glucose	a	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	b	ns	ns	ns	ns	ns	ns	**0.010↑**	ns	ns	ns	ns
	c	ns	ns	ns	**0.038↓**	ns	ns	ns	ns	ns	ns	**0.037↑**
	d	ns	ns	ns	ns	ns	ns	**0.036↑**	ns	ns	ns	ns
	e	ns	ns	ns	ns	ns	ns	**0.011↑**	ns	ns	**0.029↑**	ns
Fructose	a	**0.006↑**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	b	ns	ns	**0.039↑**	ns	ns	ns	**0.010↑**	ns	ns	ns	ns
	c	ns	ns	ns	**0.041↓**	ns	ns	ns	ns	ns	ns	**0.028↑**
	d	ns	ns	ns	ns	ns	ns	**0.040↑**	ns	ns	ns	ns
	e	ns	ns	ns	ns	ns	ns	**0.036↑**	ns	ns	ns	ns
Sucrose	a	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	b	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	c	ns	ns	ns	**0.010↓**	ns	ns	ns	**0.047↑**	**0.033↓**	ns	**0.033↑**
	d	ns	ns	ns	ns	**0.043↑**	ns	ns	ns	ns	**0.041↑**	ns
	e	ns	**0.020↑**	ns	ns	ns	**0.035↓**	ns	ns	ns	**0.028↑**	ns
Dry matter	a	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	b	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	c	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**0.019↓**
	d	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	e	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**0.008↓**

Note: - K & P2 denote Karaka and 1021/1 respectively. ns- not significant. Arrows indicate the direction of the effect of the marker allele on a trait based on mean scores in the population. The numbers in the table represent the F-values obtained from AVOVA. Upward (↑) and downward (↓) arrows explain mean scores of trait as increasing and decreasing respectively. ST – Storage Treatment and LG - Linkage group.

3.4 Discussion

This study was undertaken to investigate the genetic basis of cold-induced sweetening in potato tubers using 24 SSRs (Table 3.1) in a tetraploid mapping population segregating for pigmentation, physiological and cold-induced sweetening traits. Further studies were aimed to associate the polymorphisms observed in SSRs with the phenotypic traits. Five SSR markers involved candidate genes operating in carbohydrate metabolism, namely soluble acid invertase (Sti060 *Pain-1* - LG III), granule bound starch synthase (*STWaxy2* - LG VIII), apoplastic isoforms of acid invertase (STM1106 Inv_{ap}-*a* and STM1051 Inv_{ap}-*b* - LGs X and IX respectively) and vacuolar invertase inhibitor (*inh2*α - LG XII) (Table 3.1, Figure 3.1). Figure 3.1 represents the candidate genes studied in the present investigation. All microsatellites were single locus markers and already mapped on potato chromosomes. Associations of these markers with various pigmentation, physiological and CIS components have not been demonstrated in previous studies except for markers Sti060 (LG III), STM1051 (LG IX) and STM1106 (LG X). Figure 3.2 shows the approximate positions of the 24 microsatellites studied in the present study and aligned with previously mapped traits such as pigmentation (Jacobs et al. 1995), QTLs for starch content (Chen et al. 2001) and sugar content (Menéndez et al. 2002).

Microsatellite polymorphisms

The mapping population which segregated for various phenotypic traits was screened with 24 microsatellites markers. All 24 markers produced expected size fragments and all of them were polymorphic in the progeny (Table 3.2). However, 18 progeny lines were considered as non-hybrids and eliminated from the analysis. This was presumably either due to pollination errors, maternal seed, or seed contamination. The three monomorphic markers (Sti031, PM027 & *inh2*α, Table 3.2) tested showed no polymorphism between the parents, however segregated in the progeny. The monomorphic markers do not display sequence diversity in the microsatellite repeat and therefore may not be useful in association studies. The monomorphic DNA fragments may contain considerable variation at the level of single nucleotide polymorphisms (SNPs). Therefore sequencing and identification of SNPs could be helpful in the determination of usefulness of these markers. Out of 84 marker alleles, 49 polymorphic alleles were identified. Two markers (*STWaxy2* - LG VIII & Sti028 - LG XI, Table 3.2) were highly polymorphic. Such marker polymorphism will be an important criterion for

selecting the genetic markers underlying the CIS trait in potato and may be useful in diversity analysis and association based studies. SSRs have been shown to be highly polymorphic in potato (Milbourne et al. 1997, 1998; Ghislain et al. 2004). Both candidate and non-candidate gene markers showed polymorphism and identified several parental specific alleles at microsatellite loci (Table 3.2). Such SSR loci might link to genes or chromosomal regions of interest and therefore those specific to 1021/1 may be useful in predicting CIS resistance and therefore be used in marker-assisted selection. The marker alleles (allele 176 - STM3023 LG IV, allele 163 - Sti008 LG VII, allele 186 - PM198 LG 186 and allele 139 *inh2*α LG XII) that do not segregate for 1021/1 may not be useful in marker-trait associations and therefore they may not have any effect on the CIS trait.

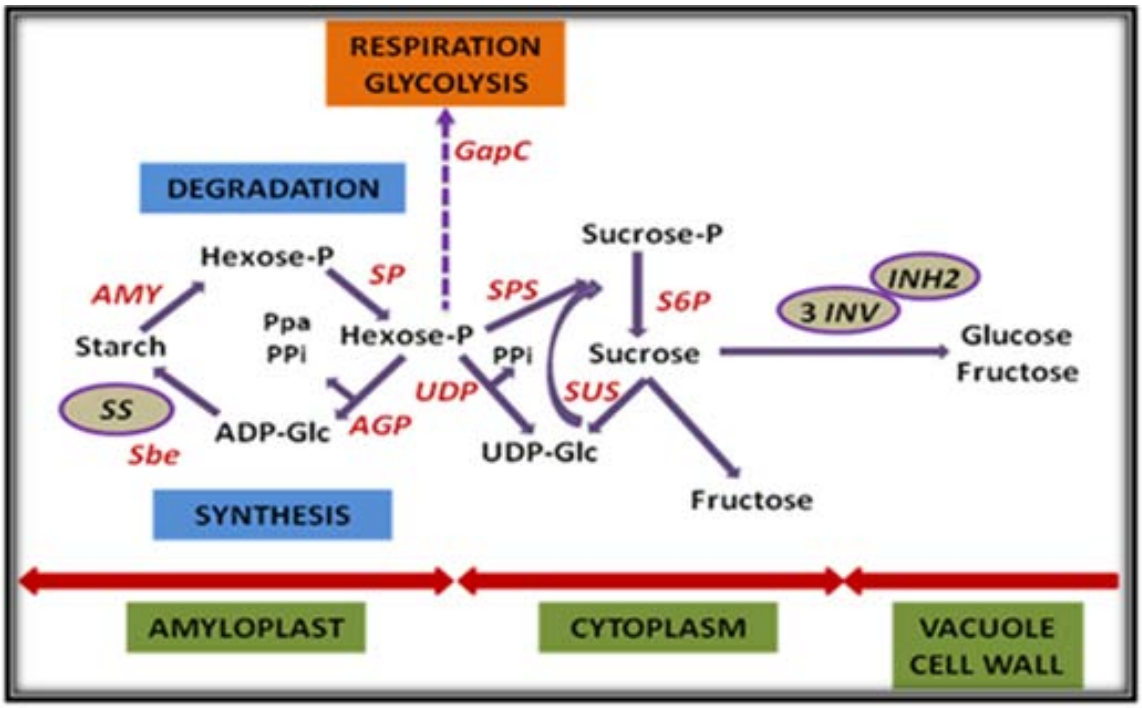


Figure 3.1 Diagrammatic representation of the candidate genes involved in the cold-induced sweetening process of potato tubers. The figure shows the simplified version of the candidate genes involved in CIS of potato tubers. The candidate genes tested in this research are *SS* - starch synthase, *INV* - invertase (one vacuolar & two apoplastic) and *inh2* - invertase inhibitor (vacuolar) are circled. Other enzymes involved in CIS process are *Sbe* - starch branching enzyme, *AMY* - β-amylase, *SP* - starch phosphorylase, *UDP* - UDP-glucose pyrophosphorylase, *AGP* - ADP-glucose pyrophosphorylase, *Ppa* – pyrophosphatase, *GapC* - glyceraldehydes 3 phosphate dehydrogenase, *S6P* - sucrose 6-P phosphatase, *SUS* - sucrose synthase and *SPS* - sucrose phosphate synthase. Adapted from Menéndez et al. (2002).

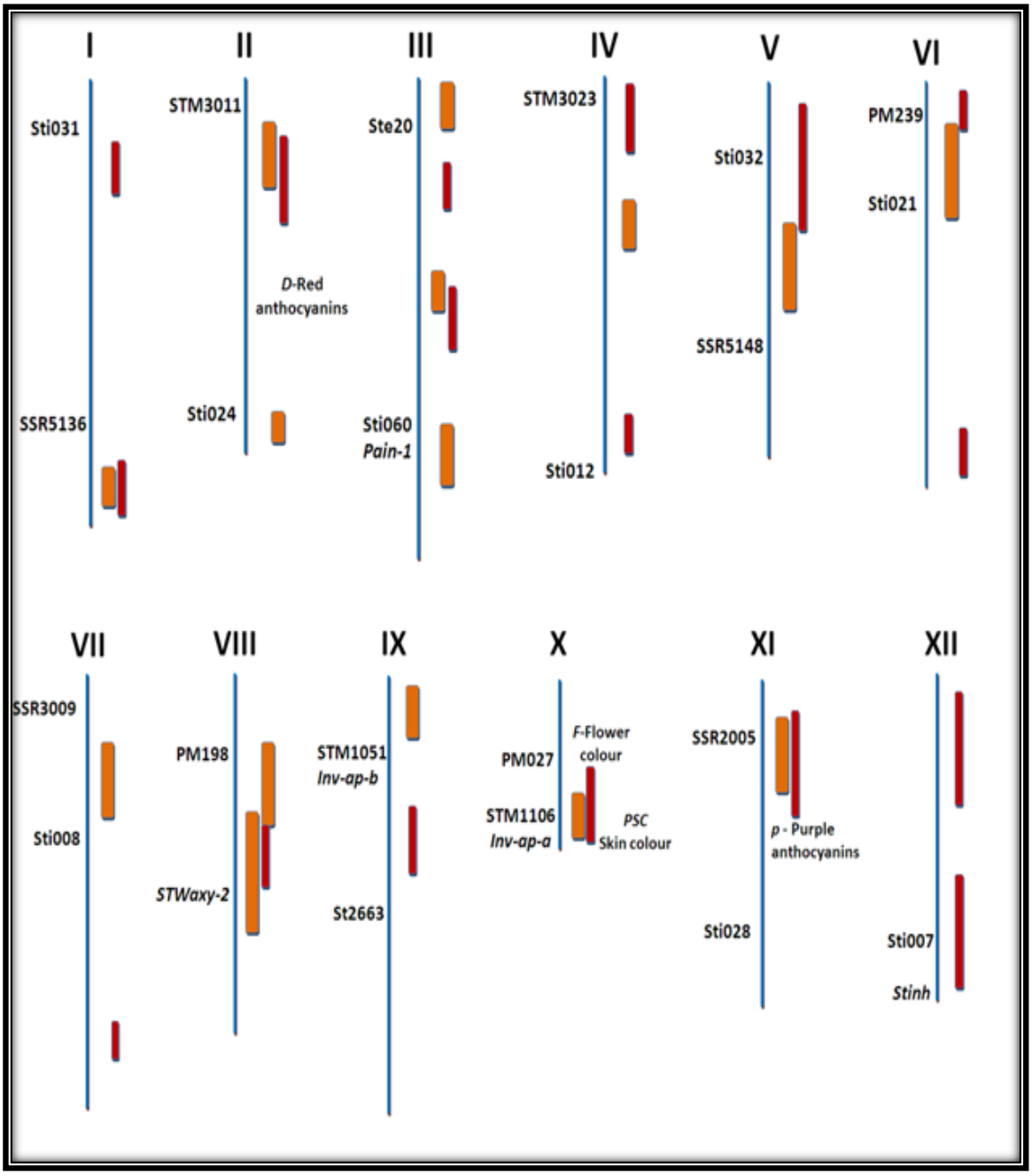


Figure 3.2 Simplified version of the twelve potato chromosomes showing molecular markers associated with pigmentation and tuber quality traits. The approximate positions of the 24 microsatellite markers analysed in the present studies are positioned on left side and the approximate positions of the QTLs for starch (Chen et al. 2001), QTLs for sugar content (Menéndez et al. 2002) and pigmentation traits (Jacobs et al. 1995) are positioned on the right side of the linkage groups. QTL for starch and sugar are shown as red and orange bars respectively. Pigmentation traits are in italics. Microsatellite positions are inferred from Milbourne et al. (1998), Feingold et al. (2005), Ghislain et al. (2004), *www.potatogenome.net* and http://solgenomics.net/.

Segregation ratios and Chi square statistics

In segregation analysis, 62 alleles out of 80 showed a good fit to the expected segregation ratios (1:1, 3:1, 5:1, 11:1 and 35:1, $P < 0.05$), while the remaining 18 marker alleles showed distorted segregation ($P < 0.05$) from the expected ratios (Table 3.2). Highly distorted segregation were evident from Sti024 (LG II), *STWaxy2* (LG VIII) and Sti007 (LG XII). Distorted segregation resulted from a single parent or from both parents. Previous results in diploid potato (Bonierbale et al. 1988 and Gebhardt et al. 1989, 1991; Jacobs et al. 1995) also showed that it is not unusual to find segregation distortion in potato and it was observed on several regions of the genome. Segregation distortion was presumably due to unidentified (sub) lethal loci (Jacobs et al. 1995) which may be due to the high genetic load and marked inbreeding depression in potato (Howard 1970). Segregating populations derived from crosses between divergent parents can result in segregation distortion caused by a number of physiological or nuclear genetic factors (Matsushita et al. 2003; Liu et al. 2008).

Association between microsatellites and pigmentation & physiological traits

Several significant marker-trait associations ($P < 0.05$) were found between DNA variants at candidate genes as well non-candidate gene loci with pigmentation and physiological traits (Table 3.3). Significant associations ($P < 0.05$) were found with the two physiological traits flowering time and emergence in field (Table 3.3). Two shared marker alleles, STM1051 (LG IX) and *inh2*α (LG XII) showed a negative effect ($\downarrow$) on emergence in the field which indicate that in the presence of these marker alleles, emergence may be inhibited or delayed. Likewise, both SSR5136 & Sti031 (LG I) showed a significant association ($P < 0.05$) with flowering time (Table 3.3). From these results it can be hypothesised that there will other genes linked to these two markers that will be responsible for these traits. Further invesitgations needed to explore these findings.

Highly significant associations ($P < 0.001$) were recorded for hypocotyl (LG XI), stem (LGs X & XI), flower (LG X) and tuber skin colour (LGs X & XI). The markers (Table 3.3) that showed highly significant associations ($P < 0.001$) may be close to the candidate genes that co-localised with the pigmentation loci (Figure 3.2). These colour characteristics have been found to be controlled by specific genes and previously mapped on potato chromosomes (Figure 3.2). Flower colour in diploid potato, was found to be controlled by three unlinked loci *D*, *F* and *P* (van Eck et al. 1993). The *P* locus is

required for the production of blue anthocyanins is mapped on chromosome XI; locus *F* is involved in the flower-specific expression of gene(s) is mapped on chromosome X, and locus *D* is involved in the biosynthesis of red anthocyanins is mapped on chromosome II. For tuber skin colour loci *R*, *E*, *I* and *PSC* have been mapped on LG X (van Eck et al. 1994b), whereas the inheritance of Loci *E* and *R* explain the two different tuber skin phenotypes in tetraploids (van Eck et al. 1994b). In addition, this study has identifed several other significant associations ($P < 0.05$) with these pigmentations traits on various LGs (Table 3.3).

The association of the scored pigmentation traits with SSR markers segregating at loci linked to known loci involved in pigmentation provides a validation of the marker analysis approach used in this study. This provides confidence in the other associations of SSR markers loci with CIS traits.

Association between microsatellites & phenotypic components of CIS

Five phenotypic components of CIS (crisp colour, glucose, fructose, sucrose and dry matter content) were evaluated in a mapping population genotyped with 24 microsatellite markers (Table 3.1). Several significant ($P < 0.05$) marker-trait associations were identified between DNA variants at candidate and non-candidate (SSR) loci and crisp quality, glucose, fructose, sucrose and dry matter contents at various storage treatments, along with the influence of the presence of marker alleles on the traits (Table 3.4). The approximate positions of microsatellites, QTLs for tuber starch and sugar are shown in Figure 3.2.

Crisp colour is the most important processing quality trait which is predominantly determined by glucose and fructose contents in tuber tissue. Processing potatoes required high dry matter and low glucose and fructose contents (Watada and Kunkel 1955; Iritani and Weller 1976a, b; McCann et al, 2010). In total 16 markers showed significant ($P < 0.05$) with crisp colour. Marker alleles originating from 1021/1 (Sti060 allele 182, LG III, SSR5148 allele 439, LG V, Sti021 allele 132, LG VI and SSR3009 allele 150, LG VII), showed significant ($P < 0.05$) and positive effects on crisp colour (cold storage treatment b) (Table 3.4) . Therefore such markers are candidates for use in marker assisted selection would be helpful in selecting genotypes with good crisping qualities. Notably SSR3009 shared allele 164 (LG VII) had a negative influence (↓) on crisp quality at all the storage treatments evaluated (Table 3.4) which indicates

that the presence of the 1021/1 specific allele (150) is important in determination of crisp colour especially after cold storage (Table 3.4). Likewise, in the presence of Sti021 allele 122 (LG VI), dry matter content was increased especially following reconditioning periods (storage treatments c & e, Table 3.4). Marker STM3023 allele 196, specific to 1021/1, showed significant association and increased dry matter content at storage treatments a to c and e (Table 3.4) and therefore, such markers are candidates for marker assisted programmes for the selection of high dry matter content. Dry matter content of potatoes is of key importance for chip making and therefore high dry matter content and low reducing sugars will be helpful in selecting potatoes for processing purposes.

A number of marker alleles (SSR5136 allele 252 LG I, Sti012 allele 201 IV, Sti032 alleles 138 & 145 LG V and SSR5148 allele 451 LG V) showed significant associations with different CIS traits at the same storage treatment (Table 3.4). These results suggest that these markers may be close to the candidate gene locus and therefore all these traits may be controlled by the same set of genes.

Results obtained by marker SSR5136 (LG-I) were noteworthy (Table 3.4) as this locus showed the best example of pleiotropic effects of individual alleles, either positive (more dry matter, better crisp colour, less glucose & fructose) or negative (less dry matter, worse crisp colour, more glucose & fructose). In addition it showed the most significant and robust associations ($P < 0.05$, $P < 0.001$) with all the traits investigated. For example in the presence of Karaka specific allele 252, crisp colour decreased, with increase in both glucose and fructose at all the storage treatments except at storage treatment a (Table 3.4). Similar negative effects were also noted in the presence of SSR5136 Karaka specific allele 272 on dry matter content at all storage treatments (except at storage treatment d). ADP-glucose pyrophosphorylase S (*AGPaseS*) (Müller-Röber et al. 1992; Preiss 1982) synthesises starch in potato tubers and is mapped on chromosome I (Chen et al. 2001). It is possible that the SSR5136 marker is may be physically close to this candidate gene locus that also co-localises with a QTL for starch (Figure 3.2). Therefore, the allelic differences of *AGPaseS*) may control the CIS phenotypic variations observed in the mapping population.

Candidate genes involved in carbohydrate metabolism with known map positions were examined for genetic associations with cold-induced sweetening and the results

agree with previous studies (Menéndez et al. 2002; Li et al. 2005; Li et al. 2008; Baldwin et al. 2011). One of the important candidate genes *waxy*, encoding granule bound starch synthase and mapped on potato chromosome VIII (Veilleux et al. 1995), is involved in starch synthesis. In the present studies, marker *STWaxy2* (Figure 3.4) showed significant associations with all the traits investigated (Table 3.4). The association of *STWaxy2* allele 219 from Karaka with crisp colour at four storage treatments (b, c, d & e) clearly indicates that the presence of this allele produced better crisp colour. The ambiguity why Karaka specific marker alleles (For example Sti012 LG IV & *STWaxy2* LG VIII) showed improved trait remains to be elucidated. This observed phenotypic effect could be due to the other candidate genes that might link these loci. Presence of a 1021/1 specific allele (*STWaxy2* allele 233) showed decreased sucrose & dry matter content at storage treatment b (Table 3.4). Therefore these effects on traits may be due to the allelic variants of *waxy* gene and explain the co-localised with QTLs for starch (Chen et al. 2001) and *Sug8a* and *Sug8b* (LG VIII) (Menéndez et al. 2002) (Figure 3.2).

Invertase is an important functional candidate gene involved in CIS which converts sucrose into hexoses i.e. glucose and fructose. Chen et al. (2001) identified three loci of cloned invertase genes on chromosomes III, IX and X, which all together identify five invertase genes in potato (Draffehn et al. 2010). Two of which are cell wall bound isoforms of acid invertase (Hedley et al. 1993, 1994), Inv_{ap}-*a* and Inv_{ap}-*b* located on chromosomes X and IX respectively, whereas the locus on chromosome III encodes a putative intra-cellular, soluble acid invertase (Zhou et al. 1994; Zrenner et al. 1996). Li et al. (2005) identified that the tandemly duplicated genes, *invGE/GF* can be assigned to locus Inv_{ap}-*b* on chromosome IX. Draffehn et al. (2010) identified *InvCD141* and *InvCD111* at Inv_{ap}-*a* locus on chromosome X. In addition to tuber starch content, both Inv_{ap}-*a* and Inv_{ap}-*b* have been shown to be associated with chip quality after harvest and cold storage (Draffehn et al. 2010; Li et al. 2005, 2008). In the present investigation STM1051 (Inv_{ap}-*b*, LG IX) identified four alleles (Table 3.4). Karaka specific allele (STM1051 allele 183) showed significant ($P < 0.05$) and negative association with sucrose (at storage treatments b & c), while 1021/1 specific allele (STM1051 allele 190) showed significant ($P < 0.05$) and positive association with sucrose (at storage treatments a & e) (Table 3.4). In addition the effect of the shared allele (STM1051 allele 250) showed significant ($P < 0.05$) and negative effect on sucrose (storage treatment e) and dry

matter content (storage treatment b) (Table 3.4). These results therefore indicate that the DNA variants of the invertase locus are responsible for these effects. Notably shared allele 156 from STM1106 (Inv_{ap}-*a*, LG X) also exhibited significant ($P < 0.05$) association with crisp colour, fructose and sucrose at storage treatment e. (Table 3.4). Therefore these two gene markers (STM1051 and STM1106) representing variation in invertase genes could explain the co-localised QTLs *Sug9a* (LG IX) and *Sug10a* (LG X) identified by Menéndez et al. (2002). Draffehn et al. (2010) identified SNPs in Inv_{ap}-*a* and Inv_{ap}-*b* from different potato genotypes and showed that the structural variations observed in different alleles might influence chip quality, tuber starch content and starch yield.

Menéndez et al. (2002) observed QTL *Sug3a* on chromosome III (Figure 3.2) and suggested that *Pain-1* (soluble acid invertase) might be a candidate gene for this QTL. Candidate gene invertase (Sti060, *Pain-1* LG III) showed significant association ($P < 0.05$) with crisp colour, glucose and fructose (Table 3.4). Furthermore, genotype specific alleles were found to play an important role. For example, 1021/1 specific allele Sti060 allele 182 showed a positive (↑) effect on crisp colour (storage treatment b) and Karaka specific allele Sti060 allele 194 showed a negative (↓) effect on glucose (storage treatments b & d). Therefore DNA variants of this candidate gene marker might play an important role in CIS. Allelic variants of *Pain-1* showed most significant and positive effects on chip quality after cold storage in tetraploid mapping populations (Li et al. 2008; Draffehn et al. 2010). Studies on CIS in potatoes indicate that increase in acid invertase activity is associated with an increase in reducing sugars (RS) contents (Matsuura-Endo et al. 2004). More specifically transgenic plants expressing vacuolar invertase gene in an antisense orientation resulted in a decrease in acid invertase activity and as consequence transgenic potato plants produced low RS content (Bhaskar et al. 2010). These results clearly indicate that vacuolar invertase is an important enzyme involved in regulating the CIS in potato (Bhaskar et al. 2010; Liu et al. 2011). These results were of special significance as 1021/1 specific allele showed a positive effect on crisp colour. Therefore sequencing of this candidate gene would be useful in determination of the SNPs responsible for observed phenotypic variation.

inh2α which is a vacuolar invertase inhibitor gene (Baldwin et al. 2011; Brummell et al. 2011) mapped on chromosome XII (Table 3.1) is known to play an important role in cold induced sweetening in potato tubers (Greiner et al. 1999;

Brummell et al. 2011). In total, two shared alleles (130 and 139) are detected from each parent, with only one (130) found to be segregating in the population. This segregating allele (130) showed significant association ($P < 0.05$) and negative influence on crisp colour (storage treatment a) and glucose, fructose and sucrose at time point e (Table 3.4). Also dry matter content was significantly and negatively influenced at both the reconditioning periods (c & e). As the allele 130 is shared by both parents, it would be difficult to determine whether the observed effect is due to 1021/1 specific allele. In this case therefore *inh2α* could be sequenced from both the parents in order to determine whether there are any SNPs in this allele which are responsible for this association. QTL *Sug12a* was mapped on chromosome XII (Figure 3.2) by Menéndez et al. (2002). Therefore it cannot be ruled out that the alleles of this gene are physically close to this QTL locus which is controlling cold-sweetening QTL *Sug12a* (Figure 3.2) or that the *inh2α* gene is underpinning this QTL. Similarly the apoplastic invertase inhibitor gene may show significant associations with CIS traits. Brummell et al. (2011) showed that both apoplastic and vacuolar invertase inhibitors are located on chromosome XII in close proximity in a tandem orientation, with the vacuolar invertase inhibitor upstream of the apoplastic invertase inhibitor. These two invertase inhibitors are separated by 5.5 kb without any intervening genes and therefore it cannot be ruled out that the apoplastic invertase inhibitor can also be physically close to or underpinning the locus which is controlling cold-sweetening QTL *Sug12a* on LG XII (Figure 3.2).

In case of SSRs which are not in candidate genes, the sequencing of genomic regions and detection of any potential SNPs could be useful in the identification of genomic regions that are associated with the CIS traits.

Concluding remarks

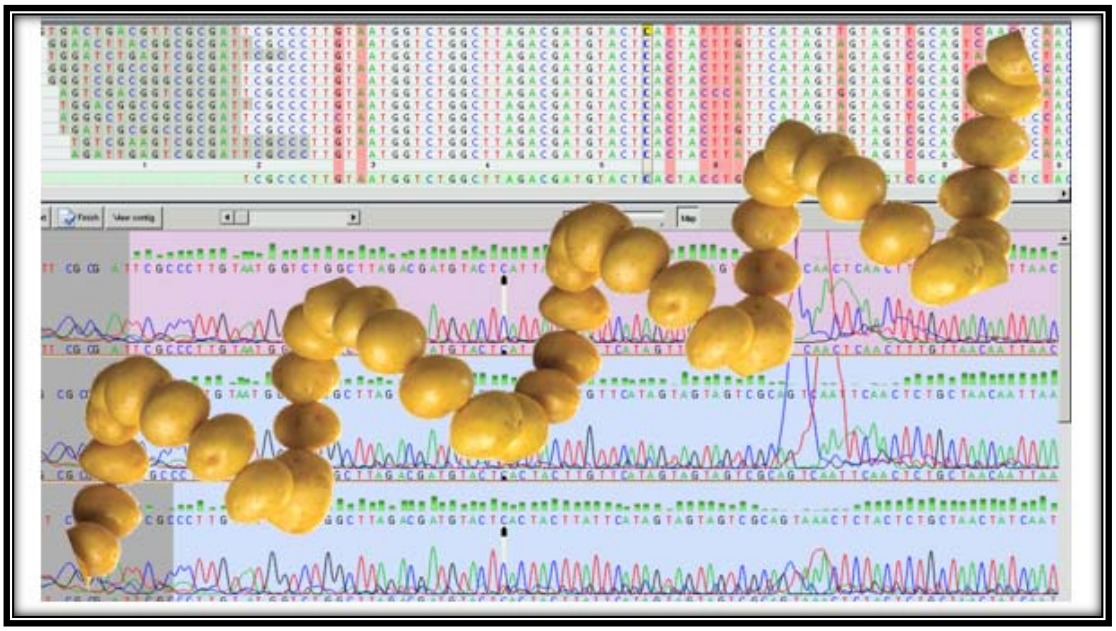
Variations in pigmentation, physiological and phenotypic components of CIS have been studied extensively in the present study, but to what extent these traits are genetically linked and/or result in phenotypic casual variations are unknown. Therefore these results demonstrating significant marker-trait associations can be further utilised in association mapping and QTL studies. Further studies on QTL mapping by analysing more number of markers or the candidate gene markers would improve the understanding of underlying genetics, physiology and biochemistry of CIS in potato tubers.

The marker alleles (Sti060 LG III, SSR514 LG V, Sti021 LG VI and SSR3009 LG VII, Table 3.4) that showed a positive effect (↑) on crisp colour and dry matter content can be used for positive selections, especially those specific to 1021/1 and therefore useful for screening CIS resistant genotypes. Transgressive segregation that was observed for crisp colour, sugars and dry matter content (Figure 2.7) can be attributed to the combined effect of marker alleles shared by both 1021/1 and Karaka (for example positive effect on crisp quality - SSR5136 LG I, Ste20 LG III, Sti032 LGV and *inh2*α LG XII, Table 3.4) or (for example negative effect on crisp quality - Sti024 LG II, SSR 3009 LG VII, STM1106 LG X). The significance of these markers needs to be subjected to validation or functional analysis by testing in other populations. The genomic regions defined by such SSR loci may be important for CIS tolerance and the SSR may be a potential target for manipulation by marker-assisted selection.

The natural variation of candidate gene alleles operating in carbohydrate metabolism have been shown to be associated with various CIS traits (Menéndez et al. 2002; Li et al. 2008; Baldwin et al. 2011). Vacuolar invertase inhibitor is one of the most important candidate genes controls the CIS in potato tubers (Greiner et al. 1999; Cheng et al. 2007; Brummell et al. 2011). In addition the present studies revealed that the candidate *inh2*α also showed significant associations with CIS traits (Table 3.4). Recently Baldwin et al. (2011) detected an interaction between specific alleles of STM1051 allele 181 and $StInh_{ap}$ allele *a* (apoplastic invertase inhibitor) and concluded that the lines those had STM1051-181 were cold tolerant if they also had $StInh_{ap}$-*a*. Invertases are known to be regulated by invertase inhibitors at posttranslational level through protein-protein interactions (Rausch and Greiner 2004). However the information on allelic variations of the apoplastic invertase inhibitor in regulation of CIS is very scanty. Therefore apoplastic invertase inhibitor is selected as a functional candidate to investigate the allelic variations of this gene from parents of the mapping population (Figure 2.1) and to elucidate its role in control of CIS in potato tubers.

Chapter 4

Allele diversity for apoplastic invertase inhibitor gene in potato



Chapter 4

Allele diversity for the apoplastic invertase inhibitor gene in potato

4.1 Introduction

Invertase inhibitors occur as vacuolar and apoplastic (cell wall) forms and post-translationally regulate invertase activity (Rausch and Greiner 2004; Reca et al. 2008; Liu et al. 2010). In potato, invertase inhibitors have been implicated to play a role in resistance to cold-induced sweetening (CIS), an important quality trait in potato breeding programmes worldwide. During cold storage starch is converted into sucrose which is enzymatically breaks down into hexoses like glucose and fructose that affects the processing quality of fried potato products (Dale and Bradshaw 2003). Many attempts have been made to further understand the biochemical and molecular backgrounds for sucrose breakdown in potato tubers, in order to identify candidate genes. *In vitro* studies conducted by Sander et al. (1996) showed that I_{NT} (apoplastic invertase inhibitor from tobacco) and I_{LE} (vacuolar invertase inhibitor from tomato) both inhibit the apoplastic/cell wall invertase (CWI) and vacuolar invertases (VI). Their experiment showed a much faster complex formation between NtCIF (apoplastic invertase inhibitor from tobacco) and NtCWI (apoplastic invertase from tobacco), compared to NtCIF and VI (vacuolar invertase) from tomato. Further, investigation leads to the conclusion that the mechanism of inactivation is clearly different for CWI and VI. *In vivo* studies have demonstrated that the cold-accumulation of reducing sugars can be reduced by overexpressing vacuolar invertase inhibitor in transgenic potato tubers (Greiner et al. 1999; Cheng et al. 2007). However, the information on *in vivo* studies on inactivation of apoplastic/vacuolar invertase by apoplastic invertase inhibitor is very scanty.

Recently Liu et al. (2010) sequenced and characterised *StInvInh* (*Solanum tuberosum* invertase inhibitor) genes from two potato genotypes. The sequencing analysis revealed four genes namely *StInvInh1*, *StInvInh2A*, *StInvInh2B* and *StInvInh3* and investigated their possible contributions to cold-induced sweetening of potato tubers by studying interactions between invertase and invertase inhibitors. Brummell et al. (2011) sequenced and characterised both apoplastic (*inh1*) and vacuolar invertase inhibitor (*inh*2) genes from cold-susceptible and resistant cultivars. Their results revealed that

during cold storage, the transcripts of invertase inhibitors were accumulated to higher abundance in cultivars resistant to cold-induced sweetening than in susceptible cultivars. Their experiment led to the conclusion that the increased amounts of invertase inhibitor may contribute to the suppression of acid invertase activity and prevent cleavage of sucrose.

CIS is a complex phenomenon and quantitative trait loci (QTLs) to explain this phenotype have been mapped by Menéndez et al. (2002). Their studies revealed that the allelic variants of enzymes operating in carbohydrate metabolism pathways contribute to the variation shown by different cultivars in cold sweetening response. A number of candidate genes operating in potato carbohydrate metabolism have been cloned, characterised, mapped and shown to be associated with cold-induced sweetening (e.g., ADP-glucose pyrophosphorylase, starch synthases, starch branching enzyme, starch phosphorylases, starch debranching enzyme, α-amylase, sucrose phosphate synthase, sucrose synthases, apoplastic and vacuolar invertases, UDP glucose pyrophosphorylase) (Chen et al. 2001; Menéndez et al. 2002; Baldwin et al. 2011). Sowokinos et al. (1997) showed a relationship between allelic polymorphism of UGPase among potato (*Solanum tuberosum*) clones and their ability to accumulate sugars in cold storage. Studies were conducted by Baldwin et al. (2011) on 161 genotypes differing in response to CIS and the potential contributions of allelic variants of the genes operating in carbohydrate metabolism. Significant associations of allelic variants of UDP-glucose pyrophosphorylase, apoplastic invertase gene and allelic interactions between apoplastic invertase & apoplastic invertase inhibitor associated with CIS in potato tubers were demonstrated (Baldwin et al. 2011).

To account for the molecular basis of CIS, it was hypothesised that the allelic variants of the apoplastic invertase inhibitor might play an important role in CIS. To date, little is known about the allelic diversity of the apoplastic invertase inhibitor in potatoes. Therefore, this study investigates the allelic diversity of the apoplastic invertase inhibitor gene from three tetraploid genotypes that differ markedly in their response to cold-induced sweetening: 1021/1 (a genotype with very high resistance to CIS), Karaka and Summer Delight (two cultivars that are highly susceptible to CIS). Gene structure and allelic diversity were investigated in detail to determine the presence of polymorphisms conferring non synonymous substitutions. Sequence differences between alleles are

hypothesised to contribute to the genetic variation in cold-induced sweetening response of the three genotypes, and if confirmed the sequence polymorphism could be exploited to develop markers to facilitate potato breeding.

4.2 Materials and methods

4.2.1 Plant material

1021/1 is an advanced breeding line from the Plant & Food Research potato breeding programme suitable for processing purposes and known to be highly tolerant to cold-induced sweetening. The Plant & Food Research cultivars Summer Delight and Karaka are non-processing cultivars and are susceptible to cold-induced sweetening. For details on the source of plant materials please see section 2.2.1. All three genotypes were grown in a greenhouse at conditions provided included heating below 15°C and ventilation above 21°C.

4.2.2 Genomic DNA isolation

Described in section 3.2.2.

4.2.3 Primer design and PCR amplification

Sequences of potato invertase inhibitor cDNA were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez, two cDNAs accessions AY864819, AY864820, & a genomic sequence GU980592 (Appendix C). The primers were designed where there no differences between the different sequences using Primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi, Rozen and Skaletsky 2000). Based on the supplied sequences the primers were expected to amplify the complete genomic region (approximately 2085 base pairs). The diagrammatic representation is presented into the results section (Figure 4.1). However, it was recognised that allelic variation may result in amplicons with a range of sizes. The primers were used to amplify products from genomic DNA of 1021/1, Karaka and Summer Delight and the assay was performed using apoplastic invertase inhibitor primers ApoInh F_1 (forward primer) ($^{5'}$-CACATTTAGTTCTTAATTTTCCCAA-$^{3'}$) and ApoInh R_1 (reverse primer) ($^{5'}$-AGAAGGGGACAAACATATATTGGA-$^{3'}$). Each 50 µl reaction mix included 1X Triple Master polymerase mix (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50 % glycerol, 0.5% Tween® 20, 0.5% Igepal® CA630) with 1.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, dTTP, 2.5 U high fidelity Triple master polymerase mix (5.0 U μl^{-1}, Eppendorf, Hamburg, Germany), 250 nM of each primer (final concentration), 10-50 ng of genomic DNA (1.0 µl). The PCR was performed on a Mastercycler (Eppendorf, Hamburg, Germany) as follows: two min initial denaturation at 94°C followed by 34 cycles of [15 s at 94°C, 20 s at 59°C, 150 s at 72°C] and a final extension of 10 min at

72°C. Amplified products were separated by electrophoresis in a 0.8% agarose gel [1X TAE buffer (Appendix A) at 100 V (5.0 volts cm^{-1}) for 80 min] and visualised under UV light after staining in an ethidium bromide solution (1 mg l^{-1}) for 30 min.

4.2.4 Cloning PCR fragments

PCR products used for cloning were excised from ethidium bromide-stained agarose gels using a sterile scalpel and extracted using QIAquick gel extraction kit (QIAGEN, Gmbh, Germany) according to manufacturer's instructions. When possible, fragments of different molecular weights were excised separately.

Allelic variants that differed in size from 1021/1, the upper fragment and two lower pooled fragments from Karaka and pooled fragments from Summer Delight were eluted in 30 µl of elution buffer (10mM Tris-Cl, pH 8.5). The approximate size of the purified products was confirmed by electrophoresis on an agarose gel and the quantity of the DNA was estimated using a NanoDrop 1000 spectrophotometer (Thermo Scientific, DE, USA) at 260 nm. Purified PCR products were ligated into pGEM®-T easy vector (Promega, Madison, WI, USA).

4.2.5 Ligation of fragments in pGEM® -T

The pGEM®-T easy vector requires a PCR product with an 'A' overhang for efficient ligation. A-tailing was not required since the high fidelity Triple master polymerase mix already adds adenosine at both ends of the amplicons. Ligation was done using pGEM®-T easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions: a ligation reaction with a total volume of 10 µl contained two µl 5 × Ligation Buffer, 0.5 µl pGEM®-T easy plasmid (50 µg μl^{-1} DNA), 1.0 - 2.3 µl of insert DNA (insert concentration was varied depending upon the concentration of DNA after gel purification, 14 to 68 ng μl^{-1}), 1 µl T_4 DNA ligase (concentration of 3 U μl^{-1}) and 2 µl - 4.5 µl of water. Depending on the size and concentration of the insert, different insert:vector ratios were used. A molar ratio of 2:1 for the fragments from 1021/1, 1:1 for the fragments from Karaka were adopted however; a molar ratio of 3:1 was adopted for the fragments from Summer Delight. The reaction was incubated at 14°C for four hr before transformation into *Escherichia coli*.

4.2.6 *E. coli* transformation & plasmid isolation

Transformation

For each transformation, 50 µl of competent cells of *E. coli* strain DH5α™ (Invitrogen, CA, USA) were thawed on ice, mixed with two µl of ligation mixture and incubated on ice for 30 mins. Cells were heat shocked for 45 s at 42°C in a water bath, then incubated on ice for two min. Three hundred µl of SOC medium (Invitrogen) was added to the cells followed by incubation at 37°C for one hr at 225 rpm on a Thermomixer (Eppendorf, Hamburg, Germany). Transformed cells (200 µl bacteria) were plated on Luria Broth (Appendix C) agar plates. The media contained 100 µg ml^{-1} ampicillin, 0.5 M Isopropyl β-D-1-thiogalactopyranoside IPTG and 80 µg ml^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) for blue-white colony selection. Petri dishes were incubated at 37°C overnight.

Colony Screening

Single white colonies were picked with a sterile tip and dipped into a PCR mix for colony PCR to amplify the target insert of the plasmid using ApoInh F_1 and R_1 primers (section 4.2.3). Each 10 µl PCR reaction included 1.0 µl ThermoPol buffer [10X, 10 mM KCL, 10 mM $(NH_4)_2SO_4$] with 200 µM of each dNTP, 0.08 µl Taq DNA polymerase (5.0 U ml^{-1}, BioLabs, New England), 0.2 µl of each primer (final concentration 0.2 µM). PCR was performed on a Mastercycler (Eppendorf, Hamburg, Germany). The conditions for PCR were as follows: initial denaturation for 5 min at 94°C followed by 34 cycles of [20 s at 94°C, 30 s at 59°C and 130 s at 72°C] and a final extension step of 10 min at 72°C. Amplified products were separated by electrophoresis in 1.0% agarose gel (1X TAE at 130 V (7 volts cm^{-1}) for 20 mins) and visualised under UV light after staining with ethidium bromide (1 mg l^{-1}) for 15 min. In parallel, the single colony was also streaked on an Luria Broth (Appendix A) agar plate containing 100 µg ml^{-1} ampicillin, 0.5 M Isopropyl β-D-1-thiogalactopyranoside IPTG and 80 µg ml^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and incubated at 37°C overnight. The PCR product obtained was confirmed by comparing the size with a molecular weight ladder (Generuler, Fermentas, Ontario, Canada) and then the corresponding colony was cultured and used for plasmid isolation and sequencing.

Plasmid isolation

The single colony was inoculated into two ml liquid Luria Broth media (Appendix A) and grown at 37°C overnight at 1,000 rpm on a Thermomixer (Eppendorf, Hamburg, Germany). The overnight culture of bacteria was pelleted by centrifugation and plasmid DNA was isolated using QuickClean 5 M Miniprep kit (GenScript, NJ, USA) following the manufacturer's protocol. The DNA was eluted in 30 µl of elution buffer (GenScript NJ, USA) and the concentration of DNA was estimated by spectrophotometry at 260 nm on a NanoDrop 1000 spectrophotometer.

4.2.7 DNA Sequencing and data analysis

The ABI PRISM Dye Terminator cycle Sequencing Ready Reaction Kit (Applied Biosystems, Australia and New Zealand) was used for sequencing. In order to sequence the complete genomic region, the sequencing experiment was divided into two steps. In the first step, the cloned fragments were sequenced using M13 forward ($^{5'}$-GTAAAACGACGGCCAG-$^{3'}$) and M13 reverse primers ($^{5'}$-CAGGAAACAGCTATGAC-$^{3'}$). Each 10 µl sequencing reaction contained 0.5 µl 1X Big Dye (Applied Biosystems Pty Ltd, Australia and New Zealand), 1.75 µl 5X Sequencing buffer, 0.16 µl of primer (M13 forward / M13 reverse at 20 µM) and 2-4 µl of plasmid DNA (45-240 ng μl^{-1}) and final volume was made to 10 µl with ddH_2O. The sequence cycling conditions were performed in 96-well plates as follows: an initial denaturing step of 96°C for 1 min then 50 cycles of [96°C for 10 s, 50°C for 5 s and 60°C for four min]. Sequencing reactions were precipitated by adding 2.5 µl 12 mM EDTA (pH 8.0) and 25 µl of 100% ethanol was added separately to each well. The plates were centrifuged at 3,200 × g for 30 min at 4°C. The plates were inverted on tissue and centrifuged again at 185 × g for 30 s and 35 µl of 70% ethanol was added to each well. The plates were centrifuged at 3,000 × g for 15 min at 4°C. Again plates were inverted on tissues and spun at 185 × g for 30 s and dried in a 37°C incubator (in the dark) for 10 min. HiDi (10 µl, Applied Biosystems Pty Ltd, Australia & New Zealand) was added to the samples and DNA were denatured at 95°C for five min and placed on ice for five min. A brief spin (185 × g for 30 s) was given to the plates. Finally septa were added and plates were loaded onto a 3130x*l* Genetic Analyzer (Applied Biosystems, Hitachi). Sixteen clones per band per cultivar were sequenced to increase the probability that all four possible alleles from each tetraploid potato cultivar were sequenced (Simko 2004). Using Staden Gap 4 software (Staden et al 1999),

sequences from each cultivar was aligned with the reference sequences (accessions AY864819, AY864820 & GU980592), to determine the exon-intron boundaries and to identify single nucleotide polymorphisms and indels. The sequences were manually checked and annotated. All the sequences were assembled, aligned, compared with each other and edited in the same contig using Pregap4 and Gap 4 programs of Staden Gap4 (http://staden.sourceforge.net/, Staden et al 1999). Individual sequences were viewed as traces for confidence and quality scores. The aligned sequences were checked for their homology and variations/mutations with the reference sequences (AY864819, AY864820 & GU980592).

In order to obtain the full genomic sequence, a second set of primers used to sequence the intron region. In this step the full genomic sequence was obtained using another set of primers [ApoInh, forward F_2 ($^{5'-}$AAGGTAATGTTATTCGTTTGCGT$^{-3'}$) and reverse R_2 ($^{5'-}$ACCAGAAGAACCAACCATTC$^{-3'}$)] (positions shown in Figures 4.1 & 4.2 in results section) and used to sequence the intron region. The sequencing reaction was repeated as described above. The overlapping sequences together with the intron region were assembled and aligned into a contiguous sequence. The complete genomic sequence was compared and exon/intron junctions were determined by alignment with the reference sequences (AY864819, AY864820, & GU980592) using WISE 2 software (http://www.ebi.ac.uk/Tools/Wise2/index.html). The putative mRNA, protein sequences (to identify amino acid polymorphisms), molecular mass and pI were predicted using Geneious Programme 5.4.6. The prediction of the signal peptide was done using http://www.cbs.dtu.dk/services/SignalP/ (Bendtsen et al. 2004). N-glycosylation sites were predicted using NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/, Gupta et al. 2004). The comparison of predicted protein sequences identified in this study also compared with the previously isolated apoplastic invertase inhibitors from potato (AY864819, GU321338, GU321339, GU321338 & GU321339), tobacco (Y12805), tomato (SGN-U332870) and *P. hybrida* (SGN-U207999). The Phylogenetic tree was constructed based on putative apoplastic invertase inhibitor protein sequences identified in this study with invertase inhibitor protein sequences from potato (AY864819, AY864821, GU321338, GU321339, GU980595, GU980594, GU980593, FJ810205, FJ810206, FJ810207, FJ810208 & FJ810209), tobacco (Y12805, Y12806 & AY145781), tomato (SGN-U317539, SGN-U332870) and *Petunia hybrida* (SGN-U207999).

The tree was constructed in MEGA 5.05 program (Tamura et. al. 2011) by neighbour-joining 1,000 bootstrap analysis.

4.3 Results

4.3.1 PCR cloning and sequencing

The PCR amplified the complete genomic region for the apoplastic invertase inhibitor gene (*inh1*) from potato genotypes 1021/1, Karaka and Summer Delight. Based on the primer positions shown in the Figure 4.1, the apoplastic invertase inhibitor gene specific primers (ApoInh F_1 and ApoInh R_1) generated two bands in 1021/1 and Summer Delight while three were amplified from Karaka (Figure 4.2). The bright major band was of a similar size (~2.1 kb) in all three genotypes. The second, smaller band of approximately 1.8 kb slightly varied in size in all three genotypes. Comparative sequence analysis revealed five alleles for invertase inhibitor namely *inh1-b*, *inh1-c, inh1-d*, *inh1-e* and *inh1-f* from the three genotypes (Figure 4.2).

4.3.2 DNA sequence structure of apoplastic invertase inhibitor alleles

The genomic sequences of five apoplastic invertase inhibitor alleles (*inh1-b* from all three genotypes, *inh1-c* from Summer Delight, *inh1-d* from 1021/1, *inh1-e* and *inh1-f* from Karaka) identified in this study and that of previously sequenced *inh1-a* (AY864819) and *inh1-b* (AY864820 and GU980592) were compared to assess the sequence polymorphism between the alleles and to identify variable regions (Table 4.1, Figure 4.3). In addition, the genome sequence from DM1-3 518 R44 (The Potato Genome Sequencing Consortium, 2011) was included and shown to be allele *inh1-a*. Alleles *inh1-b, inh1-c*, *inh1-d, inh1-e* and *inh1-f* share 83.9% sequence identity. The genomic sequences obtained for invertase inhibitor from all three genotypes exhibited 100% (*inh1-b*), 81.3% (*inh1-c*), 77.1% (*inh1-d*), 78.7% (*inh1-e*), and 79.9% (*inh1-f*) pairwise sequence identity with the previously isolated genomic clone (GU980592). All the alleles showed 70.5% pairwise sequence identity with that of *inh1-a* (AY864819). The structure of the DNA is described into three sections namely 1) upstream and downstream elements that include 5' and 3'UTRs 2) exons and 3) intron. All alleles contain two exons and a single intron. Polymorphisms were present both in the exon and intron regions (Figure 4.3). The size of *inh1-b* is 2086 bp. The remaining alleles were approximately 1.8 kb, with the smallest being *Stinvinh3* (1736 nt) and the largest *inh1-f* (1857 nt). Features of the alleles including their 5' regulatory regions and 3' untranslated regions are summarized in Figure 4.3 and Table 4.1.

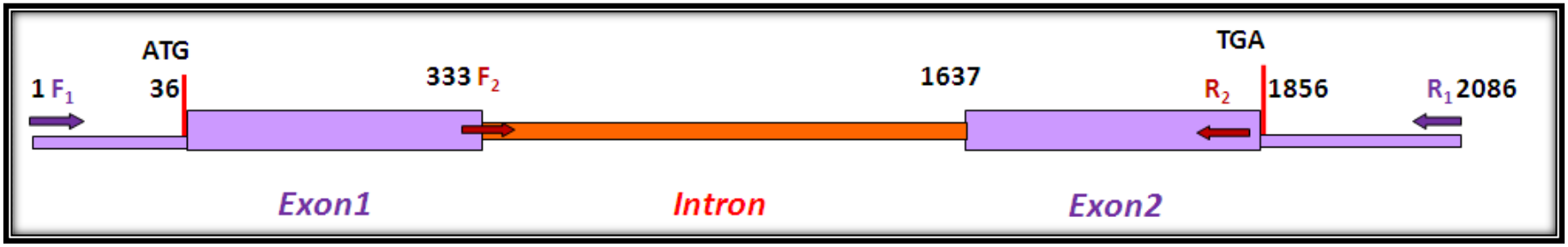


Figure 4.1: Schematic representation of the genomic organisation of the apoplastic invertase inhibitor gene. The features shown are, relative positions of primers indicated by arrows F-forward & R-reverse, exons (purple) and intron (orange), translation start (ATG) and stop (TGA) sites. Drawing is not to scale. Primers F_1, R_1 & R_2 are located in the exon while primer F_2 is located at the junction of exon1 and the intron.

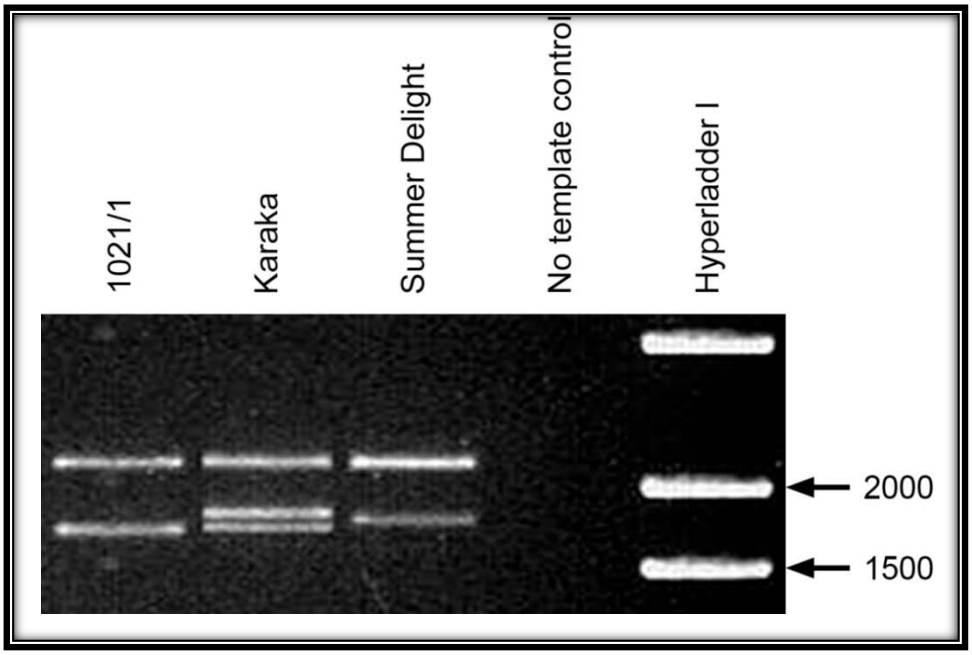


Figure 4.2: PCR amplification of the apoplastic invertase inhibitor gene. 1% agarose gel showing the PCR amplicons of invertase inhibitor alleles using primers (ApoInh F_1 and ApoInh R_1). Amplicons are approximately 2.1 bp and 1.8 bp in size. Hyper ladder I (Bioline, London, UK).

1) Upstream and downstream elements: The sequence data spans from 34 bases (*inh1-a*) & 35 bases upstream of the ATG translation start (*inh1-b*, *inh1-c*, *inh1-d*, *inh1-e* and *inh1-f*) site to 285 (*inh1-a*), 293 (*inh1-b*), 290 (*inh1-c*), 286 (*inh1-d*), 287 (*inh1-e*) and 315 (*inh1-f*) bases downstream of the TGA stop codon (Table 4.1, Figure 4.3). UTR sequences are not available for the *Stinvinh1* and *Stinvinh3* alleles. The TATA box and CAAT-like sequences were identified in the 5'- flanking region of the apoplastic invertase inhibitor gene in the potato genome sequence (Superscaffold PGSC0003DMB000000114, The Potato Genome Sequencing Consortium 2011) and are located 47 and 68 nt upstream of the transcription inititation site respectively. The 3' UTR of the alleles *inh1-a*, c*, d, e* and *f* shows a number of substitutions and indels, exhibiting 89.% sequence identity. Alleles *inh1-a*, *b, c, d* and *e* shared a deletion of 28-29 bp at nt 1968, whereas allele *inh1-f* had a repeat sequence instead (Figure 4.3).

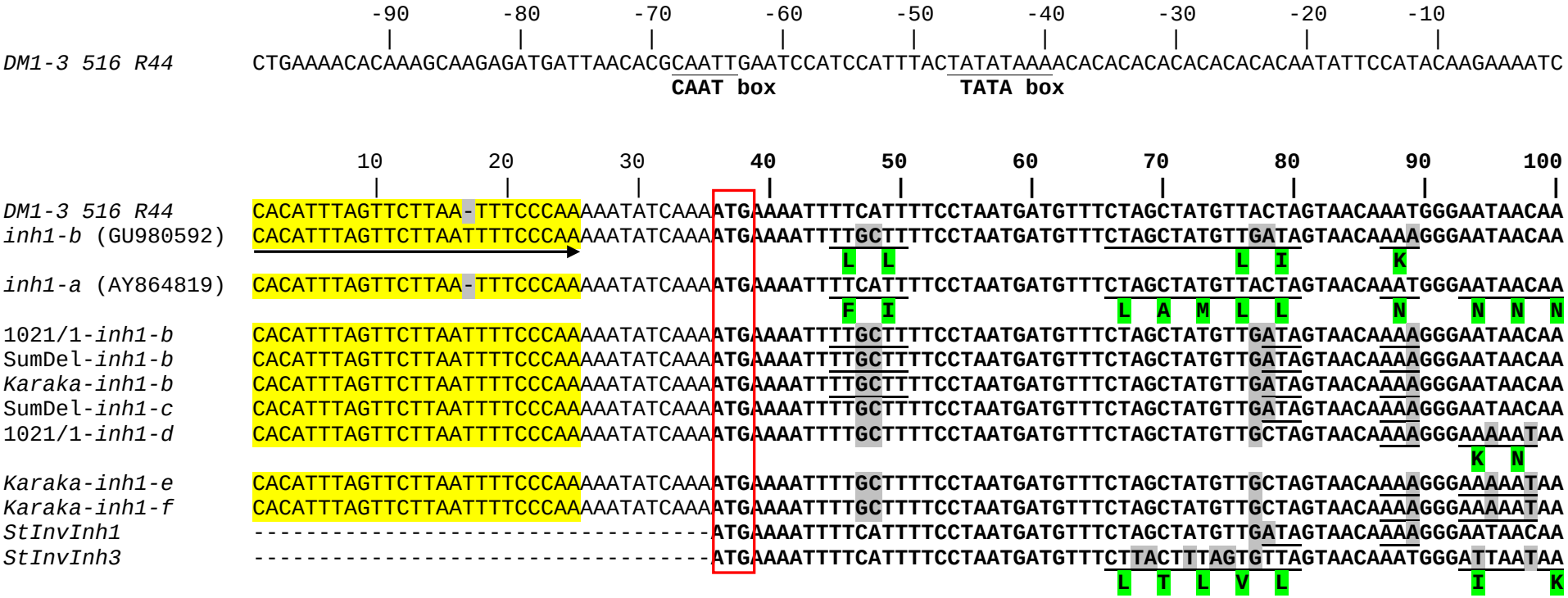
2) Allelic variations in exons: The total length of the two exons is 516 nucleotides (Figure 4.3). Exon 1 is 297 nt long in all alleles, whereas exon 2 is 219 nt in *inh1-a, b, c, d,*

e, f and *Stinvinh1* and 210 nt in *Stinvinh3*, with the ATG start codon at nt position 36 and a TGA stop codon at nt position 1885 (Figure 4.3). Comparison of the exons of the eight alleles showed 95.9% nucleotide identity with a total of 65 individual single nucleotide polymorphisms (SNPs) and an in frame deletion of nine nucleotides (Table 4.2, Figure 4.3). Only 23 SNPs resulted in silent mutations, whereas 42 conferred amino acid substitutions. Eleven SNPs were shared between two or more alleles, while 51 were unique to one allele (Table 4.2, Figure 4.3). Forty-six of the unique SNPs and the in frame deletion were exclusively observed in *Stinvinh3* (Table 4.2, Figure 4.3).

All the alleles differ from *inh1-a* by both synonymous and non-synonymous substitutions. The number of differences between allele *inh1-a* and the new alleles reported here, differed with *inh1-b/c* showing 10, *inh1-d/f* showing 8 and *inh1-e* showing 6 substitutions compared to *inh1-a*. Three non-synonymous substitutions were shared by five alleles identified here with that of *inh1-a* in the signal peptide at nt positions 47, 48, and 89 (Figure 4.3, Table 4.2) and were resulting from (Phe → Leu), (Ile → Leu) and (Asn → Lys) nucleotide (amino acid) changes, respectively (Table 4.2). In addition to these 3 non-synonymous substitutions, *inh1-a* differed by 3 additional non-synonymous substitutions with *inh1-b* at nt positions 78 (Leu → Ile), 232 (Ser → Tyr) and 1698 (Ile → Thr). Similarly in addition to 3 non-synonymous substitutions (at nt positions 47, 48 & 89) *inh1-a* differed with *inh1-c* with two additional non-synonymous changes at nt positions 150 (Lys → Glu) and 177 (Glu → Gln) (Figure 4.3, Table 4.2). Likewise in addition to these 3 non-synonymous substitutions (at nt positions 47, 48 & 89) *inh1-a* was differed with that of *inh1-d*, *e* and *f* at nt 95 (Asn → Lys). (Figure 4.3, Table 4.2). It is important to note that allele *inh1-a* was differed by one non-synonymous substitution with that of *inh1-f* at nt 151 resulting from A to G (Lys → Arg) (Figure 4.3, Table 4.2).

Alleles *inh1-b* and *c* differed by 2 non-synonymous substitutions with alleles *inh1-d, e* and *f*. These non-synonymous substitutions were at nt positions 78, 95, resulting from A to C (Leu → Ile) and T to A (Asn → Lys) nucleotide (amino acid) changes, respectively (Figure 4.3, Table 4.2). It is also important to note that the substitution at nt 232 (Ser → Tyr) was shared by alleles *inh1-b* and *StInvInh1*. The substitution at nt 151 (Lys →Arg) was exclusively found in allele *inh1-f*. The substitution of T to A at nt 95 (Asn → Lys), which is the first amino acid of the mature polypeptide, is shared by alleles *inh1-*

d, *e* and *f* (Figure 4.3, Table 4.2). In conclusion, *inh1-b & c* showed 98.2% while *inh1-d, e* & *f* showed 99.1% nucleotide identity with that on *inh1-a*.


-90 -80 -70 -60 -50 -40 -30 -20 -10
DM1-3 516 R44 CTGAAAACACAAAGCAAGAGATGATTAACACGCAATTGAATCCATCCATTTACTATATAAAACACACACACACACACACAATATTCCATACAAGAAAATC
CAAT box
TATA box
10 20 30 40 50 60 70 80 90 100
DM1-3 516 R44 CACATTTAGTTCTTAA-TTTCCCAAAAATATCAAAATGAAAATTTTCATTTTCCTAATGATGTTTCTAGCTATGTTACTAGTAACAAATGGGAATAACAA
inh1-b (GU980592) CACATTTAGTTCTTAATTTTCCCAAAAATATCAAAATGAAAATTTTGCTTTTCCTAATGATGTTTCTAGCTATGTTGATAGTAACAAAAGGGAATAACAA
L L L I K
inh1-a (AY864819) CACATTTAGTTCTTAA-TTTCCCAAAAATATCAAAATGAAAATTTTCATTTTCCTAATGATGTTTCTAGCTATGTTACTAGTAACAAATGGGAATAACAA
F I L A M L L N N N N
1021/1-inh1-b CACATTTAGTTCTTAATTTTCCCAAAAATATCAAAATGAAAATTTTGCTTTTCCTAATGATGTTTCTAGCTATGTTGATAGTAACAAAAGGGAATAACAA
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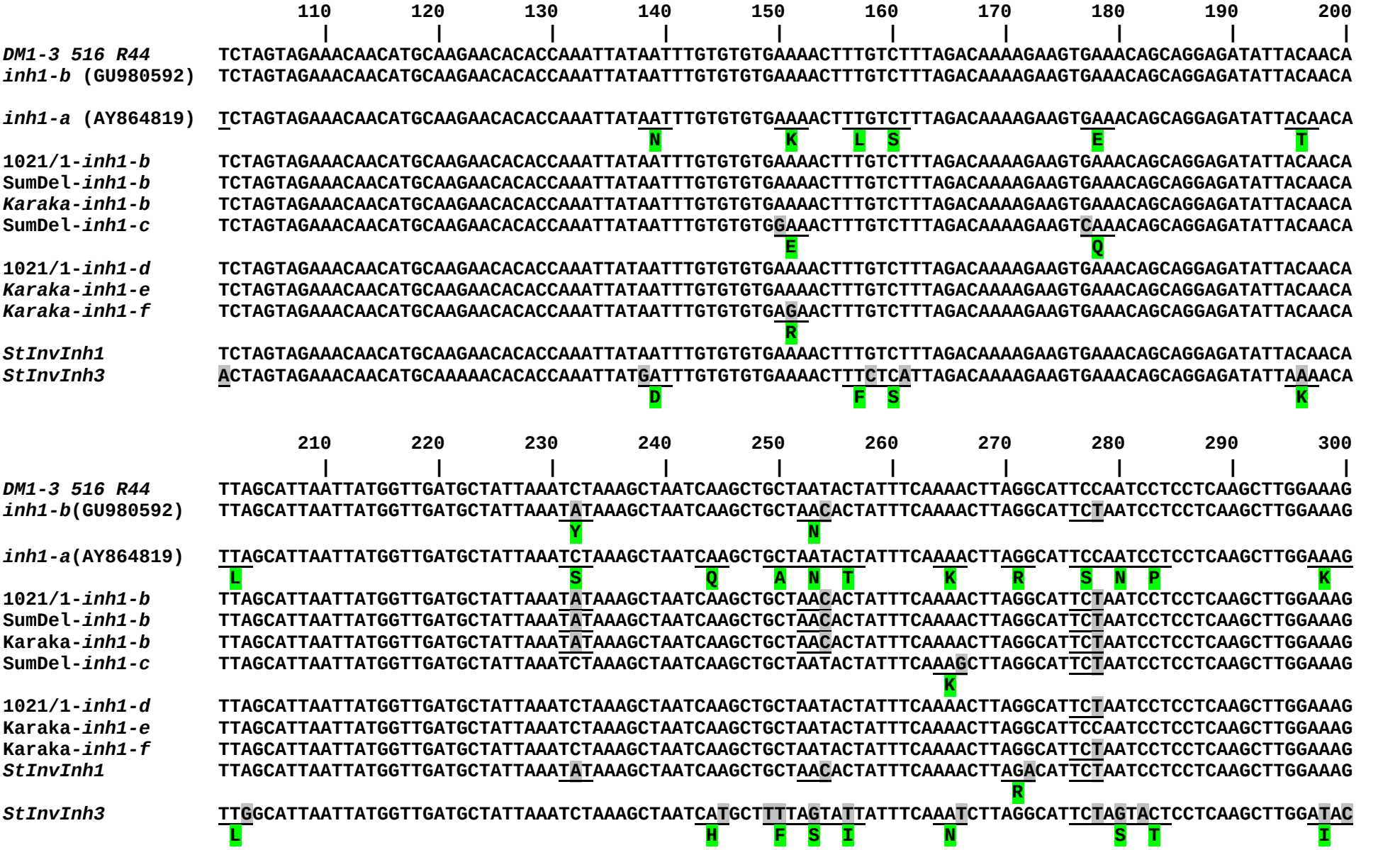
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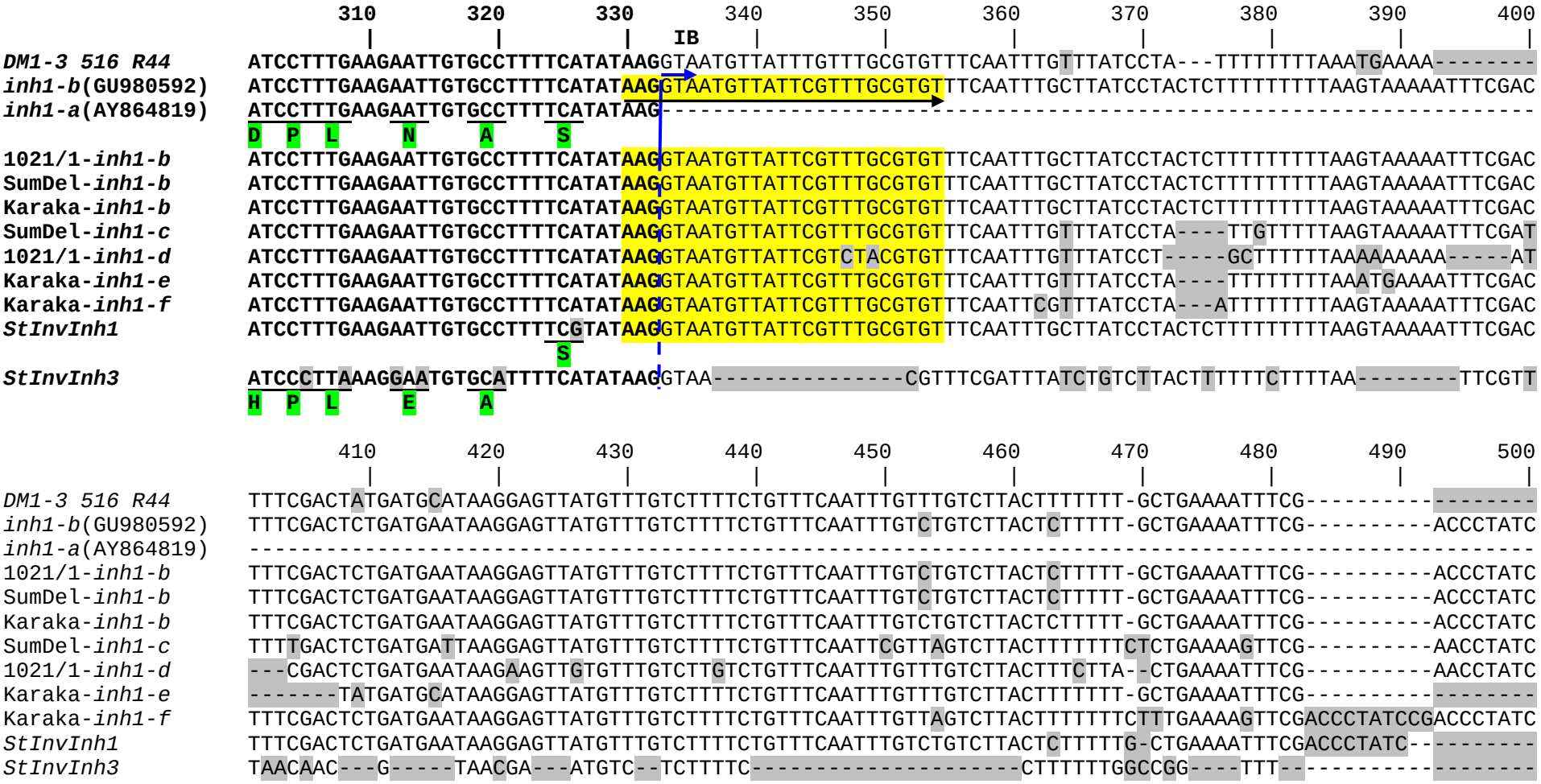
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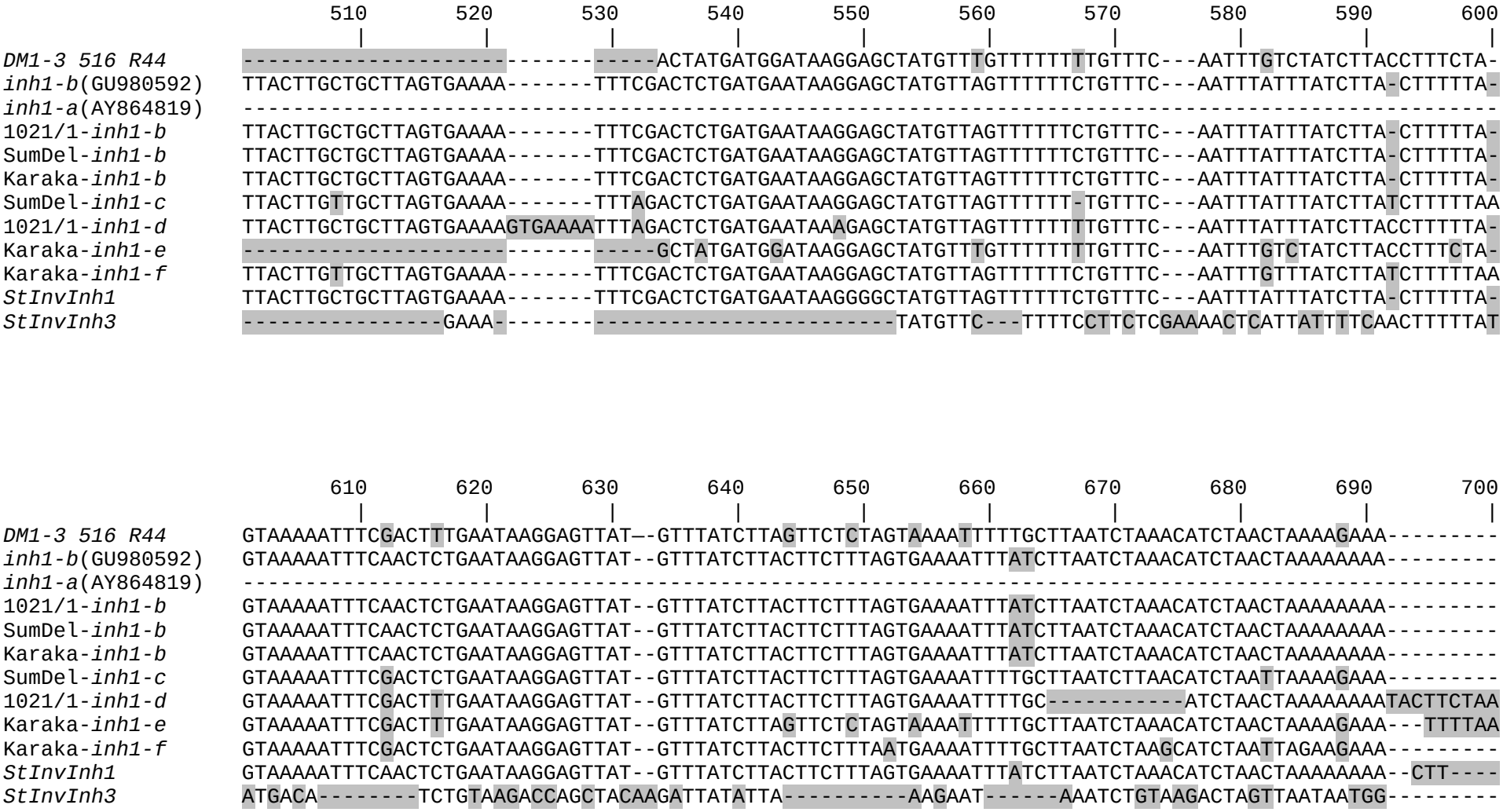
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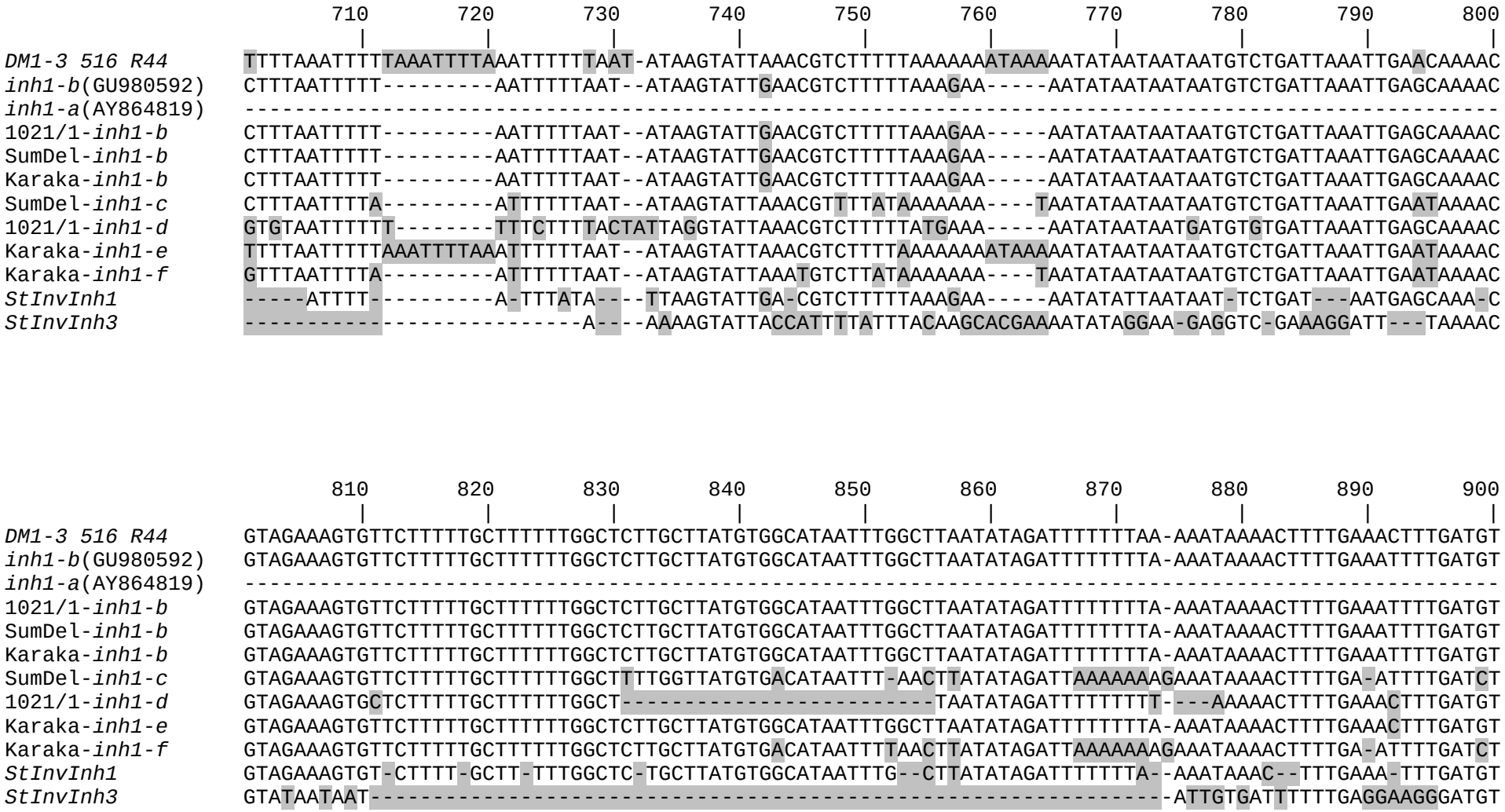
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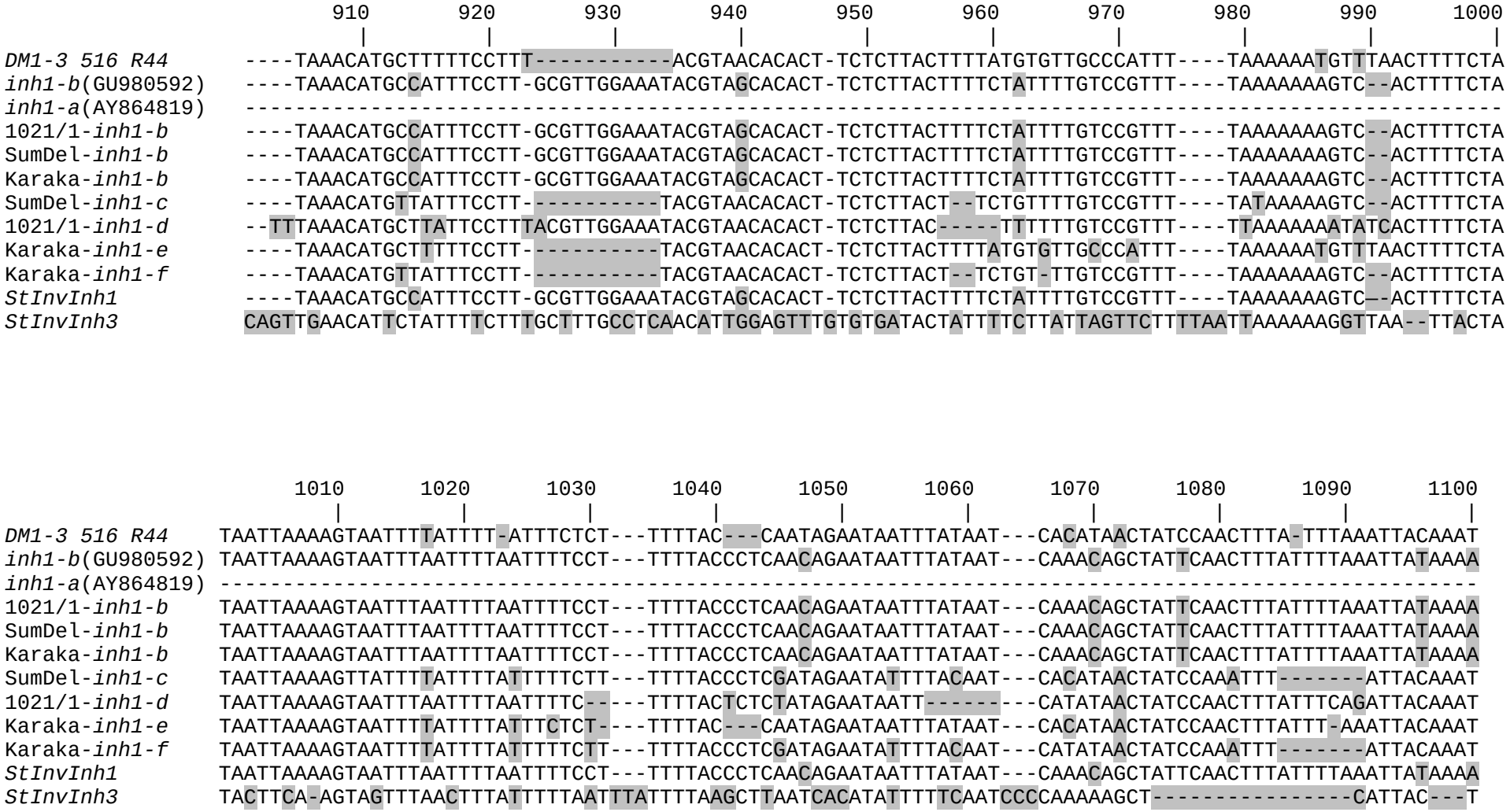
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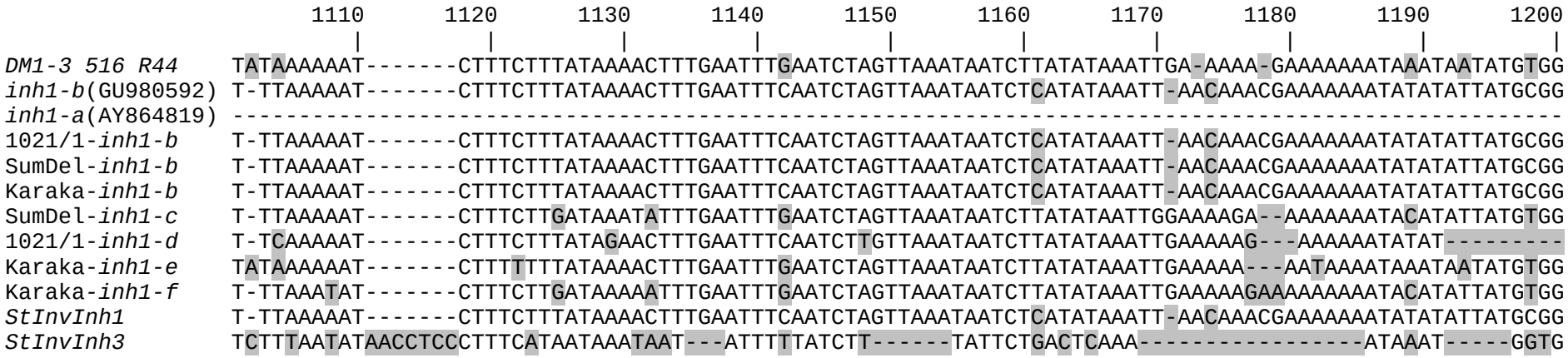
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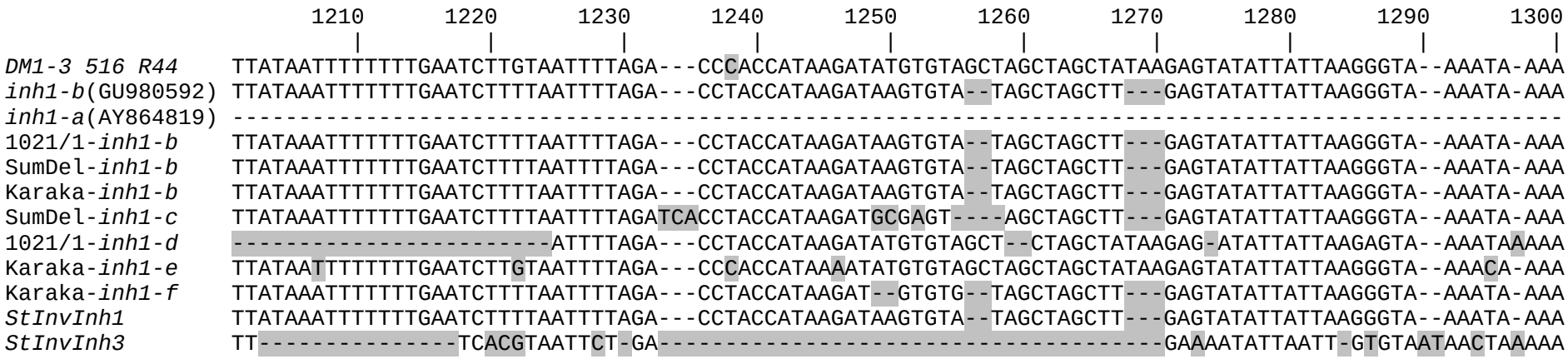
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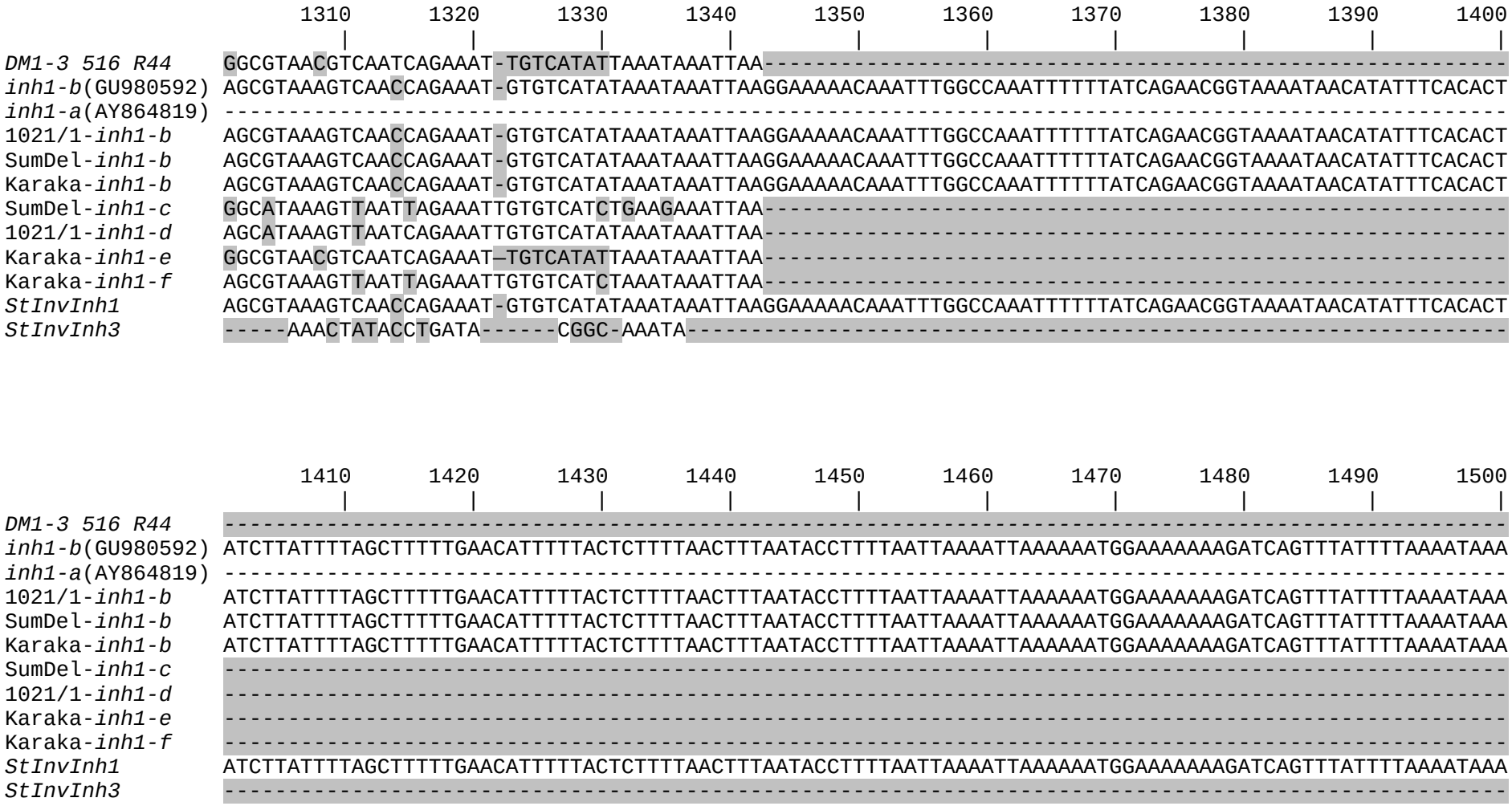
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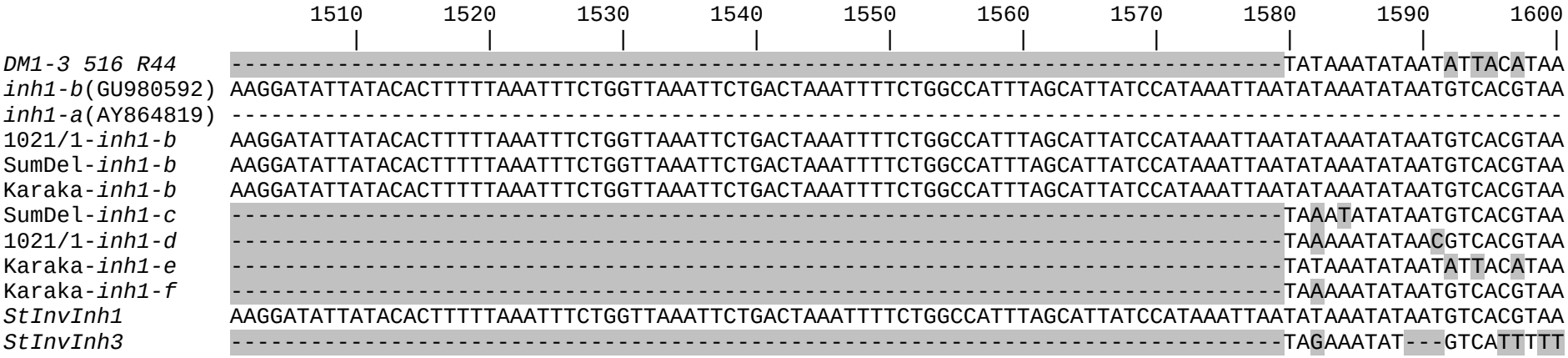
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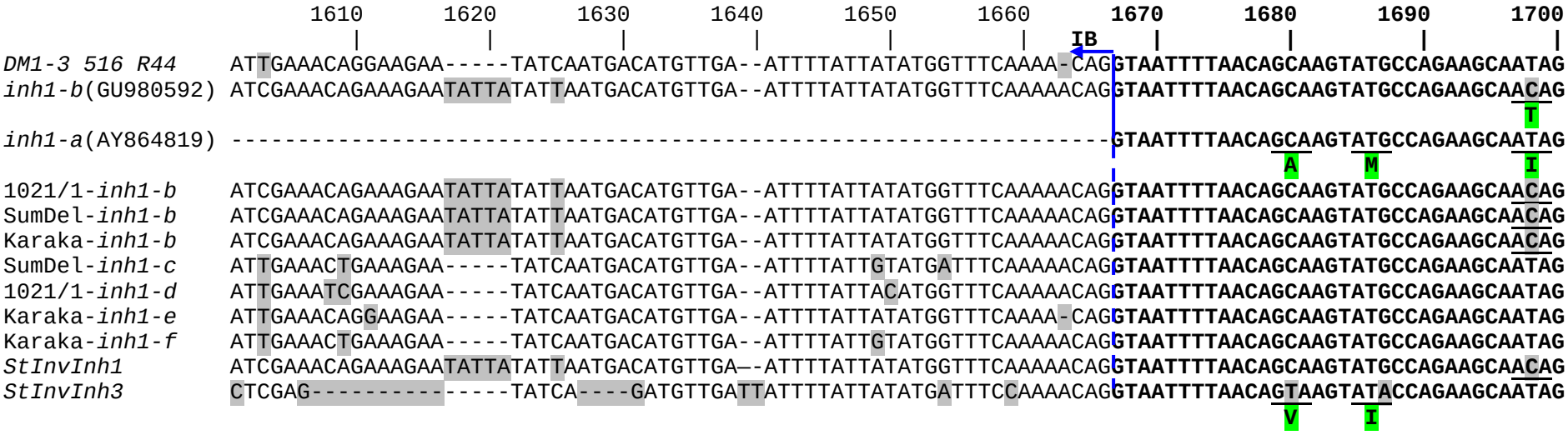
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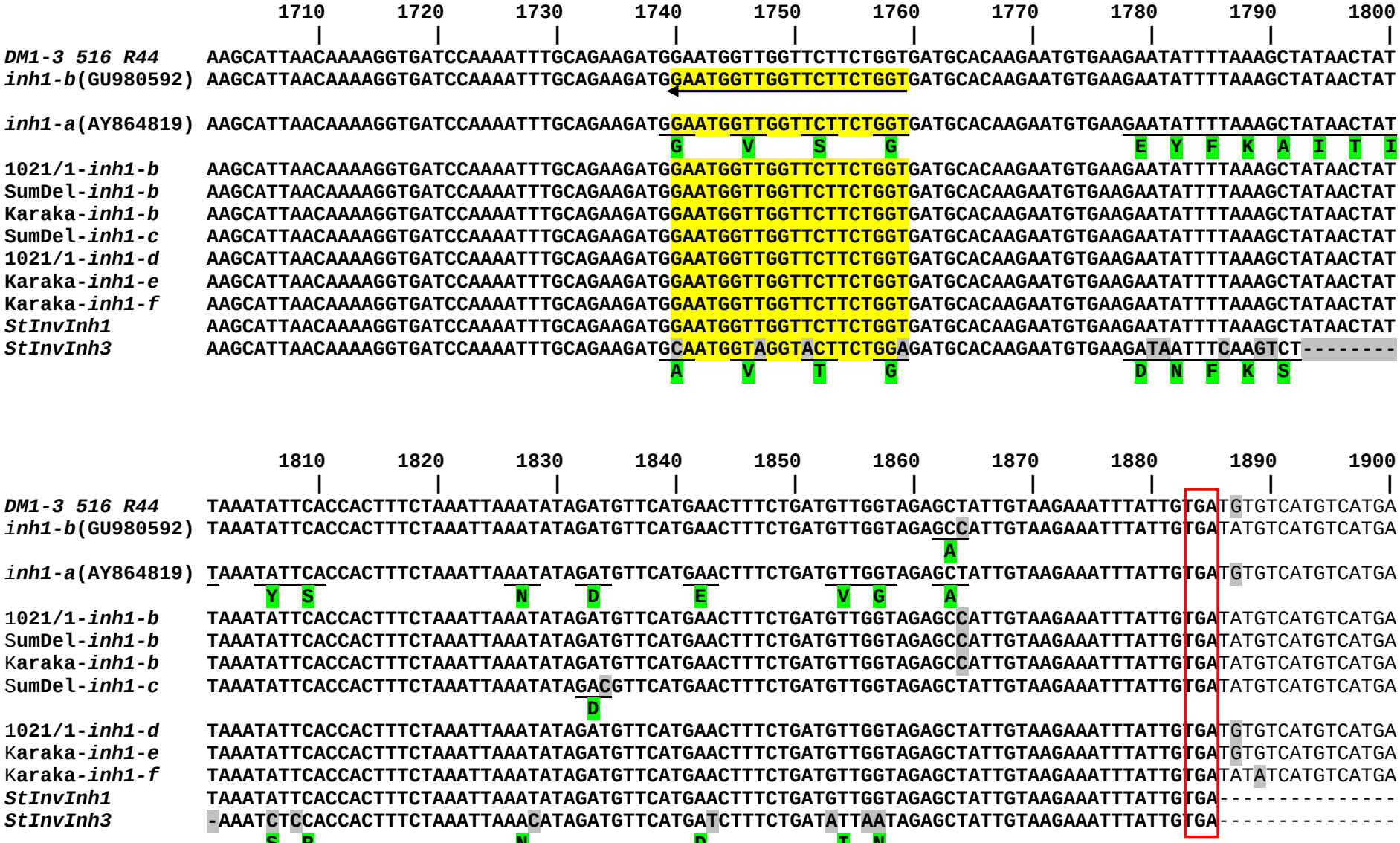
1021/1-inh1-d ATTGAAATCGAAAGAA-----TATCAATGACATGTTGA--ATTTTATTACATGGTTTCAAAAACAGGTAATTTTAACAGCAAGTATGCCAGAAGCAATAG
Karaka-inh1-e ATTGAAACAGGAAGAA-----TATCAATGACATGTTGA--ATTTTATTATATGGTTTCAAAA-CAGGTAATTTTAACAGCAAGTATGCCAGAAGCAATAG
Karaka-inh1-f ATTGAAACTGAAAGAA-----TATCAATGACATGTTGA--ATTTTATTGTATGGTTTCAAAAACAGGTAATTTTAACAGCAAGTATGCCAGAAGCAATAG
StInvInh1 ATCGAAACAGAAAGAATATTATATTAATGACATGTTGA—-ATTTTATTATATGGTTTCAAAAACAGGTAATTTTAACAGCAAGTATGCCAGAAGCAACAG
StInvInh3 CTCGAG----------TATCA----GATGTTGATTATTTTATTATATGATTTCCAAAACAGGTAATTTTAACAGTAAGTATACCAGAAGCAATAG
V I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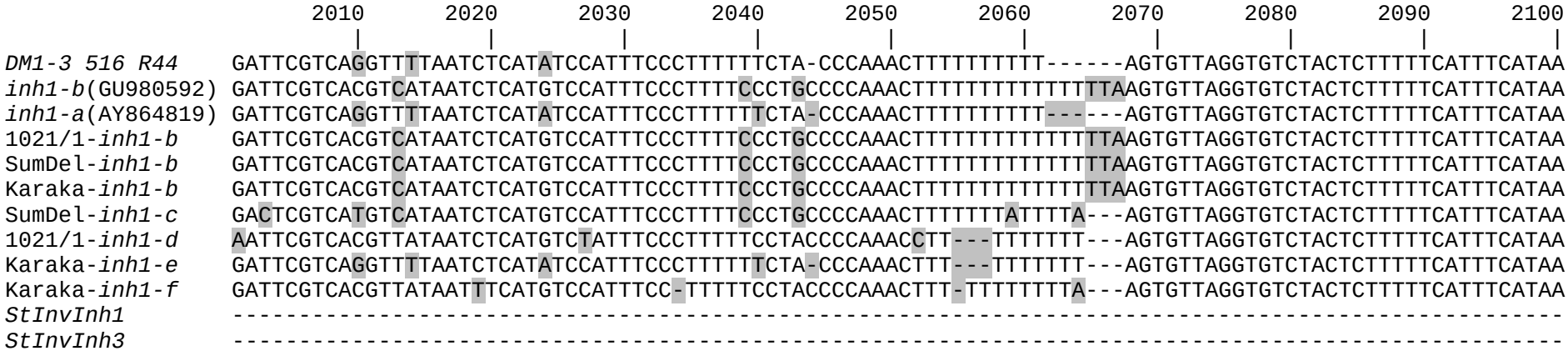
inh1-b(GU980592) AAGCATTAACAAAAGGTGATCCAAAATTTGCAGAAGATGGAATGGTTGGTTCTTCTGGTGATGCACAAGAATGTGAAGAATATTTTAAAGCTATAACTAT
inh1-a(AY864819) AAGCATTAACAAAAGGTGATCCAAAATTTGCAGAAGATGGAATGGTTGGTTCTTCTGGTGATGCACAAGAATGTGAAGAATATTTTAAAGCTATAACTAT
G V S G E Y F K A I T I
1021/1-inh1-b AAGCATTAACAAAAGGTGATCCAAAATTTGCAGAAGATGGAATGGTTGGTTCTTCTGGTGATGCACAAGAATGTGAAGAATATTTTAAAGCTATAACTAT
SumDel-inh1-b AAGCATTAACAAAAGGTGATCCAAAATTTGCAGAAGATGGAATGGTTGGTTCTTCTGGTGATGCACAAGAATGTGAAGAATATTTTAAAGCTATAACTAT
Karaka-inh1-b AAGCATTAACAAAAGGTGATCCAAAATTTGCAGAAGATGGAATGGTTGGTTCTTCTGGTGATGCACAAGAATGTGAAGAATATTTTAAAGCTATAACTAT
SumDel-inh1-c AAGCATTAACAAAAGGTGATCCAAAATTTGCAGAAGATGGAATGGTTGGTTCTTCTGGTGATGCACAAGAATGTGAAGAATATTTTAAAGCTATAACTAT
1021/1-inh1-d AAGCATTAACAAAAGGTGATCCAAAATTTGCAGAAGATGGAATGGTTGGTTCTTCTGGTGATGCACAAGAATGTGAAGAATATTTTAAAGCTATAACTAT
Karaka-inh1-e AAGCATTAACAAAAGGTGATCCAAAATTTGCAGAAGATGGAATGGTTGGTTCTTCTGGTGATGCACAAGAATGTGAAGAATATTTTAAAGCTATAACTAT
Karaka-inh1-f AAGCATTAACAAAAGGTGATCCAAAATTTGCAGAAGATGGAATGGTTGGTTCTTCTGGTGATGCACAAGAATGTGAAGAATATTTTAAAGCTATAACTAT
StInvInh1 AAGCATTAACAAAAGGTGATCCAAAATTTGCAGAAGATGGAATGGTTGGTTCTTCTGGTGATGCACAAGAATGTGAAGAATATTTTAAAGCTATAACTAT
StInvInh3 AAGCATTAACAAAAGGTGATCCAAAATTTGCAGAAGATGCAATGGTAGGTACTTCTGGAGATGCACAAGAATGTGAAGATAATTTCAAGTCT--------
A V T G D N F K S
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900
DM1-3 516 R44 TAAATATTCACCACTTTCTAAATTAAATATAGATGTTCATGAACTTTCTGATGTTGGTAGAGCTATTGTAAGAAATTTATTGTGATGTGTCATGTCATGA
inh1-b(GU980592) TAAATATTCACCACTTTCTAAATTAAATATAGATGTTCATGAACTTTCTGATGTTGGTAGAGCCATTGTAAGAAATTTATTGTGATATGTCATGTCATGA
A
inh1-a(AY864819) TAAATATTCACCACTTTCTAAATTAAATATAGATGTTCATGAACTTTCTGATGTTGGTAGAGCTATTGTAAGAAATTTATTGTGATGTGTCATGTCATGA
Y S N D E V G A
1021/1-inh1-b TAAATATTCACCACTTTCTAAATTAAATATAGATGTTCATGAACTTTCTGATGTTGGTAGAGCCATTGTAAGAAATTTATTGTGATATGTCATGTCATGA
SumDel-inh1-b TAAATATTCACCACTTTCTAAATTAAATATAGATGTTCATGAACTTTCTGATGTTGGTAGAGCCATTGTAAGAAATTTATTGTGATATGTCATGTCATGA
Karaka-inh1-b TAAATATTCACCACTTTCTAAATTAAATATAGATGTTCATGAACTTTCTGATGTTGGTAGAGCCATTGTAAGAAATTTATTGTGATATGTCATGTCATGA
SumDel-inh1-c TAAATATTCACCACTTTCTAAATTAAATATAGACGTTCATGAACTTTCTGATGTTGGTAGAGCTATTGTAAGAAATTTATTGTGATATGTCATGTCATGA
D
1021/1-inh1-d TAAATATTCACCACTTTCTAAATTAAATATAGATGTTCATGAACTTTCTGATGTTGGTAGAGCTATTGTAAGAAATTTATTGTGATGTGTCATGTCATGA
Karaka-inh1-e TAAATATTCACCACTTTCTAAATTAAATATAGATGTTCATGAACTTTCTGATGTTGGTAGAGCTATTGTAAGAAATTTATTGTGATGTGTCATGTCATGA
Karaka-inh1-f TAAATATTCACCACTTTCTAAATTAAATATAGATGTTCATGAACTTTCTGATGTTGGTAGAGCTATTGTAAGAAATTTATTGTGATATATCATGTCATGA
StInvInh1 TAAATATTCACCACTTTCTAAATTAAATATAGATGTTCATGAACTTTCTGATGTTGGTAGAGCTATTGTAAGAAATTTATTGTGA----------
StInvInh3 -AAATCTCCACCACTTTCTAAATTAAACATAGATGTTCATGATCTTTCTGATATTAATAGAGCTATTGTAAGAAATTTATTGTGA----------
S P N D I N


1910 1920 1930 1940 1950 1960 1970 1980 1990 2000
DM1-3 516 R44 TGTTACGTATCGAAAAACTTTAGTAACTTAATTGATAAATTGTCTGAATTATTATTTGCTGGTAAG----------GGTTC
inh1-b(GU980592) TGTTACGTATCGGAAAATTTTAGTAACTTAATTGATAGATTGTCTGAATTATTATCTTACTGGTGAG----------AGTTC
inh1-a(AY864819) TGTTACGTATCGAAAAACTTTAGTAACTTAATTGATAAATTGTCTGAATTATTATTTGCTGGTAAG----------GTTC
1021/1-inh1-b TGTTACGTATCGGAAAATTTTAGTAACTTAATTGATAGATTGTCTGAATTATTATCTTACTGGTGAG----------AGTTC
SumDel-inh1-b TGTTACGTATCGGAAAATTTTAGTAACTTAATTGATAGATTGTCTGAATTATTATCTTACTGGTGAG----------AGTTC
Karaka-inh1-b TGTTACGTATCGGAAAATTTTAGTAACTTAATTGATAGATTGTCTGAATTATTATCTTACTGGTGAG----------AGTTC
SumDel-inh1-c TGTTACGTATCGGTAAATTTTAGTAACTTAATTGATAGATTGTCTGAATTATTATCTTGCTGTAGAG----------AGTTC
1021/1-inh1-d TGTTACGTATTGAAAAAATTTAGTAACTTAATTGATAGATTATCTGAATTATTATCTTCCTGGTGAG----------GTTC
Karaka-inh1-e TGTTATGTATCGAAAAACTTTAGTAACTTAATTGATAAATTGTCTGAATTATTATTTTGTTGGTAAG----------GGGTTC
Karaka-inh1-f TGTTAAGTATTAGGAAATTTTAGTAACTTAATTGATAGATTATCTGAATTATTCGCTTGCTGGTGAGGGTTCGATTCGTCACGTTATAATTTCAGGGTTC
StInvInh1 ----------
StInvInh3 ----------


2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
DM1-3 516 R44 GATTCGTCAGGTTTTAATCTCATATCCATTTCCCTTTTTTCTA-CCCAAACTTTTTTTTTT------AGTGTTAGGTGTCTACTCTTTTTCATTTCATAA
inh1-b(GU980592) GATTCGTCACGTCATAATCTCATGTCCATTTCCCTTTTCCCTGCCCCAAACTTTTTTTTTTTTTTTTAAGTGTTAGGTGTCTACTCTTTTTCATTTCATAA
inh1-a(AY864819) GATTCGTCAGGTTTTAATCTCATATCCATTTCCCTTTTTTCTA-CCCAAACTTTTTTTTTT---AGTGTTAGGTGTCTACTCTTTTTCATTTCATAA
1021/1-inh1-b GATTCGTCACGTCATAATCTCATGTCCATTTCCCTTTTCCCTGCCCCAAACTTTTTTTTTTTTTTTTAAGTGTTAGGTGTCTACTCTTTTTCATTTCATAA
SumDel-inh1-b GATTCGTCACGTCATAATCTCATGTCCATTTCCCTTTTCCCTGCCCCAAACTTTTTTTTTTTTTTTTAAGTGTTAGGTGTCTACTCTTTTTCATTTCATAA
Karaka-inh1-b GATTCGTCACGTCATAATCTCATGTCCATTTCCCTTTTCCCTGCCCCAAACTTTTTTTTTTTTTTTTAAGTGTTAGGTGTCTACTCTTTTTCATTTCATAA
SumDel-inh1-c GACTCGTCATGTCATAATCTCATGTCCATTTCCCTTTTCCCTGCCCCAAACTTTTTTTATTTTA---AGTGTTAGGTGTCTACTCTTTTTCATTTCATAA
1021/1-inh1-d AATTCGTCACGTTATAATCTCATGTCTATTTCCCTTTTTCCTACCCCAAACCTT---TTTTTTT---AGTGTTAGGTGTCTACTCTTTTTCATTTCATAA
Karaka-inh1-e GATTCGTCAGGTTTTAATCTCATATCCATTTCCCTTTTTTCTA-CCCAAACTTT---TTTTTTT---AGTGTTAGGTGTCTACTCTTTTTCATTTCATAA
Karaka-inh1-f GATTCGTCACGTTATAATTTCATGTCCATTTCC-TTTTTCCTACCCCAAACTTT-TTTTTTTTA---AGTGTTAGGTGTCTACTCTTTTTCATTTCATAA
StInvInh1 ----------
StInvInh3 ----------

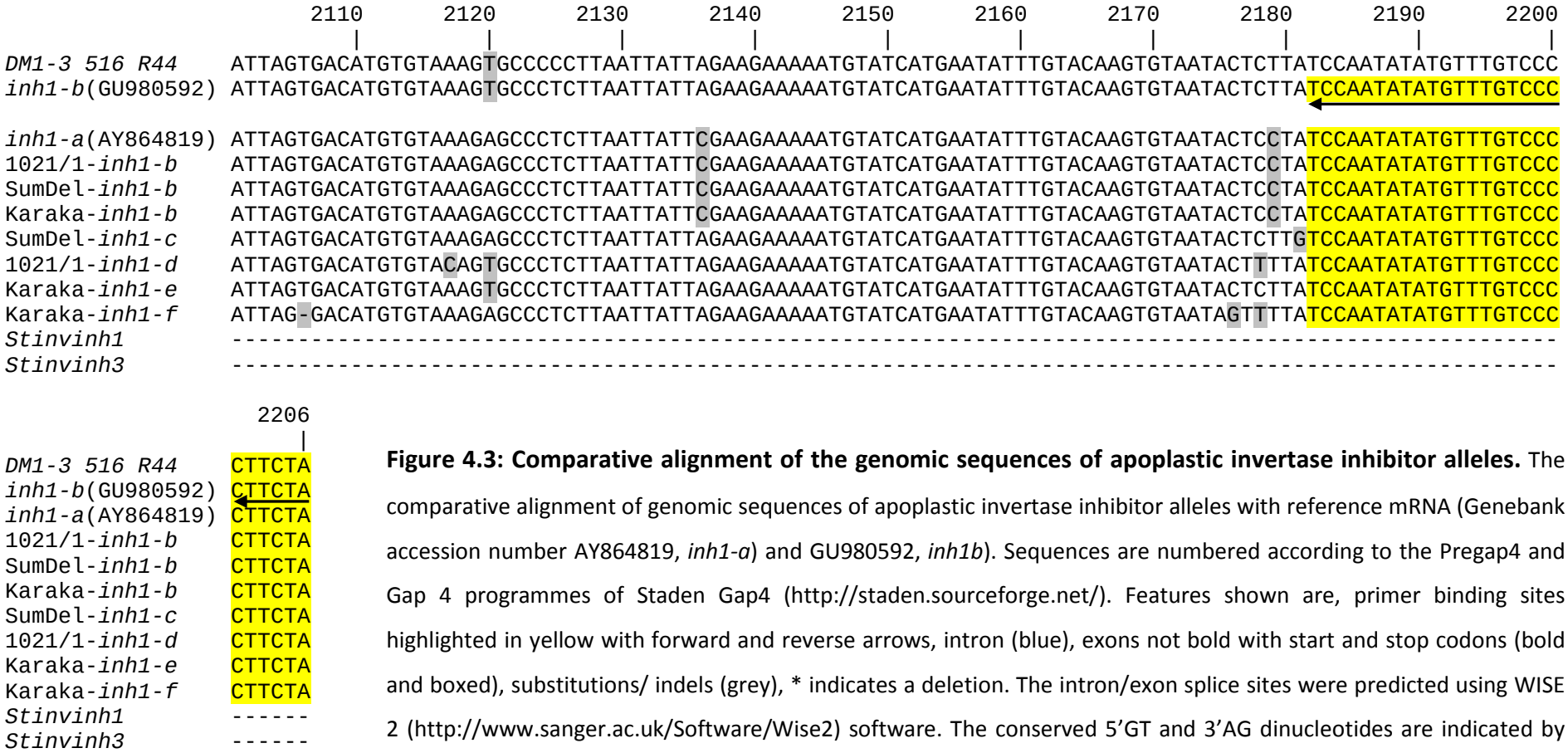


Figure 4.3: Comparative alignment of the genomic sequences of apoplastic invertase inhibitor alleles. The comparative alignment of genomic sequences of apoplastic invertase inhibitor alleles with reference mRNA (Genebank accession number AY864819, *inh1-a*) and GU980592, *inh1b*). Sequences are numbered according to the Pregap4 and Gap 4 programmes of Staden Gap4 (http://staden.sourceforge.net/). Features shown are, primer binding sites highlighted in yellow with forward and reverse arrows, intron (blue), exons not bold with start and stop codons (bold and boxed), substitutions/ indels (grey), * indicates a deletion. The intron/exon splice sites were predicted using WISE 2 (http://www.sanger.ac.uk/Software/Wise2) software. The conserved 5'GT and 3'AG dinucleotides are indicated by blue arrows. Predicted changes in the amino acid sequence are presented in a one letter code below the corresponding nucleotide codon (underlined). IB – Intron boundary. SumDel – Summer Delight.

Table 4.1 Features of the DNA sequence of invertase inhibitor alleles *inh1-a*, genomic sequence The Potato Genome Sequencing Consortium (2011), cDNA sequence Brummell et al. (2011); *StInvInh1*, *StInvInh3*, Liu et al. (2010).

Breeding line/ cultivar	Allele	Total length	5'UTR	3'UTR	Exon 1	Exon 2	Intron	% AT	
								Exon	Intron
DM1-3 518 R44	*inh1-a*	1803	34	286	297	219	967	69	78
937/3	*inh1-a*	905	34	285	297	219	--	69	--
1021/1	*inh1-b*	2086	35	293	297	219	1242	69	77
Summer Delight	*inh1-b*	2086	35	293	297	219	1242	69	77
Karaka	*inh1-b*	2086	35	293	297	219	1242	69	77
Summer Delight	*inh-1c*	1821	35	290	297	219	980	69	78
1021/1	*inh1-d*	1766	35	286	297	219	928	69	77
Karaka	*inh1-e*	1804	35	287	297	219	966	69	78
Karaka	*inh1-f*	1857	35	315	297	219	991	69	77
Shepody	*StInvInh1*	1736	--	--	297	219	1220	69	76
Shepody	*SIinvInh3*	1191	--	--	297	210	684	70	74

3) Allelic variations in intron: The extent and location of intron diversity between all eight alleles of the *inh1* locus is provided in Table 4.1 and Figure 4.3. In contrast to polymorphism in the exon regions, the single intron from the different alleles was highly polymorphic (Figure 4.3, Table 4.1). The majority of the sequence polymorphism was observed in the alleles *inh1-b* and *StInvInh1* and was primarily due to a large insertion of approximately 240 nucleotides (at nt 1342) along with substitutions and small insertions/deletions (Figure 4.3). Several insertions and deletions were apparent in allele *StInvInh3* (Figure 4.3). Likewise, two additional small deletions of 25 nt (at nt 831) and 33 nt (at nt 1192) were observed in allele *inh1-d*. Allele *inh1-e* has a deletion of 51 bp (at nt 490) in the first half of the intron. The largest intron (1242 bp) was found in *inh1-b* while it was shorter in *inh1-c*, *inh1-d*, *inh1-e* and *inh1-f* at 980, 928, 966 and 991 bp respectively (Table 4.1). Allele *inh1-e* has a deletion of 51 bp in the first half of the intron while the 7 bp deletion at nt 522 was shared by all alleles except *inh1-d*. In addition *inh1-b* also exhibited a number of deletions (< 20 nt). Mononucleotide repeats of A/T, the most common motif, were largely distributed in the non-coding region of all alleles (Figure 4.3).

It is also important to mention that intron region also showed insertions that were perfect or imperfect repeats of an adjacent sequence (at nt 482 – *inh1-f* , Figure 4.3). Alleles *inh1-b, c, d, e* and *f* have ~ 76% sequence identity amongst each other in their introns.

Exon-intron boundaries: The exon/intron structure was identical in all the alleles indicating the splicing sites are 100% conserved (Figure 4.3) and possess the conserved 5'GT and 3'AG dinucleotides at the intron boundaries. The % AT in the exon and intron regions is showed in Table 4.1.

Table 4.2: Nucleotide polymorphism in the putative mRNA of invertase inhibitor alleles

Allele	Nucleotide position															
	47	48	68	69	72	74	75	77	78	89	94	95	98	101	138	150
inh1-a	C	A	A	G	A	G	T	A	C	T	A	T	C	T	A	A
inh1-b	G	C	A	G	A	G	T	G	A	A	A	T	C	T	A	A
inh1-c	G	C	A	G	A	G	T	G	A	A	A	T	C	T	A	G
inh1-d	G	C	A	G	A	G	T	G	C	A	A	A	T	T	A	A
inh1-e	G	C	A	G	A	G	T	G	C	A	A	A	T	T	A	A
inh1-f	G	C	A	G	A	G	T	G	C	A	A	A	T	T	A	A
StInvInh1	C	A	A	G	A	G	T	G	A	A	A	T	C	T	A	A
StInvInh3	C	A	T	A	T	A	G	G	T	T	T	T	T	A	G	A
Amino acid	Phe to Leu	Ile to Leu	Leu	Ala to Thr	Met to Leu		Leu to Val	Leu	Leu to Ile	Asn to Lys	Asn to Ile	Asn to Lys	Asn	Asn to Lys	Asn to Asp	Lys to Glu

Table 4.2 continued

Allele	Nucleotide position															
	151	158	161	177	196	203	232	245	249	250	253	254	256	266	272	278
inh1-a	A	G	T	G	C	A	C	A	G	C	A	T	C	A	G	C
inh1-b	A	G	T	G	C	A	A	A	G	C	A	C	C	A	G	T
inh1-c	A	G	T	C	C	A	C	A	G	C	A	T	C	G	G	T
inh1-d	A	G	T	G	C	A	C	A	G	C	A	T	C	A	G	T
inh1-e	A	G	T	G	C	A	C	A	G	C	A	T	C	A	G	C
inh1-f	G	G	T	G	C	A	C	A	G	C	A	T	C	A	G	T
StInvInh1	A	G	T	G	C	A	A	A	G	C	A	C	C	A	A	T
StInvInh3	A	C	A	G	A	G	C	T	T	T	G	T	T	T	G	T
Amino acid	Lys to Arg	Leu to Phe	Ser	Glu to Gln	Thr to Lys	Leu	Ser to Tyr	Gln to His	Ala to Phe		Asn to Ser	Asn	Thr to Ile	Lys to Asn	Arg	Ser

Table 4.2 continued

Allele	Nucleotide position															
	280	282	298	300	305	308	312	314	320	326	1680	1687	1698	1740	1747	1751
inh1-a	A	C	A	G	T	G	A	T	C	A	C	G	T	G	T	T
inh1-b	A	C	A	G	T	G	A	T	C	A	C	G	C	G	T	T
inh1-c	A	C	A	G	T	G	A	T	C	A	C	G	T	G	T	T
inh1-d	A	C	A	G	T	G	A	T	C	A	C	G	T	G	T	T
inh1-e	A	C	A	G	T	G	A	T	C	A	C	G	T	G	T	T
inh1-f	A	C	A	G	T	G	A	T	C	A	C	G	T	G	T	T
StInvInh1	A	C	A	G	T	G	A	T	C	G	C	G	C	G	T	T
StInvInh3	G	A	T	C	C	A	G	A	A	A	T	A	T	C	A	A
Amino acid	Asn to Ser	Pro to Thr	Lys to Ile	Asp to His	Pro	Leu	Asn to Glu		Ala	Ser	Ala to Val	Met to Ile	Ile to Thr	Gly to Ala	Val	Ser to Thr

Table 4.2 continued

Allele	Nucleotide position														
	1759	**1780**	**1781**	**1786**	**1789**	**1790**	**1806**	**1808**	**1828**	**1834**	**1843**	**1853**	**1856**	**1857**	**1864**
inh1-a	**T**	**A**	**T**	**T**	**A**	**G**	**A**	**T**	**T**	**T**	**A**	**G**	**G**	**G**	**T**
inh1-b	**T**	**A**	**T**	**T**	**A**	**G**	**A**	**T**	**T**	**T**	**A**	**G**	**G**	**G**	**C**
inh1-c	**T**	**A**	**T**	**T**	**A**	**G**	**A**	**T**	**T**	**C**	**A**	**G**	**G**	**G**	**T**
inh1-d	**T**	**A**	**T**	**T**	**A**	**G**	**A**	**T**	**T**	**T**	**A**	**G**	**G**	**G**	**T**
inh1-e	**T**	**A**	**T**	**T**	**A**	**G**	**A**	**T**	**T**	**T**	**A**	**G**	**G**	**G**	**T**
inh1-f	**T**	**A**	**T**	**T**	**A**	**G**	**A**	**T**	**T**	**T**	**A**	**G**	**G**	**G**	**T**
StInvInh1	**T**	**A**	**T**	**T**	**A**	**G**	**A**	**T**	**T**	**T**	**A**	**G**	**G**	**G**	**T**
StInvInh3	**A**	**T**	**A**	**C**	**G**	**T**	**C**	**C**	**C**	**T**	**T**	**A**	**A**	**A**	**T**
Amino acid	**Gly**	**Glu to Asp**	**Tyr to Asn**	**Phe**	**Lys**	**Ala to Ser**	**Tyr to Ser**	**Ser to Pro**	**Asn**	**Asp**	**Glu to Asp**	**Val to Ile**	**Gly to Asn**		**Ala**

Note: - Nucleotide sequence and amino differences in the predicted mRNA sequences of invertase inhibitor alleles compared with the allele *inh1-a* (GenBank accession number AY864819). The amino acid changes were determined using Geneious Programme 5.4.6. Only polymorphisms in the exons are presented. The numbering is based on the Pregap4 and Gap 4 programmes of Staden Gap4 (http://staden.sourceforge.net/, Staden et al 1999). Highlighted in grey are polymorphisms. Refer Appendix D for details of three letter code amino acids. SNPs in *StInvInh3* at nt 78 (C to T) and *inh1-c* at nt 266 (A to G) do not cause a change in amino acid. The numbering is as in Figure 4.3. *StInvInh1* and *StInvInh3*, Liu et al 2010.

4.3.3 Protein comparison

Predicted protein sequences were aligned with the previously published apoplastic invertase inhibitors from potato (INH1-A, AY864819; StInvInh1, GU321338; StInvInh3, GU321339), tomato (SolyCIF, SGN-U317539), tobacco (Nt-inh1, Y12805) and petunia (PhInvInh1, SGN-U207999) (Figure 4.4). The nucleotide sequence from petunia was identified as an anonymous sequence while performing a BLAST search (SGN database, http://solgenomics.net) and was found to be identical to *PhInvInh1* (TC1812, Liu et al 2011). The secondary protein structure was predicted through comparison with the previously published structure of invertase inhibitor from tobacco (Nt-inh1, Greiner et al 1998, 1999; Nt-CIF, Hothorn et al 2004a). The apoplastic invertase inhibitors encode a predicted protein of 171 amino acid (Figure 4.4) residues (INH1-A, INH1-B, INH1-C, INH1-D, INH1-E, INH1-F, StInvInh1 and SolyCIF), with a calculated molecular mass of 18.8–18.9 kD (Table 4.3). Predicted protein sequences were slightly smaller for StInvInh3 (18.6 kD, 168 aa), PhInvInh1 (17.6 kD, 163 aa) and Nt-inh1 (18.0 kD, 166 aa). The predicted pI ranged from 7.45 to 9.26 (Table 4.3). A putative signal peptide of 19 amino acid residues at the N terminus was identified (Figure 4.4), which was confirmed through comparison with the previously isolated apoplastic invertase inhibitors from potato (INH1-A), tobacco (Nt-inh1), and tomato (SolCIF). The signal peptide preceded the N-terminal Asn-Asn-Asn sequence in all alleles except in INH1-D, INH1-E and INH1-F where a Lys-Asn-Asn sequence was found and StInvInh3 where a Ile-Asn-Lys sequence was found (Table 4.2, Figure 4.4). The Asn to Lys change at the first amino acid of the mature peptide (aa position 20) represents a non-conservative switch of an uncharged asparagine (Asn) to a positively charged lysine (Lys).

The mature proteins of INH1-A, INH1-B, INH1-C, INH1-D, INH1-E, INH1-F, StInvInh1, StInvInh3, PhInvInh1, Nt-inh1 and SolyCIF consist of 147 - 152 amino acids with a molecular weights ranged from 15.7 to 16.6 kDa (Table 4.3). The predicted pI of the mature protein ranged from 6.51 to 8.32 (Table 4.3). All amino acid sequences contain the four conserved cysteine residues characteristic of plant invertase inhibitors (Rausch and Greiner 2004). No potential *N*-glycosylation sites were identified in the predicted protein sequence.

The sequences identified in the present study were found to be high in leucine (~ 12 %) followed by alanine (9.4%) and lysine (~ 9.5%) residues. When the sequences

were aligned with previously published apoplastic invertase inhibitors from *S. tuberousm*, *S. lycopersicum*, *Petunia hybrida* and *N. tabacum*, a very high degree of homology (pair wise identity 89%) was observed and in particular the N and C-terminal end regions were highly conserved. In addition the signal sequence used for secretion into the apoplast was also identical to INH1A (Brummell et al. 2011), Nt-inh1 (Greiner et al. 1998) and SolyCIF (Reca et al. 2008).

The pairwise comparisons of deduced protein sequences between INH1B, INH1C, INH1D, INH1E, INH1F with INH1A showed 97.8% identity. The pariwise comparisons between INH1B & INH1C with INH1D, INH1E, INH1F exhibited 98.2% identity. The pariwise comparison between all five protein sequences identified in this study with StInvInh1 & StInvInh3 showed 92.3% identity. Similarly the pariwise comparisons between all five protein sequences with *S. lycopersicum* apoplastic invertase inhibitor (SolyCIF), *N. tabacum* apoplastic invertase inhibitor (Nt-inh1) and the apoplastic invertase inhibitor from *P. hybrida* (PhInvInh1), showed 97.4, 92.9 and 92% sequence identity respectively.

Table 4.3 Comparison of predicted amino acid compositions, molecular weight and pI of the apoplastic invertase inhibitors

Protein name	Accession no	Number of amino acids		Molecular weight (kD)		Predicted pI	
		Protein	Mature protein	Protein	Mature protein	Protein	Mature protein
INH1-A	AY864819	171	152	18.8	16.6	7.93	7.29
INH1-B	JQ043177	171	152	18.9	16.6	8.31	7.29
INH1-C	JQ043178	171	152	18.8	16.6	7.93	6.51
INH1-D	JQ043179	171	152	18.8	16.6	8.62	7.93
INH1-E	JQ043180	171	152	18.8	16.6	8.62	7.93
INH1-F	JQ043181	171	152	18.8	16.6	8.62	7.93
StInvInh1	GU321338	171	152	18.9	16.6	8.31	7.29
StInvInh3	GU321339	168	149	18.6	16.4	7.45	6.92
SolyCIF	SGN-U317539	171	152	18.8	16.6	8.31	7.93
Nt-inh1	Y12805	166	147	18.0	15.7	8.63	8.32
PhInvInh1	SGN-U207999	163	148	17.6	15.8	9.26	8.32

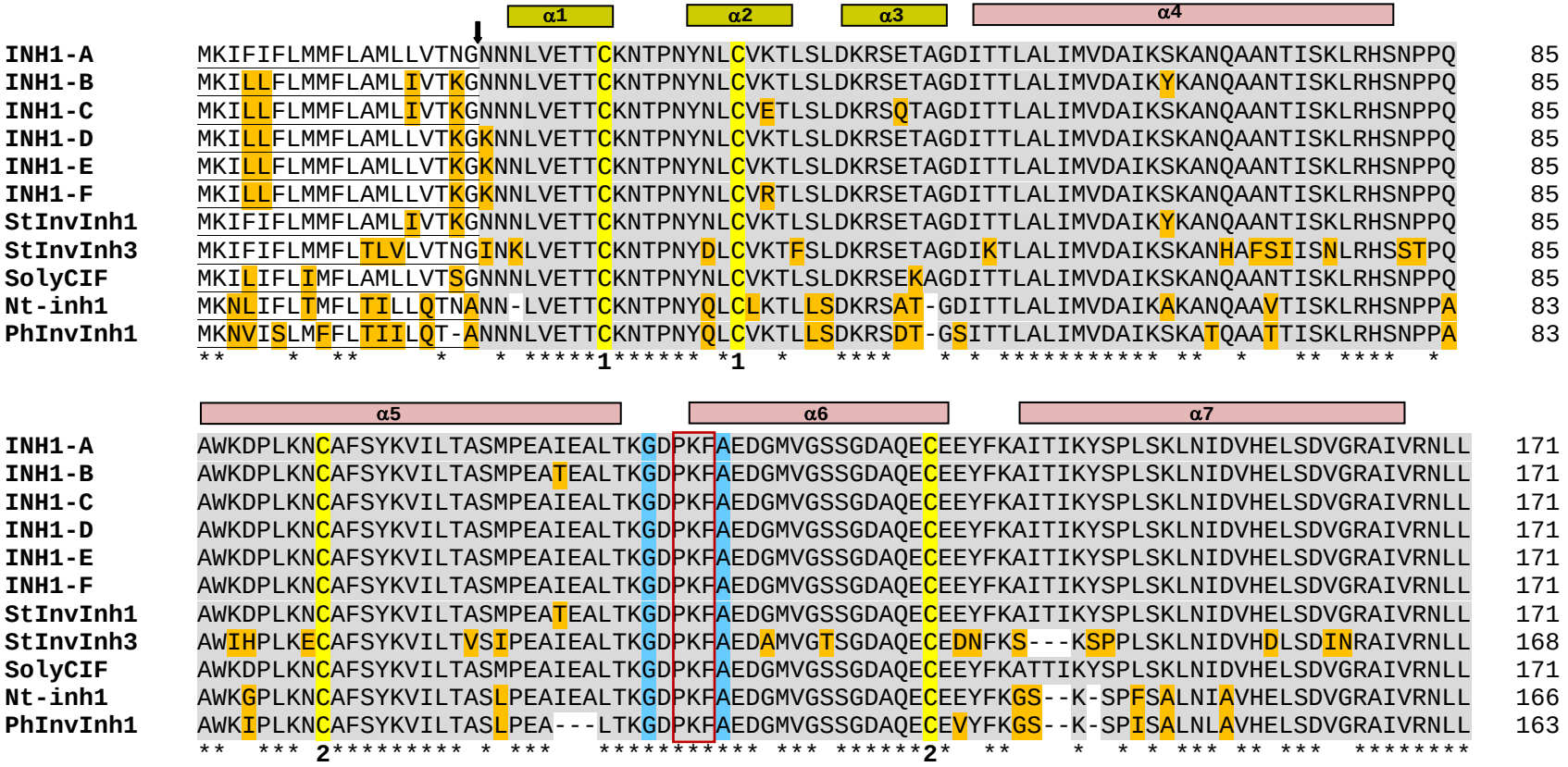

α1
α2
α3
α4
INH1-A MKIFIFLMMFLAMLLVTNGNNNLVETTCKNTPNYNLCVKTLSLDKRSETAGDITTLALIMVDAIKSKANQAANTISKLRHSNPPQ 85
INH1-B MKILLFLMMFLAMLIVTKGNNNLVETTCKNTPNYNLCVKTLSLDKRSETAGDITTLALIMVDAIKYKANQAANTISKLRHSNPPQ 85
INH1-C MKILLFLMMFLAMLIVTKGNNNLVETTCKNTPNYNLCVETLSLDKRSQTAGDITTLALIMVDAIKSKANQAANTISKLRHSNPPQ 85
INH1-D MKILLFLMMFLAMLLVTKGKNNLVETTCKNTPNYNLCVKTLSLDKRSETAGDITTLALIMVDAIKSKANQAANTISKLRHSNPPQ 85
INH1-E MKILLFLMMFLAMLLVTKGKNNLVETTCKNTPNYNLCVKTLSLDKRSETAGDITTLALIMVDAIKSKANQAANTISKLRHSNPPQ 85
INH1-F MKILLFLMMFLAMLLVTKGKNNLVETTCKNTPNYNLCVRTLSLDKRSETAGDITTLALIMVDAIKSKANQAANTISKLRHSNPPQ 85
StInvInh1 MKIFIFLMMFLAMLIVTKGNNNLVETTCKNTPNYNLCVKTLSLDKRSETAGDITTLALIMVDAIKYKANQAANTISKLRHSNPPQ 85
StInvInh3 MKIFIFLMMFLTLVLVTNGINKLVETTCKNTPNYDLCVKTFSLDKRSETAGDIKTLALIMVDAIKSKANHAFSIISNLRHSSTPQ 85
SolyCIF MKILIFLIMFLAMLLVTSGNNNLVETTCKNTPNYNLCVKTLSLDKRSEKAGDITTLALIMVDAIKSKANQAANTISKLRHSNPPQ 85
Nt-inh1 MKNLIFLTMFLTILLQTNANN-LVETTCKNTPNYQLCLKTLLSDKRSAT-GDITTLALIMVDAIKAKANQAAVTISKLRHSNPPA 83
PhInvInh1 MKNVISLMFFLTIILQT-ANNNLVETTCKNTPNYQLCVKTLLSDKRSDT-GSITTLALIMVDAIKSKATQAATTISKLRHSNPPA 83
α5
α6
α7
INH1-A AWKDPLKNCAFSYKVILTASMPEAIEALTKGDPKFAEDGMVGSSGDAQECEEYFKAITIKYSPLSKLNIDVHELSDVGRAIVRNLL 171
INH1-B AWKDPLKNCAFSYKVILTASMPEATEALTKGDPKFAEDGMVGSSGDAQECEEYFKAITIKYSPLSKLNIDVHELSDVGRAIVRNLL 171
INH1-C AWKDPLKNCAFSYKVILTASMPEAIEALTKGDPKFAEDGMVGSSGDAQECEEYFKAITIKYSPLSKLNIDVHELSDVGRAIVRNLL 171
INH1-D AWKDPLKNCAFSYKVILTASMPEAIEALTKGDPKFAEDGMVGSSGDAQECEEYFKAITIKYSPLSKLNIDVHELSDVGRAIVRNLL 171
INH1-E AWKDPLKNCAFSYKVILTASMPEAIEALTKGDPKFAEDGMVGSSGDAQECEEYFKAITIKYSPLSKLNIDVHELSDVGRAIVRNLL 171
INH1-F AWKDPLKNCAFSYKVILTASMPEAIEALTKGDPKFAEDGMVGSSGDAQECEEYFKAITIKYSPLSKLNIDVHELSDVGRAIVRNLL 171
StInvInh1 AWKDPLKNCAFSYKVILTASMPEATEALTKGDPKFAEDGMVGSSGDAQECEEYFKAITIKYSPLSKLNIDVHELSDVGRAIVRNLL 171
StInvInh3 AWIHPLKECAFSYKVILTVSIPEAIEALTKGDPKFAEDAMVGTSGDAQECEDNFKS---KSPPLSKLNIDVHDLSDINRAIVRNLL 168
SolyCIF AWKDPLKNCAFSYKVILTASMPEAIEALTKGDPKFAEDGMVGSSGDAQECEEYFKATTIKYSPLSKLNIDVHELSDVGRAIVRNLL 171
Nt-inh1 AWKGPLKNCAFSYKVILTASLPEAIEALTKGDPKFAEDGMVGSSGDAQECEEYFKGS--K-SPFSALNIAVHELSDVGRAIVRNLL 166
PhInvInh1 AWKIPLKNCAFSYKVILTASLPEA---LTKGDPKFAEDGMVGSSGDAQECEVYFKGS--K-SPISALNLAVHELSDVGRAIVRNLL 163

Figure: 4.4- **Protein sequences comparison.** Predicted amino acid sequences of the apoplastic invertase inhibitor alleles aligned with that of previously published sequences from *S. tuberousm*, *S. lycopersicum*, *P. hybrida* and *N. tabacum* using Geneious Programme 5.4.6 and adjusted manually. Based on the published information, the predicted signal peptide, cleavage cites and secondary structures were assumed. The amino acids are represented by a single letter code. The protein sequences listed in the diagram are; *S. tuberosum* INH1A (AY864819), INH1B, C, D, E and F are identified in this study, StInvInh1 (GU321338) and StInvInh3 (GU321339) from *S. tuberosum* (Liu et al, 2010), *S. lycopersicum* SolyCIF (SGN- U317539), *P. hybrida* PhInvInh1 (SGN-U207999) and *N. tabacum* Nt-inh1 (Y12805). The predictions were performed for amino acid composition (Geneious Programme 5.4.6), signal peptide (http://www.cbs.dtu.dk/services/SignalP/) and molecular mass (Geneious Programme 5.4.6) while the predicted secondary structure was manually constructed and compared with that of previously published information from *N. tabacum* (Nt-inh1 - Greiner 1998, 1999; Nt-CIF - Hothorn 2004; SolCIF - Reca et al. 2008). The predicted peptide encodes 171 amino acids with a calculated molecular weight M_r ~ 18.8 kDa. The predicted signal sequence used for secretion into the apoplast is underlined. The arrow indicates the predicted site of cleavage, which is identical to the previously demonstrated invertase inhibitors from *S. tuberosum*, *N. tabacum* and *S. lycopersicum*. Gaps introduced for alignment are represented by dashes. Asterisks indicate identical residues. Amino acid numbers are listed on right. The numbers 1 and 2 at the bottom of the sequence block denote disulfide bridges connecting the conserved two pairs of cystine (yellow) residues. Conserved amino acids of the mature peptide are shaded in grey. The mutations are shown in orange. The symbols used for the secondary structure are α1 to α6 helix. Highly conserved PFK motif in the known invertase inhibitors (Hothorn et al 2010) is boxed.

4.3.4 Phylogenetic relationship analysis

A phylogenetic tree depicted in Figure 4.5 was constructed based on the protein sequences of the apoplastic invertase inhibitors identified in this study (INH1B, INH1C, INH1D, INH1E & INH1F) and several other apoplastic and vacuolar invertase inhibitors from potato, tobacco, tomato and *Petunia*. The tree clearly showed two major branches corresponding to the two classes of invertase inhibitors from *S. tuberosum*, *S. lycopersicum*, *P. hybrida* and *N. tabacum*. Group A, consists of apoplastic invertase inhibitor sequences identified in this study (INH1B, INH1C, INH1D, INH1E, INH1F) and three members from *S. tuberosum* (INH1A, StinvInh1 and StInvInh3), one from *S. lycopersicum* (SolyCIF), one from *N. tabacum* (Nt-inh1 – apoplastic inhibitor isoform Rausch and Greiner 2004) and one from *P. hybrida* (PhInvInh1). Group B consisted of vacuolar invertase inhibitors including nine members from potato (INH2α*A1, INH2α*A2, INH2β*B1, INH2β*B2, INH2β*B, INH2β*A1, INH2β*A2, INH2α*C and INH2α*D), one from tomato (SlInvInh2) and two members from *N. tabacum* (Nt-inhh – vacuolar inhibitor isoform and vacuolar invertase inhibitor). The sequences INH2β*B1, INH2β*B2 INH2β*A1 and INH2β*A2 were boxed mainly derived from hybrid mRNAs shows close identity with vacuolar invertase inhibitors from potato (Brummell et al. 2011).

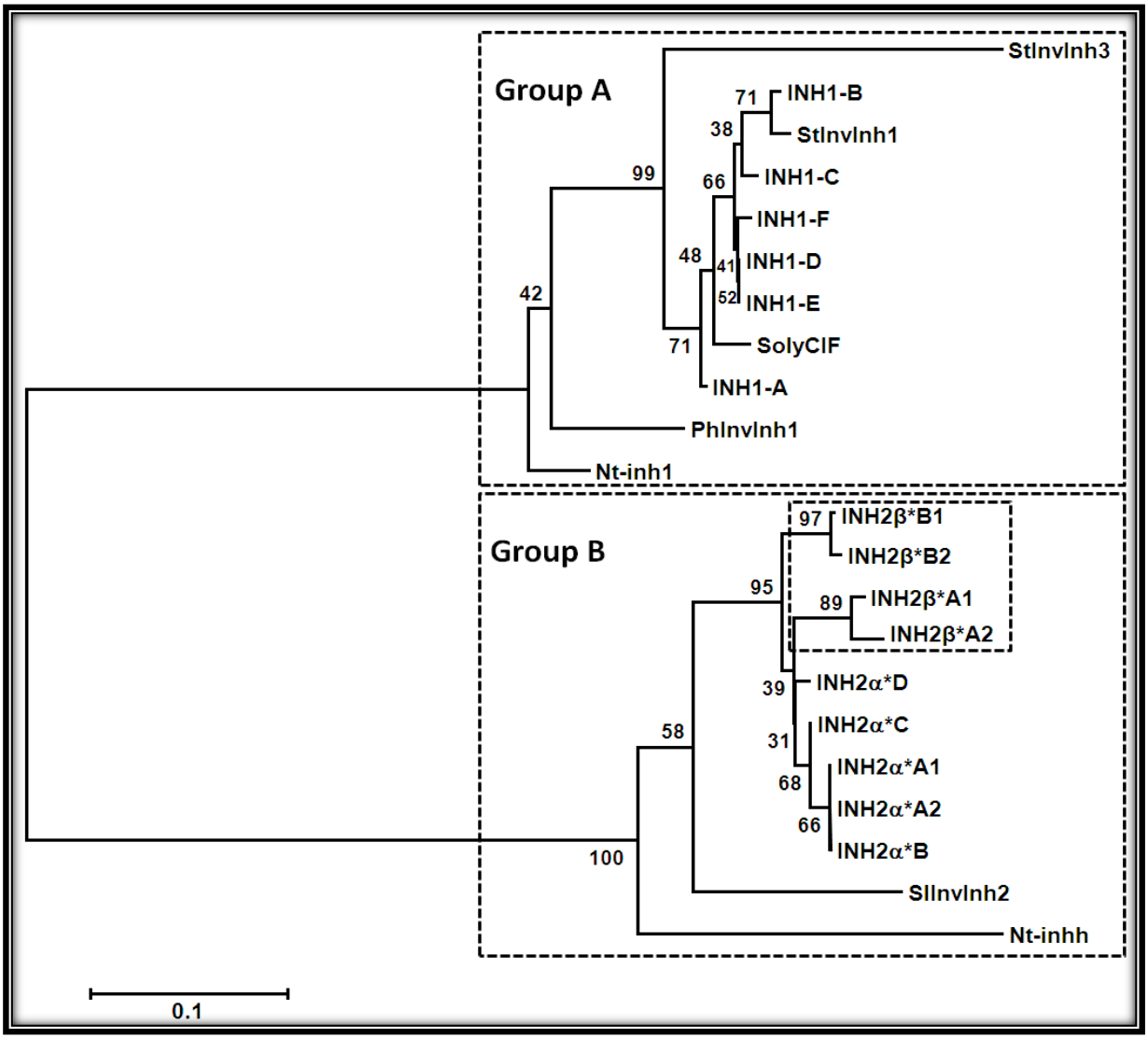


Figure 4.5

Figure 4.5: Phylogenetic tree based on amino acid sequences of invertase inhibitors. Phylogenetic tree is based on the apoplastic invertase inhibitor protein sequences identified in this study with invertase inhibitor protein sequences from *S. tuberosum*, *S. lycopersicum*, *P. hybrida* and *N. tabacum*. The tree was constructed in MEGA 5.05 program by neighbour-joining 1,000 bootstrap analysis. In the scale bar 0.1 marks 0.1 amino acid substitution per site, indicating 10 amino acid exchanges per 100 amino acids. Bootstrap values are indicated at each branch point. INH1B, INH1C, INH1D, INH1E and INH1F identified in this study. The protein sequences listed in the diagram are listed in the GenBank database (http://www.ncbi.nlm.nih.gov/) /SOL Genomics Network database (http://solgenomics.net/help/gene_search_help.pl.) under the following accession numbers:, SolyCIF (SGN-U317539) apoplastic invertase inhibitor from *S. lycopersicum*, StInvInh1 (GU321338) &, StInvInh3 (GU321339) from *S. tuberosum*, Nt-inh1 (Y12805) apoplastic invertase inhibitor from *N. tabacum*, PhInvInh1 from *P. hybrida* (SGN-U207999), vacuolar invertase inhibitors from *S. tuberosum* INH1A (AY864819), INH2β*B2 (GU980595), INH2β*B1, (GU980594), INH2α*A1 (FJ810205), INH2α*A2 (FJ810206), INH2α*B (FJ810207), INH2α*C (FJ810208), INH2α*D (FJ810209), INH2β*A1 (AY864821), INH2β*A2 (GU980593), *S. lycopersicum* (SGN-U332870), *N. tabacum* Nt-inhh (Y12806) and vacuolar invertase inhibitor (AY145781).

4.4 Discussion

In the present study, alleles encoding the apoplastic invertase inhibitor gene (*Stinh1*) isolated from three potato genotypes were characterised. This work is the first study on the structure and sequence polymorphism of the apoplastic invertase inhibitor gene in potato and has significantly increased the number of the apoplastic invertase inhibitor sequences available. A total five apoplastic invertase inhibitor alleles were identified, four of which were previously undescribed (*inh1-c*, *inh1-d*, *inh1-e* and *inh1-f*) including three variants displaying a unique substitution at the junction of the signal peptide and the mature protein (*inh1-d*, *inh1-e* and *inh1-f*). Allele diversity comparisons incorporating previously published apoplastic invertase inhibitor sequences from potato highlight the nature of polymorphisms among the various alleles of the *Stinh1* gene.

Primer design, cloning and sequencing

The primers used in this work were designed to the areas of sequence homology in the 5'UTR region of the three available apoplastic invertase inhibitor sequences from NCBI (GenBank accession numbers AY864819, AY864820 and GU980592). These primers proved capable of amplifying the apoplastic invertase inhibitor alleles with a range of fragments (Figures 4.1 & 4.2) from three genotypes used in this work. Evidence from allelic variants in other potato genes suggests that the number of alleles identified in this work is likely to be only a subset of the total alleles in potato. Recently Brummell et al. (2011) identified five alleles of *inh2α* (vacuolar invertase inhibitor) from sequencing of cDNA and genomic clones amplified from two potato cultivars (937/3 and 1021/1). As the primers described here were able to amplify five invertase inhibitor alleles they may prove useful to further studies aimed at isolating further *inh1* alleles from other potato cultivars.

DNA sequence structure and allelic polymorphism of apoplastic invertase inhibitor alleles

The diversity observed within the five alleles cloned in this study was similar both in distribution and amount to that described in other potato studies (Brummell et al. 2011). All the invertase inhibitor alleles sequenced in this study conformed to the general gene structure of other apoplastic invertase inhibitor genes (*SolyCIF* Reca et al. 2008; *StInvInh1*, *StInvInh3* Liu et al. 2010; *inh1-a*, *b* Brummell et al. 2011), with two exons interrupted by a hypervariable intron (Figures 4.1 & 4.3). To study the *SolCIF* (invertase inhibitor localised in apoplastic compartment) gene organisation, Reca et al. (2008) amplified a 1411 bp PCR fragment from genomic DNA and upon sequencing found that it contained two exons and one intron. Similar results reported by Brummell et al. (2011). Recently Liu et al. (2010)

observed two fragments, *StInvInh1* (1736 bp) and *StInvInh3* (1,191 bp) from potato genomic DNA containing two exons and one intron. These two alleles mainly varied in the intron region. In particular, the exons of all the alleles identified in this study are similar in size and sequence and confirms the well defined structure of the apoplastic invertase inhibitor. The consensus sequences at the exon-intron boundaries were 100% conserved (5'GT-3'AG on either side) in all the alleles and in full agreement with the GT-AG rule (Breathnach and Chambon, 1981). Other important features for intron splicing are the branch site and polypyrimidine tracts (as reviewed in Green 1991; Brown and Simpson 1998). Since the large deletion in the intron of alleles *c*, *d*, *e*, and *f* contains polypyrimidine tracts, these alleles may have compromised intron splicing that may influence their functionality.

Upstream and downstream elements

The 35 nucleotide 5' UTR was very highly conserved among all alleles. In contrast, the 3' UTR exhibited many SNPs and indels, especially in the first half of the 315 nucleotides recovered (Figure 4.3). Similarly Brummell et al. (2011) identified extensive differences among the 3'UTR of alleles of the *inh2α* gene. Holland et al. (2001) indicated that UTRs posses a greater potential for sequence length variability than translated regions and that the 5' UTRs may play an important role in gene expression, thus further sequencing of a greater length of the 5' UTR may be warranted.

Allelic variations in exons

Comparison of exons of all *StInh1* alleles revealed in total 65 single nucleotide substitutions, plus a nine nucleotide in frame deletion in one allele (*StInvInh3*). Of the 65 SNPs, 23 were silent and 42 led to a predicted change in an encoded amino acid (Figure 4.3, Table 4.2). These predicted changes were considered robust as 16 independent clones were sequenced for alleles *inh1-b*, *c* & *d* and 8 each in case of alleles e & f. All independent clones exhibit the same mutations. The non-synonymous SNPs in the exon regions may have functional significance as the changes in amino acid sequence may alter protein function and, as a consequence, phenotype (Jehan and Lakhanpaul 2006). The majority of SNPs (46) and the in-frame deletion were unique to the *Stinvinh3* allele (Liu et al. 2010). Given the high mutational load in this allele, it may be functionally deficient *in vivo*.

When the exon region of the alleles identified in this study was compared with that of exon region of *S. tuberosum*, *S. lycopersicum, P. hybrida* and *N. tabacum*, it revealed more than 90% pairwise sequence identity which indicates a highly conserved nature of the apoplastic invertase inhibitor across different members of the Solanaceae family. Most of

the observed amino acid substitutions in exon region were non synonymous (Figure 4.3). Chen et al. (2001) and Draffehn et al. (2010) demonstrated that the allelic variations of candidate genes operating in carbohydrate metabolism may cause functional variation that could also influence agronomic traits such as tuber starch and sugar content. In addition the studies conducted in Chapter 3 showed associations of candidate genes (Invertases, granule bound starch synthase and vacuolar invertase inhibitor) with CIS traits. Therefore, the observed allelic variations of the apoplastic invertase inhibitors could be utilised to design molecular markers that may show association with CIS. Also non-synonymous changes may further be utilised to design high resolution melting (HRM) assays that may correlate with phenotypic traits such as tuber starch and sugar contents. High resolution melting (HRM) analysis is an emerging molecular marker technology to detect genetic polymorphism between individuals based on SNPs (Gupta et al. 2001; Hayashi et al. 2004; Wu et al. 2008). The SNPs identified here may have important implications for the design of HRM markers to analyse potato mapping populations to define markers for use in breeding programmes and for biodiversity studies.

Allelic variations in intron

The intron region showed a much higher level of allelic diversity than the exon regions. This low conservation of the intron sequence between alleles (~66% nucleotide sequence identity) was primarily due to a large insertion in alleles *inh1-b* and *StInvInh1* compared with alleles *inh1-c*, *d*, *e*, *f,* the DM1-3 518 R44 genome sequence (*inh1-a*) and *StInvInh3* (Figure 4.2). A higher frequency of SNPs was observed in the intron region of the alleles identified in the present study. The location of the intron is identical to that of potato apoplastic invertase inhibitor gene (*inh1-b*- GU980592, Brummell et al. 2011). More frequent and random distribution of the SNPs was observed in the intron region of the alleles identified in the present studies. Recently Potato Genome Sequencing Consortium (PGSC 2011) has identified 3.67 million SNPs between DM clone and one or both haplotypes of RH clone. Random distribution of SNPs in the intron region is a very common feature that was also observed in other organisms (Li and Sadler 1991) including potato (Chen et al. 2001; Simko et al. 2006a). Therefore SNPs and indels can be utilised to study the allelic variants of the apoplastic invertase inhibitor gene that could be associated with the phenotypic variation in the population. In order to achieve this, allele specific markers can be developed based on the variations observed in the intron region. The SNPs and indels can be further analysed to differentiate different apoplastic invertase inhibitor alleles in “S” population

(Chapters 2 & 3). Furthermore association analysis can be carried out to detect whether there are any potential SNPs and indels from 1021/1 and Karaka are associated with CIS traits. Also, the length variation and the sequence repeats observed in the intron region of the alleles in the present study may regulate the expression of invertase inhibitor and this could be explored in future studies. Introns can regulate gene expression by enhancing transcription initiation, by increasing mRNA accumulation co-or post-transcriptionally, or by enhancing translational efficiency (LeHir et al. 2003). Introns have been observed to regulate gene expression in plants such as potato (Fu et al. 1995) and *Arabidopsis* (Casas-Mollano et al. 2006).

Another feature from the comparison of the intron region between five apoplastic invertase inhibitor alleles sequences is the nature of some of the insertions within one allele relative to another. Many of these insertions were perfect or imperfect repeats of an adjacent sequence. For example the repeat sequences observed at nt 482 – *inh1-f* (Figure 4.3) however such insertions in the introns of alleles may not have any apparent effect on gene expression. This variation between the intron of the alleles could provide a useful basis for assaying the presence of specific allelic variants of the apoplastic invertase inhibitor gene alleles in potato. Various assays such as high resolution melting (HRM) analysis and real time quantitative PCR to detect deletions would be designed to differentiate the alleles.

Protein comparison

For the comparison of protein sequences all the previously published sequences from *S tuberosum*, *S lycopersicum*, *P. hybrida* and *N. tabacum* were incorporated (Figure 4.4). The deduced amino acid sequence for the apoplastic invertase inhibitor proteins (INH1-B, INH1-C, INH1-D, INH1-E and INH1-F) established the same number of amino acids (171) with a calculated molecular mass of ~19 kD and is similar to the size of other apoplastic invertase inhibitors (SolyCIF – Reca et al. 2008; StInvInh1 – Liu et al. 2010; INH1-A – Brummell et al. 2011). StInvIn3, PhInvInh and Nt-inh1 differed in their number of amino acids and molecular mass, mainly due to indels and substitutions (Table 4.2, Figure 4.4).

Nt-inh1 from tobacco was found to be localised in the apoplast and acts as *in vitro* inhibitor of apoplastic isoform of acid invertase (Weil et al. 1994) which confirmed the apoplastic localisation of the identified proteins. In order to demonstrate this, they have extracted cell wall bound proteins from tobacco suspension cells. During cation-exchange chromatography they have detected a putative apoplastic invertase inhibitor in fractions with strong apoplastic invertase immunosignal but low or absent apoplastic invertase

activity. Brummell et al. (2011) produced a construct that fused the N-terminus quarter of INH-1 protein, including putative signal sequence to GFP (green fluorescence protein) and detected its presence in cytoplasm as well as in the cell wall of epidermal cells of both onion bulbs and lisianthus petals. Therefore based on these studies, it can be concluded that the identified sequences in the present study would target into the apoplast.

The removal of the predicted N-terminal end signal peptide generates a mature protein of 152 amino acids with a molecular weight of 16.6 kDa and a predicted pI ranging from ~ 6.5 to 8.0 (Table 4.3). An alkaline pI of ~8.0 (INH1-D, INH1-E and INH1-F) has been reported as a characteristic of cell wall bound proteins (SolyCIF, Reca et al. 2008). For INH1-D, INH1-E and INH1-F, three features of the mature protein (i.e. number of amino acids, molecular weight and pI) were identical to SolyCIF (Reca et al. 2008). INH1-A, INH1-B and INH1-C were found to differ in pI (Table 4.3) and exhibited similar features to Stinvinh1 and Stinvinh3 (Liu et al. 2010). These differences in isoelectric points result from slight differences in amino acid composition, suggesting that multiple isoforms may exist, based on variations in amino acid sequence. Whether these have functional significance remains to be established.

The predicted signal peptide and the cleavage site substantiate previous studies of apoplastic invertase inhibitors (Greiner et al. 1998; Reca et al. 2008; Liu et al. 2010; Brummell et al. 2011). The comparison confirms a 99.7% probability of a signal sequence with cleavage occurring between aa 19 and 20 and targets the mature protein to the apoplast. On the basis of an analysis of 65 eukaryotic and 20 prokaryotic signal sequences, von Heijne (1984) proposed that cleavage of signal sequences occurs at specific sites that are defined by the presence of preferred amino acids. The predicted cleavage site in the INH1 protein is in agreement with the -1/-3 rule (Von Heijne 1985).

The putative signal peptide of the apoplastic invertase inhibitor deduced from the nucleotide sequence shows a number of features which are common to the apoplastic inhibitors from *S. tuberosum* (Brummell et al. 2011; Liu et al. 2010), *N. tabacum* (Greiner et al. 1999) and *S. lycopersicum* (Reca et al. 2008). Firstly the putative signal peptide of 19 amino acid residues. The positively charged amino acid lysine (Lys) is adjacent to a methionine residue at the N terminal end of the signal peptide followed by a core of hydrophobic amino acids. The common structure of signal peptides from various proteins is described by von Heijne (1983, 1985) and indicates a positively charged n-region, followed by a hydrophobic h-region and a neutral but polar c-region. The eukaryotic h-regions are

dominated by Leu with some occurrence of Val (V), Ala, Phe (F) and Ile (I) (Nielsen et al. 1997). INH1A showed three non-synonymous substitutions (Figure 4.4, Table 4.2) with the sequences identified in this study. Similarly a number of amino acid substitutions were observed in the signal peptide of StInvInh3 (Figure 4.4). Therefore these substitutions may affect the function of signal peptide. However, functional significance of these changes remains to be elucidated. Further comparison of identified protein sequences in the present studies revealed only one peptide change occurred in the signal peptide, namely, Ile15→Leu substitution in INH1D, INH1E and INH1F (Figure 4.4). As isoleucine and leucine are both hydrophobic amino acids and are located in the putative h-region in the present studies it is unlikely that this substitution will affect the function of the signal peptide.

The amino acid substitutions observed in the N-terminal part of the protein sequences from different potato cultivars may affect the function of the signal peptide (Figure 4.4). In INH1-D, INH1-E and INH1-F a change from asparagine (neutral amino acid) to lysine (positively charged amino acid) at aa 20 caused by a single nucleotide change coincides with the junction of the signal peptide and the mature protein (Figure 4.4). This may exert an effect on signal function and may interfere with cleavage by signal peptidases. In the case of StInvInh3 the neutral asparagine at this position is replaced by isoleucine, an uncharged amino acid. Further studies on the protein expression and cellular localisation of these alleles are warranted. This can be achieved by apoplastic targeting of the proteins sequences of INH1B, INH1C, INH1D, INH1E and INH1F fused with GFP (green fluorescent protein) into potato leaves.

The predicted protein sequences identified here displayed more than 70% sequence identity to the N-terminal end portion of the mature protein (first 20 amino acids of the mature protein) and also displayed some amino acid variations with respect to *S. lycopersicum* invertase inhibitor purified by Pressey (1994). However, Reca et al. (2008) purified the invertase inhibitor (SolyCIF) from *S. lycopersicum* and after comparing the N-terminal end sequence suggested the presence of multiple isoforms in *S. lycopersicum* and concluded that the amino acid microheterogeneities or the variations in the amino acids may be due to allelic differences between inhibitors originating from different *S. lycopersicum* cultivars. Similarly Liu et al. (2010) suggested that amino acid microheterogeneties may be due to allelic differences between two inhibitors (StInvInh1 & StInvInh3) originating from different potato genotypes. Therefore, it can be hypothesised

that being highly heterozygous multiple isoforms of the apoplastic invertase inhibitors may reside in the same cultivars as well as between cultivars.

Hothorn et al. (2004a) presented a structural model of the apoplastic invertase inhibitor from tobacco that reveals an asymmetric four-helix bundle (α4 to α7) which is preceded by an N-terminal extension forming a helical hairpin along with an additional small helix (Figure 4.4). The positions of two cysteine residues (Cys-28 & Cys-37) were observed in the α1 and α2 helices while two other cysteines (Cys-94 & Cys-135) were linking helix α5 & α6. These four conserved cysteine residues are characteristic of plant invertase inhibitors and are involved in the disulfide bridge formation (Hothorn et al. 2004a; Rausch and Greiner 2004). Two disulfide bridges connecting the α5 and α6 helices as well as the α1 and α2 helices in the N-terminal end of a hairpin module contribute to the structural stabilisation of the protein (Hothorn et al. 2004a, 2004b). In addition to these four conserved cysteine residues, the well conserved N-terminal end helical hairpin extension is crucial to the structural integrity of the protein and the conserved C-terminal domain is thought to contribute to the interface stabilisation of the protein (Hothorn et al. 2004a).

Several amino acid substitutions were observed in the mature protein of the apoplastic invertase inhibitor alleles from potato (Figure 4.4). However, the functional significance of these amino acid substitutions remains unclear and therefore further research is required to confirm any alterations in protein function as a result of these amino acid substitutions. Hothorn et al. (2010) studied the formation of and interaction in the complex between *Arabidopsis* cell wall invertase 1 (INV1) and a protein inhibitor (CIF, or cell wall inhibitor of β–*fructosidase*) from tobacco. Their results suggest that the conserved amino acid motif PKF in CIF directly targets the invertase active site. This highly conserved PFK motif in the known invertase inhibitors (Hothorn et al 2010), is also conserved in the potato INH1 alleles (Pro118-Lys119-Phe120, Figure 4.4). In addition to these amino acids, the adjacent conserved Gly and Ala (Gly116 and Ala121 in Figure 4.4) have been shown to allow the insertion of consecutive loop residues into the INV1 active site cleft (Hothorn et al 2010). Khokhlova and Nesmeyanova (2003) demonstrated that not only the signal peptide, but also the downstream region corresponding to the N terminus of the mature protein is involved in the export-initiating domain and interacts with the secretory machinery in *E. coli*. Therefore it is possible that the mutations occurred in the helices which may result in destabilisation of N and C terminus regions and affect the further processing of the protein. However, further

research is required to prove any alterations in protein function as a result of these amino acid substitutions.

Amino acid sequences were checked for N-glycosylation sites. The consensus sequence Asn-X(any aa)-Ser/Thr for glycosylation was absent in all amino acid sequences of all apoplastic invertase inhibitors alleles, a key feature of apoplastic invertase inhibitors (Weil et al. 1994; Reca et al. 2008) which further substantiates that the putative amino acid sequences encodes the apoplastic invertase inhibitor.

Phylogenetic analysis

Invertase inhibitor genes can be classified into two groups either vacuolar or apoplastic depending upon their cellular localisation (Reca et al. 2008; Liu et al. 2010). The Phylogenetic tree presented in Figure 4.5 clearly differentiates between group A) apoplastic invertase inhibitors and group B) vacuolar invertase inhibitors. Similarities between the apoplastic invertase inhibitor protein sequences identified in the present study and that of previously isolated from *S. tuberosum, S. lycopersicum, P. hybrida* and *N. tabacum*, showed a strong relatedness and revealed more than 90% pairwise sequence identity which suggests the apoplastic targeting of the proteins. Therefore, it suggests that the protein sequences of invertase inhibitors may have evolved slowly and originated from the same ancestor gene and therefore the evolutionary conservation of this protein makes possible the study of similarities and differences between organisms which are closely related. The invertase inhibitor genes could have diverged into apoplastic and vacuolar forms following gene duplication. Support for this hypothesis comes from the potato genome sequence (The Potato Genome Sequencing Consortium, 2011); the vacuolar and apoplastic invertase inhibitors are located on chromosome XII in a tandem orientation, with the vacuolar invertase inhibitor 5.5 kb upstream of the apoplastic invertase inhibitor, without any intervening genes (Brummell et al. 2011).

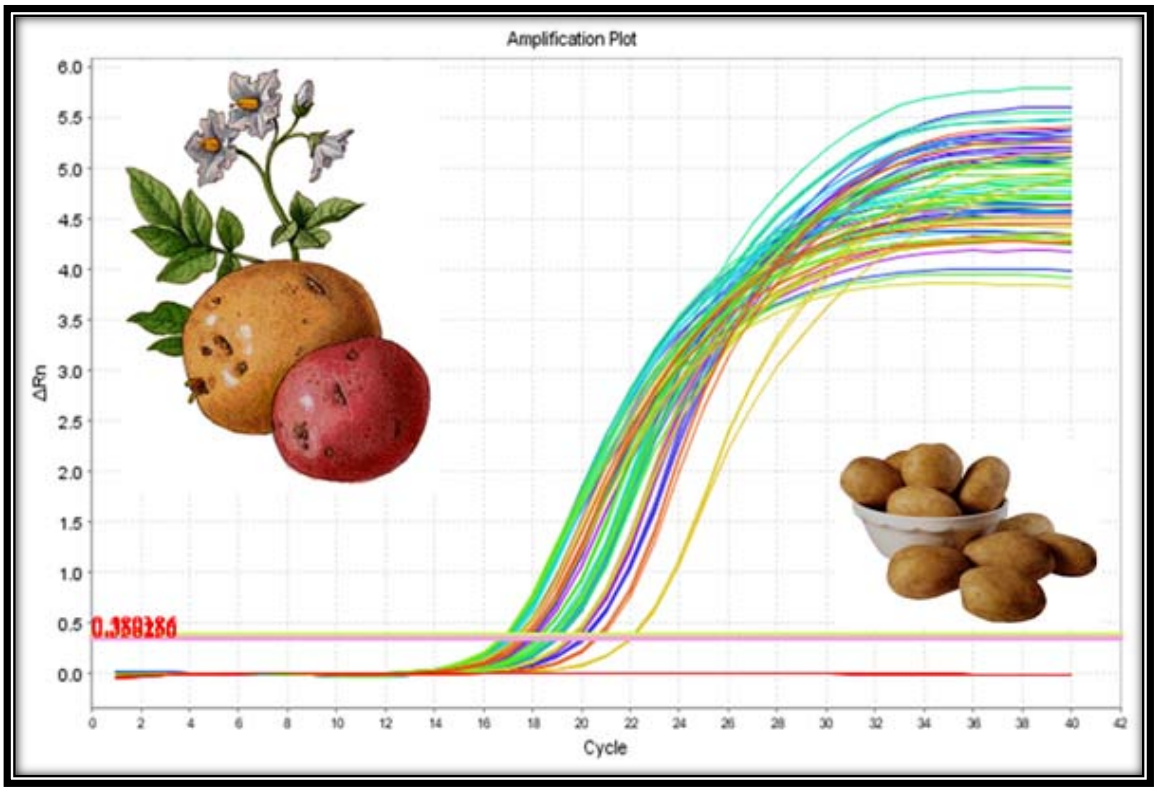
Identification of suitable alleles for transformation

The results obtained in the present study are of immense importance. The identification of genetic polymorphism in the apoplastic invertase inhibitor alleles raises the possibility that alleles of this gene may contribute to the phenotypic variation in response to cold-induced sweetening among the potato genotypes with these various alleles. The results on genetic polymorphism of candidate genes that associated with phenotypic variation in CIS have already been presented in Chapter 3. In contrast to 1021/1, which shows the highest resistance to cold sweetening, Karaka and Summer Delight are both known to be susceptible

to cold induced sweetening. A key approach to further understand the control and regulation of the apoplastic invertase inhibitor in potato, involves the overexpression of the apoplastic invertase inhibitor alleles in transgenic potato plants. The cloning and sequencing of the complete genomic sequence of all five alleles herein has provided a unique sequence resource for further characterisation of the relationship between diversity and function of the potato invertase inhibitor gene and determine the role of specific alleles in the cold-sweetening process of tubers. The present findings revealed that *inh1-b* is the most similar to previously reported sequences, whereas *inh1-c, inh1-d, inh1-e* and *inh1-f* are more divergent alleles. As *inh1-b* is the most common allele, it is therefore a key candidate for genetic transformation studies. Alleles *d*, *e* and *f* have an amino acid substitution at the junction of the signal peptide and mature protein at the first aa at the N-terminus of the mature peptide (Figures 4.3, 4.4). To study the possible functional consequence of this key substitution, allele *d* is an excellent candidate for genetic transformation because it originates from 1021/1, the genotype with the best resistance to cold-induced sweetening. Allele *c* is a third good candidate as it had several point mutations in the exon region which altered the amino acid sequence. Three alleles, namely *inh1-b* (common allele), *inh1-d* (from 1021/1), and *inh1-c* (Summer Delight), are therefore selected to test whether their overexpression results in differing responses to cold induced sweetening (Chapter 5). The activity of invertase will be reduced by overexpression studies while the complete knock down of apoplastic invertase inhibitor gene will be conducted in order to evaluate the role and the necessity of specific apoplastic invertase inhibitory proteins in transgenic potato plants in both types of genotypes.

Chapter 5

Overexpression and antisense repression of apoplastic invertase inhibitor gene in transgenic potato tubers



Chapter 5

Overexpression and antisense repression of the apoplastic invertase inhibitor gene in transgenic potato tubers

5.1 Introduction

Invertase plays an important role in the cold-induced sweetening process of potato tubers by irreversible conversion of sucrose into hexoses i.e. glucose and fructose. The resultant hexoses react in a Maillard type reaction with free amino acids at high frying temperatures (Mottram et al. 2002) and produce dark brown unacceptable crisp colour. Invertase activity (apoplastic and vacuolar) may be controlled post-translationally *in planta* by small specific invertase inhibitory proteins known to be present in potato tubers (Schwimmer et al. 1961; Pressey 1966, 1967; Pressey and Shaw 1966; Rausch and Greiner 2004). Like their target counterparts, these small inhibitory proteins, may be localised in the apoplast or vacuole (Krausgrill et al. 1998; Greiner et al. 1998).

Studies have demonstrated that the regulation of vacuolar invertase by vacuolar invertase inhibitor is an effective approach to inhibit cold sweetening of potato tubers (Greiner et al. 1999; Cheng et al. 2007). Overexpression of a putative tobacco vacuolar invertase inhibitor reduces hexose accumulation in potato tubers by 75% (Greiner et al. 1999). Likewise up to 77% reduction in reducing sugars was observed in transgenic lines expressing vacuolar invertase inhibitor from tobacco under the control of tuber class I patatin promoter (Cheng et al. 2007). Liu et al. (2010) studied the expression profiles of both apoplastic and vacuolar invertase inhibitors from different potato genotypes and concluded that expression of vacuolar rather than apoplastic invertase inhibitor explained the control of CIS process of potato tubers. Brummell et al. (2011) reported the presence of *inh1* (apoplastic invertase inhibitor), *inh2*α (vacuolar invertase inhibitor) and *inh2*β (hybrid mRNA) mRNAs in 1021/1 cold-stored potato tubers while cold-susceptible Karaka and Summer Delight had both *inh1* and *inh2*α.

Recently, Baldwin et al. (2011) conducted an association mapping studies for CIS based on 161 potato lines and eight candidate gene markers (citrate synthase - *Cis*, potato acid invertase *Pain-1*, sucrose synthase *Sus4*, apoplastic invertase - STM1051, β-amylase - *Bmy*, apoplastic invertase inhibitor – *inh1*, vacuolar invertase inhibitor *Stinh2* and UDP-glucose pyrophosphorylase - *ugp*). This study revealed that only the allelic variations in the UDP-glucose pyrophosphorylase and apoplastic invertase genes were significantly associated

with CIS. In addition the interaction between STM1051 and *inh1* in cold-tolerant potato lines demonstrated that the lines with presence of the apoplastic invertase allele *Stm1051-181* were more likely to be cold tolerant if they also had a specific apoplastic invertase inhibitor allele (*$StInh_{ap}$-a).* There is strong evidence suggesting that the vacuolar invertase inhibitor controls the activity of invertase in cold-stored potato tubers (Greiner et al. 1999; Brummell et al. 2011). In contrast little is known about the role of the apoplastic invertase inhibitor in CIS process. Therefore attempts were made to investigate the allelic diversity of the apoplastic invertase inhibitor gene from three different potato genotypes with varying resistance to CIS (Chapter 4). Based on these results the hypothesis was proposed that the functional changes observed in various alleles of the apoplastic invertase inhibitor gene (Figures 4.3, 4.4) play an important role in the post-translational modification of invertase activity in cold-stored potato tubers. For this reason three potato genotypes with varying resistance to CIS were transformed with sense and antisense constructs containing alleles of the apoplastic invertase inhibitor gene. The complete inhibition of CIS may be possible if there is a sufficient quantity of the inhibitor protein. Therefore, the overexpression experiment might increase the CIS resistance in susceptible genotypes by inducing specific invertase inhibitory proteins in transgenic potato plants. The antisense repression of the apoplastic invertase inhibitor gene in resistant genotype may reduce the levels of inhibitory proteins in transgenic potato plants and may assist in elucidating its possible role in CIS.

The specific objectives of this chapter were to (i) constitutively overexpress and knock down the expression of apoplastic invertase inhibitor alleles in 1021/1, Karaka and Summer Delight (ii) perform the molecular characterisation of transformed potato plants and (iii) perform the phenotypic assessment of tubers for cold-induced sweetening, crisp quality and carbohydrate/sugar composition.

5.2 Materials and methods

5.2.1 Vector construction

Binary vectors used in this experiment were constructed and obtained from Julie Latimer, The New Zealand Institute for Plant & Food Research Ltd, New Zealand. Four binary vectors were constructed containing full length genomic clones of three apoplastic invertase inhibitor alleles (*inh1-b*, *inh1-c* and *inh1-d*) in the sense orientation and one (*inh1-b*) in the antisense orientation. Each genomic sequence of *inh1-b*, *inh1-c* and *inh1-d* was excised from pGEMT Easy with *Eco*RI. 5' and 3' overhanging ends were removed to create a blunt fragment which was cloned into the *Sma*I site of pART7 (Gleave 1992) to clone them between the 35S promoter of the cauliflower mosaic virus (CaMV 35S) and the *ocs* 3' terminator region. The four 35S-*Inh1-ocs* 3' cassettes were then excised from pART7 with *Not*I and cloned into the *Not*I site of pART27 (Gleave 1992) to produce three binary vectors (1) pART27-*inh1-*c_s, (2) pART27-*inh1-*d_s, (3) pART27-*inh1-*b_s overexpressing *inh1-c*, *inh1-d* and *inh1-b* respectively in sense orientation, and the fourth binary vector (4) pART27-*inh1-*$b_{a/s}$ expressing *inh1-b* in an antisense orientation. The binary vectors were individually transferred to *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) by freeze-thaw method (Höfgen and Wilmitzer 1988). The *pnos-nptII-nos3'* chimeric gene on the binary vectors conferring resistance to kanamycin was used as a selectable marker. Figure 5.1 shows the schematic representation of the binary vectors used for transformation.

5.2.2 Plant material

Virus-free potato plants (*Solanum tuberosum* L., genotypes 1021/1, Karaka and Summer Delight) were multiplied *in vitro* on a multiplication medium consisting of MS salts and vitamins (Murashige and Skoog 1962) supplemented with 40 mg l^{-1} ascorbic acid, 500 mg l^{-1} casein hydrolysate, 30 g l^{-1} sucrose, and 7 g l^{-1} agar (Conner et al. 1991). The agar was added after pH was adjusted to 5.8 with 0.1 M KOH, then the medium was sterilised by autoclaving at 121°C for 15 min. Aliquots of 50 ml were dispensed into pre-sterilised plastic containers (80 mm diameter x 50 mm high; Alto Packaging Ltd., Hamilton, New Zealand). Plants were routinely sub-cultured as two to three node segments every 3-4 weeks and incubated at 26°C under cool white fluorescent lamps (80-100 µmol m^{-2} s^{-1}; 16-hr photoperiod).

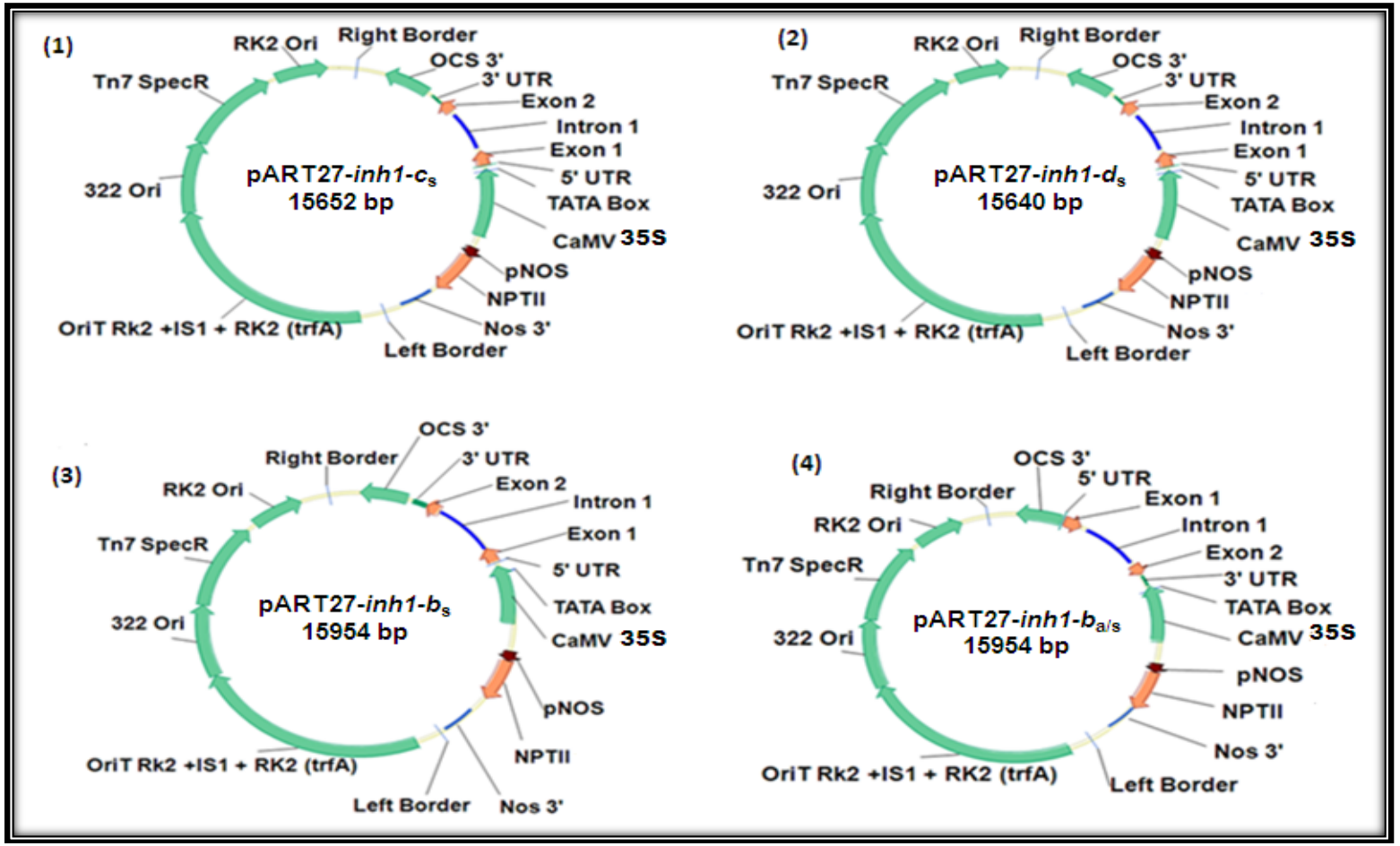


Figure 5.1: Schematic representations of four binary vectors. Three binary vectors (1) pART27-*inh1-c*$_s$, (2) pART27-*inh1-d*$_s$, (3) pART27-*inh1-b*$_s$ are overexpressing *inh1-c*, *inh1-d* and *inh1-b* in sense orientation respectively while the fourth binary vector (4) pART27-*inh1-b*$_{a/s}$ is expressing *inh1-b* in an antisense orientation. NPTII: neomycin phosphotransferase type II coding region; CaMV35S: promoter from cauliflower mosaic virus; OCS 3': terminator of octopine synthase; pNOS: promoter of nopaline synthase; NOS 3': terminator of nopaline synthase; 322 Ori: origin of replication for propagation in *E. coli*; RK2 Ori: origin of replication for propagation in *Agrobacterium*; Tn7 SpecR: transposon Tn7 DNA fragment encoding a 3"(9)-O-nucleotidyltransferase, an aminogiycoside-modifying enzyme, which mediates bacterial resistance to spectinomycin, 5' UTR – five primer untranslated region, 3' UTR - three prime untranslated region, TATA box, Exon1, Exon2, Intron 1, OriT Rk2 + IS1 + Rk2 (trfA): trfA expression cassette.

5.2.3 Potato transformation

For the transformation of 1021/1, Karaka and Summer Delight, 12 vector/potato genotype combinations were made (four constructs and three genotypes). *A. tumefaciens* strain EHA105 harbouring one of the binary vectors were cultured overnight at 28°C, on a rotary shaking table (180 rpm), in Luria Broth medium (Appendix A) supplemented with 300 mg l^{-1} spectinomycin. Bacterial suspension cultures reaching OD_{600} (optical density at 600 nm) of 0.2 - 0.6 were used for co-cultivation. Fully expanded leaves from *in vitro* grown 1021/1, Karaka and Summer Delight genotypes were excised, cut in half across midribs, while submerged in the liquid *A. tumefaciens* culture for approximately 30 s. These leaf segments were blotted dry on sterile filter paper (Whatman® No. 1, 100 mm diameter) and 10 leaf segments per Petri dish were placed on callus induction medium (multiplication medium supplemented with 0.2 mg l^{-1} naphthaleneactic acid and 2 mg l^{-1} benzylaminopurine) in standard plastic Petri dishes (9 cm diameter x 1 cm high) under reduced light intensity (5-10 µmol m^{-2} s^{-1}) by covering the Petri dishes with white paper. After two days, the leaf segments were transferred to callus induction medium supplemented with 200 mg l^{-1} Timentin™ to prevent *Agrobacterium* overgrowth. Five days later, they were transferred onto the same medium further supplemented with 100 mg l^{-1} kanamycin in order to select for transformed cell colonies. Approximately three to six weeks later, individual kanamycin resistant cell colonies (0.5-1 mm diameter), developing on the leaf segments were excised and transferred (50 per Petri dish) on to a regeneration medium (multiplication medium with sucrose reduced to 5 g l^{-1}, plus 1.0 mg l^{-1} zeatin and 5 mg l^{-1} gibberellic acid (GA_3), supplemented with 200 mg l^{-1} Timentin™ and 50 mg l^{-1} kanamycin) in plastic Petri dishes (9 cm diameter x 2 cm high).

The cell colonies were cultured under low light intensity (30-40 µmol m^{-2} s^{-1}) until shoots regenerated. Individual cell colonies, each with regenerated shoots, were transferred to potato multiplication medium containing 100 mg l^{-1} Timentin™ in plastic containers as described above. Thereafter healthy and well formed shoot clumps were excised and transferred to multiplication medium containing 100 mg l^{-1} Timentin™ and 50 mg l^{-1} kanamycin. Individual shoots (one per original shoot clump) that rooted readily in the kanamycin-supplemented medium were labelled and further sub-cultured onto potato multiplication medium with 100 mg l^{-1} Timentin™ for micro-propagation in plastic containers as described above. All antibiotics, zeatin and gibberellic acid were filter sterilised using 0.2

µm cellulose acetate filter and added, as required, just prior to dispensing the media into culture vessels or Petri dishes.

5.2.4 PCR screening of putative transformed lines

Genomic DNA extraction

Healthy leaves were collected from putative transgenic lines and control plants into 1.5 ml microfuge tubes (approximately three leaves per tube) from *in vitro* plants. Microfuge tubes were snap frozen in liquid nitrogen and stored at -80°C until use. Total genomic DNA was isolated using the cetyl trimethyl ammonium bromide (CTAB) extraction method (Doyle and Doyle 1990) with slight modifications. DNA was extracted from stored shoots by grinding to a fine powder using a pestle under liquid nitrogen. Five hundred µl of preheated (65°C) extraction buffer (Appendix A) was added and gently mixed. Samples were incubated at 60°C in a water bath for one hr and allowed to cool at room temperature. Chloroform/sec-octanol (24:1 v/v) solution (0.5 ml) was added and the sample inverted 20 times. The sample was centrifuged (Eppendorf, 5810R, Hamburg, Germany) at 10,621 x g for five min at min at 4°C. The upper aqueous phase was transferred to a fresh 1.5 ml microfuge tube and then 50 µl of 3 M sodium acetate (pH 5.2) and 500 µl ice cold (-20°C) isopropanol was added. The microfuge tube was inverted gently to precipitate DNA and centrifuged for 10 min at 20,817 x g at 4°C. The DNA was washed using 500 µl of 70% ethanol. The samples were centrifuged for 5 min at 20,817 x g at 4°C. After centrifugation the excess ethanol was pipetted out. The DNA was air dried at room temperature until the pellet turns white (15-20 min) and resuspended in 50 µl of TE^{-4} (Appendix A) and stored at -20°C.

PCR screening of putative transgenic lines

PCR analysis was performed from the putative transgenic lines to confirm the presence of the transgene, the *npt-II* gene and the endogenous potato *actin* gene. For this purpose three individual PCRs were performed.

a) Screening for the transgene in constructs (1), (2) and (3) [expected product size 1496 bp (1), 1445 bp (2) and 1758 bp (3)]: - The primers used to amplify the transgene from constructs (1), (2) and (3) were forward primer ($^{5'}$CCACTATCCTTCGCAAGACC$^{3'}$) and reverse primer ($^{5'}$ACCAGAAGAACCAACCATTC$^{3'}$). The forward primer was designed to bind to the 35S promoter region and the reverse primer was designed to bind to the apoplastic invertase inhibitor gene. Each 10 µl reaction mix included 1 µl of 10X ThermoPol buffer (100 mM KCl, 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5% Tween 20, 0.5% NP-40 and 50% glycerol), 200 µM each of dATP, dCTP, dGTP, dTTP, 0.1 µl (0.5 U) Taq DNA polymerase (5

U μl^{-1}, BioLabs, New England), 0.1 µl of each primer (stock 100 µM), 1.0 µl genomic DNA (10-50 ng). The PCR was performed on a Mastercycler (Eppendorf, Hamburg, Germany) as follows: four min initial denaturation at 94°C followed by 39 cycles of 30 s at 94°C, 30 s at 57°C, 120 s at 72°C and a final extension of five min at 72°C. Amplified products were separated by electrophoresis in a 1% agarose gel in 1X TAE buffer (Appendix A) at 5.5 V cm^{-1} for 80 min and visualised under UV light after staining with ethidium bromide (1 mg l^{-1} , Appendix A) for 15 min.

b) Screening for the antisense gene in construct (4) (product size 600 bp): -The primers used to amplify the transgene from construct (4) were forward primer ($^{5'}$CCACTATCCTTCGCAAGACC$^{3'}$) and reverse primer ($^{5'}$CAGCAAGTATGCCAGAAGCAACAG$^{3'}$). The reverse primer was targeted to the apoplastic invertase inhibitor gene. Each 10 µl reaction mix and PCR conditions were as described above except that the annealing step was 30 s at 60°C and the extension was 40 s at 72°C. Amplified products were separated by electrophoresis in a 1% agarose gel as described above (section a).

c) *npt-II* (kanamycin resistant) gene with a product size of 612 bp: - The primers used to amplify the *npt-II* gene from all four [(1), (2), (3) and (4)] constructs were the *npt-II* forward primer ($^{5'}$ATGACTGGGCACAACAGACAATCGGCTGCT$^{3'}$) and *npt-II* reverse primer ($^{5'}$CGGGTAGCCAACGCTATGTCCTGATAGCGG$^{3'}$). Each 10 µl reaction mix and the PCR conditions were as described above, except for an annealing step of 20 s at 60°C and extension step of 45 s at 72°C. Amplified products were separated by electrophoresis in a 1% agarose gel as described above.

d) *Actin* gene with a product size of 1069 bp: - The primers used to amplify the actin gene from all four [(1), (2), (3) and (4)] constructs were the *actin* forward primer ($^{5'}$GATGGCAGAAGGCGAAGATA$^{3'}$) and *actin* reverse primer ($^{5'}$GAGCTGGTCTTTGAAGTCTCG$^{3'}$). Each 10 µl reaction mix and the PCR conditions were as described above, except for an annealing step of 30 s at 58°C and extension step of 1 min 10 s at 72°C. Amplified products were separated by electrophoresis in a 1% agarose gel as described above.

5.2.5 Growth room trial

All confirmed transgenic lines (T0) and non transformed control plants were transferred to the BIOTRON (controlled growth room) Facility at Lincoln University, New Zealand (February 2010). Growth conditions were 70% humidity 16-h day/8-hr night, with a 19°C/15°C day/night temperature differential, CO_2 max 2,000 ppm, and light (max. 1150 $\mu mol.m^{-2}.s^{-1}$). Two plants were transferred from tissue culture in each of three PB¾ bags (64

mm x 64 mm x 150 mm black polythene bags) per line, with each PB¾ bag treated as a replicate. The potting mix consisted of 60% shredded pine bark, 20% crusher dust, 20% soil (supplemented with lime and slow release fertiliser (Conner et al. 1994). Tubers were harvested (June 2010) after natural senescence and immediately transferred into brown paper bags and stored in cool dark storage (temperature 6°C).

5.2.6 qRT-PCR analysis

qRT-PCR analysis was performed to test for the relative abundance of transcripts from the transgene in the leaves in T0 plants. Total RNA was isolated from these T0 plants and non transgenic control plants from approximately 30 mg of leaf material using an Illustra® RNAspin mini RNA isolation kit (GE Healthcare, Bucks, UK) following manufacturer's instructions. Healthy, youngest, fully expanded leaves were collected from each replication and pooled into a 1.5 ml microfuge tube (three leaves per tube). The microfuge tubes were snap frozen in liquid nitrogen and stored at -80°C until use. RNA was extracted by disrupting leaf tissue in SilamatS5 amalgamator (Invoclar Vivadent, NY, USA) for 10 s. The cell lysis was performed by adding 350 µl RA1 buffer (GE Healthcare, Bucks, UK) and 3.5 µl β-mercaptoethanol to the cell pellet and then mixing by vigorous vortexing (Julabo, Paramix 3, Munich, Germany). The lysate was transferred into RNA mini filter units and the mixture was centrifuged (Sigma 1-14 microcentrifuge, Bucks, UK) at 11,000 x g for one min. To this mixture, 350 µl 70% ethanol was added and mixed by vortexing the microfuge tubes twice for 5 s. The lysate was then transferred to a mini spin column (RNAspin mini RNA isolation kit, GE Healthcare, Bucks, UK) placed in two ml microfuge tube and centrifuged at 8,000 x g for 30 s. Following the addition of 350 µl MDB (Membrane Desalting Buffer, GE Healthcare, Bucks, UK) the column was centrifuged at 11,000 x g for one min to dry the membrane. After the flow was discarded, any DNA was digested by adding 95 µl DNase I (10 µl reconstituted DNase I to 90 µl DNase reaction buffer, GE Healthcare, Bucks, UK) to the column. The reaction was incubated at room temperature for 15 min. To wash and dry the membrane, 200 µl RA3 buffer (GE Healthcare, Bucks, UK) was added on to the column and centrifuged for one min at 11,000 x g. The column was transferred into a new collection tube and 600 µl RA3 buffer was added. The mixture was centrifuged at 11,000 x g for one min. Following the addition of 250 µl RA3 buffer, the column was centrifuged for two min at 11,000 x g to dry the membrane completely. To elute the RNA, 50 µl RNase-free and DNase-free sterile H_2O (GE Healthcare, Bucks, UK) was added and centrifuged at 11,000 x g for one min. RNA was quantified by spectrophotometry using a ND-1000 Spectrophotometer (NanoDrop

Technologies, DE, USA) and stored at -20°C until use. The samples were run on 1% agarose gel in 1X TAE buffer (Appendix A) at 5.5 V cm^{-1} for 40 min and visualised under UV light after staining with ethidium bromide (1 mg l^{-1}) for 15 min.

5.2.6.1 First strand cDNA synthesis

Total RNA (one µg) was reverse-transcribed to first strand cDNA using Superscript®VILO™ cDNA synthesis kit (Invitrogen, CA, USA) following manufacturer's instructions. Each 20 µl reaction included four µl of 5X VILO™ reaction mix (random primers, $MgCl_2$, and dNTPs), two µl of 10X Superscript®VILO™ enzyme mix (Superscript® III RT, RNaseOUT™ Recombinant ribonuclease inhibitor, and a proprietary helper protein), one µg RNA and DEPC-treated water. The reaction was incubated at 42°C for 60 min followed by 85°C for five min. The cDNA was stored at -20°C until further use.

5.2.6.2 Quantitative real time RT-PCR analysis

Two pairs of primers, one of which amplifies the *inh1* gene (forward primer, $^{5'}$AGGCATTCTAATCCTCCTCAAGC$^{3'}$ and a reverse primer $^{5'}$GCAGAAGATGGAATGGTTGGTT$^{3'}$) and another that amplifies a fragment of the internal reference gene elongation factor 1-alpha (EF-1α, forward primer $^{5'}$ATTGGAAACGGATATGCTCCA$^{3'}$ and reverse primer, $^{5'}$TCCTTACCTGAACGCCTGTCA$^{3'}$) were designed using Primer Express® Software Version 3.0 (Applied Biosystems, VIC, Australia). These primers were used to amplify the transcript levels of the apoplastic invertase inhibitor in the transgenic lines derived from overexpression [constructs (1) to (3)] and antisense repression [construct (4)] experiments. As the coding region of all the alleles was highly similar (98.1%), it was not possible to design the primers to detect the transcript levels of the individual alleles. For this reason the transcripts were amplified with the *inh1* gene primers that do not distinguish between the endogenous apoplastic invertase inhibitor transcripts and transcripts of the transgenes. In case of overexpression [constructs (1) to (3)] experiments the transcript levels were detected as summation of the endogenous apoplastic invertase inhibitor as well as the transgene. In order to measure the antisense expression, the values for *inh1* are derived by measuring a decrease of endogenous *inh1* expression. Every reaction was performed in triplicate per sample and results for gene expression were averaged. Each 20 µl PCR reaction contained two µl cDNA template, 10 µl Express SYBR® GreenER™ qPCR SuperMix Universal with premixed ROX reference dye (500 nM), 0.4 µl of each primer (10 µM). PCR was performed in an ABI PRISM 7700 (Applied Biosystems, VIC, Australia) using the following thermal cycle programme: 10 min at 95°C followed by 40 cycles of denaturation for 15 s at 95°C and

annealing/ extension for 60 s at 60°C. Then 15 s at 95°C, 60 s at 60°C and 15 s at 95°C for the melt curve stage. Quantification of expression of apoplastic invertase inhibitor was done using comparative C_T method (also known as ΔΔCT method, Livak and Schmittgen 2001). This involves comparing the C_T values of the apoplastic invertase inhibitor of transgenic lines with non-transgenic control. The C_T values of the apoplastic invertase inhibitor of both transgenic lines and non transgenic controls are relative to EF1-α endogenous control or reference gene. The transcript levels of the apoplastic invertase inhibitor gene of transgenic lines were then quantified normalised to the transcript levels of the apoplastic invertase inhibitor gene of non-transgenic control, which was assigned a value of 1. Data were considered significant if $P < 0.05$.

5.2.7 qRT PCR analysis and characteristics of selected transgenic lines

Based on the transcript levels in the leaves and normal phenotype of T0 plants, four mid to high expressing lines per construct for each genotype were selected for further characterisation. Four plants were established (early October 2010) from four tubers in each of PB8 bags per line (140mm x 140mm x 280mm), with each PB8 bag (containing potting mix described above) treated as a replicate. Lines were planted in three blocks. Each block consisted of a row of transgenic lines transformed with four constructs of one of the genotypes. The transgenic lines were randomised amongst the constructs within the same genotype. The growth room conditions are described in section 5.2.5. The tubers were harvested after three months, dried in the dark and stored at 6°C in brown paper bags. At the time of harvesting one tuber per replication was transferred to a sample bag (4 OZ/118ml capacity sample bags, Nasco Whirl Pack) and the samples were stored immediately at -80°C. qRT-PCR analysis was performed on these freshly harvested -80°C stored tubers using the method described in section (5.2.6) with slight modifications. The RNA was extracted from 100 mg of tuber flesh tissue ground to a fine powder using a pestle under liquid nitrogen as per the method described by Luo et al. (2011). One ml RNA lysis buffer (Appendix A) (Wan and Wilkins 1994) was added to ground tuber tissue in each tube. The lysate was mixed by vortexing and centrifuged briefly at 10,000 × g for five min to pellet the debris; 200 µl of lysate supernatant was used for RNA extraction using an Illustra® RNAspin mini RNA isolation kit (GE Healthcare, Bucks, UK) according to manufacturer's instructions as described in section 5.2.6. The quality and the quantity of the RNA were checked as described in section 5.2.6.

Crisp colour evaluation and sugar assay were performed from one month cold stored tubers using the method described in section 2.2.5. Results of four replications were averaged.

5.3 Results

5.3.1 Potato transformation

Four binary vectors (Figure 5.1) overexpressing three apoplastic invertase inhibitor alleles in sense orientation [(1) pART27-*inh1*-c_s, (2) pART27-*inh1*-d_s and (3) pART27-*inh1*-b_s)] and one [(4) pART27- *inh1*-$b_{a/s}$] expressing in an antisense orientation were introduced, via *Agrobacterium*, to produce transgenic potato plants all under the control of the constitutive CaMV 35S promoter. Putative transformants were successfully recovered for all potato genotypes, namely 1021/1, Karaka and Summer Delight, using *nptII* as a selectable marker gene on each of the four constructs. Three to four weeks following to co-cultivation, leaf explants produced cell colonies on the leaf surface and margins. The cell colonies were pale yellow to green in the case of Karaka and green in the case of Summer Delight, while those derived from 1021/1 were highly pigmented with a dark red colour. These grew as a hard compact callus, although a few cell colonies were friable. Upon transfer to regeneration medium these cell colonies were regenerated into several fully grown shoots elongated from each cell colony within four to five weeks. Single healthy shoots were excised from each shoot clump which rooted well within a week in media containing kanamycin. More than 300 putative transgenic lines, 259 lines for the three sense constructs (1), (2), (3) and 52 lines for the antisense construct (4), were obtained on kanamycin selection medium (Table 5.1).

Table: 5.1 - Independently derived putative transformants selected with kanamycin resistance

Construct	Number of kanamycin-resistant lines		
	1021/1	Karaka	Summer Delight
(1) pART27-*inh1*-c_s	34	33	31
(2) pART27-*inh1*-d_s	21	24	20
(3) pART27-*inh1*-b_s	29	25	42
(4) pART27-*inh1*-$b_{a/s}$	26	04	22

5.3.2 PCR analysis of putative transgenic lines

Putative transgenic lines regenerated on kanamycin selection medium, which showed weak or slow growth with abnormal phenotypes, were discarded. Genomic DNA from the selected putative transgenic potato lines was screened using three individual PCRs for the presence of the *nptII* gene, the apoplastic invertase inhibitor transgene, and the endogenous *actin* gene. Table 5.2 shows the number of transgenic lines positive for both

nptII gene and the apoplastic invertase inhibitor transgene selected based on their normal phenotype and healthy growth in tissue culture. PCR products from representative lines are illustrated in Figures 5.2 to 5.4. As anticipated, the expected bands sizes were detected for both *nptII* and apoplastic invertase inhibitor alleles in the selected transgenic lines and the plasmid, while the *actin* gene is produced in both transgenic and non-transgenic potato plants as a positive control. This result established that all the selected transformed potato plants contained both *npt-II* and transgene.

Table: 5.2 – Number of transgenic lines confirmed by PCR

Construct	Number of confirmed transgenic lines screened		
	1021/1	Karaka	Summer Delight
(1) pART27-*inh1*-c_s	20	14	20
(2) pART27-*inh1*-d_s	16	15	19
(3) pART27-*inh1*-b_s	20	14	18
(4) pART27-*inh1*-$b_{a/s}$	20	04	20

5.3.3 Growth room evaluation of transgenic lines

The transgenic lines that were PCR-positive for the presence of the apoplastic invertase inhibitor transgene and the kanamycin resistance gene (Table 5.2) were transferred to the BIOTRON (controlled growth room) facility available at Lincoln University, New Zealand, along with non-transformed control plants (Figure 5.5). The growth and development of all transgenic lines were phenotypically normal and similar to the wild type control plants for all the genotypes. Apoplastic invertase inhibitor transcript levels were analysed from the leaves of these transgenic lines. After their natural senescence the tubers of these transgenic lines were harvested and stored at 6°C. Although the tubers of the resulted transgenic lines did not show any visible abnormal phenotype; size of the tubers was greatly reduced in few transgenic lines.

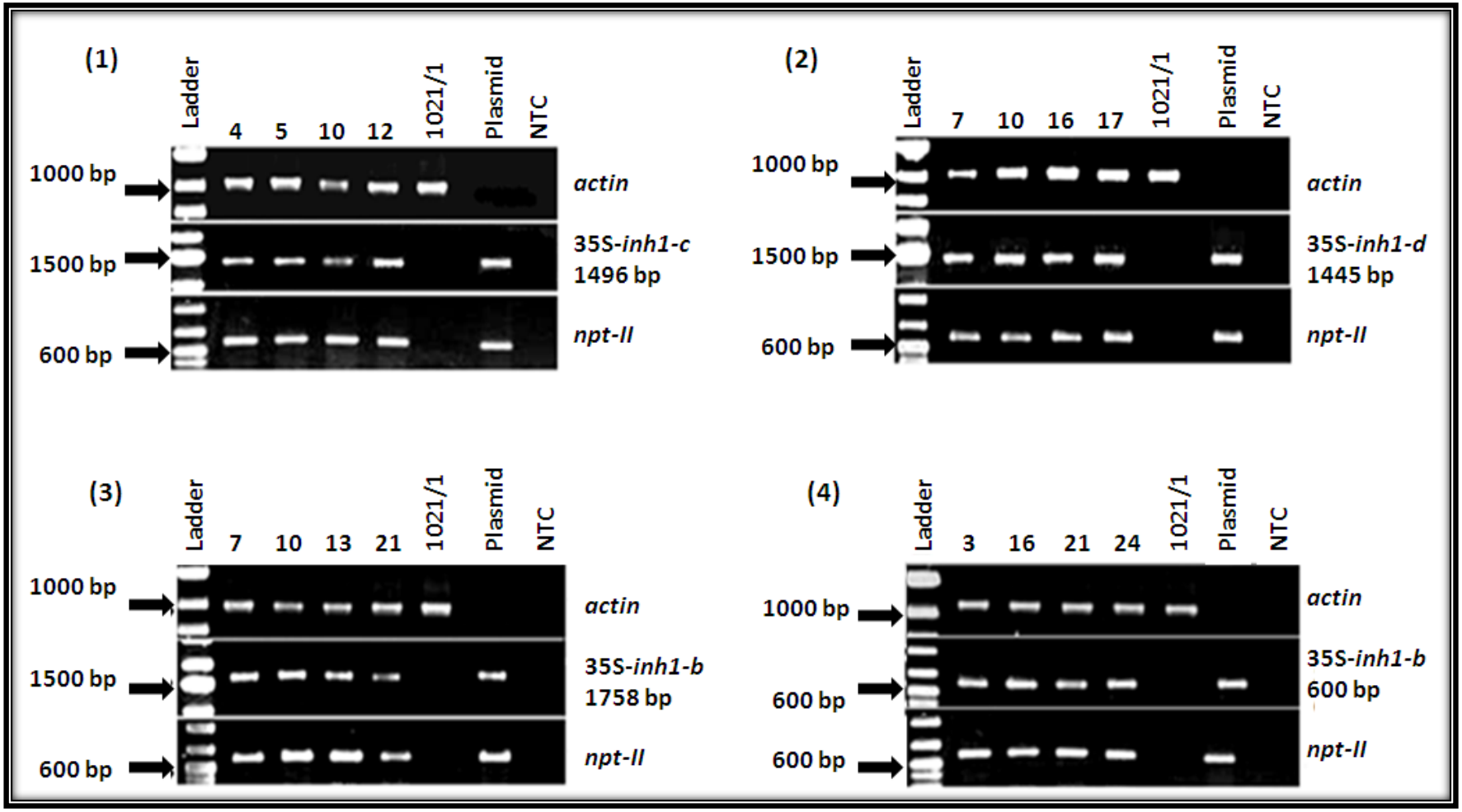


Figure 5.2 PCR screening of putative transgenic potato lines derived from 1021/1. The lines transformed with constructs (1) pART27-*inh1-c*$_s$, (2) pART27-*inh1-d*$_s$, (3) pART27-*inh1-b*$_s$ and (4) pART27-*inh1-b*$_{a/s}$ containing one of three apoplastic invertase inhibitor alleles and *npt-II* gene. Lane 1, Hyper ladder 1 (Bioline, UK) size marker; lanes 2-5 represent line number, lane no 6 non-transgenic 1021/1 control, lane no 7 is plasmid, lane no 8 is non-template control. The expected size of the 35S-*inh* transgene products are indicated on the figure, with the *nptII* primers producing an expected 612 bp product and the actin primers as an internal control producing a 1069 bp product.

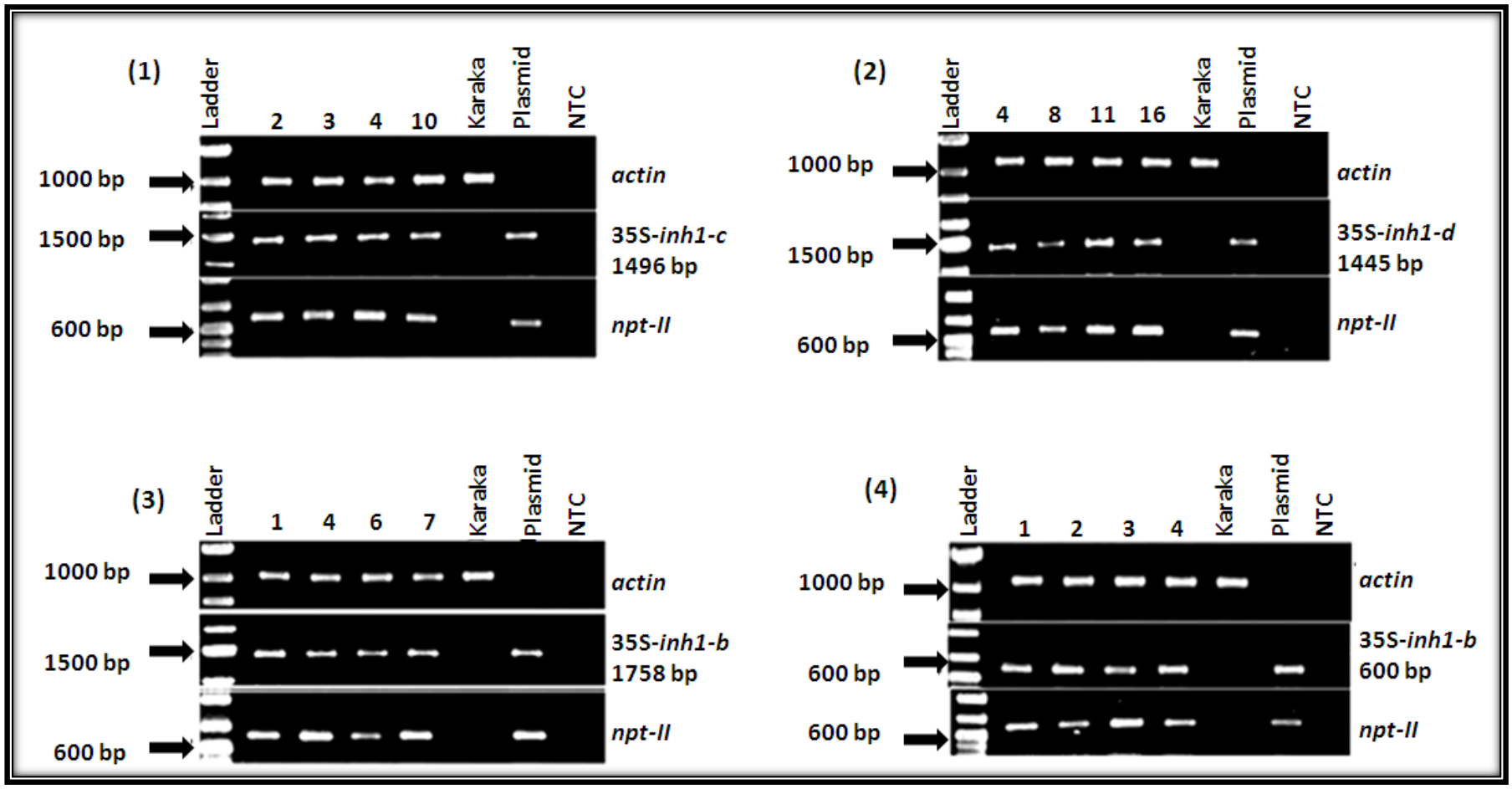


Figure 5.3 PCR screening of putative transgenic potato lines derived from Karaka. The lines transformed with constructs (1) pART27-*inh1-c*$_s$, (2) pART27-*inh1-d*$_s$, (3) pART27-*inh1-b*$_s$ and (4) pART27-*inh1-b*$_{a/s}$ containing one of three apoplastic invertase inhibitor alleles and *npt-II* gene. Lane 1, Hyper ladder 1 (Bioline, UK) size marker; lanes 2-5 represent line number, lane no 6 non-transgenic 1021/1 control, lane no 7 is plasmid, lane no 8 is non-template control. The expected size of the 35S-*inh* transgene products are indicated on the figure, with the *nptII* primers producing an expected 612 bp product and the actin primers as an internal control producing a 1069 bp product.

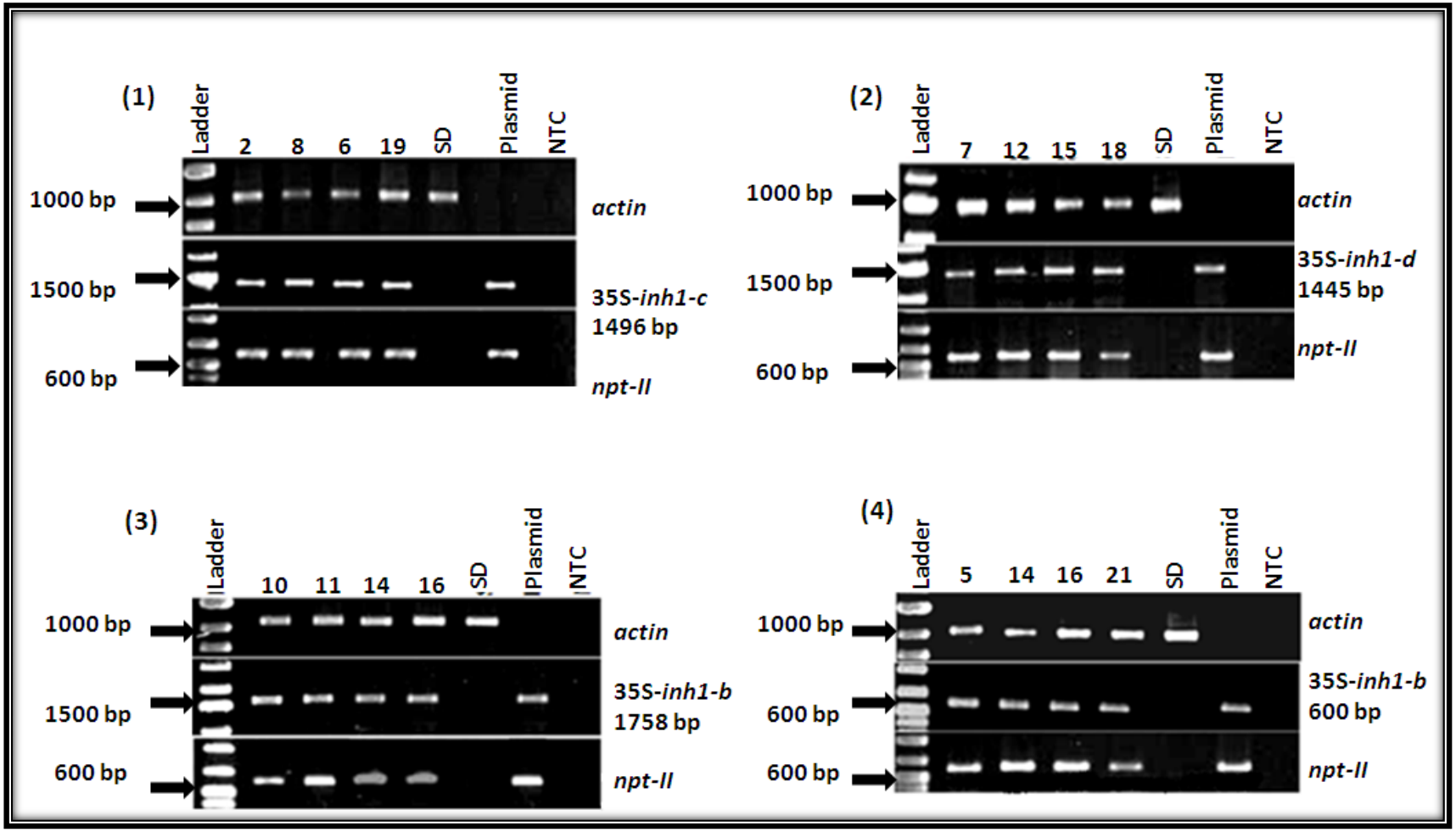


Figure 5.4 PCR screening of putative transgenic potato lines derived from Summer Delight. The lines transformed with constructs (1) pART27-*inh1-c*$_s$, (2) pART27-*inh1-d*$_s$, (3) pART27-*inh1-b*$_s$ and (4) pART27-*inh1-b*$_{a/s}$ containing one of three apoplastic invertase inhibitor alleles and *npt-II* gene. Lane 1, Hyper ladder 1 (Bioline, UK) size marker; lanes 2-5 represent line number, lane no 6 non-transgenic 1021/1 control, lane no 7 is plasmid, lane no 8 is non-template control. The expected size of the 35S-*inh* transgene products are indicated on the figure, with the *nptII* primers producing an expected 612 bp product and the actin primers as an internal control producing a 1069 bp product. SD- Summer Delight

Figure 5.5: Transgenic plants growing in BIOTRON. Three blocks were created for each genotype. Each block consisted of a row of transgenic lines transformed with four constructs of one of the genotypes. The transgenic lines were randomised amongst the constructs within the same genotype. (Photo courtesy of Robert Lamberts, the Plant & Food Research, New Zealand).

5.3.4 Quantitative RT-PCR analysis in leaves and tubers of three genotypes

Endogenous transcript levels of the apoplastic invertase inhibitor gene were measured in leaves and freshly harvested tubers of three genotypes used for transformation experiment. These transcript levels were relative to elongation factor alpha (EF-1α). As shown in Figure 5.6 relative transcript levels were high in leaves [Figure 5.6 (A)] and/ or freshly harvested tubers [Figure 5.6 (B)] of 1021/1 compared to both Summer Delight and Karaka.

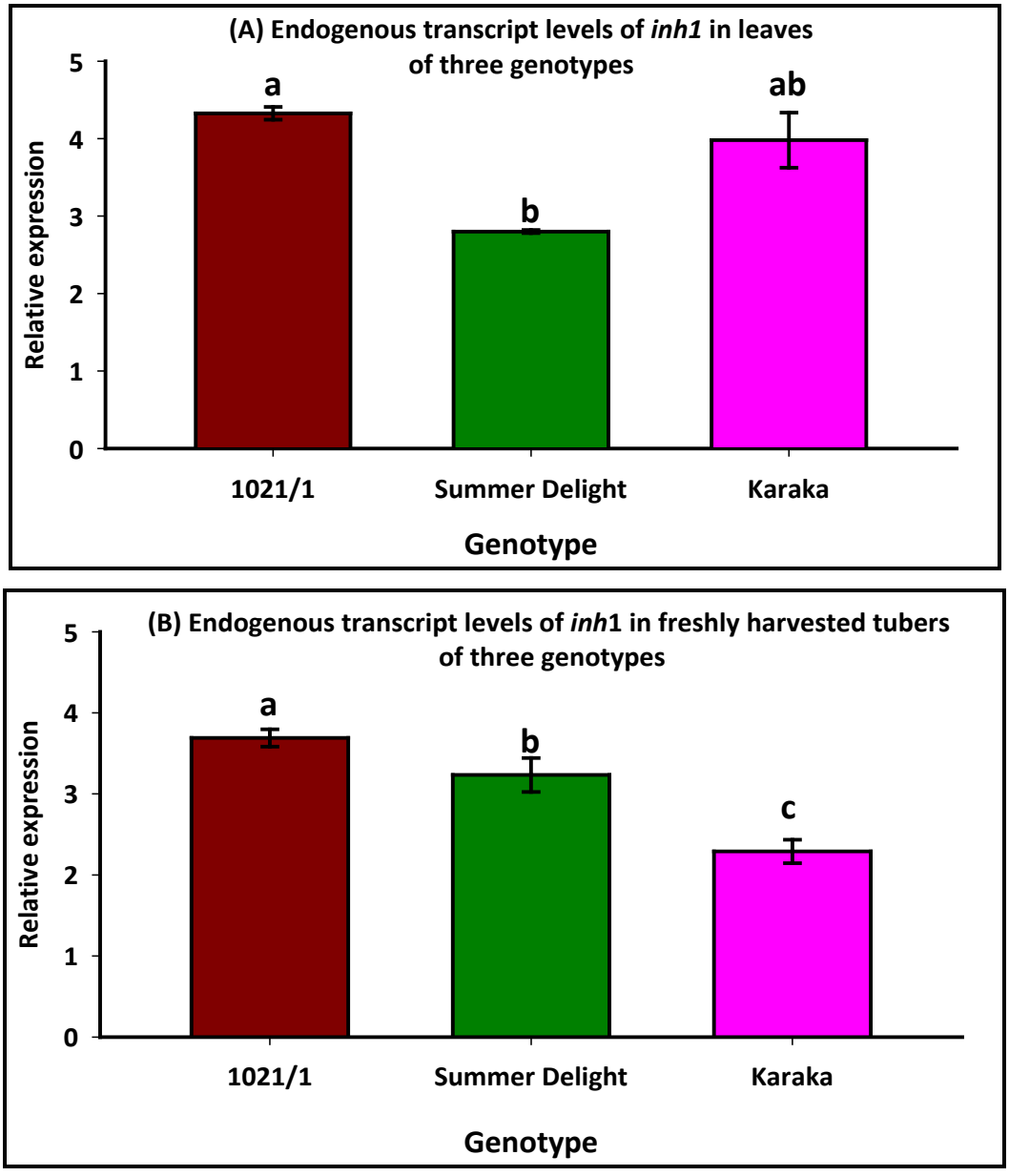


Figure 5.6 Endogenous apoplastic invertase inhibitor (*inh1*) transcript levels in leaves and tubers of three genotypes. Figures represent endogenous apoplastic invertase inhibitor (*inh1*) transcript levels in leaves and tubers of three genotypes. (A) leaves and (B) tubers of three genotypes. Data were normalized to EFlα (elongation factor alpha).

5.3.5 Quantitative RT-PCR analysis in leaves of transgenic plants

A quantitative real-time RT-PCR (qRT-PCR) assay was used to evaluate the transcript levels of all the alleles of apoplastic invertase inhibitor gene in the leaves of transgenic potato plants grown in BIOTRON facility. As the coding region of all the alleles was highly similar (98.1%), it was not possible to design the primers to detect the transcript levels of the individual alleles. For this reason the transcripts were amplified with the primers that do not distinguish between the endogenous apoplastic invertase inhibitor transcripts and transcripts of the transgenes. The transcript levels were determined relative to elongation factor alpha (EF-1α) as an internal standard gene and then normalised to the transcript levels of the control, which was assigned a value of 1.

qRT PCR analysis showed that the transcript levels of the apoplastic invertase inhibitor gene in the leaves of transgenic potato plants varied greatly (Figures 5.7 to 5.10). Most of the transgenic lines which showed increased transcript levels were significantly different from their respective controls. In general, the overexpression of all three alleles (*inh1-c*, *inh1-d* and *inh1-b*) transformed via three sense constructs [(1), (2) and (3)] was greatly enhanced and produced higher transcript levels of the apoplastic invertase inhibitor. Notably as compared to the transgenic lines derived from Karaka and Summer Delight, 1021/1 derived lines produced much higher levels of the apoplastic invertase inhibitor as a result of overexpression of *inh1-c*, *inh1-d* and *inh1-b*. 1021/1 derived transgenic lines produced greater than a tenfold increased transcript levels for 70% (1) (Figure 5.7), 94% (2) (Figure 5.8) and 60% (3) (Figure 5.9) of the transgenic lines. In the case of Karaka 43% (1) (Figure 5.7), 27% (2) (Figure 5.8) and 36% (3) (Figure 5.9) and in the case of Summer Delight 30% (1) (Figure 5.7), 37% (2) (Figure 5.8) and 15% (3) (Figure 5.9) transgenic lines showed more than tenfold increased transcript levels of the apoplastic invertase inhibitor gene. The mean fold increased in transcript levels in 1021/1 derived transgenic lines were 22.7 (1), 26 (2) and 30.6 (3) compared to Karaka 10.1 (1), 6.5 (2) and 7.9 (3) and Summer Delight 8.2 (1), 8 (2) and 7.2 (3).

Many transgenic lines derived from Karaka and Summer Delight produced apoplastic invertase inhibitor transcript levels less than or equal to their respective controls for all three constructs [(1), (2) and (3), Figures 5.7 to 5.9]. Of all the transgenic lines from the three genotypes, line 3-17 (Figure 5.9) from 1021/1 exhibited exceptionally

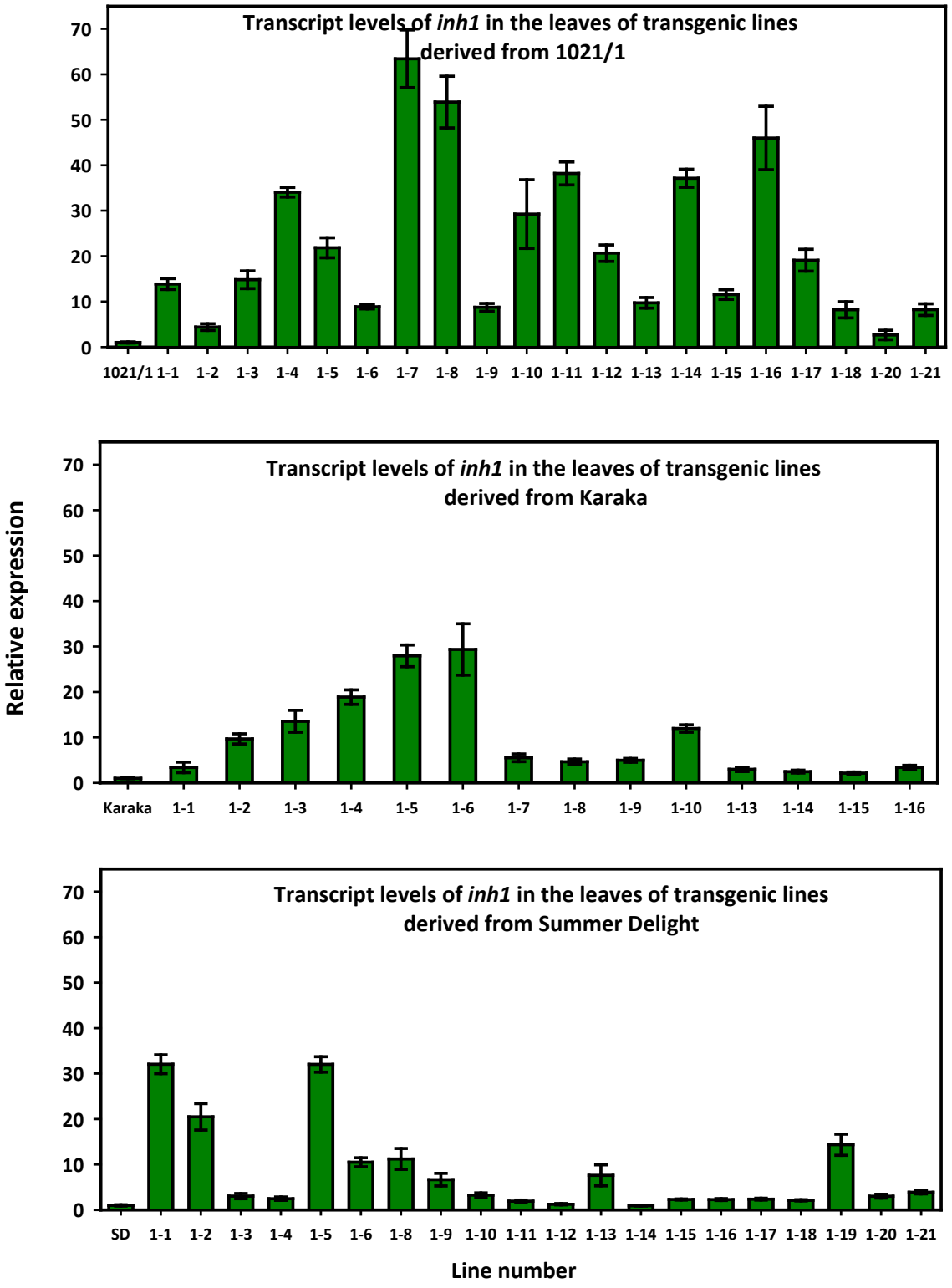


Figure 5.7: Transcript levels of *inh1* relative to elongation factor α (EF-1α) in leaves of transgenic potato lines transformed with construct (1) pART27-*inh1*-c_s. Transcript levels were determined using quantitative real time PCR and were normalised to the transcript levels of respective controls, which was assigned a value of one. Bar denotes standard errors from three replications. Data is significant at 95% confidence level. SD- Summer Delight

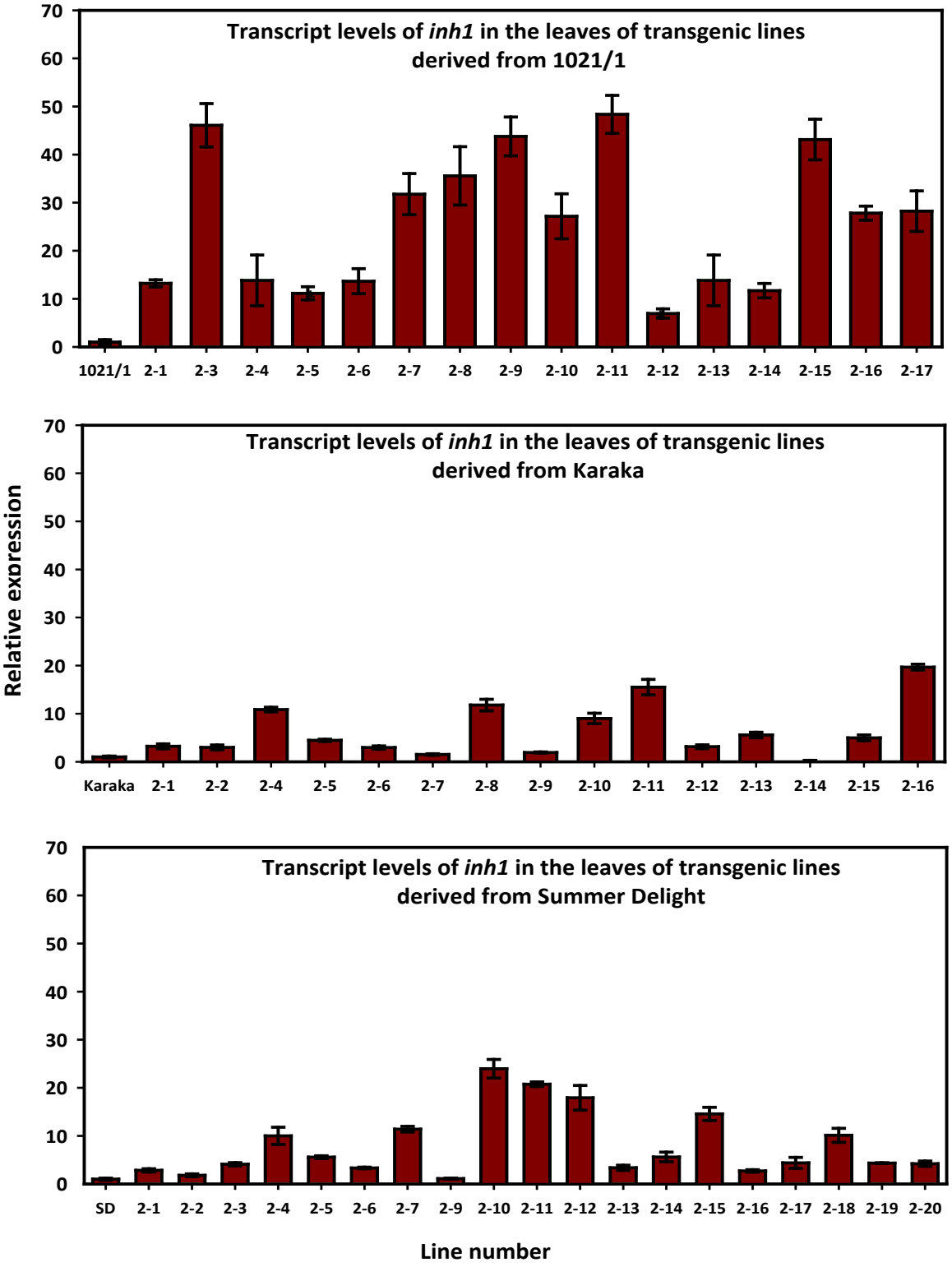


Figure 5.8: Transcript levels of *inh1* relative to elongation factor α (EF-1α) in leaves of transgenic potato lines transformed with construct (2) pART27-*inh1*-d_s. Transcript levels were determined using quantitative real time PCR and were normalised to the transcript levels of respective controls, which was assigned a value of one. Bar denotes standard errors from three replications. Data is significant at 95% confidence level. SD-Summer Delight.

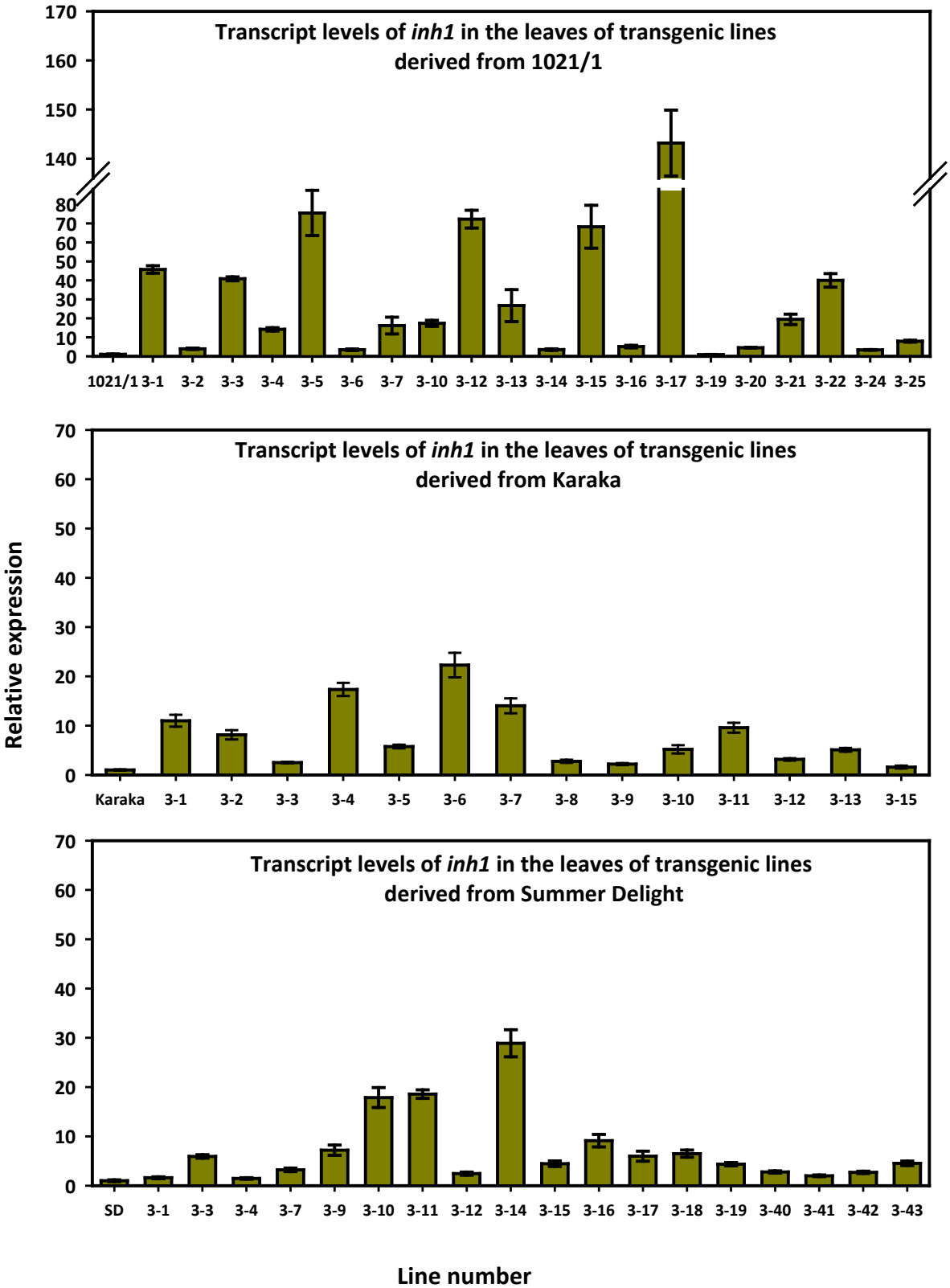


Figure 5.9: Transcript levels of *inh1* relative to elongation factor α (EF-1α) in leaves of transgenic potato lines transformed with construct (3) pART27-*inh1-b*$_s$. Transcript levels were determined using quantitative real time PCR and were normalised to the transcript levels of respective controls, which was assigned a value of one. Bar denotes standard errors from three replications. Please note the scale difference. Data is significant at 95% confidence level. SD- Summer Delight.

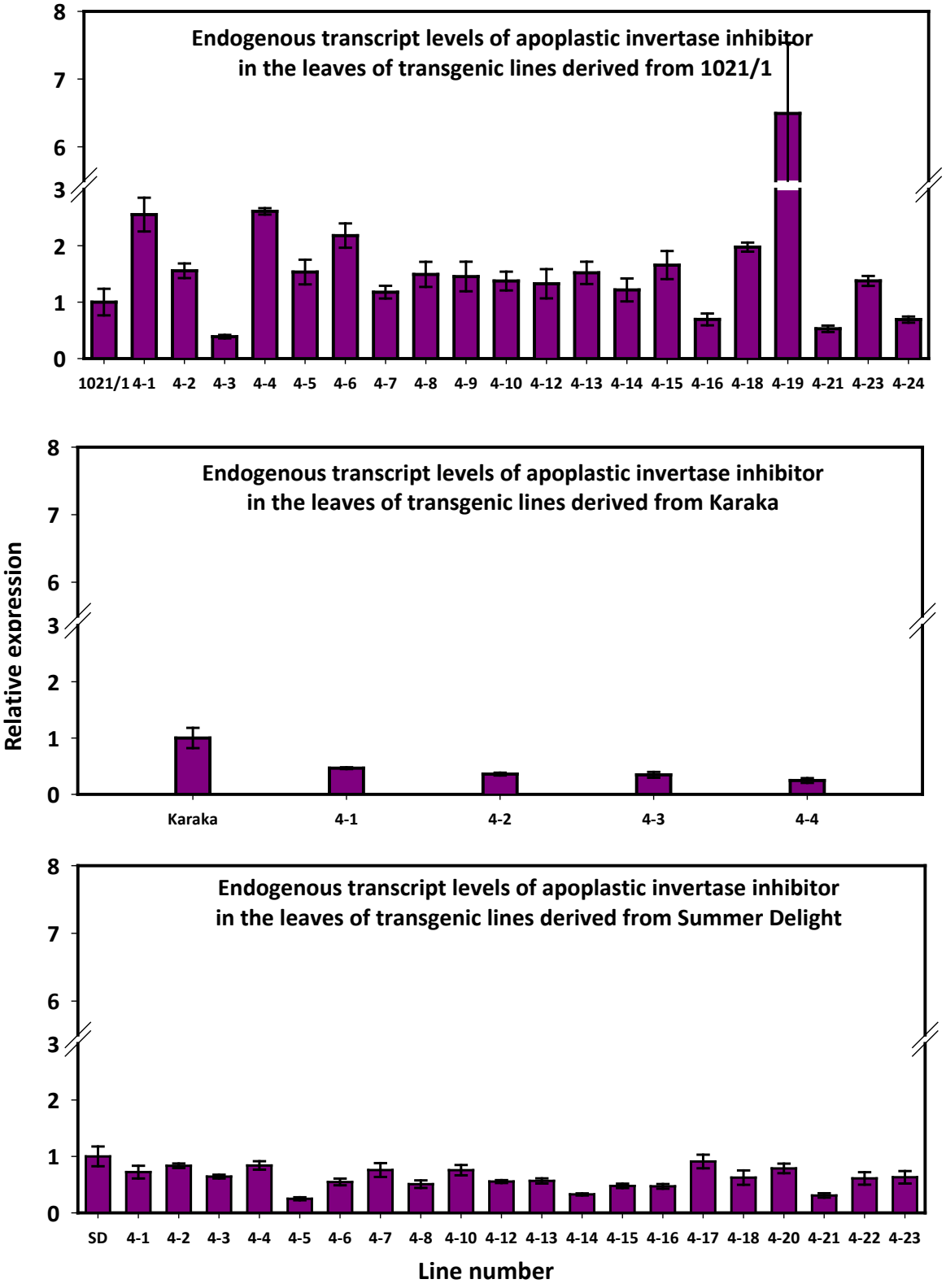


Figure 5.10: Endogenous transcript levels of apoplastic invertase inhibitor in the leaves of transgenic lines. Antisense transcript levels of *inh1-b* relative to elongation factor α (EF-1α) in leaves of transgenic potato lines transformed with construct (4) pART27-*inh1b*$_{a/s}$ determined using quantitative real time PCR. Transcript levels were normalised to the transcript levels of respective controls, which was assigned a value of one. Bar denotes standard errors from three replications. Please note the scale difference. Data is significant at 95% confidence level. SD- Summer Delight.

high transcript levels of the *inh1-b* allele with a 143 fold increase over the non-transgenic 1021/1 control (Figure 5.9).

In order to investigate the antisense suppression of the apoplastic invertase inhibitor gene, the endogenous transcript levels of the apoplastic invertase inhibitor from the transgenic lines transformed with construct (4) containing *inh1-b* (Figure 5.10) were detected. In this case the expression of the antisense construct containing *inh1b* was not detected. For this purpose, the transcripts were amplified with the same primers that were used to detect the transcript abundance of only the endogenous apoplastic invertase inhibitor gene. The reduction in the transcript levels of the endogenous gene indicates the suppression of the apoplastic invertase inhibitor gene. A total 44 transgenic lines were transformed with antisense construct (4) (Table 5.2, Figure 5.10).

The transcript levels of the apoplastic invertase inhibitor were significantly reduced for 3 of 20 lines in 1021/1 (15%), all 4 derived from Karaka (100%) and 10 out of 20 (50%) derived from Summer Delight (Figure 5.10). Overall the results obtained from antisense repression of the apoplastic invertase inhibitor gene indicated that the transcript levels were hardly affected and most of the lines had increased transcript levels in case of 1021/1 derived transgenic lines compared to those derived from Karaka and Summer Delight.

5.3.6 Quantitative RT-PCR analysis in tubers of transgenic plants

A quantitative real-time RT-PCR (qRT-PCR) assay was used to evaluate the transcript levels of all the alleles of apoplastic invertase inhibitor gene in freshly harvested tubers of transgenic potato plants grown in the BIOTRON (controlled growth room) facility. For this reason lines were selected with mid to high transcript levels of apoplastic invertase inhibitor in leaves and good looking tubers for the sense constructs [(1), (2) and (3)], whereas lines with lower expression and good looking tubers were selected from the antisense construct (4). Four lines from each construct per genotype (total 48 lines) were further planted in the BIOTRON for the phenotypic assessment of tuber traits associated with cold-induced sweetening and analysis of the apoplastic invertase inhibitor transcript levels in tuber tissue.

Freshly harvested tubers from all these 48 selected transgenic lines were analysed using qRT-PCR along with the appropriate non-transgenic controls. The results are summarised in Figures 5.11 (E), 5.12 (E) and 5.13 (E). Freshly harvested transgenic tubers showed varied transcript levels of the apoplastic invertase inhibitor gene. Under the control of the 35S promoter all 36 lines (12 per genotype) from the three genotypes transformed

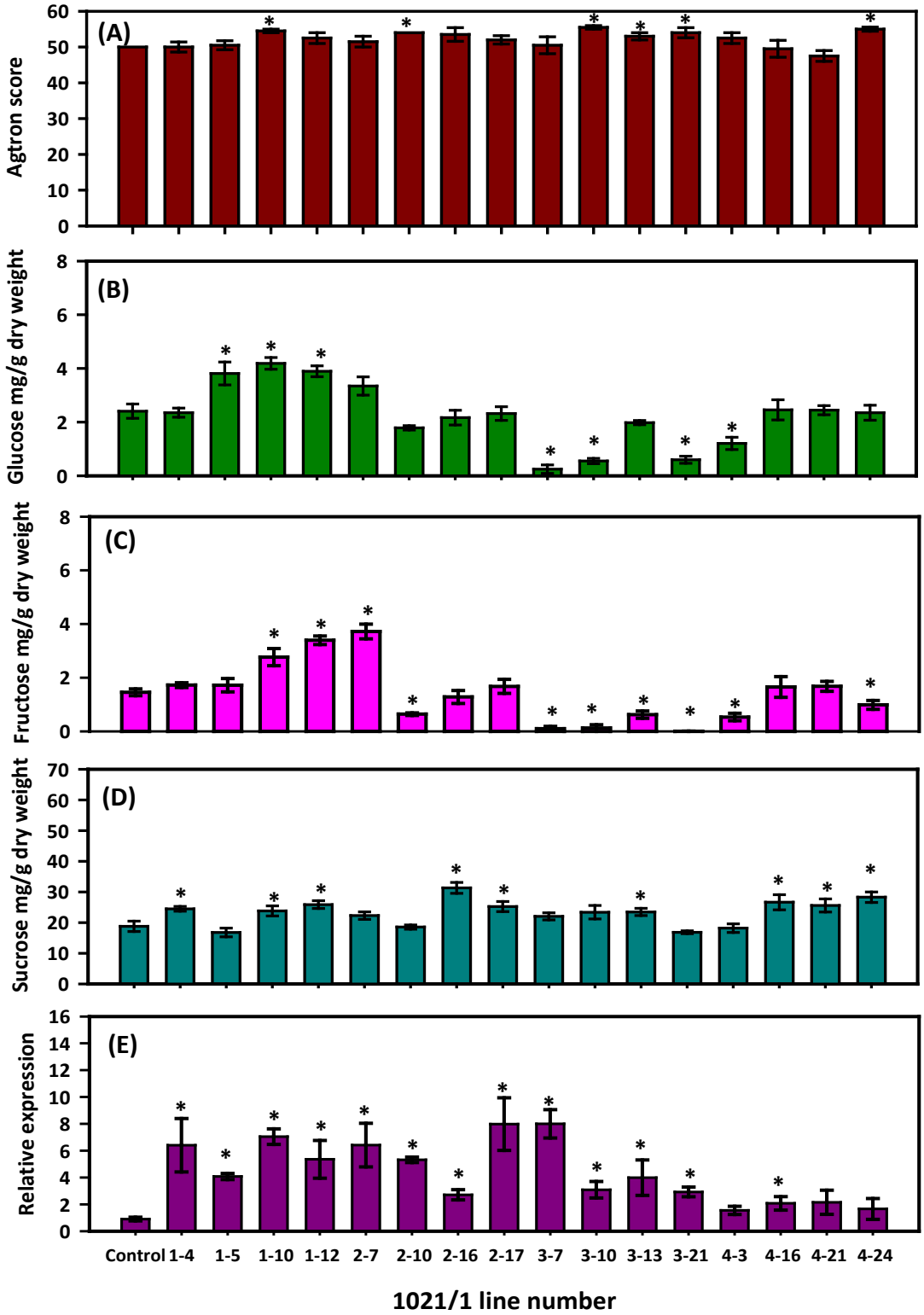


Figure 5.11 Phenotypic characterisation of CIS traits and qRT PCR results from transgenic tubers of 1021/1. Effects of over-expression [constructs (1) to (3)] and antisense repression [construct (4)] of apoplastic invertase inhibitor alleles in independently derived transgenic lines of 1021/1. (A) crisp colour - the values are the "Agtron" scores, (B) glucose, (C) fructose, and (D) sucrose contents in cold stored tubers. Numbers 1, 2, 3 & 4 are associated with construct numbers. The values are average of four replicates. Bars represent the standard error from four preparations. X axis represents line number/name while Y axis represents parameter glucose, fructose and sucrose, expressed as mg/g dry weight. (E) Transcript levels of apoplastic invertase inhibitor alleles relative to elongation factor α (EF-1α) in freshly harvested tubers of transgenic potato lines determined using quantitative real time PCR. * denote significance at $P < 0.05$. Note the scale difference.

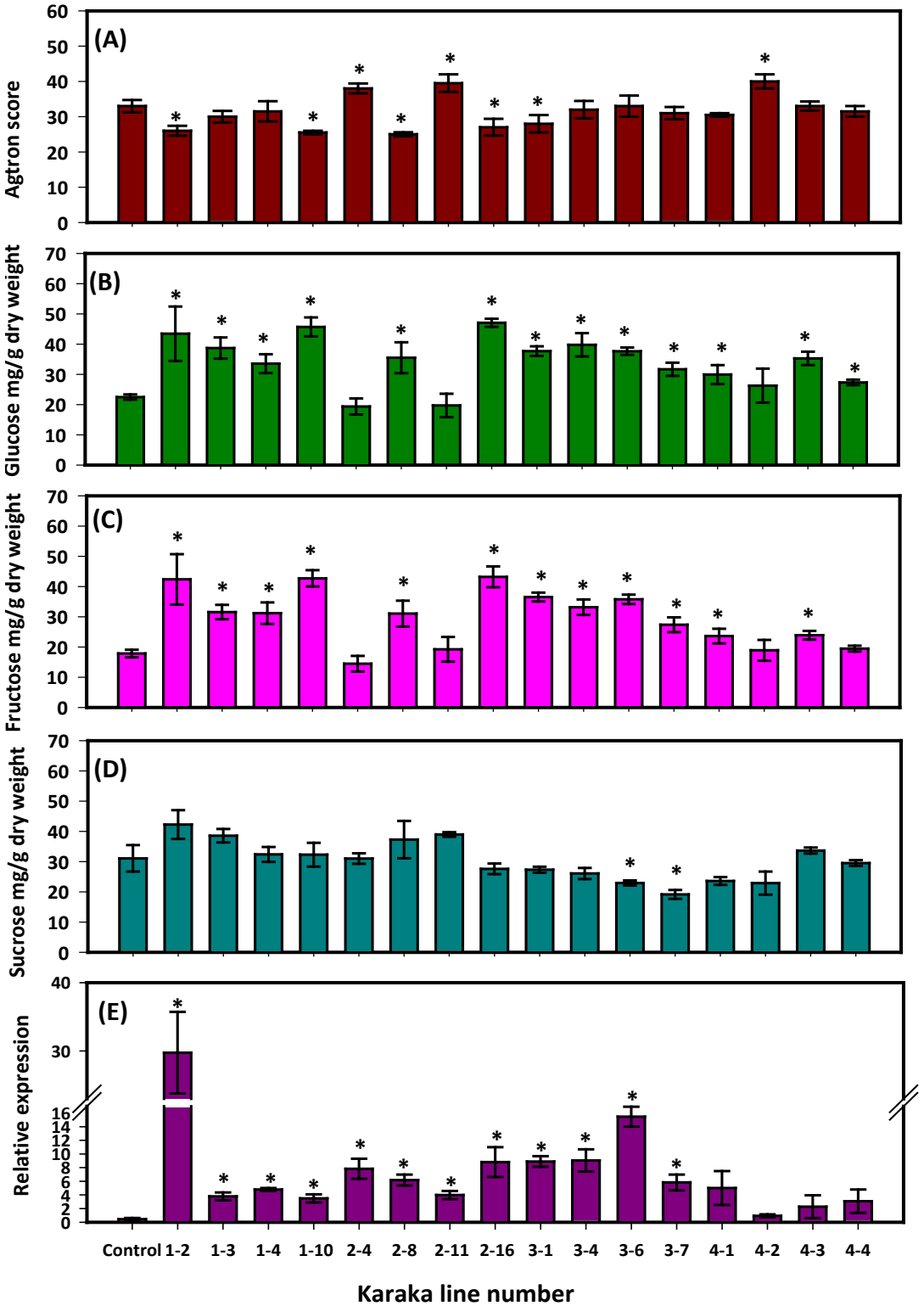


Figure 5.12 Phenotypic characterisation of CIS traits and qRT PCR results from transgenic tubers of Karaka. Effects of over-expression [constructs (1) to (3)] and antisense repression [construct (4)] of apoplastic invertase inhibitor alleles in independently derived transgenic lines of Karaka. (A) crisp colour - the values are the "Agtron" scores, (B) glucose, (C) fructose, and (D) sucrose contents in cold stored tubers. Numbers 1, 2, 3 & 4 are associated with construct numbers. The values are average of four replicates. Bars represent the standard error from four preparations. X axis represents line number/name while Y axis represents parameter glucose, fructose and sucrose, expressed as mg/g dry weight. (E) Transcript levels of apoplastic invertase inhibitor alleles relative to elongation factor α (EF-1α) in freshly harvested tubers of transgenic potato lines determined using quantitative real time PCR. * denote significance at $P < 0.05$.Note the scale difference.

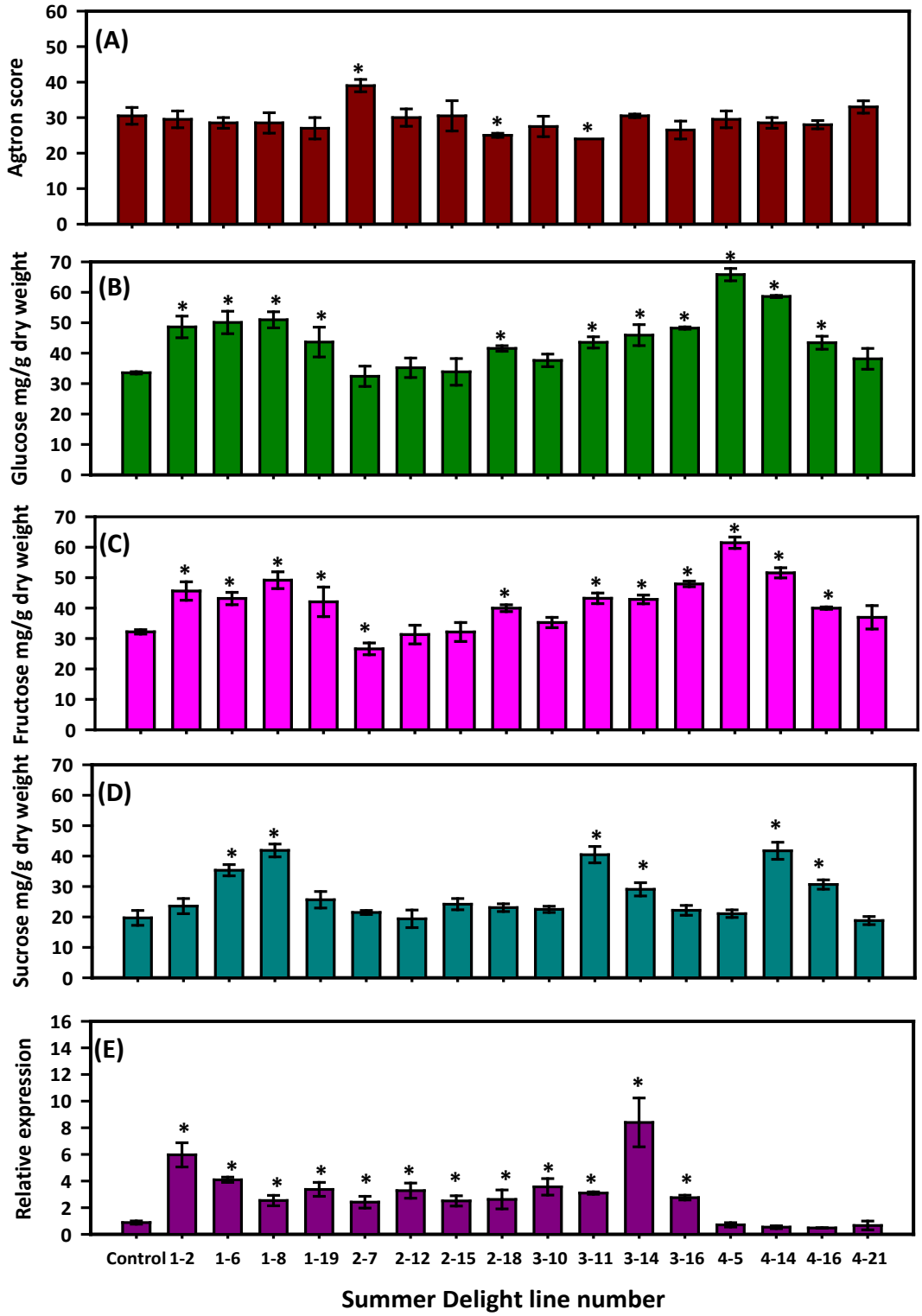


Figure 5.13 Phenotypic characterisation of CIS traits and qRT PCR results from transgenic tubers of Summer Delight. Effects of over-expression [constructs (1) to (3)] and antisense repression [construct (4)] of apoplastic invertase inhibitor alleles in independently derived transgenic lines of Summer Delight. (A) crisp colour - the values are the "Agtron" scores, (B) glucose, (C) fructose, and (D) sucrose contents in cold stored tubers. Numbers 1, 2, 3 & 4 are associated with construct numbers. The values are average of four replicates. Bars represent the standard error from four preparations. X axis represents line number/name while Y axis represents parameter glucose, fructose and sucrose, expressed as mg/g dry weight. (E) Transcript levels of apoplastic invertase inhibitor alleles relative to elongation factor α (EF-1α) in freshly harvested tubers of transgenic potato lines determined using quantitative real time PCR. * denote significance at $P < 0.05$. Note the scale difference.

with constructs (1) to (3) showed significantly higher expression levels (>2.5 fold) of apoplastic invertase inhibitor gene in tuber tissue [Figures 5.11 (E), 5.12 (E) & 5.13 (E)]. From all 36 lines, line 1-2 from Karaka showed almost 30 fold increased transcript levels of *inh1-c* compared to Karaka control. Line 3-6 from the same group showed more than 15 fold increased transcript levels of *inh1-b* compared to Karaka control [Figure 5.12 (E)].

An effect of antisense transformation on the apoplastic invertase inhibitor gene was detected from the transgenic lines transformed with construct (4). None of the lines [a total of 12 lines, four per genotype] showed significantly decreased transcript level of the apoplastic invertase inhibitor gene from the three genotypes when transformed with antisense construct (4) [Figures 5.11 (E), 5.12 (E) and 5.13 (E)]. Overall results from the transcript quantification revealed much higher transcript levels of the apoplastic invertase inhibitor alleles in the leaves compare to the freshly harvested tubers of the transgenic potato plants.

5.3.7 Phenotypic evaluation of selected transgenic lines

In order to evaluate the phenotypic effects from the anticipated overexpression and antisense repression of apoplastic invertase inhibitor alleles, transgenic potato tubers were assessed for tuber quality traits such as crisp colour, glucose, fructose and sucrose after one month cold storage. The results are summarised in Figures 5.11 to 5.13.

All 12 transgenic lines derived from 1021/1 transformed with constructs (1) to (3) showed better than or equal crisp quality compared to the control 1021/1 indicated by high Agtron scores [Figure 5.11 (A)]. These lines showed significantly increased or decreased glucose and fructose contents compared to the control 1021/1 [Figure 5.11 (B) & (C)]. Interestingly all four lines overexpressing the *inh1-b* allele [construct (3)] showed very low levels of glucose [Figure 5.11 (B)] and almost negligible amount of fructose [Figure 5.11 (C)] compared to the control 1021/1. All four antisense lines (4) did not alter the crisp colour and levels of glucose and fructose only marginally fluctuated. Mean sucrose levels (all 16 lines) were either significantly increased in nine lines or equal to the non-transgenic 1021/1 control (seven lines) [Figure 5.11 (D)].

In contrast to 1021/1 derived transgenic lines, those derived from Karaka exhibited a wide range of variability for crisp colour after one month of cold storage when transformed with one of the four constructs [Figure 5.12 (A)]. Only three of the 16 lines showed improved crisp colour compared to control Karaka. Two of these lines (2-4 and 2-11) resulted from overexpression of the *inh1-d* allele [construct (2), Figure 5.12 (A)], while peculiarly the third

line (4-2) resulted from antisense repression of apoplastic invertase inhibitor gene [construct (4) Figure 5.12 (A)]. No significant reduction in the levels of glucose and fructose was observed in these lines [Figure 5.12 (B) & (C)]. The lines overexpressing the *inh1-c* allele [construct (1)] and the *inh1-b* allele [construct (3)] showed more than a 1.5 fold increased levels of glucose [Figure 5.12 (B)] and fructose [Figure 5.12 (C)], reflected by low Agtron scores [Figure 5.12 (A)]. Sucrose levels remained unchanged or had a slight increase or decrease irrespective of construct type when compared to the non-transgenic Karaka control [Figure 5.12 (D)].

In the case of Summer Delight, out of the 16 lines, only one line derived showed significantly improved crisp colour [Figure 5.13 (A)] as an effect of overexpression of *inh1-d*. The majority of these lines also produced significantly higher levels of glucose [Figure 5.13 (B)] and fructose [Figure 5.13 (C)] compared to control Summer Delight. Lines overexpressing the *inh1-c* allele [construct (1)] or the *inh1-b* allele [construct (3)] showed almost a 1.5 fold increased levels of both glucose and fructose (except for line 3-10). Lines produced from antisense repression of apoplastic invertase inhibitor [construct (4)] also exhibited significantly high (except 4-21) levels of glucose and fructose compared to control Summer Delight. In general the mean sucrose levels were either significantly greater than (6 of 16 lines) or equal (10 of 16 lines) to control, Summer Delight [Figure 5.13 (D)].

5.4 Discussion

The overall goal of this chapter was to study the effects of overexpression and antisense repression of apoplastic invertase inhibitor alleles identified in Chapter 4 on CIS responses in transgenic potato tubers. To date, there has been no *in vivo* evidence on the role of different alleles of apoplastic invertase inhibitor gene in regulation of cold-induced sweetening process in transgenic potato tubers. To achieve this three specific alleles were transformed in sense orientation for overexpression, while one allele was also transformed in an antisense orientation to attempt knock down of apoplastic invertase inhibitor expression. A thorough phenotypic assessment was made of selected transgenic lines to evaluate the contribution of each allele to the cold-induced sweetening process of potato tubers.

Potato Transformation

A total 311 putative transgenic lines were selected for kanamycin resistance (Table 5.1). Of these, 201 PCR confirmed transgenic lines with a healthy and normal appearance in tissue culture were established in a growth room to obtain tubers (Table 5.2). No consistent visible detrimental phenotype was observed in most of the tissue culture regenerated plants in response to expression of *inh1*. The only exception was slow growth with abnormal phenotype in a few lines probably due to the somaclonal variations.

PCR analysis of putative transgenic lines

The presence of the transgene was confirmed by screening the genomic DNA of selected putative transgenic lines grown in a growth room (Table 5.2). During the PCR confirmation of the transgenic status of the putative transformants, care was taken to avoid the amplification of native apoplastic invertase inhibitor gene. This problem was circumvented by designing PCR primers over the junction between the 35S promoter and the apoplastic invertase inhibitor alleles. PCR screening of the selected 201 independently derived transgenic lines (T0) showed the presence of both the kanamycin resistance gene and the transgenic apoplastic invertase inhibitor gene in all lines (Figures 5.2-5.4). The endogenous potato *actin* gene was used as an positive control and was detected in both transgenic and non-transgenic potato plants (Figures 5.2-5.4).

Growth room evaluation of transgenic lines

T0 transgenic potato plants, showed no abnormal phenotypes during the vegetative growth compared to control genotypes. However, some transgenic lines produced some off-type or very small tubers that became apparent after harvest. This effect on size of the

transgenic potato tubers is probably a consequence of somaclonal variation. It is unlikely to be a response to *inh1* overexpression since the abnormal phenotypes were not observed in most high expressing lines. The occurrence of individuals producing off-type tubers, including a large number of small, elongated and knobbly tubers, is commonly reported among populations of transgenic potato lines (Belknap et al. 1994; Conner et al. 1994; Davidson et al. 2002b). Such off-types have often been attributed to somaclonal variation, which involves epigenetic or genetic changes during the tissue culture phase of plant transformation (Conner 2007). Barrell and Conner (2011) established that this somaclonal variation occurs during tissue culture and is independent of transgene insertion.

Quantitative transcript analysis of *inh1* in leaves of transgenic plants

Because of the high level of nucleotide identity in the coding region of the alleles, the results of qRT-PCR were expected to detect the total (endogenous + transgene) transcript levels of the apoplastic invertase inhibitor gene. Quantitative real time PCR was used to estimate the transcript levels of the apoplastic invertase inhibitor gene in leaves of transgenic potato plants. The levels of *inh1* expression varied substantially among individual transformants (Figures 5.7-5.10).

The wide variation in the levels of the *inh1* expression in independent transformants might have resulted from the random insertion of a transgene at different positions in the plant genome and such quantitative differences are referred to as resulting from 'position effects' (van Leeuwen et al. 2001). The expression of the transgene therefore can be influenced by surrounding endogenous plant DNA in which the transgene is randomly integrated (Grant et al. 1991; Conner and Christey 1994). Transcript levels can be affected also by the copy number of an integrated gene (Gallie 1998; Matzke and Matzke 1998). Such variation in transgene expression between independently derived transgenic lines has been commonly reported in potatoes (Davidson et al. 2002a, 2004a; Meiyalaghan et al. 2006).

Although overexpression of all three alleles (*inh1-c* Figure 5.7, *inh1-d* Figure 5.8 and *inh1-b*, Figure 5.9) resulted in high transcript levels in the leaves of most of the transgenic lines derived from all three genotypes, the results obtained from 1021/1 were highly notable. 1021/1 derived transgenic lines had higher transcript levels of *inh1* as a result of overexpression of *inh1-c* (Figure 5.7), *inh1-d* (Figure 5.8), and *inh1-b* (Figure 5.9) compared to those derived from Karaka and Summer Delight. A marked increase in transcript levels in the leaves of 1021/1 derived transgenic lines suggest that the transcripts levels were probably more stable in 1021/1 derived lines than those obtained from Karaka and Summer

Delight. Transcripts of the apoplastic invertase inhibitor gene relative to the elongation factor alpha gene (EF1α) were determined from three control genotypes to investigate the endogenous levels. The results clearly demonstrated that 1021/1 had higher transcripts in leaves and freshly harvested tubers relative to Karaka or Summer Delight (Figure 5.6). These results suggest that the transcript levels of the apoplastic invertase inhibitor gene might remain high and stable after cold storage in 1021/1. This observation is supported by the results of Brummell et al. (2011), who observed that the cold-stored potato tubers of susceptible and resistant genotypes showed strongly induced and varied levels of *inh1* (apoplastic invertase inhibitor) mRNA. These studies indicate that the presence of *inh1* transcripts is genotype specific and may play an important role in CIS in potato tubers.

Similar to overexpression experiments, the results obtained from antisense transformation (Figure 5.10) of the apoplastic invertase inhibitor gene for the 1021/1 derived transgenic lines were highly noticeable. The transgenic lines were expected to show complete reduction of transcript levels as an effect of antisense repression of the apoplastic invertase inhibitor gene. However, only three out of 20 lines produced from 1021/1 showed significant reduction in the endogenous transcript levels (Figure 5.10) of the apoplastic invertase inhibitor gene. In many 1021/1 derived transgenic lines antisense repression of the apoplastic invertase inhibitor gene failed to impact on the mRNA levels; in fact seven of the 20 lines exhibited a significant increase in *inh1* transcript levels (Figure 5.10). All four transgenic lines derived from Karaka and 10 (50%) derived from Summer Delight showed the reduction of the apoplastic invertase inhibitor transcript levels in leaves. However, in general, none of the transgenic line showed complete suppression of the apoplastic invertase inhibitor transcripts (Figure 5.10). These results further support the earlier observation that the transcripts stability of the apoplastic invertase inhibitor in 1021/1 is much greater than in Karaka and Summer Delight.

Quantitative transcript analysis of *inh1* in tubers of transgenic plants

The key objective of this study was to evaluate the effects of overexpression and antisense repression on transcript levels of the apoplastic invertase inhibitor in freshly harvested transgenic tubers. The hypothesis was that allelic variations of the apoplastic invertase inhibitor gene might play an important role in the post-translational modification of invertase activity in cold-stored potato tubers. Only four lines per construct per genotype were selected based on the mid to high transcript levels in leaves and normal looking tubers.

These lines were grown in larger bags in the available space to produce sufficient tuber material for transcript level detection and the phenotypic analysis of CIS traits.

The transcript levels were not determined from cold-stored transgenic potato tubers mainly because all the alleles were constitutively expressed under the 35S promoter and therefore the transcript levels of the apoplastic invertase inhibitor may not alter in response to cold. The constitutive expression of three apoplastic invertase inhibitor alleles (*inh1-b*, *inh1-c* and *inh1-d*) resulted in high transcript levels in freshly harvested tubers of 1021/1 [Figure 5.11 (E)], Karaka [Figure 5.12 (E)] and Summer Delight [Figure 5.13 (E)] compared to their respective controls. Therefore it is expected that the transgenic lines derived from all the three genotypes might accumulate high levels of the apoplastic invertase inhibitor upon cold storage, resulting in an improved response to CIS in potato tubers.

Antisense repression of apoplastic invertase inhibitor did not result in significant reduction in the transcript levels in the tubers of the transgenic lines [Figures 5.11 (E), 5.12 (E) and 5.13 (E)]. As noted in leaves, overexpression and antisense repression of the specific alleles resulted in highly varied transcript levels in freshly harvested transgenic potato tubers, which might be also attributed to the position effects.

Effects of *inh1* overexpression on CIS traits after tuber cold storage

Overexpression of three genotype specific apoplastic invertase inhibitor alleles (*inh1-b* common allele of 1021/1, Karaka and Summer Delight, *inh1-c* from Summer Delight and *inh1-d* from 1021/1) in general did not consistently alter crisp colour in the transgenic lines derived from 1021/1 (12 lines), whereas the levels of glucose and fructose fluctuated significantly above or below the levels of control (Figure 5.11). Similar to 1021/1, overexpression of three alleles (*inh1-b*, *inh1-c* and *inh1-d*) did not show better crisp quality or lower levels of glucose and fructose in lines derived from Summer Delight (11 lines, except for crisp colour in 2-7) and Karaka (10 lines, except for crisp colour in 2-4 & 2-11) (Figures 5.12 and 5.13). These results were of particular importance in evaluating the biological role of *inh1* in the regulation of CIS in potato tubers. No consistent response was observed in response to overexpression of genotype specific alleles of *inh1* in these traits.

Effects of antisense repression of *inh1* on CIS traits after tuber cold storage

The hypothesis behind the generation of antisense lines was to test whether the *inh1* transcript levels is required for maintaining the cold sweetening resistance phenotype, especially in 1021/1. Complete knock down of the apoplastic invertase inhibitor should result into higher invertase activity and result in high levels of reducing sugars and

concomitant darker crisp colour. However, significant repression of *inh1* transcripts was not achieved in tubers in either 1021/1 (Figures 5.11), Karaka (Figure 5.12) or Summer Delight (Figure 5.13). This is also reflected in no consistent changes in the CIS phenotypic traits in these lines (Figures 5.11 – 5.13).

Does *inh1* play a role in CIS traits?

Transgenic potato lines were developed to investigate whether overexpression of specific *inh1* alleles was involved in the control of CIS in potato tubers. The hypothesis was that the differences in the coding sequence of the alleles might play an important role in the cold induced sweetening process of the cold susceptible and resistant genotypes. As a result, crisp colour would be improved by specific alleles in transgenic Karaka and Summer Delight, while the antisense repression was expected to result in reduced crisp quality in 1021/1. Although no consistent results were obtained to support the proposed hypothesis, other results on transcript levels of *inh1* in 1021/1 offer other potential explanations for the high performance of 1021/1 in CIS response.

Overall results showed that there is much more remains to be learned about the mechanism by which apoplastic invertase inhibitor remains more stable in 1021/1. From the results obtained from transcript levels and phenotypic characterisation of transgenic potato plants, it can be speculated that there could be three levels controlling the CIS process in potato tubers. Phenotypic differences may have arisen due to the differential mRNA stability in the three genotypes that probably responded differently due to the physiological and genetic makeup of the genotypes. The second level of regulation was possibly at post-transcriptional level while yet another level of control could be at the level of protein turnover or the enzyme stability.

The higher and stable transcript levels of *inh1* in leaves of 1021/1 compared to Karaka and Summer Delight suggest the post-transcriptional regulation of *inh1*. The levels of mRNA observed in freshly harvested tubers of 1021/1 derived transgenic lines may have remained relatively stable and unaltered in cold storage compared to Karaka and Summer Delight derived transgenic lines. This could be due the constitutive expression of all the alleles under the 35S promoter. Previous studies in transgenic potato tubers showed that the alteration of invertase inhibitor gene expression plays an important role in CIS process in potato tubers (Greiner et al. 1999; Cheng et al. 2007). Greiner et al. (1999) transformed potato with a vacuolar invertase inhibitor (*Nt-inhh*) under a CaMV 35S promoter and observed 75% reduction in hexose accumulation. Their results of northern blot analysis

revealed that the *Nt-inhh* mRNA levels in tubers were similar to those found in leaf tissue, and cold-storage of tubers did not significantly alter *Nt-inhh* transcript levels. Likewise the transcripts of other important genes involved in CIS such as vacuolar invertase, sucrose phosphate synthase and UDP-glucose pyrophosphorylase were found to be high in cold-stored potato tubers (Zrenner et al. 1993; Deiting et al. 1998; Bagnaresi et al. 2008) and found to play an important role in CIS. One possible mechanism to explain the transcripts of the apoplastic invertase inhibitor in 1021/1 could be due to the differential regulation of apoplastic invertase inhibitor by their micro-RNAs (miRNAs). These miRNAs bind to the mRNA transcripts and result into translational repression or target mRNA degradation and gene silencing (Bartel, 2004, 2009). Several studies have demonstrated that miRNAs are involved in abiotic and biotic stresses such as oxidative stress (Sunkar et al. 2006), cold (Liu et al. 2008; Zhou et al. 2008), salt, nutrient deficiency (Chiou et al. 2006; Kawashima et al. 2009). Therefore RNA sequencing experiments may be helpful for analysing expression and identifying differentially regulated transcripts in response to cold. Functional significance and interactions between miRNA and their target mRNA could be investigated further in order to demonstrate whether *inh1* play a role in CIS of potato tubers.

Chapter 6

General discussion

Chapter 6

General Discussion

Cold storage of potatoes is unavoidable to ensure year-round supply of potatoes, but this leads to CIS. CIS susceptibility limits the processing of tubers into acceptable crisps and French fries due to the accumulation of reducing sugars. Biotechnological tools have led to the identification of genes underlying QTLs for sugar and starch content in potato tubers (Chen et al. 2001; Menéndez et al. 2002). Although research has provided significant gains in understanding the physiological, biochemical and molecular factors involved in CIS there is still a large gap between genetics and physiology/biochemistry of CIS. Comprehensive studies are required to further investigate the genetic basis of CIS using DNA sequencing, microarray techniques, molecular markers and transgenics. This thesis provides a step forward by using genetic and biotechnological tools to investigate the genetic basis of CIS in potato tubers. It comprises four distinct objectives with each of the respective empirical chapters describing efforts that were taken to contribute further understanding of CIS in potato tubers.

Two mapping populations were established to study the CIS phenomenon using tetraploid genotypes displaying varying degrees of response to CIS and these provided the foundation of the thesis. Both mapping populations had phenotypes with varied pigmentation traits. Due to time constraints only one mapping population was phenotyped for various CIS traits and genotyped with 24 SSR markers including candidate genes of known position on potato chromosomes. The next step was to investigate the association between the phenotype and the genotype. From this data the candidate gene apoplastic invertase inhibitor was selected to investigate the allelic diversity in the parents of the mapping populations. Finally, to complete the study, transgenic potato plants were produced to investigate the role of apoplastic invertase inhibitor alleles in the CIS process of potato tubers. This final chapter provides a thesis overview and discusses the thesis findings in a broader context and present an outlook for future research using the work described herein as a foundation.

Potato cultivars with high dry matter content and low glucose and fructose levels are suitable for processing into French fries and crisps. The correlation between reducing sugars (glucose and fructose) and crisp quality was initially studied in the parents of the mapping population (“S”). Genotype 1021/1 showed best processing quality (indicated by high Agtron scores) at all the five storage treatments as compared to the other parents and

grandparents. Further analysis of the 300 progeny of the mapping population provided a resource to study pigmentation, physiological, biochemical and genetic traits. This was the first study that has provided the comprehensive data on pigmentation, physiological and CIS traits in a tetraploid mapping population. The purpose of this objective was to gather phenotypic data and study the segregation patterns and to provide the basis for association studies. The mapping population was shown to vary in pigmentation, physiological traits, crisp colour and other tuber quality parameters such as sugars and dry matter content (Sections 2.3.2 and 2.3.4). Marked segregation was observed for various components of CIS such as crisp colour, sugars and dry matter content at different storage treatments (Section 2.3.4). Cold storage durations affected mean crisp quality indicated by low Agtron scores and high glucose and fructose levels suggesting that there is a strong correlation between the sugar content and crisp quality. Studies on reconditioning periods helped to improve the mean crisp colour while some progeny did not respond at all. Overall the results obtained in this study endorsed a strategy to identify CIS tolerant lines and lines with high dry matter content that could be utilised in the future potato breeding programme in New Zealand.

Correlation studies between glucose, fructose, sucrose, dry matter and crisp colour provided additional information on the degree of relationships between variables (Section 2.3.5). Although glucose and fructose contents in the tuber were the major determinants of crisp quality of the cold-stored potato tubers, the present studies revealed that fructose has more influence on crisp colour than glucose. Similar results showed by Song (2004). Knowledge and information on genetic variation of these traits in different potato populations is very scanty. Therefore present studies suggest that there are ample opportunities for the genetic dissection of these traits and also selection and development of potato genotypes for various pigmentation and CIS traits.

The information gained on CIS traits helped to gain insight into further understanding the genetic dissection of the CIS pathway using molecular marker based association studies (Chapter 3). Therefore, it was important to understand the genetic and enzymatic reasons behind the phenotypic variability. Following the phenotypic assessment the progeny was profiled with 24 simple sequence repeat (SSR) loci of known map position including candidate genes [apoplastic invertases (markers STM1106 LG X & STM1051, LG IX), vacuolar invertase (*Pain-1*, LG III), vacuolar invertase inhibitor (*Stinh2*α) and granule bound starch synthase (*STWaxy2* LG VIII)] operating in carbohydrate metabolism of potato tubers. Molecular marker studies provided an approach that facilitated the identification of genomic

loci and candidate genes playing a key role in CIS process of potato tubers. This research showed that all the 24 markers were polymorphic in the progeny, producing 84 alleles (Sections 3.3.1 and 3.3.2). Of the 84 alleles, 80 marker alleles were segregating while 62 were segregated in expected ratios. These studies helped to elucidate the segregation pattern of the marker alleles in the progeny and also identified the cultivar-specific alleles. The phenotypic data (Chapter 2) and the genotypic data (Chapter 3) were used in association based studies that were conducted with genotype-phenotype correlations using single marker allele ANOVA (Kreike et al. 1994) analysis.

The association based studies led to an identification of DNA markers underlying phenotypic variation. The marker data that was associated with the phenotype data detected highly significant associations ($P < 0.05$) with various pigmentation, physiological and CIS traits (Sections 3.3.3 and 3.3.4). There is a growing interest in applying association based studies to a wide range of plants including potatoes to identify genes/QTLs responsible for quantitative variation of complex traits. The candidate gene approach was widely used in the search of CIS (Menéndez et al. 2002, Li et al. 2005), tuber quality traits (D'hoop et al. 2008), tuber bruising and enzymatic discoloration (Urbany et al. 2011) in potato. Jacobs et al. (1995) constructed a molecular map for various morphological traits along with isozymes and transposons that provided a reference framework for mapping quantitative trait loci. The research reported here suggests there are ample opportunities for the genetic dissection of these traits and also selection and development of potato genotypes for various pigmentation traits. Crisp quality, high dry matter content and low glucose and fructose are important for processing of potatoes into crisps. Associations between several markers (e.g. SSR5136 LG I, STM3023 LG IV, SSR5148 LG V etc.) with various CIS traits have been first time demonstrated in this research (Section 3.3.4). Such markers could be further investigated to study whether they are physically close to the candidate gene loci which are controlling QTLs for sugars and starch. The markers that showed significant association and positive effect on crisp colour and dry matter content as a presence of a 1021/1 specific alleles (STM3023 LG IV; SSR5148 LG V; Sti021 LG VI) could be utilised for future marker assisted breeding programmes. In particular, these alleles could be further sequenced to identify the SNPs that are involved in the phenotypic variation. The candidate gene markers Sti060 (*Pain1*- LG III), STWaxy2 (LG VIII), STM1051 (Inv_{ap}-*b* LG IX), STM1106 (Inv_{ap}-*a* LG X) and Stinh2α (vacuolar invertase inhibitor LG XII) showed significant

associations ($P < 0.05$) with cold sweetening traits and the direction of the effect was either positive or negative on these traits (Section 3.3.4).

As a number of marker alleles showed a borderline effect, further work is required to develop definitive conclusions. Although the studies were conducted with limited marker sets in this thesis it was possible to obtain robust associations between SSRs and phenotypic components of CIS. Adding additional markers especially candidate gene markers such as those operating in carbohydrate metabolism would assist in future association mapping and/ QTL mapping studies. In addition, several molecular markers have been demonstrated to be in-depth associated with single or multiple traits and showed a borderline effect on CIS traits (Section 3.3.4). The association studies provided a basis to study and investigate the genomic loci contributing to phenotypic variation in CIS. By characterising the allelic variability associated with CIS traits found in this study, new and existing candidate gene alleles involved in starch and sucrose metabolism can be assessed. Deeper sequence characterisation could enhance the understanding of allelic impact on CIS mechanism of potato tubers. For these reasons the candidate gene apoplastic invertase inhibitor was selected as a case study because of the following reasons. Firstly, the results from association based studies (Chapter 3) showed the contribution and association of different candidate gene alleles of invertases, vacuolar invertase inhibitor and granule bound starch synthase with CIS traits. Secondly, the functional interaction between apoplastic invertase (marker STM1051) and apoplastic invertase inhibitor and the presence of specific alleles of these two genes in cold tolerant potato lines has been demonstrated (Baldwin et al. 2011). These experiments revealed the importance of the involvement of the apoplastic invertase inhibitor in the regulation of CIS. In addition, invertase inhibitors have already been shown to play an important role in CIS in potato tubers (Greiner et al. 1999; Brummell et al. 2011). However, the impact of natural variation of invertase inhibitor genes in CIS in potato tubers among different genotypes was completely unknown. To explore this opportunity, the cloning and sequencing of different alleles of the apoplastic invertase inhibitor was necessary and new information is provided by this thesis.

The various apoplastic invertase inhibitor alleles that were cloned from the parents of the mapping population were studied in detail for sequence polymorphism. The results identified a total of five alleles (*inh1-b*, *inh1-c*, *inh1-d*, *inh1-e* and *inh1-f*) that had high sequence polymorphism especially in the intron region (Section 4.3.2). Of five alleles, four were novel alleles (*inh1-c*, *inh1-d*, *inh1-e* and *inh1-f*). The exon region showed non-

synonymous SNPs that altered the amino acids. The results revealed that the predicted protein sequences of alleles *inh1-d*, *inh1-e* and *inh1-f* has a unique substitution at the predicted junction of the signal peptide and mature protein (Section 4.3.3). The significance of these SNPs and the contribution of each allele to the CIS process remain to be elucidated. These results provided opportunities to investigate the role of the apoplastic invertase inhibitor in CIS process in potato tubers. Future research could focus on utilising sequencing information of the apoplastic invertase inhibitor alleles to design allele specific markers and SNP based association studies. In addition, transgenic plants could be created to demonstrate the role of the apoplastic invertase inhibitor by overexpression and antisense suppression of this gene. Based on the overall studies, the hypothesis was proposed that the natural variation in the apoplastic invertase inhibitor gene may contribute to the phenotypic variability in CIS. To support this, suggestion, genetic transformation of the vacuolar invertase inhibitor genes have already shown the importance of vacuolar invertase inhibitor in the CIS process (Greiner et al. 1999; Cheng et al. 2007). On the other hand the allelic diversity of the genes (e.g. invertases and UDP-glucose pyrophosphorylase, Baldwin et al. 2011 and invertases, vacuolar invertase inhibitor and granule bound starch synthase), operating in carbohydrate metabolism have been shown to be associated with genetic variability of CIS. However, the impact and the variability in the CIS response due to allelic variations of apoplastic invertase inhibitor have not been studied in detail. In addition, information on overexpression or knock down expression of the apoplastic invertase inhibitor gene in general, and of allelic variations, in controlling CIS in potato tubers was completely unknown.

Therefore, to investigate the role of various alleles of the apoplastic invertase inhibitor in controlling the CIS process of potato tubers, transgenic plants were produced from three cultivars (1021/1, Karaka and Summer Delight). This was the first study to demonstrate the role of various alleles of the apoplastic invertase inhibitor in the CIS process of potato tubers. To accomplish this, three specific alleles were overexpressed and knock down expression of the apoplastic invertase inhibitor gene was undertaken (Chapter 5). The results on qRT-PCR clearly showed that the transcript levels of the apoplastic invertase inhibitor were higher and more stable in the leaves of 1021/1 derived transgenic lines compared to those derived from Karaka and Summer Delight (Section 5.3.5). Freshly harvested tubers showed a wide range of variability in the transcript levels of the apoplastic invertase inhibitor from the transgenic lines derived from all three cultivars (Section 5.3.6).

Notably the analysis showed that cold-stored transgenic tubers did not have altered crisp colour and sugar levels in the 1021/1, Karaka and Summer Delight overexpressed or antisense transgenic lines (Section 5.3.7). These findings suggest the higher levels of the apoplastic invertase inhibitor transcript in 1021/1 and this may be one of the key determinants in CIS in 1021/1. The results did not provide firm evidence as to whether allelic variations of the apoplastic invertase inhibitor were responsible for CIS resistance or susceptibility, but rather that the contribution of this gene to CIS was complex and likely to involve other factors. Firstly, based on the transcript level data the post-transcriptional regulation of invertase inhibitor can be presumed and further studies could be carried out using microarray techniques. Bagnaresi et al (2008) conducted transcript profiling of early events associated with CIS in potato tubers. Their results have identified several cold-responsive genes and genes involved in the CIS process of potato tubers that has revealed a complex network of events involving sugars, redox and hormone signalling. Secondly, invertase could be under post-translation regulation by invertase inhibitor. Although the functional significance remains to be elucidated, it would be worth to test interactions between miRNAs and these allelic variants in the turnover of the corresponding transcripts. miRNA mediated transcript regulation may play an important role in maintaining the stability of *inh1* in 1021/1. Sequence variations in miRNAs and their target binding sites have been reported (McConnell et al 2001; Ehrenreich and Purugganan, 2008). Microarray-based experiments may be helpful for analysing expression and identifying differentially regulated transcripts in response to cold. Functional significance and interactions between miRNA and their target mRNA could be investigated further. More studies on these aspects are required to demonstrate the importance of the apoplastic invertase inhibitor in CIS process of potato tubers.

Current efforts to understand CIS by biotechnological tools have resulted in important achievements. In this work the use of natural variation at candidate gene loci allows a comprehensive approach to analyse the regulation and function of molecular networks involved in the CIS process. However, the nature of the genetically complex mechanisms of CIS is difficult to understand, probably complicated by the multigenic nature of this trait and other unknown factors. A full elucidation of the CIS mechanism requires clear answers to the following questions: Which genes and proteins are up regulated or down regulated in response to cold storage of tubers? What are the functions of these genes or allelic variants of these genes and proteins in regulation of CIS? Which genes can be used

as genetic markers for the breeding and selection of CIS tolerant genotypes or successfully engineered in transgenic plants? The recently published potato genome sequence (The Potato Genome Sequencing Consortium, 2011) will provide new avenues for advances and analysis of complex traits in potatoes. More than 39,000 protein coding genes are predicted in potato (Potato Genome Sequencing Consortium, 2011). CIS could be controlled by a network of genes and therefore the potato genome sequence provides opportunities to understand and discover the genomic loci or additional candidate genes. For example, the candidate gene neutral invertase is present in several commercially important crop plants including sugarcane and sugarbeet. Therefore, it could be possible that upon cold storage sucrose can be cleaved by sucrose synthase and neutral invertase in the cytoplasm. Presence of neutral invertase has not been reported previously in potato. However, a sequence search of the potato genome sequence revealed the presence of neutral invertase in potato. Further studies are required to investigate the role of neutral invertase in the CIS process of potato tubers. In addition SNP-based assays will allow high-throughput genotyping of potato germplasm and populations. Such studies will enable more efficient marker-assisted breeding efforts in potato (Hamilton et al. 2011). The studies conducted in this research will likely prove highly informative towards understanding the underlying network of metabolism in potato tubers with the recent advances in potato genome sequencing and single nucleotide polymorphisms discovery.

Directions for future research

In addition to suggestions in the above discussion the following suggestions for future research are given.

a) Analysis of starch quality and quantity: - Analysing the starch quality and quantity by selecting genotypes with high dry matter content and/ starch would be an alternative approach for GM (Genetically Modified) crops and identification of the gene sequences of the enzymes involved in starch metabolism would be useful in investigating starch for nutritional and industrial applications.

b) Microarray based studies: - Quantify the expression of the genes operating in starch and sucrose metabolism (synthesis and breakdown) using microarray techniques. Correlate the different expression patterns of the genes with the metabolic profile in terms of crisp colour, sugar and dry matter contents at all the five storage treatments. The presence of apoplastic invertase inhibitor (*inh1*), vacuolar invertase inhibitor (*inh2*α), and hybrid mRNA (*inh2*β) transcripts have been detected in CIS resistance and susceptible potato genotypes

(Brummell et al. 2011). However, little is known about the events that trigger CIS and the association of these invertase inhibitors with CIS. Microarray experiments may allow the contribution and identification of events associated with CIS in potato tubers could also be undertaken. Transcript profiling of early events associated with CIS and several cold-responsive genes and carbohydrate associated genes such as β-amylase have been reported (Bagnaresi et al. 2008).

c) Association mapping / QTL mapping: - Cloning and sequencing various alleles of the genes involved in starch and sucrose metabolism followed by developing molecular markers based on the sequence variations for SNPs, insertions and deletions. Studies of allelic sequences and their effect on phenotype might be useful to elucidate the mechanisms of specific candidate gene alleles and identification of QTLs for sugar and starch. The Potato Genome Sequencing Consortium (2011) has provided a resource in terms of the whole genome sequence assembly. Therefore, the database can be searched for the possible presence of new genes involved in CIS. For example, a neutral invertase that could cleave sucrose into glucose and fructose in the cytoplasm.

d) SNPs based association studies: – The sequence polymorphism observed in the alleles of apoplastic invertase inhibitor could be used to design allele specific assays. High resolution melting analysis can be performed to distinguish different alleles. Furthermore, association studies that can identify the association of specific SNPs in the apoplastic invertase inhibitor alleles with CIS traits could be developed. In addition, in the case of those SSRs which were not segregating for 1021/1, sequences of those genomic regions can be searched for any potential SNPs involved in phenotypic variations. For this research the SNPs can be identified from the genotype specific alleles and the likely impact of SNPs on the CIS trait can be determined by association mapping. The natural diversity of potato invertases (both apoplastic and vacuolar) using SNPs have already been shown to be associated with CIS traits in potato (Draffehn et al. 2010).

e) Post-translational modification and interaction of apoplastic invertase and apoplastic invertase inhibitor upon cold storage: - Proteolytic cleavage or degradation of apoplastic invertase inhibitor upon cold storage could be studied. The proteolysis of invertase and invertase inhibitors upon wounding has been shown (Reca et al. 2008; Jansen 2009). The present study has identified non-synonymous SNPs in different apoplastic invertase inhibitor alleles. Therefore, it could be worth studying the interaction of apoplastic invertase with different apoplastic invertase inhibitor alleles. Extracellular concentrations of sucrose and pH

dependant regulation of the complex between *Arabidopsis* apoplastic invertase and tobacco apoplastic invertase inhibitor have already been established (Hothorn et al. 2010). Such studies have opened opportunities to study the interactions between invertases and invertase inhibitors.

f) Role of miRNA (micro-RNA) in regulation of invertase inhibitors: -The presence of apoplastic invertase inhibitor specific miRNA and their regulation in response to cold temperatures and cultivar specific background could be studied. Low temperature dependant regulation of miRNA has been reported in plants (Szittya et al. 2003).

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Appendices

Appendix A – Culture media, buffers, solutions and enzymes

Culture media, buffers and solutions
ABI3100 loading buffer: For one 96-well plate, mix 960 µl HiDi (Applied Biosystems Pty Ltd, Australia and New Zealand) + 7.2 µl of Genescan GS 400 HD ROX ladder (Applied Biosystems Pty Ltd, Australia and New Zealand).
β-fructosidase (Invertase, Boehringer): Activity is 94.2 Units ml^{-1}. Weigh 2 g β-fructosidase in an Eppendorf tube and add 0.7 ml 0.1 M citrate buffer (pH 4.6). Further dilution is made by adding 900 µl of ddH_2O.
0.1 M Citrate buffer (pH 4.6): In 100 ml ddH_2O mix 44.5 ml 0.1 M citric acid (Analar) solution with 55.5 ml 0.1 M trisodium citrate (Analar) solution.
DNA extraction buffer (Chapter 4): In 400 ml ddH_2O add 31.885 g sorbitol (final concentration 350 mM), 50 ml 1 M Tris pH 7.5 (final concentration 0.1 M), 5 ml 0.5 M Na_2-EDTA disodium salt (final concentration 5 mM). Make final volume to 500 ml with ddH_2O and autoclave. Add 3.8 g L^{-1} sodium bisulphite just prior to use.
DNA extraction buffer (Chapter 5): In 400 ml ddH_2O add 20 ml 0.5 M EDTA pH 8.0 (final concentration 20 mM), 10 ml 1 M Tris pH 8.0 (final concentration 0.02 mM), 40.9 g NaCl (final concentration 1.4 M) and make final volume to 500 ml with ddH_2O. Add 10 g CTAB after autoclaving.
0.5 M EDTA: Add 46.53 g EDTA disodium salt to 200 ml distilled water. Adjust to pH 8.0 by adding ~ 5 g NaOH, dissolve and then add ddH_2O to a final volume of 250 ml and autoclave.
Ethidium bromide 10 mg ml^{-1}: Add 10 mg ethidium bromide to 1 ml ddH_2O. Store in dark at 4°C. For electrophoresis use a final concentration of 1 mg l^{-1}.
Loading buffer (10x): Weigh 2 g Ficoll 400 and 2 ml 0.5 M EDTA in 7 ml ddH_2O, add 0.02 g Bromophenol blue, 0.02 g Xylene cyanol and 1 ml 10 % SDS (w/v). Heat @ 65°C for 4 hrs.
Luria Broth (LB): Add 10 g Bacto-tryptone, 5 g Yeast extract, 10 g NaCl, 15 g bactoagar to 1,000 ml ddH_2O. Adjust to pH 7.0 with 5 M NaOH and autoclave.
1 M $MgCl_2$ (Sigma): Used in preparation of solution 1 (Chapter 2, Section 2.2.5.3) Dissolve 9.521 g in 100 ml ddH_2O (final concentration in the sugar assay is 3.8 mM).
Nuclear lysis buffer (Chapter 4): In 300 ml ddH_2O add 100 ml 1 M Tris pH 7.5 (final concentration 0.2 mM), 50 ml 0.5 M Na_2-EDTA disodium salt (final concentration 50 mM), 58.44 g NaCl (final concentration 2 M) & 10 g CTAB. Final volume to 500 ml with ddH_2O.

Culture media, buffers and solutions
Precipitation mix: Mix 11 ml of 95 % ethanol + 0.9 ml 3 M Sodium acetate pH 4.8. Prepare fresh before the DNA precipitation step (Chapter 3, Section 3.2.4).
Potato multiplication media: MS salts & vitamins (Murashige & Skoog 1962) supplemented with 40 mg l^{-1} ascorbic acid, 500 mg l^{-1} casein hydrolysate, 30 g l^{-1} sucrose, and 7 g l^{-1} agar.
RNA lysis buffer: In 200 ml warm DEP-C H_2O add 15.25 g sodium borate decahydrate (Borax, final concentration 80 mM), 2.3 g ethylene glycol bis(β-aminoethyl ether)-N-N'-tetraacetic acid (final concentration 12 mM), 2 g sodium dodecyl sulphate, 2 g sodium deoxycholate (final concentration 10 mM). Adjust to pH 9.0 with 5 N NaOH and autoclave for 20 min. Add 31 mg 20 ml^{-1} dithiothreitol and 0.4 g 20 ml^{-1} polyvinylpyrrolidone (Mw 40,000) after autoclaving.
100 mM Sodium maleate buffer pH 6.5: Dissolve 5.805 g Maleic acid in approximately 400 ml ddH_2O water. Add ddH_2O to 400 ml, adjust to pH 6.5 with NaOH and make up the final solution to 500 ml.
Solution 1 (Chapter 2): Used in sugar assay (Section 2.2.5.3). Add 30 mg NAD (Roche; to give a final assay concentration of 1.18 mM) or NADP (Roche; to give a final assay concentration of 1.41 mM.), 60 mg ATP ($ATP.Na_2.3H_2O$ Sigma), 100 µl 1 M $MgCl_2$, and 13.0 ml 0.2 M triethanolamine Buffer (pH 7.6) to ddH_2O to give a final volume of 26 ml**.**
50X TAE Buffer: Add 242 g Tris base (final concentration 2 M) in 750 ml ddH_2O, 37.2 g EDTA (final concentration 0.1 M), 57.1 ml glacial acetic acid and ddH_2O to 1,000 ml. The working solution of 1x TAE buffer is made by diluting the stock solution 50 times in ddH_2O. To make 1X working stock, dilute 80 ml 50 X TAE in 4 L ddH_2O.
10x TBE: Add 108 g Tris (final concentration 890 mM), 55 g H_3BO_3 (final concentration 890 mM), 40 ml of 0.5 M EDTA (final concentration 20 mM) in 800 ml ddH_2O. Make up to 1 L with ddH_2O and autoclave. For 0.5x TBE dilute 50 ml of 10x TBE to 1 L.
0.2 M Triethanolamine Buffer pH 7.6: Dissolve 29.84 g of triethalolamine (BDH) in 900 ml ddH_2O. Adjust to pH 7.6 with HCl and make up the final volume to 1 litre with ddH_2O.
TE^{-4}: In 90 ml ddH_2O add 1 ml 1 M Tris-HCl (pH 7.5) and 20 µl 0.5 M EDTA (pH 8.0). Make up final volume 100 ml with ddH_2O and autoclave.

Enzymes used in sugar analysis
Hexokinase/ Glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*) HK/G6PDH (Roche, Solution contains, 340 U Hexokinase ml^{-1} & 170 U G6PDH ml^{-1}). Add 260 µl of HK/G6PDH suspension to 800 µl of Solution 1. (G6P-DH sourced from *Leuconostoc mesenteroides* is used in this assay and is highly specific for D-glucose-6-phosphate).
Phosphoglucose Isomerase PGI (3500 U ml^{-1} Roche) - Add 30 µl of PGI suspension to 120 µl ddH_2O & 450 µl Solution 1.

Appendix B: Table of mean values for various CIS traits

	Agtron score					Glucose mg g^{-1} dry weight					Fructose mg g^{-1} dry weight					Sucrose mg g^{-1} dry weight					% Dry matter				
ST	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e
Line no																									
S001	48	30	38	31	43	0.64	8.98	3.49	6.16	1.04	0.00	5.26	1.91	5.07	0.82	5.28	8.68	7.37	7.04	3.53	25.58	25.23	30.93	30.92	31.35
S002	46	28	36	27	37	1.05	14.21	9.75	13.06	0.74	0.00	9.84	5.39	9.62	0.06	5.30	6.20	9.14	9.01	2.88	21.90	23.13	28.67	28.21	28.02
S003	54	26	33	29	30	0.76	9.02	2.19	6.81	2.06	0.00	9.40	2.31	6.68	1.84	6.83	9.99	5.40	9.63	6.11	24.06	23.85	29.19	28.36	29.82
S004	46	33	42	42	42	2.44	19.05	5.82	5.04	8.82	0.28	5.24	2.06	1.54	1.79	10.80	9.89	4.82	5.46	4.54	24.38	24.50	24.85	28.47	27.18
S005	50	29	32	30	35	1.94	11.71	13.25	14.86	6.96	0.00	8.74	3.29	7.43	2.24	7.25	8.16	5.20	5.16	4.83	22.55	23.53	30.22	28.14	28.59
S006	50	32	40	32	36	0.39	10.81	6.67	14.12	6.10	0.00	5.79	4.41	9.30	3.51	8.70	8.04	5.06	9.84	3.40	20.65	23.33	27.82	28.02	29.24
S007	51	40	40	46	53	0.56	2.55	3.95	2.46	0.22	0.00	1.87	2.36	2.11	0.00	8.63	6.26	5.15	7.88	0.64	20.80	21.35	26.99	29.34	29.90
S009	50	32	38	36	37	0.50	6.62	2.09	3.20	4.11	0.00	3.09	2.50	1.76	2.06	5.57	5.53	4.47	5.44	3.81	24.37	25.20	30.11	32.24	31.34
S010	50	24	24	36	27	0.38	14.32	13.67	4.32	11.40	0.20	12.60	11.37	3.97	11.88	5.03	11.18	8.30	8.27	11.94	22.21	22.98	27.06	28.96	28.09
S011	50	37	38	34	35	0.83	5.05	3.55	5.87	3.20	0.00	2.02	2.50	4.24	2.49	9.97	7.88	6.62	6.41	5.23	21.24	23.34	27.64	27.77	29.30
S012	55	34	45	38	55	0.04	5.75	0.95	3.17	0.43	0.00	6.00	1.59	3.82	0.00	8.18	8.99	5.45	7.90	4.00	22.36	23.83	29.56	32.55	31.90
S013	44	31	39	32	36	2.00	10.57	8.96	12.16	3.05	0.00	3.15	2.13	3.64	0.91	7.96	8.66	7.32	11.16	6.94	22.74	25.26	30.16	29.86	31.70
S014	48	41	45	38	38	0.00	2.38	0.02	1.57	1.08	0.36	1.99	0.66	1.17	0.08	7.13	10.48	6.77	9.10	4.98	28.92	28.55	33.66	33.39	31.69
S015	52	30	33	26	27	0.25	6.23	2.53	8.13	2.21	0.00	5.61	3.47	7.74	2.79	6.56	10.08	7.80	10.10	6.38	21.97	22.32	27.18	26.81	27.64
S016	49	35	38	34	45	0.66	4.88	1.29	3.50	1.02	0.00	4.08	0.80	3.97	0.62	7.10	7.37	4.13	7.40	3.62	22.70	24.03	29.68	31.69	31.54
S017	41	33	41	33	38	0.23	10.65	9.64	15.46	9.58	0.22	6.74	5.34	7.89	5.49	5.91	6.95	5.11	6.76	2.02	21.36	23.16	29.23	29.26	30.05
S018	52	35	38	28	42	0.52	6.93	5.88	12.32	1.73	0.00	7.38	6.01	6.77	1.25	10.62	12.19	8.72	7.72	3.93	19.24	19.81	24.67	25.03	28.52
S019	50	37	49	39	51	1.39	4.56	0.52	1.31	0.50	0.00	3.76	0.00	1.18	0.00	4.87	5.94	4.85	5.49	3.58	22.74	24.43	30.46	31.24	31.84
S020	47	29	40	27	39	2.48	16.31	1.54	18.86	1.81	0.00	8.80	1.78	13.11	1.90	7.26	8.47	3.44	6.62	2.66	22.52	19.86	27.29	27.13	26.21
S021	49	24	30	28	29	0.33	16.36	7.16	12.96	6.81	0.00	11.38	5.71	8.12	5.96	23.17	10.59	10.00	10.11	6.02	22.01	23.21	27.85	27.07	29.10
S022	50	24	25	25	33	0.65	19.29	11.64	20.51	14.21	0.00	10.45	8.00	12.29	11.27	18.96	12.77	7.35	7.39	7.17	21.77	23.56	26.85	28.29	29.85
S023	52	32	42	36	39	0.68	7.14	1.65	4.45	1.63	0.00	3.32	0.36	2.35	1.06	5.07	8.40	4.24	8.28	5.13	25.20	24.89	31.12	33.39	33.96
S024	43	28	35	31	34	9.58	20.49	9.84	19.31	11.67	0.36	13.70	2.71	9.80	5.53	5.06	6.95	4.59	7.65	2.59	21.19	23.40	28.60	26.47	27.49
S025	49	32	34	28	32	4.11	5.72	4.99	13.75	5.78	0.00	5.32	4.72	9.91	5.69	5.96	12.76	5.59	6.04	5.66	19.57	20.21	27.24	27.10	28.05
S026	48	26	25	29	28	4.26	12.78	14.87	13.11	7.87	0.01	11.87	13.68	11.06	8.68	12.83	7.65	9.02	11.65	10.08	21.06	22.90	28.48	27.17	26.90

Appendix B: Table of mean values for various CIS traits

	Agtron score					Glucose mg g^{-1} dry weight					Fructose mg g^{-1} dry weight					Sucrose mg g^{-1} dry weight					% Dry matter				
ST	a	b	c	d	e	a	b	c	d	e	A	b	c	d	e	a	b	c	d	e	a	b	c	d	e
Line no																									
S027	54	31	39	35	46	0.62	6.93	3.75	6.95	3.41	0.00	6.80	4.61	5.60	2.21	5.76	8.92	6.02	3.72	2.87	23.01	21.84	26.40	27.83	30.02
S028	42	24	24	24	26	6.13	20.66	14.67	19.72	14.89	0.48	20.71	16.82	11.51	8.92	12.38	12.10	11.86	8.34	8.65	17.08	21.51	24.79	26.55	25.44
S029	50	29	32	34	30	0.37	6.74	2.77	5.99	4.54	0.00	6.20	2.34	7.08	4.65	4.64	11.68	4.18	8.66	5.57	22.58	22.09	27.06	29.12	27.42
S030	44	24	35	25	32	0.32	15.78	1.90	13.26	2.50	0.00	11.11	1.93	11.96	2.34	9.31	12.94	4.91	9.54	3.94	23.83	23.81	28.49	28.58	29.56
S031	47	30	40	40	49	3.53	8.19	3.75	2.96	1.20	0.00	4.47	1.08	1.59	0.00	8.57	10.90	5.16	7.34	3.44	18.72	21.99	28.27	29.48	32.47
S032	49	29	28	34	32	3.23	20.53	21.72	18.68	15.94	0.57	10.22	10.06	7.01	4.98	7.13	8.33	10.03	5.96	6.72	17.27	18.59	25.35	27.89	26.24
S033	47	24	32	25	28	0.26	18.30	3.28	17.28	5.25	0.00	14.28	0.00	16.95	1.80	7.93	13.65	6.43	13.07	3.97	21.10	24.62	25.89	27.11	28.87
S034	49	32	44	38	47	5.21	13.01	1.14	11.26	3.16	0.00	5.72	1.04	5.96	1.21	10.53	16.82	5.03	13.50	4.25	21.33	21.46	27.74	28.96	28.62
S035	44	26	27	33	35	5.79	11.34	11.25	10.95	8.18	0.14	9.52	7.40	6.10	5.92	6.35	9.94	6.00	10.06	6.92	23.01	23.60	26.43	30.00	30.86
S037	44	27	39	31	37	3.28	16.56	0.25	15.98	12.85	0.00	10.95	3.48	8.73	7.81	6.43	10.83	6.87	11.56	8.05	23.32	18.39	29.71	30.48	30.62
S038	52	30	34	36	42	0.10	4.18	7.74	1.77	0.97	0.00	3.82	4.34	1.93	1.09	3.94	7.27	5.45	6.03	2.96	21.19	23.62	29.07	30.65	30.24
S039	50	34	43	36	39	0.70	11.46	1.69	4.99	3.71	0.00	7.09	1.16	3.20	2.38	9.83	18.64	7.34	10.24	6.57	20.87	22.84	29.28	28.54	27.39
S040	43	24	34	27	37	1.94	17.11	6.39	14.58	5.77	0.00	9.56	7.16	9.90	3.49	8.11	9.88	7.18	10.24	6.50	20.40	22.58	29.70	30.03	30.12
S042	48	41	43	41	51	2.15	5.15	0.17	3.63	1.41	0.00	1.84	0.00	1.55	0.25	5.91	7.28	4.55	6.99	4.74	22.41	25.05	29.15	30.92	30.42
S043	48	30	42	33	50	0.24	6.15	1.70	4.68	0.70	0.00	5.98	1.48	4.86	0.10	9.73	11.47	6.00	8.85	5.18	23.43	23.10	28.75	29.25	31.56
S044	45	26	36	29	31	0.96	16.62	3.05	12.58	5.03	0.00	13.17	3.19	8.85	5.21	11.73	8.99	8.00	9.70	4.44	22.10	24.54	28.63	29.32	28.61
S045	51	30	50	42	56	0.60	12.84	0.99	0.68	0.21	0.00	6.49	0.10	0.22	0.00	6.23	8.34	6.30	8.32	5.61	24.42	24.82	29.88	31.09	30.94
S046	51	42	40	33	41	0.52	3.97	3.08	6.95	2.20	0.00	3.67	2.50	6.70	1.84	7.26	7.55	6.15	8.11	5.60	23.97	26.14	30.93	32.64	34.02
S047	48	29	38	31	45	2.15	8.64	4.94	10.83	2.06	0.00	12.90	0.00	5.81	1.17	5.34	10.09	6.89	10.70	4.85	22.29	22.10	31.12	30.10	31.16
S048	34	28	31	30	26	2.96	21.43	14.05	16.16	14.11	0.39	13.64	8.28	11.46	11.60	7.51	13.83	8.32	7.15	8.55	17.39	20.19	24.54	25.21	23.36
S050	44	33	34	32	30	1.14	10.86	4.94	16.13	21.95	0.00	6.05	2.85	6.36	7.49	6.34	9.96	7.42	8.41	8.02	21.86	21.83	28.29	28.70	26.43
S051	49	32	41	35	44	0.34	5.85	2.21	2.91	1.86	0.00	3.14	0.91	2.28	0.59	4.35	4.71	3.45	5.09	3.10	25.33	27.02	30.31	32.44	31.84
S052	46	24	34	26	34	6.34	13.05	5.64	16.53	3.50	0.00	9.24	3.28	11.56	3.31	14.21	14.15	6.97	12.11	6.41	21.97	23.01	29.90	30.43	31.31
S053	56	37	30	25	28	0.35	8.32	5.18	17.41	6.09	0.00	7.41	4.92	16.05	6.37	6.67	9.35	6.81	12.63	6.17	23.96	23.98	29.60	28.92	30.94
S054	44	33	34	31	48	4.87	10.92	13.60	8.30	2.06	0.16	4.03	6.27	3.47	0.02	14.07	12.84	8.94	12.61	7.35	18.87	19.96	26.11	27.59	27.12

Appendix B: Table of mean values for various CIS traits

	Agtron score					Glucose mg g^{-1} dry weight					Fructose mg g^{-1} dry weight					Sucrose mg g^{-1} dry weight					% Dry matter				
ST	a	b	c	d	e	a	b	c	d	e	A	b	c	d	e	a	b	c	d	e	a	b	c	d	e
Line no																									
S055	49	34	42	36	46	0.93	8.49	1.32	3.91	1.54	0.00	7.59	0.24	2.85	0.00	9.82	19.68	7.61	9.74	6.56	22.41	24.40	29.88	32.28	30.80
S056	52	26	34	26	28	0.84	10.69	4.47	9.25	3.58	0.05	10.25	4.60	9.57	4.02	9.63	11.44	6.42	8.99	5.88	21.91	21.90	26.89	27.45	27.80
S057	46	34	44	42	47	2.81	5.44	1.24	2.15	1.17	0.00	1.64	0.06	1.08	0.03	2.93	6.38	3.04	6.78	2.75	19.89	21.36	27.02	27.00	27.75
S058	45	29	34	27	30	7.13	12.41	4.95	13.50	4.76	0.18	8.70	3.72	9.28	4.54	9.23	9.13	6.52	9.30	7.30	21.54	23.74	28.81	30.27	32.10
S059	53	31	43	32	40	0.46	6.74	1.54	4.29	1.23	0.00	7.90	1.12	4.03	1.42	4.22	9.95	5.15	7.33	3.40	21.81	22.56	28.89	30.48	29.21
S060	50	30	37	32	37	6.13	14.34	2.55	14.88	4.68	0.00	11.18	3.53	11.51	4.97	6.78	12.16	5.64	8.54	5.04	23.69	22.94	31.32	28.83	31.15
S061	54	36	43	42	44	0.13	3.54	2.50	2.53	1.17	0.00	2.67	1.06	1.35	0.30	7.99	12.56	6.67	7.98	5.80	21.42	24.86	28.40	30.58	28.48
S062	50	30	30	31	31	0.19	10.01	7.35	6.76	5.15	0.00	9.68	6.29	6.96	5.86	6.83	17.05	12.13	11.15	6.70	19.57	21.70	25.97	28.02	26.46
S063	49	35	43	37	39	0.23	9.35	4.57	2.20	1.69	0.05	2.61	1.33	1.61	1.59	4.06	4.21	3.55	4.30	2.12	23.79	24.32	30.46	30.38	30.02
S064	48	30	39	31	39	0.56	6.43	1.40	4.08	1.35	0.00	7.01	1.45	3.23	0.45	4.99	10.32	6.01	6.72	3.30	23.30	25.15	28.85	30.30	30.04
S065	50	36	43	34	42	1.04	9.82	1.59	9.65	1.80	0.00	6.57	0.00	5.27	0.30	10.41	10.75	7.83	4.13	7.64	25.09	26.94	32.14	32.14	31.77
S066	45	27	35	29	36	4.33	16.58	8.56	13.35	4.38	0.44	8.12	5.04	9.23	2.97	17.78	14.00	3.54	9.43	7.93	23.61	24.71	28.41	30.42	33.30
S068	53	32	36	34	36	0.31	5.92	3.66	4.88	1.47	0.00	5.07	2.11	4.49	0.82	8.20	12.26	7.80	8.61	6.07	22.31	21.35	27.76	28.02	30.01
S069	50	28	34	29	28	0.55	15.04	2.43	17.56	9.06	0.00	10.76	4.91	9.78	6.64	8.35	14.97	11.55	13.53	6.37	24.86	25.13	29.63	28.82	28.70
S070	51	25	32	27	33	0.18	13.21	8.26	11.75	5.14	0.00	4.42	4.23	10.70	7.00	5.21	5.39	3.65	9.46	5.13	20.38	22.95	26.40	27.27	27.35
S072	53	29	30	39	43	0.06	10.11	2.05	3.57	1.26	0.00	8.06	2.57	4.01	1.63	7.23	14.56	6.50	6.38	3.84	24.92	22.97	30.00	31.60	31.68
S073	49	27	37	34	38	0.30	11.13	1.36	7.76	1.59	0.00	10.84	1.43	7.61	1.44	7.69	10.41	5.45	6.72	2.61	22.97	25.65	29.66	31.96	30.20
S074	52	41	55	38	43	0.37	1.33	0.27	2.01	1.67	0.00	0.72	0.00	1.74	1.18	8.03	6.24	4.75	6.13	4.68	23.38	27.59	32.66	33.64	33.40
S078	36	27	30	27	34	9.51	9.99	8.34	10.76	3.41	0.32	6.95	5.22	7.52	2.82	6.37	7.03	4.26	6.53	5.49	20.18	21.85	27.37	28.24	29.64
S081	49	27	37	30	41	1.19	15.82	3.64	9.29	1.59	0.00	14.72	0.00	5.59	2.20	6.40	11.35	10.20	5.20	4.53	25.68	27.11	30.84	33.01	32.82
S082	54	36	37	37	34	0.45	6.18	3.47	6.06	5.93	0.00	5.27	3.83	5.96	5.33	7.36	9.54	6.96	7.72	5.06	23.54	22.84	28.02	29.06	28.14
S083	53	32	41	40	45	1.86	6.23	4.75	3.26	1.66	0.00	5.17	3.53	2.56	0.30	9.15	13.55	7.71	8.68	4.03	21.62	24.80	30.02	31.35	30.20
S084	46	24	33	32	26	1.79	15.31	9.34	14.74	15.97	0.88	7.65	7.39	6.58	9.06	11.55	15.76	4.38	10.66	10.00	22.87	25.91	29.02	29.74	29.07
S085	50	28	40	37	36	0.72	16.23	6.54	8.21	2.69	0.00	14.53	6.39	5.98	2.01	12.88	15.51	8.87	10.81	4.88	22.99	22.82	27.68	30.09	28.29
S086	51	26	27	30	28	0.51	8.74	5.67	10.44	5.63	0.00	8.63	6.83	10.20	6.71	3.38	9.65	4.77	6.70	4.31	23.80	25.42	30.31	30.70	32.28

Appendix B: Table of mean values for various CIS traits

	Agtron score					Glucose mg g^{-1} dry weight					Fructose mg g^{-1} dry weight					Sucrose mg g^{-1} dry weight					% Dry matter				
ST	a	b	c	d	e	a	b	c	d	e	A	b	c	d	e	a	b	c	d	e	a	b	c	d	e
Line no																									
S087	50	30	40	32	46	0.23	6.34	1.62	7.94	0.99	0.00	5.26	1.41	4.78	0.85	8.22	10.00	4.68	9.38	3.27	25.41	24.20	29.10	29.76	30.60
S088	38	24	35	32	34	0.75	20.98	9.93	8.83	2.65	0.97	16.93	11.96	5.06	2.75	9.27	8.76	5.54	9.73	7.02	18.19	18.90	27.14	28.88	28.35
S089	50	28	37	32	45	0.53	12.20	4.76	11.11	0.86	0.00	7.50	3.92	6.51	0.35	14.68	7.20	7.59	9.42	3.56	24.83	25.45	31.54	30.52	33.09
S090	42	49	54	53	52	0.02	1.99	1.61	1.72	0.97	0.00	0.93	0.15	1.32	0.29	3.08	12.25	6.25	8.07	5.13	21.98	24.36	29.52	33.52	32.82
S091	49	30	41	31	44	0.70	6.14	3.35	7.30	1.06	0.00	5.71	2.81	3.47	0.00	8.08	19.82	1.93	11.61	6.35	25.33	24.27	29.57	31.28	31.73
S092	50	31	35	33	38	0.18	6.54	5.32	4.83	2.54	0.00	3.78	4.10	3.98	2.22	9.06	13.24	10.72	6.50	3.54	22.58	22.95	30.15	32.82	30.01
S093	42	34	40	32	40	0.99	2.24	1.31	6.71	2.50	0.06	1.61	0.36	4.20	1.06	8.12	5.96	3.29	8.08	4.43	25.46	26.96	30.78	32.65	32.46
S095	47	32	35	26	31	0.89	3.89	2.66	10.36	5.01	0.00	2.88	2.03	6.72	2.67	4.41	4.13	3.18	5.79	2.26	21.03	23.88	26.81	27.23	27.45
S096	49	30	37	28	32	0.29	7.83	3.06	10.84	8.30	0.00	4.40	2.47	7.84	6.65	8.21	13.14	7.00	8.91	3.78	25.50	26.45	29.73	33.22	31.81
S098	47	32	40	38	38	1.17	4.70	4.61	5.41	3.73	0.00	2.71	2.66	3.19	2.87	9.23	9.32	5.46	9.11	5.12	19.12	21.24	26.26	25.31	23.98
S099	47	28	32	30	30	1.24	13.60	11.35	15.55	11.83	0.28	11.85	6.85	10.38	7.63	4.11	7.06	3.75	7.35	4.60	22.75	23.26	27.85	29.31	30.46
S100	49	31	43	42	44	0.65	5.55	6.77	3.54	4.31	0.00	3.82	2.71	2.49	1.43	5.80	9.43	8.62	11.87	5.29	23.81	23.00	28.47	31.48	30.00
S101	42	24	31	28	34	4.91	21.84	4.91	12.12	3.04	0.88	11.64	4.45	9.33	3.16	5.81	8.09	5.39	9.46	5.23	20.43	21.10	29.04	29.19	29.42
S103	54	24	28	24	29	0.33	10.76	10.99	11.20	4.79	0.00	12.76	11.75	10.26	5.39	7.30	6.41	6.16	6.47	3.48	23.01	22.31	28.56	28.04	29.17
S104	44	32	35	30	33	2.43	15.04	7.32	9.78	5.19	0.00	5.75	3.45	7.05	2.42	11.50	14.60	8.13	6.45	5.83	22.25	25.09	29.97	26.10	28.62
S105	50	36	46	51	54	0.29	3.73	0.99	0.71	0.70	0.00	1.21	0.01	0.01	0.00	6.93	7.71	4.71	5.36	3.33	27.64	27.78	33.74	34.54	34.46
S106	55	32	35	28	41	0.01	6.98	2.97	8.40	1.89	0.00	6.70	3.73	3.19	1.84	7.06	11.10	6.31	6.61	4.40	21.61	22.24	27.31	29.48	29.26
S107	53	32	40	31	42	0.10	3.25	1.47	4.51	1.76	0.00	2.72	0.95	3.79	1.33	9.16	9.72	6.73	9.36	6.64	22.92	23.22	28.81	29.61	28.84
S108	49	35	36	28	37	1.31	9.68	12.54	10.86	7.89	0.00	5.64	5.86	6.24	2.49	6.74	9.32	5.07	8.87	5.84	25.24	25.25	28.91	31.39	31.47
S109	49	32	40	34	32	0.29	3.85	0.23	6.08	1.38	0.00	3.66	0.00	5.43	1.53	0.52	7.24	4.81	8.41	5.01	24.74	24.61	29.31	29.03	29.97
S110	53	29	33	33	34	0.10	12.33	5.10	11.76	4.03	0.00	11.77	4.49	6.34	2.41	5.24	9.13	6.57	6.79	4.91	20.79	22.36	27.17	26.80	28.21
S111	54	28	42	32	42	0.00	9.26	2.48	7.37	1.59	0.06	3.01	1.22	3.21	0.08	8.31	11.80	6.82	9.99	5.82	27.26	25.24	31.41	30.98	31.51
S112	45	26	32	28	32	1.70	17.49	11.75	12.82	10.22	0.00	10.15	6.35	8.44	7.91	7.71	11.89	11.01	8.85	7.73	23.75	24.78	29.26	29.55	28.86
S113	52	30	40	34	39	0.13	10.95	3.37	10.73	1.80	0.00	9.21	2.83	7.70	1.95	5.64	10.31	6.75	8.43	5.81	22.94	23.36	28.07	28.38	30.84
S114	51	26	42	31	38	0.54	10.45	1.70	8.32	1.38	0.00	8.54	1.55	7.42	1.28	9.19	11.31	6.36	8.09	5.04	23.92	28.53	32.76	31.96	32.25

Appendix B: Table of mean values for various CIS traits

	Agtron score					Glucose mg g^{-1} dry weight					Fructose mg g^{-1} dry weight					Sucrose mg g^{-1} dry weight					% Dry matter				
ST	a	b	c	d	e	a	b	c	d	e	A	b	c	d	e	a	b	c	d	e	a	b	c	d	e
Line no																									
S115	43	24	24	28	33	4.08	22.07	13.81	11.26	12.76	0.00	15.38	11.23	9.06	9.34	8.97	10.21	5.93	10.23	6.68	21.24	21.66	28.00	29.48	26.96
S116	54	38	53	38	49	0.42	3.28	0.82	3.50	0.91	0.00	1.86	0.00	1.03	0.00	4.70	8.12	5.52	7.38	4.65	23.23	24.92	29.24	34.70	34.31
S117	48	28	36	32	38	0.17	10.84	3.40	8.59	4.47	0.00	5.78	1.61	5.64	1.91	8.69	11.38	6.24	8.55	5.91	22.06	23.81	32.13	28.63	45.36
S118	51	38	36	29	45	1.63	5.36	3.80	7.20	3.63	0.34	5.19	3.23	3.23	2.91	7.23	11.63	8.72	19.66	8.72	19.15	19.87	25.12	27.96	27.41
S119	48	29	40	28	32	0.55	15.04	2.43	11.03	4.47	0.00	6.18	1.89	6.76	2.83	7.54	6.38	4.99	7.94	5.21	21.87	22.73	27.12	28.74	27.92
S120	52	47	42	42	54	0.24	2.01	1.86	2.99	0.94	0.15	1.09	6.64	1.48	0.01	5.84	15.49	5.56	10.87	7.47	26.27	27.61	29.69	32.51	33.70
S121	52	25	34	28	38	0.13	11.81	5.17	10.60	3.60	0.00	12.65	7.13	7.01	2.94	7.03	14.32	7.14	9.02	6.10	20.89	22.49	27.93	26.53	29.30
S122	49	31	40	36	48	0.12	5.58	2.62	4.11	0.71	0.00	2.62	1.01	2.87	0.24	5.47	7.13	5.52	7.45	4.71	25.56	27.10	28.62	30.85	31.50
S123	48	41	46	42	45	0.69	4.32	1.95	3.35	1.42	0.00	2.27	0.20	2.23	0.44	4.97	7.02	5.32	6.45	4.45	25.41	26.54	29.59	32.66	32.50
S124	50	29	34	32	33	3.87	9.26	7.06	10.11	9.71	0.42	9.58	6.04	6.46	6.14	10.03	14.16	8.87	9.20	5.50	18.88	20.43	24.66	27.00	27.34
S125	49	32	48	40	45	0.29	4.01	0.60	5.03	4.05	0.00	2.26	0.00	2.36	1.34	7.17	12.27	7.30	6.87	6.05	24.72	25.10	34.24	31.34	31.08
S127	46	32	32	38	45	0.45	15.01	5.23	7.09	1.31	0.30	9.02	3.98	6.95	1.32	7.92	18.28	7.91	6.34	4.26	26.39	23.92	31.33	32.15	33.35
S128	49	29	38	33	42	0.42	10.02	3.97	11.13	1.82	0.02	9.39	2.99	5.92	1.60	5.26	14.83	7.48	9.82	5.21	22.13	22.30	27.38	29.49	29.02
S129	47	24	25	25	25	1.82	16.66	16.00	11.03	5.08	0.00	10.00	14.35	10.55	4.65	6.38	11.89	8.23	8.34	3.84	20.42	21.93	26.38	26.75	28.73
S130	51	24	24	25	30	0.37	20.41	15.50	14.31	6.13	0.00	11.85	7.13	11.65	4.61	6.02	7.12	3.43	6.23	4.30	20.20	21.70	27.14	26.45	28.62
S131	51	32	30	34	46	0.14	5.15	2.15	2.86	0.65	0.00	1.77	2.02	2.95	0.50	4.75	10.64	6.84	9.07	4.70	23.28	25.47	30.44	31.86	32.89
S132	40	24	37	30	33	2.94	24.12	13.10	13.59	12.35	0.00	9.37	3.24	6.92	5.45	2.76	9.02	4.66	4.57	4.25	19.86	18.67	26.97	24.76	25.56
S133	52	26	36	24	30	0.20	12.14	1.22	21.72	13.07	0.00	11.80	2.14	11.49	3.19	6.42	9.87	5.53	8.30	6.17	23.07	24.16	30.37	30.00	29.90
S134	51	37	50	38	54	0.26	5.46	1.10	5.43	1.32	0.00	2.17	0.29	2.40	0.17	6.20	9.36	7.13	7.89	4.59	26.01	25.04	30.29	31.50	33.21
S135	54	38	50	38	45	0.83	4.30	1.17	6.84	2.55	0.00	4.00	0.73	5.42	1.06	4.82	8.76	3.60	9.97	3.43	22.74	24.84	28.33	29.46	29.14
S136	49	26	30	26	35	0.66	11.28	6.97	15.59	2.30	0.00	10.03	5.10	12.18	2.30	8.99	13.90	5.75	13.01	6.98	26.42	25.14	30.52	30.06	31.12
S137	51	38	37	38	45	0.64	3.54	3.15	5.30	1.07	0.00	2.87	2.92	4.36	0.63	5.30	10.12	7.02	7.86	5.65	22.16	23.49	28.10	29.21	28.80
S138	49	32	45	37	39	0.38	10.37	3.95	6.77	4.65	0.00	8.55	3.00	4.50	2.97	10.98	20.56	10.73	9.22	4.96	19.95	21.50	27.48	31.83	29.85
S139	53	31	48	36	45	0.73	4.34	1.23	3.20	0.92	0.00	3.88	0.76	2.76	0.00	4.58	13.57	5.05	6.90	3.35	22.16	24.68	26.94	27.83	28.18
S140	54	48	48	44	42	0.37	1.93	1.37	1.68	1.80	0.00	1.35	1.03	0.81	0.50	6.35	9.41	8.80	6.80	5.13	19.07	20.50	27.22	26.37	26.97

Appendix B: Table of mean values for various CIS traits

	Agtron score					Glucose mg g^{-1} dry weight					Fructose mg g^{-1} dry weight					Sucrose mg g^{-1} dry weight					% Dry matter				
ST	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e
Line no																									
S141	50	32	46	37	44	0.32	12.06	1.91	9.95	6.25	0.00	5.10	1.85	6.60	3.70	6.39	12.73	6.49	8.80	6.20	23.96	26.06	30.59	29.28	30.97
S142	48	29	38	32	34	1.79	10.53	1.63	4.79	2.90	0.00	8.46	1.47	2.62	1.45	7.07	13.85	2.99	8.13	2.69	22.71	25.28	30.61	31.62	32.95
S143	44	32	40	32	40	4.19	6.95	2.92	6.98	3.96	0.00	2.94	1.75	7.03	1.29	9.36	8.80	5.49	5.82	4.79	23.17	28.10	31.43	28.56	34.30
S144	48	32	38	27	34	0.27	6.15	3.59	7.92	3.67	0.00	3.90	1.87	5.62	3.16	6.28	5.11	3.99	7.24	4.66	22.26	21.50	27.10	27.95	27.20
S145	52	28	30	29	31	0.26	9.41	7.74	8.27	3.81	0.00	9.60	7.99	9.28	4.69	4.27	8.22	5.96	5.88	2.67	19.84	19.95	25.57	25.66	26.95
S146	48	32	33	28	30	1.12	9.32	6.78	10.34	7.03	0.00	9.71	6.32	9.60	4.73	8.62	12.73	10.36	10.85	7.03	22.37	24.37	28.28	28.16	27.06
S147	48	26	26	28	43	0.62	10.29	0.28	13.34	2.99	0.13	8.32	0.00	4.83	3.24	8.28	10.16	6.91	8.69	2.69	25.02	26.83	33.32	33.44	32.08
S148	50	33	43	35	48	0.55	15.04	2.43	7.61	0.71	0.00	6.96	2.63	5.93	0.00	7.79	10.08	6.88	9.28	4.22	24.27	24.90	29.21	29.80	29.63
S149	50	38	49	42	50	0.58	3.10	1.54	2.11	0.40	0.00	2.10	0.69	0.89	0.12	5.82	7.41	5.26	7.68	3.50	20.85	22.12	28.85	28.88	29.69
S151	50	32	39	35	33	0.56	3.70	1.59	5.16	3.48	0.00	3.07	0.97	4.45	3.21	6.26	10.24	7.07	6.25	4.76	22.28	23.23	30.40	29.75	30.18
S152	39	29	28	30	28	0.73	15.79	9.07	18.35	13.42	0.19	13.03	8.74	12.05	11.80	20.77	9.66	4.79	9.41	7.39	21.79	22.55	28.42	28.47	30.52
S153	51	28	31	24	28	0.55	15.04	2.43	15.10	2.57	0.00	11.58	9.85	11.43	3.60	5.50	12.32	6.98	6.45	4.17	21.37	20.93	26.58	26.23	27.16
S155	50	26	36	32	33	0.69	10.49	8.29	10.63	6.67	0.00	10.14	4.98	6.17	4.39	6.38	12.54	12.36	12.54	7.88	25.24	24.36	28.79	32.06	30.18
S156	50	27	36	26	32	0.21	6.63	2.57	10.86	2.92	0.00	7.09	3.17	10.28	4.50	6.57	8.37	5.15	9.66	4.48	21.13	22.63	28.57	27.62	27.56
S157	51	30	38	33	39	0.63	5.47	2.79	5.65	1.03	0.00	5.70	3.20	5.70	0.03	8.37	12.97	8.77	7.93	3.80	20.76	22.29	27.26	25.92	27.40
S158	48	41	40	31	33	5.07	4.56	3.92	7.37	5.71	0.04	3.33	3.97	6.06	5.52	13.13	10.82	5.92	7.41	6.20	23.09	24.44	27.74	29.11	27.12
S159	46	28	34	29	28	4.14	18.88	11.00	17.96	18.68	0.00	13.08	6.69	11.73	11.19	7.36	17.13	5.66	9.05	10.50	23.26	24.86	28.25	27.06	31.55
S160	44	32	34	37	42	0.05	7.90	4.50	6.93	3.12	0.03	2.47	0.84	3.56	1.31	11.01	8.81	5.73	9.56	4.73	19.68	24.88	26.72	28.22	30.61
S161	54	34	34	45	44	0.55	9.58	1.08	3.87	1.91	0.00	2.50	0.18	0.84	0.33	7.30	8.45	6.18	7.24	4.03	24.00	25.52	32.93	31.42	31.96
S162	52	44	50	42	54	0.27	1.75	0.27	8.97	0.62	0.00	0.52	0.00	4.24	0.00	4.35	6.13	4.21	5.65	3.49	23.16	24.94	28.75	29.12	29.78
S163	49	28	37	31	35	0.66	6.68	1.81	5.64	1.83	0.28	6.82	2.77	6.22	2.85	3.32	7.60	4.05	6.07	3.80	21.34	21.42	28.80	28.87	27.75
S164	46	39	48	36	44	1.99	3.00	2.05	3.29	2.25	0.00	1.91	1.63	2.14	1.35	12.66	9.89	4.90	6.48	5.13	25.74	27.41	31.24	31.55	32.48
S165	49	29	31	28	33	2.84	20.41	8.33	19.23	6.09	0.18	14.70	10.13	12.39	6.42	6.17	11.40	3.94	7.60	2.64	21.33	22.56	26.58	28.03	27.63
S166	47	42	52	50	52	0.64	1.89	0.66	1.05	0.54	0.00	0.54	0.00	0.06	0.00	8.11	7.71	5.72	9.28	5.19	23.70	23.12	30.84	31.50	31.74
S167	47	29	34	32	34	9.59	10.82	9.02	13.92	7.38	0.22	10.07	8.33	10.46	7.13	8.10	9.32	7.26	6.56	3.22	21.70	23.22	28.79	30.27	28.88

Appendix B: Table of mean values for various CIS traits

	Agtron score					Glucose mg g^{-1} dry weight					Fructose mg g^{-1} dry weight					Sucrose mg g^{-1} dry weight					% Dry matter				
ST	a	b	c	d	e	a	b	c	d	e	A	b	c	d	e	a	b	c	d	e	a	b	c	d	e
Line no																									
S168	46	29	41	37	40	1.12	11.88	1.56	9.95	4.67	0.00	7.37	0.80	6.47	1.58	9.94	14.97	6.68	6.45	5.72	23.38	27.45	32.00	29.85	31.87
S169	50	32	38	31	42	4.79	11.43	9.33	14.65	4.16	1.03	6.44	1.49	7.15	2.75	5.69	5.85	4.11	6.23	3.38	24.88	24.39	29.54	30.93	31.04
S170	56	37	42	50	50	0.50	5.47	0.76	1.04	1.75	0.03	4.43	0.26	0.29	0.37	5.17	10.51	4.71	7.01	7.71	26.06	29.25	33.23	36.40	35.39
S171	54	31	30	33	38	0.20	5.58	2.61	5.33	2.03	0.00	5.78	3.67	5.01	2.48	5.34	13.46	7.63	11.01	5.68	22.54	23.89	31.16	33.33	30.93
S172	49	31	33	34	38	0.29	4.18	2.78	5.30	1.45	0.00	2.55	2.94	4.37	0.76	5.81	7.63	7.92	7.66	3.92	24.54	26.10	28.56	29.74	30.99
S173	50	42	38	34	41	0.26	0.74	1.34	10.49	4.36	0.00	1.28	2.38	7.61	3.18	7.93	6.41	6.73	4.26	2.42	19.39	22.74	27.93	29.00	28.45
S174	47	36	45	42	40	0.83	2.69	1.11	2.79	0.63	0.00	1.67	0.34	1.44	0.01	4.34	7.66	3.47	5.65	0.36	21.71	23.32	28.01	29.18	27.80
S175	48	35	44	48	51	0.20	1.92	0.49	1.00	0.71	0.00	1.18	0.00	0.32	0.00	7.05	8.13	5.66	8.37	4.78	24.82	26.22	32.18	32.83	31.61
S176	48	29	39	28	45	0.23	11.48	0.67	12.07	1.13	0.00	7.88	0.15	7.53	0.00	11.06	13.08	6.79	5.80	4.54	22.29	20.90	29.51	29.76	30.36
S177	50	41	45	44	43	0.39	1.17	0.95	1.07	1.48	0.00	0.46	0.19	0.62	0.87	6.28	7.07	5.95	6.55	4.92	22.09	20.39	26.99	27.85	28.44
S178	48	40	42	40	44	0.12	0.55	0.08	1.45	0.71	0.00	1.28	0.34	0.72	0.12	7.78	8.65	7.97	8.56	4.45	21.71	26.08	29.67	30.53	31.77
S179	52	33	37	31	28	0.21	4.44	3.01	3.76	10.10	0.00	4.29	3.12	4.52	9.63	4.51	9.42	5.17	9.74	5.74	24.27	24.17	28.84	28.09	30.25
S180	49	32	46	34	42	1.36	10.28	1.48	3.59	2.07	0.11	4.50	0.31	2.16	0.27	5.94	10.36	7.77	8.72	4.33	24.73	24.48	31.13	29.06	29.51
S181	51	32	34	34	39	0.66	7.96	5.72	5.08	3.63	0.00	7.21	4.25	4.81	2.82	5.13	8.61	6.00	6.38	4.61	23.88	23.76	30.70	33.07	32.47
S182	48	32	47	39	53	0.26	9.20	2.87	6.19	0.73	0.00	7.42	0.53	1.84	0.00	9.76	7.32	6.07	11.03	4.22	25.34	25.74	29.92	31.52	33.18
S183	49	30	45	31	32	1.80	8.23	3.06	10.12	4.29	0.00	3.97	3.18	6.82	3.19	6.29	8.95	5.23	6.67	2.88	23.59	24.78	31.80	33.37	33.49
S184	50	31	32	42	40	0.92	8.82	7.54	5.67	3.71	0.00	8.82	7.44	2.29	2.22	5.79	9.01	5.65	7.50	4.51	22.96	22.17	29.04	31.76	31.10
S185	52	26	26	28	25	1.77	11.26	7.85	15.32	8.91	1.82	10.11	5.41	12.31	9.35	6.10	8.47	5.50	10.68	6.36	22.88	21.61	27.04	27.30	28.09
S186	47	32	36	31	32	0.63	6.91	5.07	12.52	10.36	0.03	3.53	4.00	5.33	5.26	7.80	6.54	5.50	6.21	4.08	21.66	21.60	28.06	25.99	27.40
S187	52	50	47	56	56	0.56	1.42	1.32	0.67	0.00	0.00	0.85	0.62	0.10	0.00	6.37	8.90	8.44	8.79	5.25	23.81	25.22	33.76	34.54	35.48
S188	47	33	41	39	48	1.65	2.63	0.93	3.91	0.94	0.00	2.43	0.03	2.73	0.12	11.04	4.88	4.36	7.72	4.46	23.36	22.33	30.69	29.94	30.29
S189	55	41	46	50	52	0.59	5.36	1.35	1.43	0.85	0.00	4.79	0.87	1.19	0.31	4.18	9.09	6.84	6.05	3.74	20.70	22.18	27.32	29.33	29.69
S190	47	24	24	24	24	1.27	20.09	10.81	22.25	20.21	0.33	11.07	9.45	12.99	9.32	2.90	8.56	7.74	13.17	9.84	16.56	17.04	24.24	21.30	23.34
S191	46	32	36	30	41	1.22	10.01	5.31	14.18	10.13	0.00	6.92	3.62	11.41	7.41	6.55	8.56	4.40	12.88	5.30	22.01	22.05	26.71	27.45	29.32
S192	41	25	32	29	31	5.34	12.98	7.38	7.70	3.34	0.00	8.76	5.94	6.31	2.40	9.00	8.01	5.26	8.04	3.96	21.69	24.82	32.84	30.29	32.16

Appendix B: Table of mean values for various CIS traits

	Agtron score					Glucose mg g^{-1} dry weight					Fructose mg g^{-1} dry weight					Sucrose mg g^{-1} dry weight					% Dry matter				
ST	a	b	c	d	e	a	b	c	d	e	A	b	c	d	e	a	b	c	d	e	a	b	c	d	e
Line no																									
S193	40	27	36	24	30	11.52	7.97	2.46	9.32	5.31	0.50	6.27	2.74	6.87	3.67	17.05	11.24	6.03	8.82	5.07	23.89	24.78	28.56	28.94	30.23
S194	38	26	32	29	32	0.18	16.45	10.43	13.28	9.41	0.35	10.90	6.46	9.09	8.12	15.34	12.42	7.97	10.01	7.03	19.64	22.09	28.98	28.05	27.54
S195	48	31	38	30	34	0.63	12.37	4.76	8.61	5.59	0.00	8.70	4.87	6.63	4.14	9.26	9.95	7.88	10.52	4.47	21.66	23.95	28.45	29.25	30.03
S196	46	37	47	42	48	3.37	6.12	3.59	3.70	2.01	0.00	2.47	0.47	2.32	0.83	5.67	10.87	5.80	6.22	4.05	23.17	23.78	29.70	31.19	30.60
S203	47	31	34	33	38	2.02	11.47	9.30	11.64	3.46	0.08	9.02	6.43	8.96	3.02	6.09	9.41	7.93	9.18	3.87	22.17	22.94	28.17	28.97	31.79
S204	46	27	43	28	40	0.22	7.65	0.57	6.33	0.48	0.00	7.42	0.87	6.16	0.96	9.71	13.14	6.36	10.10	5.35	23.34	23.81	28.40	30.04	30.75
S205	54	28	28	26	33	0.20	10.65	10.12	13.89	7.32	0.00	9.36	9.48	11.76	7.32	4.88	10.61	5.56	10.17	4.29	22.97	23.08	27.14	26.54	28.38
S206	52	38	34	27	38	0.32	5.00	2.61	4.75	2.50	0.00	4.69	1.87	3.61	1.46	4.25	12.66	9.03	9.98	5.86	25.40	26.01	31.94	32.03	28.71
S207	54	35	38	36	33	0.43	3.99	4.38	4.57	5.74	0.00	3.85	3.92	3.49	4.30	4.94	7.20	5.10	7.35	4.44	24.18	24.62	31.07	29.65	28.98
S208	52	43	46	38	43	0.97	2.58	1.20	4.86	2.33	0.00	1.98	0.56	4.67	1.60	7.77	6.95	4.77	6.00	3.82	27.30	28.47	32.66	31.20	33.36
S209	50	46	42	39	49	0.46	1.01	0.78	1.73	0.83	0.00	0.50	0.23	1.51	0.06	6.78	7.94	5.36	4.76	3.26	26.55	26.13	31.44	31.81	34.31
S210	48	30	32	27	33	1.02	6.46	5.68	6.93	4.06	0.00	5.91	5.34	7.82	4.39	8.99	11.67	8.26	10.48	8.15	20.65	23.28	28.70	28.32	28.26
S211	52	28	38	31	41	0.70	9.39	5.20	6.13	1.66	0.00	10.10	6.08	6.45	1.90	9.02	16.44	12.44	10.23	6.69	20.74	21.16	25.97	27.71	27.78
S212	47	26	33	26	33	1.64	13.49	2.28	9.70	5.72	0.14	10.35	1.38	7.43	4.55	10.59	7.27	6.60	9.04	5.00	20.95	22.06	28.95	28.41	27.48
S213	48	24	32	28	30	3.09	15.56	5.27	16.18	8.12	0.00	12.13	4.25	11.44	4.80	9.77	12.44	3.71	8.96	4.67	23.66	25.35	30.98	29.35	30.24
S214	48	28	41	36	47	2.66	7.45	6.33	4.07	0.71	0.00	7.40	2.54	3.45	0.00	6.66	13.92	7.44	11.17	5.30	22.60	26.40	28.80	32.06	31.16
S215	49	31	28	26	29	0.41	8.60	7.12	11.05	4.54	0.00	7.49	5.49	9.57	3.84	7.06	10.09	8.70	8.91	4.99	21.26	22.18	29.63	29.24	28.94
S217	54	49	49	51	50	0.27	1.46	0.61	2.38	1.32	0.00	0.28	0.00	1.10	0.11	4.59	5.68	4.12	3.75	2.75	23.27	24.76	29.07	30.17	31.47
S218	46	31	34	27	33	0.86	13.50	4.68	15.17	2.80	0.00	9.91	4.42	8.46	2.96	11.22	13.06	8.36	10.14	4.59	23.76	22.97	28.91	29.59	30.33
S219	47	27	29	26	30	1.04	12.89	7.96	17.33	2.44	0.06	10.80	4.63	8.56	2.29	7.49	12.27	5.60	9.77	5.44	23.17	23.46	29.36	31.00	33.40
S220	51	32	43	44	48	0.70	3.72	1.73	1.38	0.46	0.01	2.42	0.34	0.93	0.00	6.48	6.87	3.72	6.17	2.95	26.64	27.68	32.49	34.95	33.44
S221	48	30	36	28	32	2.59	7.89	13.32	8.37	5.51	0.00	6.95	8.38	5.49	2.35	10.02	10.53	6.68	8.33	7.28	20.51	22.78	25.95	26.41	27.64
S222	54	33	42	32	44	0.08	1.83	0.72	3.61	1.35	0.00	1.10	0.00	2.60	0.54	8.44	7.35	6.01	7.74	2.85	21.02	21.82	26.94	27.15	25.02
S223	46	24	30	25	25	0.93	17.00	6.21	16.54	15.55	0.00	12.71	5.03	10.27	8.27	10.46	12.75	8.49	8.51	7.45	21.80	21.96	27.15	37.52	25.01
S224	52	30	38	28	34	0.42	9.94	3.23	12.84	5.06	0.00	4.85	2.94	8.39	3.91	8.12	10.73	4.91	9.21	5.59	24.88	27.31	34.57	31.54	32.34

Appendix B: Table of mean values for various CIS traits

	Agtron score					Glucose mg g^{-1} dry weight					Fructose mg g^{-1} dry weight					Sucrose mg g^{-1} dry weight					% Dry matter				
ST	a	b	c	d	e	a	b	c	d	e	A	b	c	d	e	a	b	c	d	e	a	b	c	d	e
Line no																									
S225	53	41	42	41	49	0.22	3.46	1.46	1.93	0.52	0.00	4.01	1.36	1.93	0.16	8.93	5.34	7.31	7.61	4.57	22.87	23.96	27.30	28.47	29.15
S226	48	27	40	44	52	0.70	5.19	1.23	1.27	0.98	0.00	3.74	0.04	0.64	0.00	5.30	8.20	6.01	9.52	5.83	23.99	27.52	30.54	33.73	32.85
S229	56	37	41	36	49	0.01	2.62	1.11	3.36	0.86	0.00	1.75	0.00	2.99	0.00	5.03	10.15	5.69	11.03	4.81	22.04	24.84	32.16	31.69	32.84
S230	54	43	46	36	47	0.25	3.12	0.65	2.07	1.66	0.00	3.23	0.20	1.88	0.87	6.66	6.57	4.19	5.78	3.18	23.99	21.75	28.03	37.92	28.19
S231	47	30	40	33	41	1.48	6.02	2.09	5.62	0.82	0.00	5.78	1.74	5.55	0.39	9.13	10.75	6.69	10.13	4.53	27.17	23.95	30.06	29.24	30.29
S232	48	34	46	39	44	0.77	4.88	0.99	3.01	2.02	0.00	1.83	0.00	1.24	0.17	9.89	8.82	3.41	5.48	2.95	25.37	26.47	30.20	29.66	31.73
S233	50	24	24	26	29	0.63	14.70	10.63	13.72	7.68	0.00	13.72	10.64	14.17	9.16	4.04	6.52	3.96	6.71	3.31	18.19	19.81	26.17	26.94	27.52
S235	44	36	48	39	41	6.80	4.09	1.69	6.81	5.08	0.00	0.00	0.00	1.75	0.70	8.59	9.42	7.54	7.66	4.10	22.02	28.35	31.02	29.78	31.80
S236	53	32	39	38	48	0.70	5.75	1.08	2.88	0.66	0.00	3.26	0.72	2.60	0.00	4.54	10.45	5.36	7.06	3.81	22.06	21.35	28.61	28.08	28.85
S237	46	28	41	32	37	0.48	13.21	1.89	10.74	6.88	0.00	9.62	0.36	4.05	1.66	6.32	9.30	5.35	6.56	5.23	26.76	26.81	31.67	32.10	33.04
S245	54	28	37	30	46	2.04	10.81	2.59	4.70	2.90	0.00	8.79	1.66	3.27	1.44	5.55	9.93	5.25	7.61	4.26	25.64	25.99	29.35	31.40	32.38
S246	47	24	24	26	28	1.91	13.79	8.38	10.01	6.97	0.08	13.31	9.42	8.67	6.18	8.07	19.98	9.36	11.09	6.82	19.71	21.60	24.59	27.74	26.01
S247	52	25	37	30	36	0.23	12.55	4.15	12.11	3.13	0.00	12.66	5.50	11.59	3.63	2.94	9.44	5.01	7.67	4.92	21.20	21.04	27.47	29.09	31.43
S248	48	34	50	48	40	0.44	11.42	1.29	7.86	3.72	0.00	3.59	0.16	0.80	0.13	9.41	11.47	5.00	5.31	4.84	23.63	25.30	23.39	26.31	24.35
S249	50	28	46	29	42	0.55	6.87	1.08	8.16	1.85	0.00	6.91	1.30	6.92	1.93	5.07	10.60	5.03	8.01	3.77	21.55	24.27	28.85	29.95	31.77
S250	52	27	30	29	30	0.21	10.75	6.65	10.59	5.01	0.00	11.57	6.91	9.58	6.15	4.16	8.39	4.49	6.62	3.64	21.44	22.50	27.60	27.81	27.90
S251	50	40	43	37	42	0.82	2.06	2.72	10.79	5.20	0.01	1.12	0.59	4.92	1.73	6.24	7.99	6.73	7.50	5.50	24.33	27.52	31.80	30.81	32.36
S252	52	36	38	31	42	0.37	2.80	2.09	3.83	1.12	0.00	2.09	1.69	3.96	0.10	5.46	9.38	4.90	6.65	4.51	23.33	26.73	30.11	30.66	32.77
S253	46	30	33	32	34	1.60	5.31	5.13	6.95	3.24	0.00	4.53	3.39	6.11	2.87	4.78	9.33	7.90	9.92	6.96	21.96	24.77	28.66	31.31	28.93
S254	50	34	42	40	43	2.41	2.65	1.86	2.78	1.81	0.50	2.01	0.59	1.68	1.32	5.89	6.98	4.73	8.11	5.05	22.21	26.92	30.15	32.99	32.92
S255	52	34	44	35	41	0.43	9.63	0.69	4.02	2.94	0.00	3.32	0.56	2.62	2.47	6.05	5.57	4.80	7.02	3.12	21.18	22.53	28.37	27.24	28.17
S256	48	35	50	43	49	0.22	4.37	0.95	4.89	2.93	0.27	3.51	0.38	3.25	1.61	8.57	6.66	3.67	5.86	3.53	20.96	23.14	29.54	29.00	28.55
S257	54	34	46	49	50	0.34	3.42	1.37	2.16	1.30	0.00	2.73	0.84	0.92	0.15	8.32	15.82	10.26	10.68	7.22	24.91	28.22	32.56	32.20	33.08
S258	56	29	42	31	40	0.69	10.27	1.44	11.89	1.95	0.00	6.57	1.11	8.58	0.97	4.29	11.23	4.15	6.31	3.03	21.31	22.29	27.37	26.95	28.09
S259	52	33	40	34	40	0.44	8.54	3.05	6.98	4.15	0.00	7.56	3.39	6.91	4.51	9.92	9.00	5.16	6.50	2.82	20.45	21.30	27.06	25.98	28.19

Appendix B: Table of mean values for various CIS traits

	Agtron score					Glucose mg g^{-1} dry weight					Fructose mg g^{-1} dry weight					Sucrose mg g^{-1} dry weight					% Dry matter				
ST	a	b	c	d	e	a	b	c	d	e	A	b	c	d	e	a	b	c	d	e	a	b	c	d	e
Line no																									
S260	52	30	32	26	37	0.20	7.83	6.09	10.52	4.30	0.00	6.85	6.29	9.28	4.55	6.29	15.66	8.45	12.22	5.50	23.60	23.81	29.37	30.10	29.75
S263	44	31	31	26	29	2.63	11.80	4.75	16.55	12.67	0.00	5.75	2.08	9.83	7.06	5.13	10.03	6.28	9.64	8.48	22.93	23.49	28.79	27.82	28.64
S264	52	30	33	28	34	1.39	18.59	6.62	14.64	4.62	0.00	6.02	5.26	15.95	5.19	9.18	10.57	6.74	10.76	3.96	19.78	20.76	27.58	26.63	26.98
S265	46	24	31	31	30	0.58	16.22	14.80	14.11	12.53	0.52	14.57	11.97	11.71	9.85	10.03	9.86	4.84	8.38	5.76	20.39	20.38	25.74	27.97	27.69
S266	51	32	42	31	36	0.88	4.10	2.13	5.09	1.83	0.00	4.33	2.60	4.94	1.50	6.30	12.18	7.84	11.53	6.94	22.95	27.94	32.65	33.35	31.40
S267	49	24	34	24	26	0.46	19.21	6.64	19.52	6.57	0.00	11.99	7.58	19.00	8.83	13.49	9.89	7.49	10.80	5.56	23.37	21.33	26.23	28.14	29.48
S268	52	44	41	41	46	0.57	4.62	5.90	9.02	2.31	0.00	3.73	2.08	7.50	1.67	8.70	7.71	7.12	8.27	5.57	20.59	22.28	26.25	27.23	28.18
S269	48	26	36	28	26	1.40	17.68	9.07	13.86	16.47	0.10	15.01	7.72	9.28	9.90	11.25	14.57	7.48	9.26	7.87	23.52	25.99	36.48	38.82	41.44
S270	48	30	33	28	33	1.39	8.98	5.38	6.57	2.16	0.00	3.61	1.90	4.41	0.81	12.60	14.54	10.19	9.47	6.98	24.34	22.69	28.00	29.88	30.05
S272	54	32	45	33	42	0.15	4.03	1.60	3.94	3.61	0.00	3.57	1.20	3.57	3.30	2.94	5.55	3.91	5.63	2.57	24.92	25.94	27.80	30.62	31.01
S273	49	29	41	39	41	0.40	5.27	1.17	3.12	1.62	0.00	5.14	0.39	2.45	0.45	8.12	8.89	6.13	7.68	4.93	26.42	24.97	31.36	33.39	34.20
S274	48	32	42	34	39	0.69	6.03	1.61	7.86	1.80	0.00	4.09	0.78	5.48	1.36	5.98	9.34	4.23	8.24	4.25	23.33	25.99	31.42	31.61	32.46
S275	52	32	42	39	40	0.00	6.58	1.85	5.59	1.22	0.00	1.26	1.78	4.87	0.92	7.58	5.90	7.42	9.69	6.36	21.37	24.20	28.55	28.80	30.27
S276	53	36	45	41	44	0.00	3.11	0.90	6.52	2.09	0.00	1.27	0.00	6.43	0.35	7.05	12.24	6.30	11.62	5.51	24.80	24.41	29.89	31.40	32.95
S278	52	41	41	36	43	1.42	7.51	4.70	8.47	2.33	0.00	4.66	1.86	4.51	0.67	6.96	6.65	9.34	10.08	6.16	23.53	24.96	31.13	29.59	29.71
S280	55	34	36	32	30	0.12	6.06	3.54	6.61	4.58	0.00	5.62	2.92	5.68	4.20	2.42	9.50	7.63	7.76	5.64	22.55	23.55	29.46	28.91	28.53
S282	47	25	36	26	30	5.01	13.20	5.65	13.77	6.31	0.33	13.03	6.48	11.68	6.62	5.41	13.49	8.38	9.48	5.32	21.67	24.97	28.97	31.88	31.67
S283	47	29	39	30	35	0.47	12.73	1.69	3.42	2.07	0.00	11.54	1.92	4.23	1.70	6.18	14.50	6.37	8.50	5.46	25.57	27.57	31.39	32.35	23.84
S284	46	30	40	31	41	1.73	6.33	2.74	5.10	0.94	0.00	5.48	2.19	4.77	1.01	6.69	10.34	6.23	10.37	5.29	23.64	25.59	30.26	31.43	30.11
S287	52	28	29	24	25	0.56	7.89	5.77	12.07	7.61	0.21	8.12	6.26	11.03	8.51	4.12	10.40	4.97	8.81	4.93	21.45	20.83	27.37	28.05	28.93
S288	52	30	41	32	38	2.48	5.32	1.54	5.04	1.51	0.00	4.80	1.78	4.27	1.23	7.26	12.56	3.44	8.49	5.28	23.19	25.60	30.06	31.43	32.60
S289	48	24	28	28	29	1.38	18.18	20.61	17.85	12.47	0.00	15.37	14.78	11.10	11.00	9.62	4.87	5.09	9.33	6.98	21.43	23.44	28.22	29.74	29.23
S292	56	30	38	40	38	0.65	5.51	1.31	1.33	1.51	0.13	5.16	1.11	0.91	0.59	9.22	14.30	6.94	10.00	7.46	24.90	25.72	32.33	33.57	32.88
S293	50	44	49	38	49	0.23	1.34	0.27	2.15	0.69	0.00	1.15	0.04	1.71	0.10	0.00	6.64	5.55	6.24	4.58	25.49	26.68	31.31	31.99	33.13
S294	53	24	31	27	32	1.94	11.55	5.39	13.56	3.81	0.00	11.38	4.57	11.39	3.03	12.48	12.81	5.20	8.46	5.10	20.17	19.88	24.98	24.54	27.33

Appendix B: Table of mean values for various CIS traits

	Agtron score					Glucose mg g^{-1} dry weight					Fructose mg g^{-1} dry weight					Sucrose mg g^{-1} dry weight					% Dry matter				
ST	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e
Line no																									
S295	48	32	42	42	40	0.52	2.00	0.92	1.51	1.07	0.00	1.26	0.09	0.67	0.48	5.64	8.54	7.34	9.22	7.60	26.67	28.77	33.80	34.61	33.30
S296	47	27	41	28	44	8.76	17.54	2.25	16.63	3.10	0.15	15.29	1.94	11.38	1.08	10.36	13.86	7.53	9.25	6.72	22.92	24.21	31.25	31.05	32.74
S297	50	30	35	31	32	2.36	12.64	4.87	10.94	5.49	0.00	9.58	4.05	9.63	4.39	2.45	7.69	3.98	9.38	3.05	21.43	24.07	29.63	26.36	28.63
S298	44	31	43	38	38	0.67	4.58	1.36	1.92	3.26	0.00	2.15	0.86	1.28	0.76	8.60	8.84	4.07	8.64	3.86	20.72	22.90	27.64	29.80	28.46
S299	50	33	28	26	30	0.67	8.34	17.12	10.82	9.72	0.24	0.24	13.37	7.27	7.52	8.06	18.69	8.06	14.45	11.57	23.28	23.48	29.69	31.68	32.19
S300	49	31	37	38	39	1.91	5.91	6.85	4.07	1.09	0.00	4.27	4.09	3.84	0.99	5.88	7.43	7.26	7.79	4.41	23.63	24.54	29.47	31.74	30.83
S301	50	40	44	40	45	0.21	2.38	1.28	2.24	2.52	0.00	1.34	0.74	1.55	0.00	6.25	6.73	6.41	5.53	5.92	26.94	29.13	33.24	33.56	34.58
S302	53	36	36	27	30	0.70	3.97	3.61	6.28	1.72	0.00	4.55	3.91	5.95	1.78	5.19	12.94	10.25	10.93	5.64	23.24	23.58	29.17	28.97	29.68
S303	52	37	42	40	43	0.22	2.20	1.32	2.72	0.75	0.00	1.68	1.19	2.68	1.06	4.70	7.12	4.82	6.26	4.39	22.53	23.59	27.47	29.66	30.74
S304	49	31	45	33	47	1.39	4.03	0.33	2.95	0.84	0.00	3.01	0.41	2.70	0.88	5.60	8.68	5.31	7.22	4.33	24.28	27.03	30.11	31.94	33.39
S305	44	24	30	25	35	7.66	18.41	11.67	14.49	7.54	0.00	13.31	6.17	10.93	5.58	7.50	6.22	8.52	12.44	5.80	21.49	21.13	28.77	29.91	30.37
S306	44	24	28	24	24	3.85	24.66	23.28	21.93	18.95	0.06	15.09	12.19	11.96	15.72	4.57	5.32	5.01	9.44	6.41	18.90	18.05	26.24	28.31	28.73
S307	46	34	49	40	37	0.15	5.82	1.19	3.08	3.41	0.00	3.72	0.00	2.91	1.53	4.81	10.20	6.40	4.94	3.15	23.82	26.24	31.55	32.05	30.58
S308	50	30	42	38	39	0.55	11.24	4.77	5.23	2.91	0.00	8.14	5.28	4.39	2.79	4.46	10.76	2.90	5.77	3.88	23.52	23.74	29.00	31.28	28.06
S310	56	37	42	37	42	0.50	3.23	5.87	6.38	2.99	0.00	3.34	5.64	4.76	2.17	5.07	8.75	10.11	7.00	4.32	22.57	22.24	29.48	29.83	29.30
S311	54	41	44	43	41	0.22	1.65	0.75	1.64	1.75	0.00	0.80	0.30	1.32	1.45	4.50	9.89	5.98	9.54	6.00	21.74	20.84	27.73	28.23	28.78
S312	52	34	50	52	51	2.93	4.01	0.44	1.28	1.53	0.00	3.32	0.00	0.41	0.03	6.97	12.35	5.94	8.37	6.07	23.04	24.86	32.24	32.41	31.94
S313	51	30	34	28	28	0.41	9.44	4.37	10.90	4.40	0.00	9.31	3.74	8.67	5.12	4.67	9.48	5.22	6.65	4.06	21.02	22.42	25.75	26.49	26.55
S314	48	34	42	56	53	0.23	1.39	1.38	0.45	0.66	0.00	0.56	0.33	0.00	0.00	9.11	7.13	5.69	6.61	5.05	25.39	27.83	31.98	35.03	33.77
S315	47	31	36	32	33	3.81	7.02	5.01	3.46	6.85	0.00	4.74	3.92	3.74	3.90	8.52	8.60	4.11	5.43	4.12	21.97	20.72	22.42	19.67	20.09
S317	48	24	32	27	32	3.47	20.93	11.39	9.21	5.11	0.00	10.21	9.35	10.01	3.62	5.70	11.52	9.67	9.81	4.77	23.83	23.88	29.80	30.51	32.20
S318	52	33	43	33	39	0.30	6.40	1.34	3.20	1.62	0.00	5.01	0.15	2.00	0.42	3.64	7.60	5.38	9.06	5.13	19.87	21.13	27.36	28.01	27.53
S320	53	35	34	29	33	1.89	12.12	5.00	10.20	8.50	0.14	9.76	4.17	8.65	6.64	8.75	12.51	4.67	8.45	6.41	22.33	23.91	27.08	28.36	28.03
S322	47	24	38	30	33	0.00	18.10	11.77	16.30	8.63	0.00	15.67	3.79	9.09	4.51	0.00	9.40	10.09	7.04	6.27	19.99	23.64	27.54	28.11	28.34
S327	52	36	44	34	42	0.50	4.94	1.26	5.77	1.35	0.00	3.72	0.75	2.67	0.56	4.85	10.29	4.52	8.18	4.33	24.27	23.74	30.69	30.46	32.04

Appendix B: Table of mean values for various CIS traits

	Agtron score					Glucose mg g^{-1} dry weight					Fructose mg g^{-1} dry weight					Sucrose mg g^{-1} dry weight					% Dry matter				
ST	a	b	c	d	e	a	b	c	d	e	A	b	c	d	e	a	b	c	d	e	a	b	c	d	e
Line no																									
S328	48	24	29	29	33	0.37	14.49	4.15	12.28	5.30	0.00	13.75	6.69	8.41	4.12	7.57	6.47	7.42	10.37	6.78	21.97	26.72	29.33	31.88	32.64
S330	53	30	29	32	35	0.23	11.57	6.45	8.63	5.91	0.00	10.50	6.93	9.37	5.89	5.66	7.86	6.02	7.35	4.70	20.90	22.92	29.25	29.45	29.52
S331	50	35	40	33	35	0.40	4.62	1.73	4.78	1.16	0.00	4.32	0.96	4.08	0.57	7.35	14.09	7.45	10.57	5.60	21.94	24.26	28.56	28.86	28.48
S332	52	38	43	34	36	0.02	2.23	0.61	4.06	1.61	0.00	1.63	0.00	3.60	1.81	4.28	7.60	5.44	7.30	5.61	22.46	24.79	28.60	29.30	28.96
S334	48	32	46	33	48	2.18	3.79	1.38	4.73	0.63	0.00	2.15	0.01	2.26	0.00	6.96	9.49	4.92	6.77	3.94	21.69	26.69	29.16	30.60	32.56
S335	48	32	39	38	32	1.23	6.25	1.71	5.62	5.75	0.00	2.47	0.94	3.21	3.70	5.06	8.23	5.53	9.59	5.54	19.13	19.82	26.24	26.38	27.27
S336	52	32	38	35	36	0.21	4.70	2.73	3.96	1.18	0.00	3.26	2.11	3.41	1.05	4.05	7.02	4.27	4.99	3.89	23.23	23.46	27.04	29.80	30.26
S337	48	32	41	30	41	0.24	3.83	2.34	3.80	2.03	0.00	3.36	0.95	3.45	1.29	5.19	4.43	4.53	7.06	3.55	25.81	25.46	31.76	32.79	32.65
S338	50	29	36	34	35	0.35	7.59	2.38	4.04	2.43	0.00	6.99	2.65	3.82	2.42	10.01	14.33	6.75	10.88	7.56	28.05	25.98	32.68	34.02	33.82
S340	43	24	24	26	27	2.91	18.61	18.93	16.07	8.93	0.43	13.94	12.92	11.32	8.25	20.14	7.06	5.28	9.53	5.32	20.80	24.28	27.57	29.47	29.62
S341	54	48	52	49	54	0.56	0.49	0.21	0.62	0.03	0.00	0.00	0.00	0.24	0.00	5.11	7.05	5.65	7.56	6.23	20.14	22.28	25.87	27.30	26.91
S343	50	24	26	24	24	0.93	10.65	9.92	11.19	10.27	0.00	10.48	12.44	11.08	11.09	7.76	9.19	7.41	6.08	5.95	22.56	23.42	28.04	29.50	28.93
1021/1	48	41	46	47	51	0.35	2.77	1.17	0.91	0.72	0.04	1.91	0.80	0.31	0.19	9.63	12.51	6.46	7.00	5.64	23.60	25.29	28.93	29.11	30.07
Karaka	46	25	27	25	27	4.75	20.06	14.17	19.41	16.84	0.37	13.13	10.33	12.40	11.90	5.94	9.85	5.72	9.54	8.75	19.41	21.89	25.26	25.58	24.79

Notes:

1) Agtron score measures the colour of the crisps and the values for crisp colour and dry matter (%) are average of five biological replications

3) The values for glucose, fructose and sucrose are average of three technical replications measured as mg g^{-1} dry weight

4) The values of 1021/1 and Karaka are averaged from several plots

5) The first column indicates line number and a, b, c, d and e represents the ST = storage treatments described below

a = fresh harvest, **b** = after one month of cold storage at 6°C; c = after one month of cold storage at 6°C, plus 10 days of reconditioning at 18°C

d = after four months of cold storage 6°C (d); **e** = after four months of cold storage 6°C, plus 10 days of reconditioning 18°C

Appendix C

Apoplastic invertase inhibitor sequences (http://www.ncbi.nlm.nih.gov/)

GenBank accession AY864819 (*inh1-a* allele - mRNA)

```
   1 cgcaattgaa tccatccatt tactatataa aacacacaca cacacacaca atattccata
  61 caagaaaatc cacatttagt tcttaatttc ccaaaaatat caaaatgaaa attttcattt
 121 tcctaatgat gtttctagct atgttactag taacaaatgg gaataacaat ctagtagaaa
 181 caacatgcaa gaacacacca aattataatt tgtgtgtgaa aactttgtct ttagacaaaa
 241 gaagtgaaac agcaggagat attacaacat tagcattaat tatggttgat gctattaaat
 301 ctaaagctaa tcaagctgct aatactattt caaaacttag gcattccaat cctcctcaag
 361 cttggaaaga tcctttgaag aattgtgcct tttcatataa ggtaatttta acagcaagta
 421 tgccagaagc aatagaagca ttaacaaaag gtgatccaaa atttgcagaa gatggaatgg
 481 ttggttcttc tggtgatgca caagaatgtg aagaatattt taaagctata actattaaat
 541 attcaccact ttctaaatta aatatagatg ttcatgaact ttctgatgtt ggtagagcta
 601 ttgtaagaaa tttattgtga tgtgtcatgt catgatgtta cgtatcgaaa aactttagta
 661 acttaattga taaattgtct gaattattat tttgctggta agggttcgat tcgtcaggtt
 721 ttaatctcat atccatttcc ctttttttcta cccaaacttt ttttttagt gttaggtgtc
 781 tactcttttt catttcataa attagtgaca tgtgtaaagt gccccccttaa ttattagaag
 841 aaaaatgtat catgaatatt tgtacaagtg taatactctt atccaatata tgtttgtccc
 901 cttct
```

GenBank accession AY864820 (*inh1-b* allele - mRNA)

```
   1 tccacattta gttcttaatt ttcccaaaaa tatcaaaatg aaaattttgc ttttcctaat
  61 gatgtttcta gctatgttga tagtaacaaa agggaataac aatctagtag aaacaacatg
 121 caagaacaca ccaaattata atttgtgtgt gaaaactttg tctttagaca aaagaagtga
 181 aacagcagga gatattacaa cattagcatt aattatggtt gatgctatta aatataaagc
 241 taatcaagct gctaacacta tttcaaaact taggcattct aatcctcctc aagcttggaa
 301 agatcctttg aagaattgtg ccttttcata taaggtaatt ttaacagcaa gtatgccaga
 361 agcaacagaa gcattaacaa aaggtgatcc aaaatttgca gaagatggaa tggttggttc
 421 ttctggtgat gcacaagaat gtgaagaata ttttaaagct ataactatta aatattcacc
 481 actttctaaa ttaaatatag atgttcatga actttctgat gttggtagag ccattgtaag
 541 aaatttattg tgatatgtca tgtcatgatg ttacgtatcg gaaaatttta gtaacttaat
 601 tgatagattg tctgaattat tatcttactg gtgagagttc gattcgtcac gtcataatct
 661 catgtccatt tccctttcc ctgccccaaa ctttttttt ttttttaagt gttaggtgtc
 721 tactcttttt catttcataa attagtgaca tgtgtaaaga gccctcttaa ttattcgaag
 781 aaaaatgtat catgaatatt tgtacaagtg taatactcct atccaatata tgtttgtccc
 841 cttct
```

Appendix C

Genomic DNA sequence (*inh1-b* allele- GenBank accession GU980592)

```
   1 atccatttac tatataaaac acatacacac acactatact aacatacaag aaaaatccac
  61 atttagttct taatttccc aaaaatatca aaatgaaaat tttgcttttc ctaatgatgt
 121 ttctagctat gttgatagta acaaaaggga ataacaatct agtagaaaca acatgcaaga
 181 acacaccaaa ttataatttg tgtgtgaaaa ctttgtcttt agacaaaaga agtgaaacag
 241 caggagatat tacaacatta gcattaatta tggttgatgc tattaaatat aaagctaatc
 301 aagctgctaa cactatttca aaacttaggc attctaatcc tcctcaagct tggaaagatc
 361 ctttgaagaa ttgtgccttt tcatataagg taatgttatt cgtttgcgtg tttcaatttg
 421 cttatcctac tctttttttt taagtaaaaa tttcgacttt cgactctgat gaataaggag
 481 ttatgtttgt cttttctgtt tcaatttgtc tgtcttactc tttttgctga aaatttcgac
 541 cctatcttac ttgctgctta gtgaaaattt cgactctgat gaataaggag ctatgttagt
 601 tttttctgtt tcaatttatt tatcttactt tttagtaaaa atttcaactc tgaataagga
 661 gttatgttta tcttacttct ttagtgaaaa tttatcttaa tctaaacatc taactaaaaa
 721 aaactttaat ttttaatttt taatataagt attgaacgtc tttttaaaga aaatataata
 781 ataatgtctg attaaattga gcaaaacgta gaaagtgttc tttttgcttt tttggctctt
 841 gcttatgtgg cataatttgg cttaatatag atttttttta aaataaaact tttgaaattt
 901 tgatgttaaa catgccattt ccttgcgttg gaaatacgta gcacacttct cttacttttc
 961 tattttgtcc gttttaaaaa aagtcacttt tctataatta aaagtaattt aattttaatt
1021 ttccttttta ccctcaacag aataatttat aatcaaacag ctattcaact ttattttaaa
1081 ttataaaatt taaaaatctt tctttataaa actttgaatt tcaatctagt taaataatct
1141 catataaatt aacaaacgaa aaaaatatat attatgcggt tataaatttt tttgaatctt
1201 ttaattttag acctaccata agataagtgt atagctagct tgagtatatt attaagggta
1261 aaataaaaag cgtaaagtca accagaaatg tgtcatataa ataaattaag gaaaaacaaa
1321 tttggccaaa ttttttatca gaacggtaaa ataacatatt tcacactatc ttattttagc
1381 tttttgaaca tttttactct tttaacttta atacctttta attaaaatta aaaaatggaa
1441 aaaaagatca gtttatttta aaataaaaag gatattatac actttttaaa tttctggtta
1501 aattctgact aaattttctg gccatttagc attatccata aattaatata aatataatgt
1561 cacgtaaatc gaaacagaaa gaatattata ttaatgacat gttgaatttt attatatggt
1621 ttcaaaaaca ggtaatttta acagcaagta tgccagaagc aacagaagca ttaacaaaag
1681 gtgatccaaa atttgcagaa gatggaatgg ttggttcttc tggtgatgca caagaatgtg
1741 aagaatattt taaagctata actattaaat attcaccact ttctaaatta aatatagatg
1801 ttcatgaact ttctgatgtt ggtagagcca ttgtaagaaa tttattgtga tatgtcatgt
1861 catgatgtta cgtatcggaa aattttagta acttaattga tagattgtct gaattattat
1921 cttactggtg agagttcgat tcgtcacgtc ataatctcat gtccatttcc cttttccctg
1981 ccccaaactt ttttttttt tttaagtgtt aggtgtctac tctttttcat ttcataaatt
2041 agtgacatgt gtaaagagcc ctcttaatta ttcgaagaaa aatgtatcat gaatatttgt
2101 acaagtgt
```

Appendix D: Amino acids

Amino acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	ILe	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Try	W
Tyrosine	Tyr	Y
Valine	Val	V

Appendix E: List of presentations/ publications /awards

Sagar S. Datir, Julie M. Latimer, Susan J. Thomson, Hayley J. Ridgway, Anthony J. Conner, Jeanne M. E. Jacobs. 2012. Allele diversity for the apoplastic invertase inhibitor gene from potato – Accepted for publication - Molecular Genetics and Genomics

Datir, Sagar, Latimer, Julie, M., Ridgway, Hayley, J., Jacobs, Jeanne, M.E. and Conner, Anthony, J. "Allelic diversity of invertase inhibitor genes in potato" 19th Biennial Meeting for the New Zealand Branch of the International Association of Plant Biotechnology (IAPB), 8th - to 11th February 2011, New Zealand.

Datir, S., Shaw, M.L., Monaghan, K., Wright, K.M., Ridgway, H.J., Jacobs, J.M.E., & Conner, A.J. "Genetic basis of cold-induced sweetening in potato tubers". COMBIO 2009, 6th to 10th December 2009, Christchurch, New Zealand.

Sagar Datir, Martin Shaw, Hayley Ridgway, Jeanne Jacobs and Anthony Conner "Genetic analysis of candidate genes for cold-induced sweetening in potato. The Sixth **"***Solanaceae Genome* Workshop" at *New Delhi* from 08 - 13 November, *2009.*

Sagar Datir, Martin Shaw, Hayley Ridgway, Jeanne Jacobs and Anthony Conner "Genetic analysis of candidate genes for cold-induced sweetening in potato". 18th Biennial Meeting for the New Zealand Branch of the International Association of Plant Biotechnology (IAPB) and Metabolomics Workshop, 16th to 18th February 2009, New Zealand.

Sagar Datir, Hayley Ridgway, Jeanne Jacobs and Anthony Conner "Genetic analysis of resistant to cold-induced sweetening in potato" Post graduate conference, 2009, 14th & 15th August, Lincoln University, New Zealand.

List of awards/ scholarships

- New Zealand International Doctoral Research Scholarship (NZIDRS)
- Howard Bezar Memorial Rotary Prize
- MacMillan Brown Agricultural Research Scholarship Lincoln University
- Lincoln University Graduate Scholarship