Elucidating the mechanisms of senescent sweetening in stored potato tubers

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Abstract

Senescent sweetening is a storage disorder that typically occurs following medium to long-term storage of potato tubers in the presence of sprout suppressors at moderate storage temperatures. It represents a significant issue for the processing industry where reducing sugar accumulation results in problems of dark fry colour. Furthermore, the Maillard reaction between reducing sugars and asparagine results in the accumulation of the potential neurotoxin and carcinogen acrylamide in processed products. At present almost nothing is known regarding the mechanisms promoting senescent sweetening which differs from cold-induced sweetening in that it is not reversible by transfer of tubers to higher temperatures. In the present work we set out to test the hypothesis that oxidative damage caused during long term storage is linked to senescent sweetening. A marked difference in storage induced reducing sugar accumulation was observed between a sweetening resistant and a sweetening sensitive cultivar. However, markers of oxidative damage and activities of antioxidant enzymes did not exhibit any specific correlation with reducing sugar accumulation indicating that oxidative damage and senescent sweetening may not be linked. To identify the underlying biochemical causes of sugar accumulation GC/MS was used to quantify a range of primary metabolites in sweetened and unsweetened tubers. Few differences were observed in metabolite profiles however, labelling with [^{13}C] glucose indicated a greater capacity for sucrose synthesis in the sweetening resistant compared with the sweetening sensitive cultivar. In addition, differences in specific activity of carbohydrate metabolism enzymes as well as microarray data suggest starch re-synthesis and alternative metabolic sinks for carbon as potential traits linked to sweetening resistance. Moreover, we identified *GPT2* as a potential candidate gene associated with the accumulation of sugars during long-term storage. These findings will lead to a better understanding of the mechanisms, processes and genes involved in senescent sweetening and will provide insights into improved storage management in the short-term and the development of senescent sweetening resistant cultivars in the longer term.

I would like to dedicate this to my family, especially to my grandparents Antonio and Concha. You are always in my heart.

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Abbreviations

$^1O_2$	singlet oxygen
ABA	abscisic acid
ADP	adenosine diphosphate
ADP-Glc	ADP-glucose
AGPase	ADP-glucose pyrophosphorylase
AMP	adenosine monophosphate
AMY	α-amylase
ANOVA	analysis of variance
APX	ascorbate peroxidase
ARF	ADP-ribosylation factor
AsA	ascorbate
ATP	adenosine triphosphate
BAM	β-amylase
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
C4H	cinnamate-4-hydroxylase
CAT	catalase
cDNA	complementary DNA
CHS	chalcone synthase
CIPC	chloropropham
CIS	cold-induced sweetening
CKs	cytokinins
CO_2	carbon dioxide
cwInv	cell wall invertase
cytInv	cytosolic invertase
DAB	diaminobenzidine tetrahydrochloride
ddH2O	deionized distilled water
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DTT	dithiothreitol
DW	dry weight

EDTA	ethylenediaminetetraacetic acid
EF1α	elongation factor-1-α
ETC	electron transport chain
EU	European Union
F1,6BP	fructose-1,6-bisphosphate
F3H	flavanone-3-hydroxylase
F6P	fructose-6-phosphate
FE	Feature Extraction software
FFA	free fatty acids
FK	fructokinase
FW	fresh weight
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
GAs	gibberellins
GC/MS	gas chromatography/mass spectrometry
GPT	glucose-6-phosphate/phosphate translocator
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
H_2O	water
H_2O_2	hydrogen peroxide
HK	hexokinase
$HO^{\bullet}$	hydroxyl radical
HPLC	high-performance liquid chromatography
IARC	International Agency for Research on Cancer
Inv	invertase
JA	jasmonic acid
JAO2	jasmonic acid oxidase 2
KRP3	kip-related protein 3
LSD	least significant difference
MDA	malondialdehyde
MDHAR	monodehydroascorbate reductase

MnSOD	mitochondrial superoxide dismutase
MSTFA	*N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide
NAT	Acyl-CoA N-acyltransferases
NBT	nitroblue tetrazolium
NCED	9-cis-epoxycarotenoid dioxygenase
O_2	oxygen
$O_2^{\bullet -}$	superoxide anion radical
PCA	principal component analysis
PGA	3-phosphoglyceric acid
PGI	phosphoglucoisomerase
PGM	phosphoglucomutase
PIP	plasma membrane intrinsic protein
PME	pectin methyl esterase
PMSF	phenylmethylsulphonyl
PP2A	protein phosphatase 2A
PPi	inorganic pyrophosphate
PSII	photosystem II
PVPP	polyvinylpolypyrrolidone
qRT-PCR	quantitative real-time polymerase chain reaction
QTL	quantitative trait loci
RCC1	regulator of chromosome condensation
RNAi	RNA interference
ROS	reactive oxygen species
RRTF1	redox responsive transcription factor 1
RT-IVT	reverse transcription and *in vitro* transcription
S6P	sucrose-6-phosphate
SGAs	steroidal glycoalkaloids
SLs	strigolactones
SNF	sucrose non-fermenting
SnRK	sucrose non-fermenting 1-related protein kinase
SOD	superoxide dismutase
SPP	sucrose phosphate phosphatase
SPS	sucrose phosphate synthase
SS	senescent sweetening

SuSy	sucrose synthase
T6P	trehalose-6-phosphate
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TBE	tris-borate/EDTA
TCA	tricarboxylic acid
TCP	teosinte branched1/cycloidea/proliferating cell factor
TFs	transcription factors
TIP	tonoplast intrinsic protein
TPI	triose phosphate isomerase
TPP	trehalose-6-phosphate phosphatase
TPS	trehalose-6-phosphate synthase
TrP	triose phosphate
UDP-Glc	UDP-glucose
UGPase	UDP-glucose pyrophosphorylase
UK	United Kingdom
UV	ultraviolet radiation
vacInv	vacuolar invertase

Chapter 1: Introduction

1.1 Introduction

Potato (*Solanum tuberosum* L.) is a member of the Solanaceae family of flowering plants. It is the third most important food crop (Birch *et al*., 2012) with 381 million tons produced annually for human consumption (FAO 2014). It originated and was first domesticated in the Andes Mountains of South America (Birhman & Kaul, 1989).

In the United Kingdom (UK), the potato processing industry is worth £3.9 billion at retail and supports more than 20,000 jobs. Approximately 3.5 million tonnes of tubers are stored for up to 8 months each season in the UK (Cunnington, 2008). Cooking quality and appearance are important to the consumer and industry in terms of post-cooked appearance and taste. Much depends upon variety choice but also the way the potato is grown and stored. Maintenance of tuber quality during long-term storage, more specifically, the prevention of sugar accumulation, is necessary to maintain acceptable fry colour and prevent acrylamide formation in processed products.

1.2 Storage, sugars and quality

The level of sugars in potato tubers is an essential factor affecting quality in potatoes. Storage for processing is typically undertaken at relatively high temperatures (8–12°C) in the presence of sprout suppressors to prevent cold-induced sweetening (CIS) (Marquez & Anon, 1986; Burton, 1989; Sowokinos, 1990). However, tubers can undergo the distinct physiological process of senescent sweetening (SS) after prolonged storage, leading to significant losses (Burton, 1989). The principal reason is the fact that reducing sugars such as glucose and fructose react with free amino acids during frying to produce distasteful dark processed fries and crisps via non-enzymatic Maillard-type reactions (Figure 1-1) (Shallenberger *et al*., 1959). These reactions are related to aroma, taste, and colour, playing an important role in the appearance and taste of foods. Moreover, acrylamide is present in different foods processed at high temperature, and it is formed from asparagine and reducing sugars (carbonyl compounds) in the process of Maillard reactions (Mottram *et al*., 2002; Stadler *et al*., 2002).

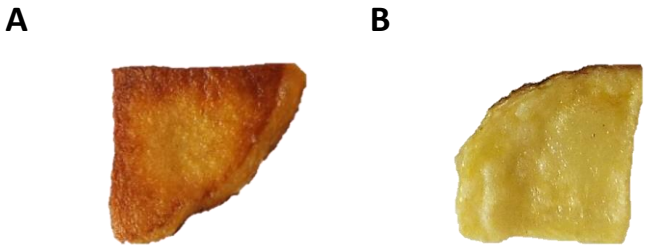


Figure 1-1. Fry colour of crisps from tubers after 53 weeks of storage. Frying was performed using the standard PepsiCo protocol at 177°C. **A.** Crisp from susceptible to senescent sweetening cultivar (Arsenal) presenting high reducing sugars content. **B.** Crisp from senescent sweetening resistant cultivar (VR 808) with low reducing sugars content.

1.2.1 Maillard reactions and quality

The Maillard reaction was described in 1912 by the French chemist Louis Camille Maillard (Maillard, 1912). The chemistry underlying the Maillard reaction is very complex encompassing a whole network of various reactions, and different factors involved in food processing influence it (Figure 1-2) (Hodge, 1953). The Maillard reaction consists of several non-enzymatic reactions between sugars and amino groups, enhanced by high temperature (>120°C) and low moisture content. For this reason, it occurs mainly in cooked foods prepared by frying, baking, roasting, and toasting (Hodge, 1953). Maillard reaction generates melanoidin pigments and complex mixtures of compounds imparting flavour and aroma heterocyclic compounds. These compounds include pyrazines, pyrroles, furans, oxazoles, thiazoles, and thiophenes (Mottram, 2007; Halford *et al*., 2011). The compounds formed during the Maillard reaction give to cooked foods their signature flavour and aroma, meaning that any attempts to reduce acrylamide formation are likely to affect the characteristics that define the different product types.

The Maillard reaction is a complex, multi-step reaction which is initiated by the condensation of the carbonyl group of a reducing sugar (such as glucose or fructose) with the amino group of an amino acid or another amino compound, generating a Schiff base. A Schiff base is a type of imine, a compound containing a carbon-nitrogen double bond, in the case of a Schiff base with the nitrogen atom attached to an organic group. Cyclisation and acid-catalysed rearrangement generate Amadori rearrangement products from glucose and maltose, and

Heyns rearrangement products from fructose, which undergo enolisation, deamination, dehydration, and fragmentation to produce sugar dehydration as well as fragmentation products, which contain one or more carbonyl groups, including deoxyosones, heterocyclic furfurals, furanones, pyranones, dicarbonyls (C2-C3) and hydroxycarbonyls (Hellwig & Henle, 2014). The carbonyl compounds may contribute to flavour characteristics. However, they are highly reactive and can undergo further reactions with free amino acids and other amines. One of these reactions is Strecker degradation, involving the deamination and decarboxylation of an amino acid to generate an aldehyde, an α-aminoketone, and carbon dioxide (CO_2), and it is a Strecker-type degradation of asparagine that is responsible for acrylamide production (Zyzak *et al.*, 2003). The asparagine reacts with dicarbonyl or hydroxycarbonyl compounds derived from the Maillard reaction to produce a Schiff base. This can be converted either to acrylamide by decarboxylation followed by the removal of a substituted imine, or it can be converted to 3-aminopropionamide by the elimination of a carbonyl group, and the 3-aminopropionamide converted to acrylamide by the removal of ammonia (Granvogl & Schieberle, 2006; Granvogl *et al.*, 2007).

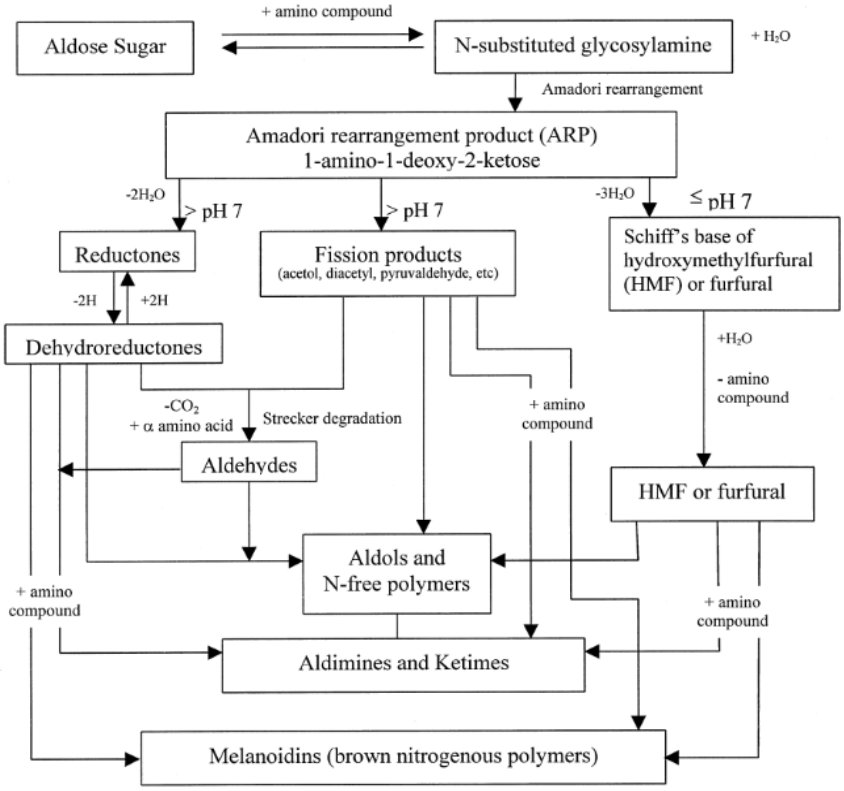


Figure 1-2. Maillard reaction scheme. Taken from Martins *et al.* (2000).

Maillard reactions affect various food quality parameters: organoleptic properties, colour, and protein functionality. In some cases, these reactions lead to desired changes, such as the generation of delicate flavours. However, in other cases, undesired quality changes are obtained, especially if the Maillard reactions are too pronounced, producing bitter and burnt tastes. The fact of being able to control Maillard reactions during food production and storage is essential from a food quality perspective. Nonetheless, elucidating the progress of Maillard reactions in foods is complicated since the presence of multiple reactants and the dynamic conditions found in food matrices, processing, and storage conditions all contribute to a complex chemical landscape.

Since sugar content and Maillard reactions are related to the appearance and taste of foods, regarding potatoes, several studies about fry colour have been reported. Fry colour tests of Russet Burbank and Shepody potatoes are more closely correlated with glucose than with fructose concentrations, total reducing sugars, sucrose, or total sugars (Pritchard & Adam, 1994). Besides, Coleman *et al*., (1993) indicated that the crisp colour was associated with tuber glucose content regardless of detection method, cultivar, growing site, or storage temperature.

1.2.2 Acrylamide in potato tubers

Acrylamide (C_3H_5NO) is a white, odourless, crystalline, water-soluble solid. Acrylamide forms from free asparagine and reducing sugars such as glucose or fructose within the Maillard reaction at low water activity and high temperatures (Figure 1-3) (Mottram *et al*., 2002; Stadler *et al*., 2002). Hence, both free asparagine and reducing sugars are widely referred to as the precursors for acrylamide. Since potatoes contain relatively high levels of both asparagine and reducing sugars, this is the most likely route to acrylamide formation in potato crisps. For that reason, factors which affect the concentration of precursors such as variety (Hebeisen *et al*., 2005), storage temperature and time (Amrein *et al*., 2004) and level of nitrogen and phosphorus in the soil (Heuser *et al*., 2005) affect acrylamide formation in the cooked product.

The chemical reaction leading to acrylamide formation is also responsible for the development of fry colour in potato crisps, and correlations between instrumental colour parameters and levels of acrylamide in cooked potato products have been reported (Pedreschi *et al*., 2006). Both the temperature and duration of heating have a significant influence on acrylamide levels in potato crisps (Rydberg *et al*., 2003; Pedreschi *et al*., 2004).

In potato products, the limiting factor for acrylamide formation is usually the concentration of reducing sugars (Amrein *et al*., 2004; Becalski *et al*., 2004; de Wilde *et al*., 2005; Burch *et al*., 2008; Shepherd *et al*., 2010, 2013). However, an effect of free asparagine concentration on acrylamide formation has also been observed (Becalski *et al*., 2004, Shepherd *et al*., 2010, 2013).

Furthermore, two aspects of potato composition affecting acrylamide formation are the ratio of glucose to fructose and the concentration of free proline. Although both glucose and fructose can contribute to the creation of colour as well as acrylamide, fructose has been observed to favour the production of acrylamide over colour during the cooking of French fries, in comparison to glucose (Mestdagh *et al*., 2008; Higley *et al*., 2012). Also, free proline has been shown to inhibit acrylamide formation in model systems (Koutsidis *et al*., 2009).

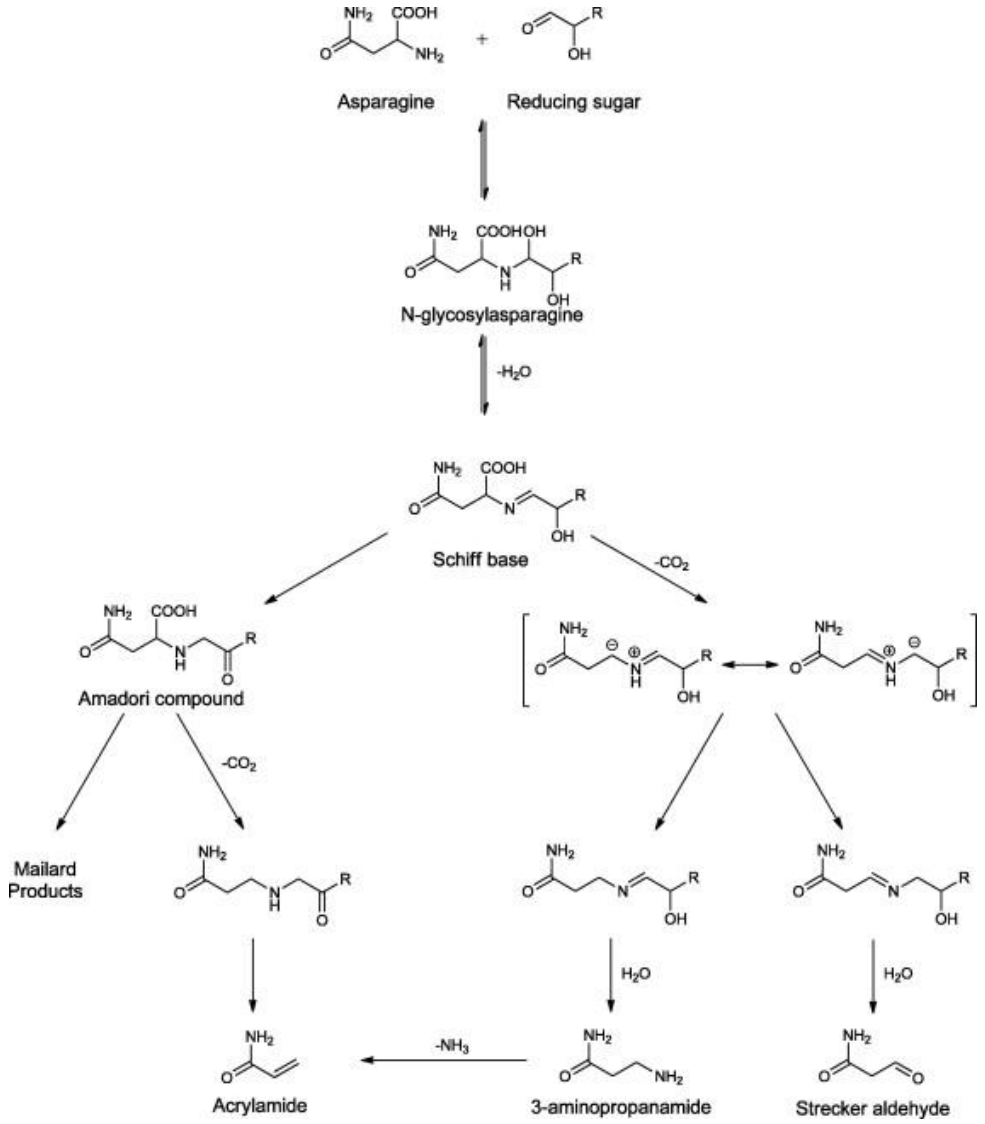


Figure 1-3. A proposed mechanism for acrylamide formation as a side reaction of the Maillard reaction. Based on Mottram *et al.* (2002); Stadler *et al.* (2004); and Granvogl & Schieberle (2006). Taken from Medeiros Vinci *et al*. (2012).

1.2.2.1 Biological effects of acrylamide

The Scientific Committee on Toxicity, Ecotoxicity and the Environment demonstrated in 2001 the neurotoxicity, genotoxicity, carcinogenicity and reproductive toxicity of acrylamide (Carere, 2006; Keramat *et al*., 2011; Semla *et al*., 2017). Acrylamide is a potent neurotoxin affecting male reproduction as well as causing birth defects. In addition, it has been reported to be carcinogenic in laboratory animal trials (reviewed by Friedman, 2003; CONTAM Panel, 2015) and considered as a Group 2A carcinogen by the International Agency for Research on

Cancer (IARC), the specialised cancer agency of the World Health Organization (IARC, 1994). Furthermore, the estimated average dietary exposure of acrylamide in humans might be linked to morphological changes in nerves (JECFA, 2006; 2011; CONTAM Panel, 2015). The toxic effects of acrylamide are mediated by the formation of oxidative stress, genotoxic metabolites, affected propagation of neural signals, ultrastructural, and histological defects in the central neural system (LoPachin, 2004; El-Sayyad *et al*., 2011; Pingot *et al.*, 2013). As acrylamide is both genotoxic and carcinogenic, the margins of exposure indicate a health concern. Therefore, Commission Regulation (European Union (EU)) 2017/2158 establishing mitigation measures and benchmark levels for the reduction of the presence of acrylamide in food has been adopted in November 2017 and entered into force in April 2018.

1.2.3 Sprout suppressants during storage

Potato tubers for the processing industry are stored at 8–12°C and relative humidity of 85–90% the world over, which is the most common way of long-term storage of potatoes. The benefit of storing the potatoes within the temperature range of 8–12°C is the minimum accumulation of sugars (Smith, 1987; Ezekiel *et al*., 2007a,b) as well as the minimum rate of respiration in stored potato tubers (Burton, 1989). This storage method keeps the stored potatoes suitable for table and processing purposes. However, once the natural dormancy period of potato is over, the prevailing temperatures in these storage methods favour sprouting and sprout growth. In 2012, the United Kingdom recorded overall losses of 17% (770,000 tons), being the leading cause of wastage the premature sprouting and rotting during storage (Terry *et al*., 2011; Pritchard *et al*., 2012). For that reason, the use of some sprout suppressants to check the sprout growth becomes essential under these methods of potato storage. World over isopropyl N-(3-chlorophenyl) carbamate (CIPC, also referred to as chlorpropham) is the most commonly used sprout suppressant on potatoes when stored at 8–12°C (Smith & Bucher, 2012). However, it has now been banned by the EU. The European Commission does no longer allow the use of CIPC since January 1, 2020.

1.2.3.1 Factors affecting dormancy

Dormancy break in potato tubers is a physiological mechanism that is regulated by both environmental factors and endogenous signals (Sonnewald & Sonnewald, 2014). The onset of dormancy break and its further development are believed to be affected by the relative

concentration of different biochemical compounds such as plant growth regulators: abscisic acid (ABA), auxins, cytokinins (CKs), ethylene, gibberellins (GAs), and strigolactones (SLs); and other compounds such as carbohydrates and organic acids (Sonnewald, 2001; Viola *et al*., 2007; Pasare *et al*., 2013).

Ethylene is required during the earliest stage of dormancy initiation (Suttle, 1998) and it has been reported to break endo-dormancy following short-term treatments (Foukaraki *et al*., 2014) as well as to inhibit sprout growth and promote eco-dormancy (Foukaraki *et al*., 2016). However, Suttle (2009) suggested ethylene is not involved in hormone-induced dormancy break, supporting the fact that the effect of ethylene depends on the physiological state of potato tubers.

A sustained synthesis and action of ABA are required for dormancy induction and maintenance (Suttle, 2004; Mani *et al*., 2014). Cross-talk between ABA and other phytohormones is known (Chang *et al*., 2013), as well as with sugar metabolic pathways, which facilitates the onset of dormancy break and further sprouting (Brady, 2013). Nevertheless, the increase in ABA as a result of exogenous ethylene application has been suggested to delay dormancy break (Foukaraki *et al*., 2016). Associated with the ABA decline, there is an increase in sucrose contents, which is considered a pre-requisite for bud outgrowth (Viola *et al*., 2007; Sonnewald & Sonnewald, 2014). In this regard, auxins are essential for their role in vascular development. Auxins support the symplastic reconnection of the apical bud region: a discrete cell domain that remains symplastically isolated throughout tuberisation. Therefore, this reconnection is essential for sucrose to reach the meristematic apical bud. Furthermore, high sucrose levels promote trehalose-6-phosphate accumulation (T6P), which favours sprouting, probably decreasing sensitivity to ABA (Debast *et al*., 2011; Tsai & Gazzarrini, 2014).

Furthermore, CKs and GAs are required for the reactivation of meristematic activity and sprout growth (Hartmann *et al*., 2011). An increment in both cytokinin concentration and sensitivity have been observed prior to dormancy break as critical factors for meristematic reactivation (Suttle, 2004). Additionally, coordination between CKs and auxins induce sprout elongation (Aksenova *et al*., 2013). Moreover, sensitivity to GAs, which is negatively affected by SLs, increases throughout post-harvest storage, and it is possibly responsible for sprout vigour (Roumeliotis *et al*., 2012). Since SLs are essential as regulators of lateral bud development,

they may be related to para-dormancy establishment instead of eco- and endo-dormancy (Pasare *et al*., 2013).

1.2.3.2 Adverse consequences of sprouting of potato tubers during storage

As previously described, the control of sprout growth is a crucial factor for long-term storage of potato tubers. Sprouting leads to a higher rate of respiration, remobilization of storage compounds in the potato tubers (mainly starch and proteins) as well as causing shrinkage due to loss of water (Burton, 1955; Sonnewald & Sonnewald, 2014). Also, sprouting is highly detrimental to the nutritional status and quality aspects of potatoes (van Es & Hartmans 1987; Mani *et al*., 2014). These changes also cause deterioration in processing quality due to loss in mass, decreased turgor, structural change due to growth of sprout tissue, and increase in sugar concentrations due to hydrolysis of starch (van Es & Hartmans, 1987; Davies, 1990; Burton *et al*., 1992; Daniels-Lake *et al*., 2005). Sprouting also affects adversely potato quality parameters such as firmness, and content of vitamin C (Rezaee *et al*., 2011). In this context, to reduce weight loss and other undesirable physiological and biochemical changes that can adversely affect the quality of potatoes, the use of sprout suppressants has become an integral part of potato storage and potato industry.

1.2.3.3 Use of CIPC during storage

Suppression of sprout growth in potato tubers is a crucial step to managing potato quality during storage. World over, CIPC is the most utilized sprout suppressant chemical due to its high efficacy. CIPC is a selective and systemic herbicide with an ability to translocate acropetally in the plant system (Ashton & Crafts, 1981). CIPC acts as a mitotic inhibitor by interfering with the process of spindle formation during cell division (Ashton & Crafts, 1981; Vaughn & Lehnen, 1991; Kleinkopf *et al*., 2003). In this way, the absence of cellular division prevents sprouting, targeting the essential and indispensable cellular process. Besides this, CIPC also causes an alteration in cellular structure and functions. It is known to inhibit RNA synthesis, protein synthesis, the activity of β-amylase along with suppression of transpiration and respiration, and interfere with oxidative phosphorylation and photosynthesis (Vaughn & Lehnen, 1991). CIPC is considered as the most effective sprout suppressant for potatoes. It can be converted into an emulsifiable concentrate, fogging concentrate, granules, and dustable

powder (van Vliet & Sparenberg, 1970; Corsini *et al*., 1979; Conte *et al*., 1995). It is usually applied as a post-harvest fogging treatment on stored potatoes.

CIPC has little or no adverse effect on quality parameters (Rastovski, 1987; Tayler *et al*., 1996; Blenkinsop *et al*., 2002; Ezekiel *et al*., 2005; Mehta *et al*., 2010). However, concerns about CIPC usage have increased after studies have reported toxic and carcinogenic properties (Balaji *et al*., 2006; El-Awady Aml *et al*., 2014). The EU Commission has published its Implementing Regulation (EU) 2019/989 concerning the non-renewal of approval of the active substance CIPC. Legislation constraints are leading the potato industry to seek alternative technologies that can extend post-harvest storage while maintaining tuber quality.

1.2.3.4 Alternatives to CIPC

In general, the sprout suppressant CIPC is commercially applied as a thermal hot fog during prolonged potato storage (Blenkinsop *et al*., 2002). However, legislative bodies are constraining its use. Alternatives to traditional sprout control include hydrogen peroxide plus (Al-Mughrabi, 2010; Mani *et al*., 2014), 1,4-dimethyl naphthalene (de Weerd *et al*., 2010), UV-C (Cools *et al*., 2014), essential oils (Teper-Bamnolker *et al*., 2010), and ethylene. Continuous exogenous ethylene supplementation has been commercially approved as a sprout suppressant in the United Kingdom by the Chemicals Regulation Directorate (Briddon, 2006); how ethylene inhibits sprout growth has not been completely clarified yet. Supplementation of ethylene can increase the content of reducing sugars in tubers (Daniels-Lake *et al*., 2005), which negatively affects processed potato quality. However, late ethylene supplementation was efficacious at delaying tuber sprouting and more effective preventing accumulation of reducing sugars when compared to early supplementation (Foukaraki *et al*., 2014). Hence, late ethylene supplementation may reduce storage costs while providing high-quality tubers. The increase in ABA levels induced by ethylene may explain the delay of dormancy break (Foukaraki *et al*., 2016).

1.3 Cultivars, sugar content and the onset of sweetening

The amount of free sugar that tubers accumulate depends on the cultivar (van Vliet & Schriemer, 1960; Burton, 1969; Samotus *et al*., 1974; Coffin *et al*., 1987; Richardson *et al*., 1990; Zrenner *et al*., 1996). Potato cultivars differ considerably in the timing of onset of

senescent sweetening. Table 1-1 shows a range of important UK processing varieties in terms of how long they can be stored before the onset of senescent sweetening. Dormancy characteristics are also included. Although the cultivars that are most susceptible to senescent sweetening tend to have short dormancy, there are important exceptions to this rule, such as Maris Piper and Record (Colgan *et al*., 2012). In addition to the effect of cultivar, growing conditions that affect the maturity of tubers at harvest can impact the timing of the onset of senescent sweetening, and an effect of storage temperature is also evident (Groves *et al*., 2005).

Table 1-1. Classification of potato processing varieties by onset of senescent sweetening.

Variety	Main market	Development of senescent sweetening	Length of dormancy
Lady Rosetta	Crisp	Early onset	2
Crisps4all	Crisp	Medium onset	3*
Hermes	Crisp	Medium onset	3
Pentland Dell	Chip	Medium onset	3
Cabaret	Chip	Late onset	5
Maris Piper	Chip	Late onset	2
Record	Crisp	Late onset	3
Saturna	Crisp	Late onset	4
Verdi	Crisp	Late onset	6
VR 808	Crisp	Late onset	4*
Lady Claire	Crisp	Very late onset	6
Markies	Chip	Very late onset	5
Russet Burbank	Chip	Very late onset	8

Dormancy (1-9 scale, 9 = Long, NIAB Pocket Guide, 2008, NIAB, Cambridge. *For newer varieties, dormancy periods have been estimated. Adapted from Colgan *et al*. (2012)

Processing potatoes directly into crisps or fries from cold storage (2-4°C) presents several benefits including less shrinkage, retention of dry matter, decreased disease loss, extended marketability, and the elimination of chemical applications for dormancy-prolonging. The problem arises when at low temperature tubers undergo a phenomenon in which both glucose and fructose accumulate by the process of CIS (Marquez & Anon, 1986; Burton, 1989; Sowokinos, 1990).

1.4 Carbohydrate metabolism in tubers

The major sugars in potato tubers are glucose, fructose, and sucrose (Burton, 1989). Sugar levels in tubers are conditioned by several factors, including genotype, environmental and growing conditions, and different post-harvest factors such as storage. During storage, carbohydrates are converted from starch for respiration purposes, and sugars start accumulating when their net production exceeds their use.

The amount of starch accumulated in mature potato tubers is the net result of photosynthetic carbon fixation, the synthesis of transient starch and its conversion into sucrose in photosynthetically active source leaves, the vascular transport of sucrose from the leaves to the developing-sink tuber, and starch synthesis and degradation in the tuber during the growth period (reviewed in Frommer & Sonnewald, 1995). When the enzymes and transport proteins participating in these processes are altered, effects on the morphology and the carbohydrate partitioning affecting tuber starch content are observed (Frommer & Sonnewald, 1995).

Photosynthesis is the major source of fixed carbon. During plant photosynthesis, CO_2 is fixed in the chloroplasts via the Calvin cycle to produce triose-phosphates (TrP). In the cytosol, TrP can be transported to the cytosol by a TrP/phosphate translocator. Two TrP molecules result in one fructose-1,6-bisphosphate (F1,6BP) molecule in a reaction catalysed by aldolase. F1,6BP is then further metabolized to generate other hexose phosphates, such as fructose-6-phosphate (F6P) and glucose-6-phosphate (G6P). G6P can be used to generate nucleotide sugars such as UDP-glucose (UDP-Glc), and UDP-Glc is combined with F6P to produce sucrose-6-phosphate (S6P) in a reaction catalysed by sucrose phosphate synthase (SPS). S6P is dephosphorylated by sucrose phosphate phosphatase (SPP) to form sucrose. Sucrose is the primary product of photosynthetic tissues, and the main sugar transported from the source tissues through the phloem to non-photosynthetic tissues (sink tissues) (Ruan, 2014). In non-photosynthetic tissues, such as potato tubers, the transported sucrose is used for many metabolic pathways, providing energy, as well as carbon skeletons, to produce organic matter such as amino acids, nucleotides and structural carbohydrates.

After arriving to sink tissues, sucrose can enter the sink cells via different pathways (Ma *et al*., 2018). Sucrose can be unloaded from the phloem to the apoplast by sucrose transporters. Then, it can enter the sink cells via sucrose transporters or hydrolysed by cell wall invertases (cwInv,

EC 3.2.1.26) to generate glucose and fructose, which can enter the sink cells via hexose transporters (Ruan, 2014). In addition, sucrose can pass directly from the phloem to sink cells through plasmodesmata, which is the main route during tuber bulking (Viola *et al*., 2001). Inside sink cells, sucrose can be metabolized or transported to the vacuole, where it can be stored as sucrose, transformed into fructans by fructosyltransferases, or hydrolysed by vacuolar invertases (vacInv, EC 3.2.1.26) and stored as hexoses. To be metabolized, sucrose must be cleaved by either cytosolic invertases (cytInv, EC 3.2.1.26) or sucrose synthases (SuSy, EC 2.4.1.13). While cytInv catalyses the irreversible hydrolyzation of sucrose into glucose and fructose, SuSy catalyses the reversible cleavage of sucrose using UDP to generate fructose and UDP-Glc.

In potato tubers, most of the incoming sucrose is converted to starch as a long-term carbon store for reproductive growth. Starch is the major carbon store in plants, formed of an insoluble polymer of linked glucose units (Martin & Smith, 1995). ADP-glucose (ADP-Glc) pyrophosphorylase (AGPase, EC 2.7.7.27) catalyses the first committed step of starch synthesis in the plastid, converting glucose-1-phosphate (G1P) and ATP to ADP-Glc and inorganic pyrophosphate (PPi). Then, ADP-Glc is used by starch synthases and branching enzymes to elongate the glucan chains of the starch granule. Work with Arabidopsis mutants (Neuhaus & Stitt, 1990) and potato tubers (Geigenberger *et al*., 2004) showed that the enzyme catalyses a near rate-limiting step in the pathway of starch synthesis.

AGPase has been reported to be subject to post-translational redox regulation. There is evidence for the in vivo role of post-translational redox modulation of AGPase in regulating starch synthesis in heterotrophic potato tubers (Tiessen *et al*., 2002). Post-translational redox activation of AGPase increases the rate of starch synthesis in response to external factors without the support of any increase in the levels of glycolytic intermediates (Tiessen *et al*., 2002).

1.4.1 Trehalose-6-phosphate and its role in carbohydrate metabolism

Sugars can act as messengers in signal transduction. This is the case for trehalose, a non-reducing disaccharide composed of two glucose moieties. The biosynthesis of trehalose in plants involves the generation of T6P from G6P and UDP-Glc by trehalose-6-phosphate synthase (TPS; EC 2.4.1.15), and the subsequent dephosphorylation of T6P to trehalose by

trehalose-6-phosphate phosphatase (TPP; EC 3.1.3.12; Cabib & Leloir, 1958; O'Hara *et al*., 2013). Both trehalose and T6P have been reported to play a role in regulating carbohydrate metabolism (Ponnu *et al*., 2011). It has been postulated that T6P is transported into plastids by an unknown mechanism, inducing starch synthesis via activation of AGPase mediated by thioredoxin (Kolbe *et al*., 2005). T6P may be converted into trehalose, which has been reported to regulate starch breakdown in plastids (Ponnu *et al*., 2011).

Transgenic potato lines over-expressing *E. coli* TPS displayed decreased starch content and reduced ATP, coupled with an increased respiration rate revealing high metabolic activity (Debast *et al*., 2011). In addition, over-expressed TPS lines showed delayed sprouting. On the contrary, lines that over-expressed the *E. coli* TPP gene exhibited reduced T6P content and accumulated soluble carbohydrates, hexose-phosphates, and ATP. However, over-expressed TPP lines displayed no changes in starch content and/or early sprouting (Debast *et al*., 2011). In this context, Ponnu *et al*. (2011) postulated that T6P functions as a critical regulator of plant growth in response to environmental factors by regulating the central carbon metabolism.

T6P has been reported to control sucrose utilization (Schluepmann *et al*., 2004) and starch metabolism in plants (Wingler *et al*., 2000). Transgenic Arabidopsis lines expressing *E. coli* TPS or TPP genes exhibited differences in T6P accumulation and showed different behaviours to exogenous sucrose. In these plants, rising concentration of T6P increased the utilization of sucrose (Schluepmann *et al*., 2004). On the contrary, it has been observed that the T6P content is strongly related to sucrose availability in Arabidopsis wild-type plants. Sucrose feeding rapidly induces T6P in carbon-starved seedlings (Lunn *et al*., 2006). This increase of T6P in response to exogenous sucrose may be due to a rise in the amount of available G6P and UDP-Glc, which have been observed to be important in determining biomass accumulation and plant growth (Meyer *et al*., 2007). Therefore, T6P indirectly reflects sucrose concentrations and has been widely accepted as an indicator of sucrose status in plants (Lunn *et al*., 2006; reviewed in Paul *et al*., 2008).

Besides, T6P has been reported to inhibit the sucrose non-fermenting 1-related protein kinase1 (SnRK1) complex of the sucrose non-fermenting-1 (SNF1)-related group of protein kinases in Arabidopsis (Zhang *et al*., 2009), in wheat grain extracts (Martínez-Barajas *et al*., 2011), and potato tubers (Debast *et al*., 2011). In potato tubers, sucrose and glucose lead to redox activation of AGPase via two different signalling pathways involving SnRK1, and hexokinase,

respectively (Tiessen *et al*., 2003). Hexokinase and SnRK1 are both implicated in a regulatory network that controls the expression and phosphorylation of cytosolic enzymes in response to sugars (Smeekens, 2000).

1.5 Cold-induced and senescent sweetening in stored potato tubers

Tubers are typically stored at cold temperatures to reduce shrinkage due to respiration and to minimize losses to tuber-borne pathogens. Cold-stored tubers, however, accumulate the reducing sugars glucose and fructose (Fitzpatrick & Porter, 1966; Schippers, 1975; Ewing *et al*., 1981). CIS is a heritable trait (Hayes & Thill, 2002, 2003; Menendez *et al*., 2002; Jansky & Hamernik, 2009; Jansky *et al*., 2011).

Ohad *et al*. (1971) suggested that cold storage temperatures may also damage the amyloplast membrane. This would make the membrane more permeable to starch hydrolysis enzymes. The amount of free fatty acids (FFA) in cell membranes can increase several fold in tubers subject to stress such as low temperature for cold sweetening or ageing for senescent sweetening. Furthermore, reduced potato tuber membrane integrity has been reported to be correlated with an increase of membrane electrolyte leakage, previously implicated in the accumulation of sugars. Generally, increases in FFA reduce membrane fluidity leading to a breakdown in membrane permeability. And this, in turn, increases fry colour. Spychalla and Desborough (1990) found that changes to the FFA content of lipids during storage had little bearing on permeability. However, the amount of linolenic acid in membrane lipids appears to confer potatoes with better processing quality. In these instances, either the physical status or the chemical composition changes of the tuber membranes must account for the increased electrolyte leakage. Increased membrane lipid unsaturation is one chemical change that appears to confer resistance to increased electrolyte leakage in stored potato tubers (Isherwood, 1976; Knowles & Knowles, 1989; Spychalla & Desborough, 1990; O'Donoghue *et al*., 1994). Changes in membrane structure and function could result in cellular adjustments in the compartmentalization of key ions, substrates, and enzyme effector molecules (Isherwood & Kennedy, 1975).

Since key starch metabolism enzymes are associated with CIS (Sowokinos, 1990; Li *et al*., 2005, 2008; Bhaskar *et al*., 2010; Wu *et al*., 2011), studies of the effects of cold temperature storage on processing quality have focused on the activity of enzymes involved in the

conversion of starch to sugars. The hydrolytic pathway of starch degradation involves α-amylase (AMY) and β-amylase (BAM). Multiple genes encode different amylase isoforms that may have different roles depending on plant tissues and species. It has been reported that different amylases such as StAmy23, StBAM1, and StBAM9 regulate CIS of tubers (Hou *et al*., 2017). Zhang *et al*. (2014) have reported the expression of the StAmy23, which is localized in the cytoplasm, was strongly induced by low temperature in potato tubers and after RNA interference (RNAi) silencing resulted in the lower accumulation of reducing sugars in tubers stored at 4°C for 15 days and improved crisp colour, implying that StAmy23 is involved in potato CIS. Moreover, StBAM1 may play a role in the potato CIS process by hydrolysing soluble starch in the amyloplast stroma whilst StBAM9 plays vital and distinct roles in the starch degradation pathway of potato CIS by acting on starch granules (Hou *et al*., 2017).

The pattern of the reducing sugars content might change depending on the mechanism of starch breakdown. Sucrose hydrolysis by invertase and starch degradation have been reported to be the main pathways involved in potato CIS (Blenkinsop *et al*., 2003; Bhaskar *et al*., 2010; Zhang *et al*., 2014; Lin *et al*., 2015). The invertase activity is considered critical for sucrose cleavage (Bhaskar *et al*., 2010). Lin *et al*. (2015) revealed an evidence of a protein complex (StvacInv1–StInvInh2B–SbSnRK1) is implicated in the regulation of the enzyme activity in cold-stored tubers. The study confirmed the protein complex by pairwise interactions using biomolecular fluorescence complementation assays. The inhibition of StvacInv1 by StInvInh2B is blocked by SbSnRK1β and is restored by the phosphorylated form of SbSnRK1α. Inactivated SbSnRK1α is thus critical to maintaining invertase activity for promoting potato CIS (Lin *et al*., 2015). A higher level of SbSnRK1α expression has been reported to be accompanied by elevated SbSnRK1α phosphorylation, reduced acid invertase activity, a higher sucrose-hexose ratio, and improved crisp fry colour (Lin *et al*., 2015).

During cold-induced sweetening in stored potatoes, starch degradation occurs and eventually reducing sugars accumulate through several enzymatic reactions (Figure 1-4) (Mares *et al*., 1985; Morrell & ap Rees, 1986). Tuber starch amylose content has been reported to be higher in CIS-resistant varieties than in susceptible ones (Barichello *et al*., 1990; Jansky & Fajardo, 2014). Starch properties, such as the amylose:amylopectin ratio, may influence starch hydrolysis rates in granules, where starch is stored in tubers. Eventually, this would have an impact on the conversion of starch to free sugars (Barichello *et al*., 1990). However, Cottrell

et al. (1995) found no association between CIS resistance and amylose content. Amylose content is influenced not only by variety but also by storage, production year, and field location.

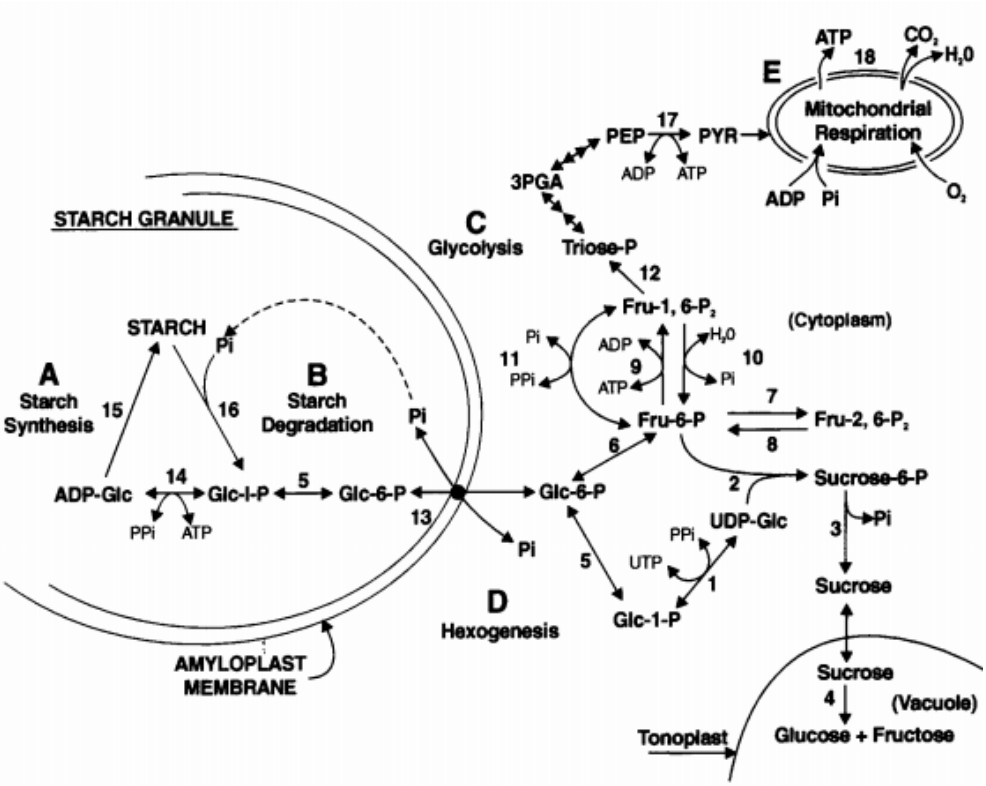


Figure 1-4. Related carbohydrate pathways in potato tubers. Enzymes represented: 1, UDP-glucose pyrophosphorylase; 2, sucrose-6-phosphate synthase; 3, sucrose-6-phosphate phosphatase; 4, acid invertase; 5, phosphoglucomutase; 6, phosphohexoseisomerase; 7, fructose-6-phosphate 2-kinase; 8, fructose-2,6-bisphosphatase; 9, ATP-phosphofructokinase; 10, fructose-l,6-bisphosphatase; 11, PPi-phosphofructokinase; 12, aldolase; 13, glucose-6-phosphate/phosphate translocator protein; 14, ADP-glucose pyrophosphorylase; 15, starch synthase; 16, starch phosphorylase; 17, pyruvate kinase and 18, mitochondrial electron transport and oxidative phosphorylation reactions. Taken from Sowokinos (2001).

Genetic mapping studies have reported that CIS is associated with a large number of quantitative trait loci (QTL) (Menéndez *et al*., 2002; Li *et al*., 2008). QTL have been linked to genes encoding invertase, SuSy 3, SPS, AGPase, sucrose transporter 1, and a putative sucrose sensor (Menéndez *et al*., 2002). The genetic complexity of the CIS trait is consistent with the involvement of numerous enzymes in the metabolic pathways linking starch synthesis and breakdown to sugar formation and utilization in plants (Nguyen-Quoc & Foyer, 2001; Sowokinos, 2001; Nägele *et al*., 2010). Genes associated with any of these enzymes and their

regulation could influence the amounts of glucose and fructose. Several studies have associated molecular markers with genes involved in starch or sugar metabolism have been linked to QTL for sugar content or crisp colour (Menéndez *et al*., 2002).

Several methods have been developed to improve cold storage and avoid CIS. RNA interference technology has been used to silence the vacInv gene, minimizing the accumulation of reducing sugars and improving cold storage (Clasen *et al*., 2016). In addition, it has been reported that silencing the potato vacInv prevents reducing sugar accumulation in cold-stored tubers, and crisps processed showed a significant acrylamide reduction and lighter colour even when tubers were stored at 4°C (Bhaskar *et al*., 2010). In this term, low levels of vacInv gene expression have been observed in cold-stored tubers from wild potato germplasm stocks that are resistant to cold-induced sweetening as well. Hence, both processing quality and acrylamide problems in potato can be controlled effectively by suppression of the vacInv gene through biotechnology or targeted breeding. This fact has a greater relevance for the fresh market as well as home processing.

Cold-induced sweetening problem has been mostly solved since the storage for processing is typically undertaken at relatively high temperatures (> 8°C) while the use of suppressors such as CIPC is required to prevent sprouting. Furthermore, breeding and new biotechnological methods have been developed to avoid the accumulation of sugars at cold storage. However, tubers can undergo a distinct physiological process called senescent sweetening after prolonged storage (> 5 months) (Burton, 1989), leading to significant losses for the potato processing industry. Though cold-induced sweetening can be reconditioned by increasing the storage temperature (Pritchard & Adam, 1994), the reducing sugar accumulation produced by senescent sweetening is considered irreversible (Isherwood & Burton, 1975). Hence, senescent sweetening in stored potato tubers remains a problem.

Though almost nothing is known regarding the mechanisms of senescent sweetening, the most widely accepted hypothesis is that tissue senescence in terms of the breakdown of cellular function is responsible for sweetening. The disruption of the amyloplast membrane could increase the rate of phosphorolytic and/or hydrolytic breakdown of the starch granule leading to degradation of starch as a consequence of starch has been exposed to amylolytic enzymes. This process could lead to an accumulation of free sugars driving to senescent sweetening as a

result of the amylolytic membrane, and subcellular organization are lost (Ohad *et al*., 1971; Sowokinos *et al*., 1987; Kumar & Knowles, 1993).

1.6 SnRK and metabolic signalling

In plants, sugar production through photosynthesis is a vital process, and sugar status modulates and coordinates internal regulators and environmental signals that control growth and development (Koch, 1996; Sheen *et al*., 1999; Smeekens, 2000). Biochemical, molecular, and genetic experiments have supported a central role of sugars in the control of plant metabolism, growth, and development and have revealed interactions that integrate light, stress, and hormone signalling (Roitsch, 1999; Sheen *et al*., 1999; Smeekens, 2000; Gazzarrini & McCourt, 2001; Finkelstein & Gibson, 2002) and coordinate carbon and nitrogen metabolism (Stitt & Krapp, 1999; Coruzzi & Bush, 2001; Coruzzi & Zhou, 2001).

One of the most common mechanisms in signal transduction is protein phosphorylation and dephosphorylation, and the use of specific inhibitors has indicated the involvement of a variety of protein kinases and protein phosphatases in plant sugar signalling (Rolland *et al*., 2002). Sucrose non-fermenting 1-related protein kinase1 (SnRK1) is a serine/threonine-protein kinase that takes its name from sucrose non-fermenting-1 (SNF1) protein kinase, its homologue in yeast (*Saccharomyces cerevisiae*) (Celenza & Carlson, 1986).

SnRK1 is a plant protein kinase with a catalytic domain similar to that of SNF1 of yeast and AMP-activated protein kinase of animals. The SNF1 family of protein kinases are a distinct group within the protein kinase superfamily but are closely related to the calcium-dependant protein kinase group, which includes the animal calmodulin-dependent protein kinases and the plant calmodulin-like domain protein kinases (Hardie, 2000). The first plant SnRK1 sequence to be reported was a cDNA (complementary DNA) isolated from a rye endosperm cDNA library (Alderson *et al*., 1991). SnRK1 genes have since been identified and characterized in many plant species (reviewed by Halford & Hardie, 1998).

Plants contain two other subfamilies of protein kinases, SnRK2 and SnRK3, containing catalytic domains with sequences that place them clearly within the SNF1 family. The SnRK2 and SnRK3 gene subfamilies appear to be unique to plants and are relatively large and diverse compared with SnRK1. SnRKs 2 and 3 have been implicated in stress and ABA-mediated

signalling pathways. This subfamilies have been associated with responses to abiotic stresses such as drought, salinity, cold, and osmotic stress (Hey *et al*., 2010). The SnRK2 subfamily includes PKABA1 from wheat, which is involved in mediating ABA-induced changes in gene expression (Anderberg & Walker-Simmons, 1992; Gómez-Cadenas *et al*., 1999). Moreover, there is evidence that ABA promotes degradation of SnRK1 in wheat and activates a putative calcium-dependent SnRK2 (Coello *et al*., 2012).The SnRK3 gene family includes SOS2, an Arabidopsis protein kinase involved in conferring salt tolerance (Halfter *et al*., 2000; Liu *et al*., 2000).

1.6.1 SnRK1 and the regulation of carbohydrate metabolism

SnRK1 indirectly controls carbohydrate metabolism because it modulates the transcription of several genes such as sucrose synthase (sucrose degradation) and α-amylase (starch degradation) (Purcell *et al*., 1998; Laurie *et al*., 2003). SnRK1 has also been found to be involved in starch biosynthesis (Geigenberger, 2003). It stimulates the redox activation of AGPase, the key regulatory enzyme of this biosynthesis pathway, in response to high sucrose levels. This result is consistent with data showing that SnRK1 disruption in the moss *Physcomitrella patens* leads to a defect in starch accumulation (Thelander *et al*., 2004). SnRK1-antisense transgenic pea seeds have been shown to have a higher carbon/nitrogen ratio than wild type (Radchuk *et al*., 2006). The various phenotypes observed (e.g., maturation defects, lower globulin content, higher sucrose level) suggest that SnRK1 might be implicated in the coordination of cell division and expansion, a process involving sugar signals.

In vitro, SnRK1 phosphorylates and inactivates four important plant metabolic enzymes: (i) 3-hydroxymethyl-3-methylglutaryl-CoA reductase (Dale *et al*., 1995) (ii) SPS, which catalyses sucrose biosynthesis; (iii) nitrate reductase, which catalyses the first step of nitrogen assimilation into amino acids (Sugden *et al*., 1999) and (iv) trehalose phosphate synthase 5 (TPS5), a key enzyme in the synthesis of trehalose-6-phosphate, a signalling sugar that regulates plant metabolism and development (Harthill *et al*., 2006).

1.6.2 Regulation of SnRK1 activity

There is evidence of differential transcriptional regulation of SnRK1 gene expression. In potato, the highest levels of expression occur in stolons as they begin to develop into tubers (Man *et al*., 1997). Expression gradually declines in maturing tubers but is lowest in leaves. The exact nature of the signal that brings about changes in the SnRK1 gene expression or activation state is not known. Dephosphorylation and inactivation of spinach SnRK1 have been found to be inhibited by low concentrations of 5'-AMP (Sugden *et al*., 1999a). There is also evidence that SnRK1 may be inhibited by G6P (Toroser *et al*., 2000), although others have attributed the apparent inhibition of SnRK1 by G6P to the presence of a contaminant (Sugden *et al*., 1999). This has led to the hypothesis that SnRK1 is activated in response to high intracellular sucrose and/or low intracellular glucose levels (Halford & Dickinson, 2001). Zhang *et al*. (2009) suggested the effects on SnRK1 activity are specific to T6P, as SnRK1 was not inhibited by the other tested sugars or sugar phosphates.

SnRK1 is a hetero-trimeric protein complex composed of an AKIN10 or AKIN11 catalytic α-subunit, β-, and γ-subunits, which together form the active kinase complex (Polge & Thomas, 2007). Over-expression of SnRK1 promotes plant survival under low light and starvation conditions, in addition to altering inflorescence development and delaying senescence. In contrast, akin10 akin11 virus-induced gene silencing double mutant plants showed growth arrest coupled with premature senescence. The AKIN10/AKIN11 signalling cascade seems to be crucial for plant survival under stress, e.g., darkness and sugar deprivation (Baena-González *et al*., 2007).

1.6.3 SnRK1 and plant stress responses

Reported data suggest the involvement of this plant kinase complex in the global regulation of metabolism as well as in developmental and stress responses (reviewed by Polge & Thomas, 2007).

The yeast SNF1 kinase is a heterotrimeric enzyme consisting of α(SNF1), β(SIP1, SIP2, or GAL83) and γ(SNF4) subunits. In high glucose, the SNF1 kinase complex is inactive, and the SNF1 regulatory domain auto-inhibits the catalytic domain. In low glucose, this auto-inhibition is relieved and the SNF4-activating subunit binds to the regulatory domain. As a third

component, the kinase complex contains one of the related scaffolding proteins SIP1, SIP2 or GAL83 (Carlson, 1999). The β-subunits are required for kinase function and substrate definition in yeast (Schmidt & McCartney, 2000). GAL83 mediates the association of SNF1 with SIP4, a SNF1-regulated transcription activator of gluconeogenic genes (Vincent & Carlson, 1999). GAL83 directs SNF1 to the nucleus in a glucose-regulated manner (Vincent *et al*., 2001). The first indication of involvement of SnRK1 in plant stress response was the salt hypersensitivity of the antisense StubGAL83 transgenic potato plants, which suggests that SnRK1 might activate protection systems against this stress (Lovas *et al*., 2003). Furthermore, several lines of evidence indicate that SnRK1 is involved in plant-pathogen interactions (Hao *et al*., 2003). The expression of an antisense sequence of Arabidopsis SnRK1 in tobacco increased its sensitivity to virus attack. In contrast, over-expression of a sense sequence increased its resistance, suggesting that SnRK1 might be a component of plant antiviral defence. The plant-specific AKINβγ subunit also interacts with two proteins involved in nematode resistance through its glycogen-binding domain (Gissot *et al*., 2006).

The consequences of changes in SnRK1 levels on plant development and stress response constitute a further indication that SnRK1 might play an important role in the regulation of global metabolism, the disturbance of which might lead to developmental or adaptation defects. This speculation is supported by data showing that the allocation of carbon to roots by SnRK1 kinases allows better tolerance to herbivore attacks (Schwachtje *et al*., 2006).

1.7 Sugars and phytohormone responses

The field of plant sugar response is complicated by the fact that plants appear to respond to soluble sugar levels or flux by several response pathways. Many plant developmental, physiological, and metabolic processes are partially regulated by nutrient availability. Particularly, alterations in soluble sugars availability, such as glucose and sucrose, help regulate a diverse array of processes. Multiple studies indicate that many of these processes are also regulated in response to other signalling molecules, such as phytohormones.

Sugar- and phytohormone-response pathways are involved in the regulation of many processes. However, little is known about the mechanisms by which different response pathways interact. The available evidence suggests that these interactions may be quite direct in some cases and indirect in others. For example, a component of one response pathway might interact directly

with components of another response pathway to form a complex. Alternatively, response pathways might interact indirectly by altering the levels of the same second messenger.

1.7.1 Phytohormones effect on sugar metabolism and transport

Several phytohormones are involved in the regulation of sugar metabolism and/or transport. ABA is implicated in the regulation of sugar transport and metabolism. Treatment of germinating rice seeds with ABA plus glucose results in a higher accumulation of sugars in the scutellum than treatment with glucose alone, suggesting that ABA stimulates glucose uptake from the media (Kashem *et al*., 1998). ABA and GAs also help regulate sugar concentrations by altering α-amylase levels, thereby affecting the rate at which sugars are produced from starch (reviewed by Bethke *et al*., 1997).

Senescence is an internally programmed degenerative process leading to death in plants. CKs are involved in plant growth and developmental processes, including senescence (Mok, 1994). These processes are related to the demand for carbohydrates, regulation of assimilate partitioning (Brenner & Cheikh, 1995), sink strength (Kuiper, 1993), and source-sink relationships (Roitsch & Ehneß, 2000).

CKs affect the distribution of nutrients and further modulate sink strength as indicated by their ability to establish local metabolic sinks, which has been demonstrated by mobilisation of radiolabelled nutrients, such as sugars, from other parts of the plant to CK-treated areas (Kuiper, 1993).

Sucrose metabolism and transport are very important for growth and senescence. These processes depend on the activities of SPS, SuSy, and invertase (cytInv, vacInv, cwInv). SPS and SuSy are involved in regulating the synthesis of sucrose (Huber & Israel, 1982; Stitt *et al*., 1988). Invertase activities has been observed to be dominant during the initiation and expansion of sink tissues (Koch, 2004). Plants contain neutral invertases, localized in the cytosol (cytInv), and acidic invertases, localized in the vacuoles (vacInv), and cell wall (cwInv) in the apoplast (Roitsch & González, 2004). In particular, extracellular invertase (cwInv) has essential functions both in source-sink regulation and in supplying carbohydrates to sink tissues. Therefore, it is a central modulator of sink activity (Tang *et al*., 1999; Goetz *et al*., 2001;

Roitsch *et al.*, 2003). In addition, CKs are involved in the regulation of invertase activity; extracellular invertase activity is usually high in tissues with an elevated cytokinin concentration (Lefebre *et al.*, 1992).

1.8 Plants and stress factors

Stress in plants can be defined as any external factor that negatively influences plant growth, productivity, reproductive capacity, or survival. This includes a wide range of factors that can be broadly divided into two main categories: abiotic or environmental stress factors and biotic or biological stress factors.

In many cases, the abiotic stresses do not occur independently, and thus the stress environment may involve a complex of interacting stress factors. The abiotic stress factors that most commonly influence plant performance include deficiencies or excesses of water, extremes of irradiance, excessively low or high temperature, deficiencies or excesses of several nutrients, high salinity, and extremes of soil pH. Abiotic stresses may also include mechanical stresses and stresses associated with compounds that may be toxic for the plants in high concentrations, which is the case of oxidative stress produced by reactive oxygen species (ROS).

1.8.1 Respiration and oxidative stress

Plant growth and development are driven by electron transfer reactions, involving both a reduction and a complementary oxidation process. Redox reactions are the metabolic processes through which cells convert and distribute the energy that is necessary for growth and maintenance. Even though most forms of life are oxygen-dependent for respiration, oxygen (O_2) can be a damaging chemical in certain forms. ROS, such as singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$), are partially reduced or excited forms of atmospheric oxygen (Halliwell & Gutteridge, 2007). ROS are required for many important signalling reactions but are toxic products of aerobic metabolism as well (Konig *et al.*, 2012; Foyer & Noctor, 2013; Mignolet-Spruyt *et al.*, 2016). In plants, ROS are continuously produced as products of several metabolic processes such as respiration and photosynthesis, and they are localized in different cellular compartments (Foyer & Harbinson, 1994). Plants also generate ROS by activating various oxidases and peroxidases,

producing them in response to different environmental changes (Allan & Fluhr, 1997; Schopfer *et al*., 2001; Bolwell *et al*., 2002).

When plants are exposed to unfavourable environmental conditions, this increases the production of ROS. Moreover, one of the changes associated with ageing in potato tubers includes an increase in tuber respiration rate resulting in oxidative stress and lipid peroxidation (Kumar & Knowles, 1993; Kumar & Knowles, 1996).

The ROS at high concentrations can behave as extremely reactive molecules. The ability of ROS to react indiscriminately with almost all cellular components leads to cause oxidative damage to proteins, DNA, and lipids (Beckman & Ames, 1997; Berlett & Stadtman, 1997). Consequently, high ROS levels produce cellular damage such as membrane leakage and cell lysis by an indiscriminate attack.

Hence, the process of ROS detoxification in plants is essential for the protection of plant cells as well as their organelles against the toxic effect of these ROS (Mittler, 2002; Apel & Hirt, 2004). The ROS detoxification systems include enzymatic and non-enzymatic antioxidant components (Table 1-2) (Scandalios, 2005). In plant tissues, the non-enzymatic antioxidant components such as ascorbate and glutathione can exist in either their reduced or oxidised forms. The redox status of these compounds both act as a marker of plant oxidative stress and also influence plant signalling, gene expression, and metabolism (Noctor, 2006).

Oxidative stress is a central factor in abiotic and biotic stress phenomena and is the result of an imbalance between the production of ROS and antioxidant defence, causing significant damage (Halliwell & Gutteridge, 2007).

Table 1-2. Major antioxidant components of plant cells.

Component	*Cellular location*	*Antioxidant functions*
Enzymatic antioxidants		
Superoxide dismutase	Chloroplasts, mitochondria, peroxisomes, cytosol	Catalyzes the reaction $2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$
Catalase	Peroxisomes, mitochondria[a], cytosol[a]	Catalyzes the reaction $2H_2O_2 \rightarrow 2H_2O + O_2$
Ascorbate peroxidase	Chloroplasts, mitochondria, peroxisomes, cytosol	Catalyzes the reaction $H_2O_2 + 2AsA \rightarrow 2H_2O + 2MDHA$
Monodehydroascorbate reductase	Chloroplasts, mitochondria, cytosol	Catalyzes the reaction $2MDHA + NAD(P)H \rightarrow 2AsA + NAD(P)$
Dehydroascorbate reductase	Chloroplasts, mitochondria, cytosol	Catalyzes the reaction $DHA + 2GSH \rightarrow AsA + GSSG$
Glutathione reductase	Chloroplasts, mitochondria, cytosol	Catalyzes the reaction $GSSG + NAD(P)H \rightarrow 2GSH + NAD(P)$
Peroxiredoxins	Chloroplasts, mitochondria, nucleus, cytosol	Reduce peroxides with the concomitant oxidation of active site–cysteine residues
Glutaredoxins	Chloroplasts, mitochondria, cytosol, apoplast	Glutathione-dependent reduction of oxidized cysteine residues in target proteins
Thioredoxins	Chloroplasts, mitochondria, nucleus, cytosol, apoplast	Ferredoxin/NADPH-dependent reduction of oxidized cysteine residues in target proteins
Nonenzymatic antioxidants		
Ascorbate	Soluble antioxidant found in all cellular compartments	Ascorbate–glutathione cycle, direct scavenging of $O_2^{\cdot-}$, $OH^{\cdot}$, regeneration of tocopheroxyl radical
Glutathione	Soluble antioxidant found in all cellular compartments	Ascorbate–glutathione cycle, reduction of oxidized protein cysteine residues
Carotenoids	Lipid soluble antioxidants located in plastids	Scavenging of $^1O_2$
Tocopherols	Lipid soluble antioxidants located in plastids, vacuoles, nuclei, oil bodies	Scavenging of $^1O_2$, quenching lipid peroxides

[a]Evidence for mitochondrial and cytosolic catalase localization remains uncertain. Taken from Hancock (2017).

1.8.2 Mitochondria and oxidative stress

Respiration is an energy-conserving process that couples the transfer of potential energy from the oxidation of reduced organic matter to high-energy intermediates and heat and is known as the primary function of mitochondria.

In aerobic respiration, mitochondria carry out the final steps to generate the bulk of the needed ATP for growth and cellular maintenance. Moreover, mitochondrial metabolism is significant for many other important cellular processes such as photosynthesis, photorespiration, nitrogen metabolism, redox regulation, and signalling (Heazlewood *et al.*, 2004; Bauwe *et al.*, 2012).

The different steps of the respiratory apparatus in plant mitochondria can be framed as a sequential set of processes involving the transport of reduced glycolytic products from the cytosol into the mitochondrion, including a series of reactions leading to the release of CO_2 and reduction of O_2 to water.

The major carbon metabolising machinery present in plant mitochondria is represented by the eight enzymes of the tricarboxylic acid (TCA) cycle. TCA cycle and associated enzymes link the product of the oxidation of pyruvate and malate to CO_2 with the generation of NADH for the oxidation by the mitochondrial respiratory chain leading the substrate-level phosphorylation of ADP to ATP (Figure 1-5) (Fernie *et al*., 2004).

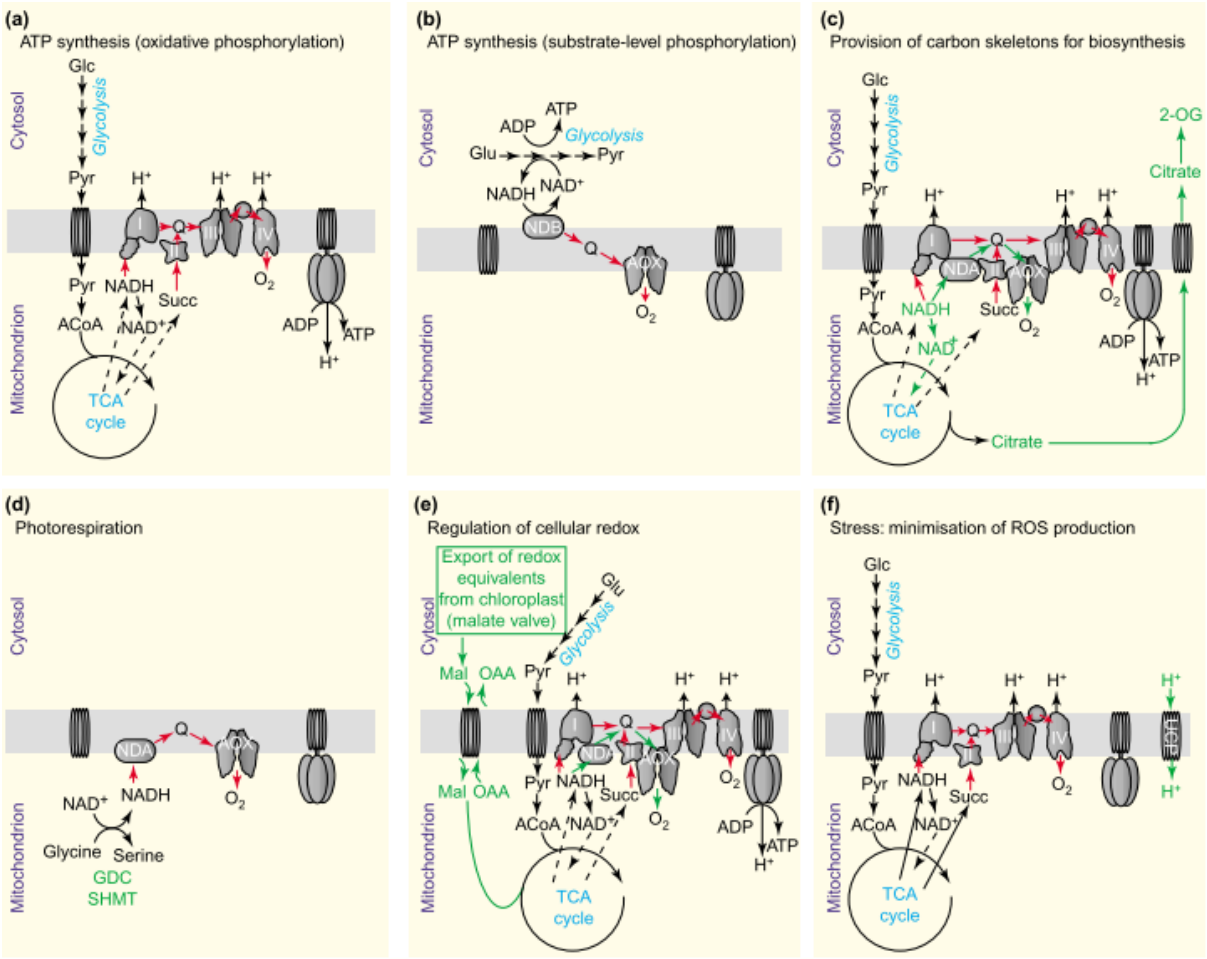


Figure 1-5. Possible interactions of mitochondrial electron transport with other pathways. **(a)** ATP synthesis (i.e. oxidative phosphorylation). Pyruvate (Pyr) supplied by glycolysis is oxidised by the mitochondrial TCA cycle, and electrons from the resulting reductant are transferred through the electron transport chain with a chemiosmotically coupled synthesis of ATP. Complexes I–IV of the electron transport chain are shown. **(b)** ATP synthesis (substrate-level phosphorylation). Glycolysis may also contribute to ATP production, particularly under conditions in which the oxidative phosphorylation pathways are impaired. Glycolytic flux is dependent upon the recycling of cytosolic NAD^+, which can be achieved via the external NADH dehydrogenase (NDB). The activity of AOX could provide an entirely non-proton-pumping electron transport pathway, in which electron flux is not limited by mitochondrial ATP synthesis.

Fig. 1-5. Continuation. (c) Provision of carbon skeletons for biosynthesis. Withdrawal of TCA-cycle intermediates (the export of citrate to support nitrogen assimilation is illustrated) may necessitate a higher flux of portions of the TCA cycle and a higher rate of entry of electrons into the electron transport chain. These extra electrons may be accommodated by a non-proton-pumping pathway that consists of the internal NDA and AOX, such that electron flow is not restricted by the rate of ATP synthesis. **(d)** Photorespiration. The oxidation of photorespiratory glycine in the mitochondrial matrix requires the recycling of NAD^+. This can be achieved by the entry of electrons from NADH into the electron transport chain. The non-phosphorylating pathway explained above may operate to avoid electron flow being limited by the rate of ATP synthesis. **(e)** Regulation of cellular redox. Photosynthesis requires a precise balance between the generation of NADH and ATP. One way in which this may be achieved is to export excess NADH via metabolite shuttles. The 'malate valve' exports excess chloroplastic reductant as malate and imports it into the mitochondrion via oxaloacetate (OAA) exchange. Mitochondrial malate dehydrogenase releases the NADH. The extent to which this NADH supports ATP synthesis depends on the route of electrons through the electron transport chain (red arrows represent the phosphorylating pathway; green arrows represent the non-phosphorylating pathway). **(f)** Stress: minimisation of ROS production. A high mitochondrial membrane potential restricts electron flow and increases the leakage of electrons to form superoxide. This can be minimised by the activity of mitochondrial UCP, which dissipates the proton gradient. UCP is activated by superoxide, providing a regulatory loop for this pre-oxidant defence mechanism. AcoA, acetyl-CoA; GDC, glycine decarboxylase; Glu, glucose; 2-OG, 2-oxoglutarate; Mal, malate; SHMT, serine hydroxymethyltransferase; Succ, succinate. Taken from Fernie *et al*. (2004).

Mitochondria are surrounded by two membranes which have very different permeability properties. The outer membrane allows relatively non-specific transport of small molecules from the cytosol into the inter-membrane space (Mannella, 1992; Mannella *et al*., 2001). The inner membrane contains very selective transporters for small molecules to the matrix space. This allows a complex set of inner membrane carrier functions to have a large influence on the functions of mitochondria (Laloi, 1999). Alterations in cellular metabolism and energy demand such oxidative damage can undergo changes in their morphology and respiratory capacity by regulation the composition and abundance of the protein machinery (Jacoby *et al*., 2012). Moreover, mitochondria are key agents in how plants respond to oxidative stress.

In general, stress can alter the rate at which some of the mitochondria electron transport chain (ETC) complexes donate or accept electrons, ultimately leading to over-reduction of certain sites of the ETC which produce electron leakage to O_2 following to ROS production (Jacoby

et al., 2011). The primary sites of mitochondrial ROS production are the ETC complexes I, II, and III (Møller *et al*., 2007; Gleason *et al*., 2011).

Mitochondrial antioxidants and detoxification enzymes play crucial roles in stress tolerance by alleviating the effects of excess ROS production. The ETC components in mitochondria are particularly damaged by ROS, and this can be problematic since protection is crucial to maintain ATP production during stress (Jacoby *et al*., 2011).

The accumulation of ROS can induce lipid peroxidation in the mitochondria, referring to free radical autoxidation of polyunsaturated fatty acids of membrane lipids (such as arachidonic, and linoleic and linolenic acids) to generate various cytotoxic aldehydes, alkenals, and hydroxyalkenals (Jacoby *et al*., 2012). The interaction of $HO^{\bullet}$ with polyunsaturated fatty acids initiates lipid peroxidation that by a sequential series of reactions leads to a number of toxic lipid peroxidation end products by a non-enzymatic, metal ion enhanced process (Noordermeer *et al*., 2000).

Several studies have reported overall changes in abundance of mitochondrial proteins following conditions which induce oxidative stress in several plant species (Sweetlove *et al*., 2002; Taylor *et al*., 2005; Taylor *et al*., 2009; Jacoby *et al*., 2010; Huang *et al*., 2011; Hossain *et al*., 2012; Tan *et al.*, 2012). Besides, the large respiratory subunits of the ETC also coordinate protein changes to alter respiration response to oxidative stress conditions (Tan *et al*., 2012). These changes involve proteins responding to ROS and their damage. In this instance, the mitochondria are protected by antioxidant enzymes that detoxify ROS, and many have been observed to change their rate during oxidative stress including ascorbate peroxidase (Dooki *et al*., 2006), monodehydroascorbate reductase (Sarry *et al*., 2006) and peroxiredoxins (Sweetlove *et al*., 2002; Sarry *et al*., 2006) among others.

Increased oxidative damage in tuber cellular membranes has been associated with long term-storage while potato mitochondria have been reported to be highly oxidised under stress conditions (Kumar & Knowles, 1993; Salvato *et al*., 2014). This damage in mitochondria could lead to malfunction of the respiratory machinery in aged tubers, reducing the capacity for respiration. In terms of sweetening, starch hydrolysis may also be triggered by a requirement for increased respiration to provide ATP and reducing equivalents for membrane repair (Kumar & Knowles; 1996). Senescent sweetening may be induced as a result of a reduced capacity for

respiration leading to an accumulation of sugars and reduced availability of ATP required for starch resynthesis.

1.8.3 Amyloplasts and oxidative stress

Mitochondria are not the only organelles that can be affected by different stresses. Amyloplasts have been reported to be damaged as a result of stress during storage (Ohad *et al*., 1971; Sowokinos *et al*., 1987). These organelles are responsible for the synthesis and storage of starch granules.

As mentioned previously, the storage of potato tubers at 4°C can result in cold-induced sweetening by the degradation of starch and accumulation of free sugars. This sweetening is minimized when the tubers are stored at 25°C (Smith, 1968). The process of degradation of starch may be related to a change in the distribution of different enzymes or substrates within the subcellular compartments of the potato tuber (Ohad *et al*., 1971).

The activities of both lipoxygenase (EC 1.13.11) and lipolytic acyl hydrolase (EC 3.1.2) affecting both starch granule breakdown and lipid metabolism are reduced in the prematurely sweetened tissue (Sowokinos *et al*., 1985; Lulai *et al*., 1986).

Alterations in lipid metabolism may influence the structure and transport properties of cellular membranes. Furthermore, the double-walled plastid membrane surrounding the amyloplasts has been suggested to play an important function in the regulation of carbon partitioning between the granule and free sugars in the cytoplasm (Mares *et al*., 1985). Loss of cellular compartmentalization during senescence has been associated with significant physical changes affecting the integrity of the amyloplast membrane (Isherwood, 1976; Wetstein & Sterling, 1978). Studies suggest the observed sweetening that has been demonstrated to occur by handling stress may be mediated by an accelerated disruption of the amyloplast membrane (Orr *et al*., 1985; Sieczka & Maatta, 1986).

In this context, oxidative stress could induce the premature disruption of the amyloplast membrane and increase the rate of phosphorolytic and/or hydrolytic breakdown of the starch granule in an irreversible way. This process could lead to degradation of starch and accumulation of free sugars driving to senescent sweetening as a result of the amylolytic

membrane, and subcellular organization are lost (Figure 1-6) (Ohad *et al*., 1971; Sowokinos *et al*., 1987).

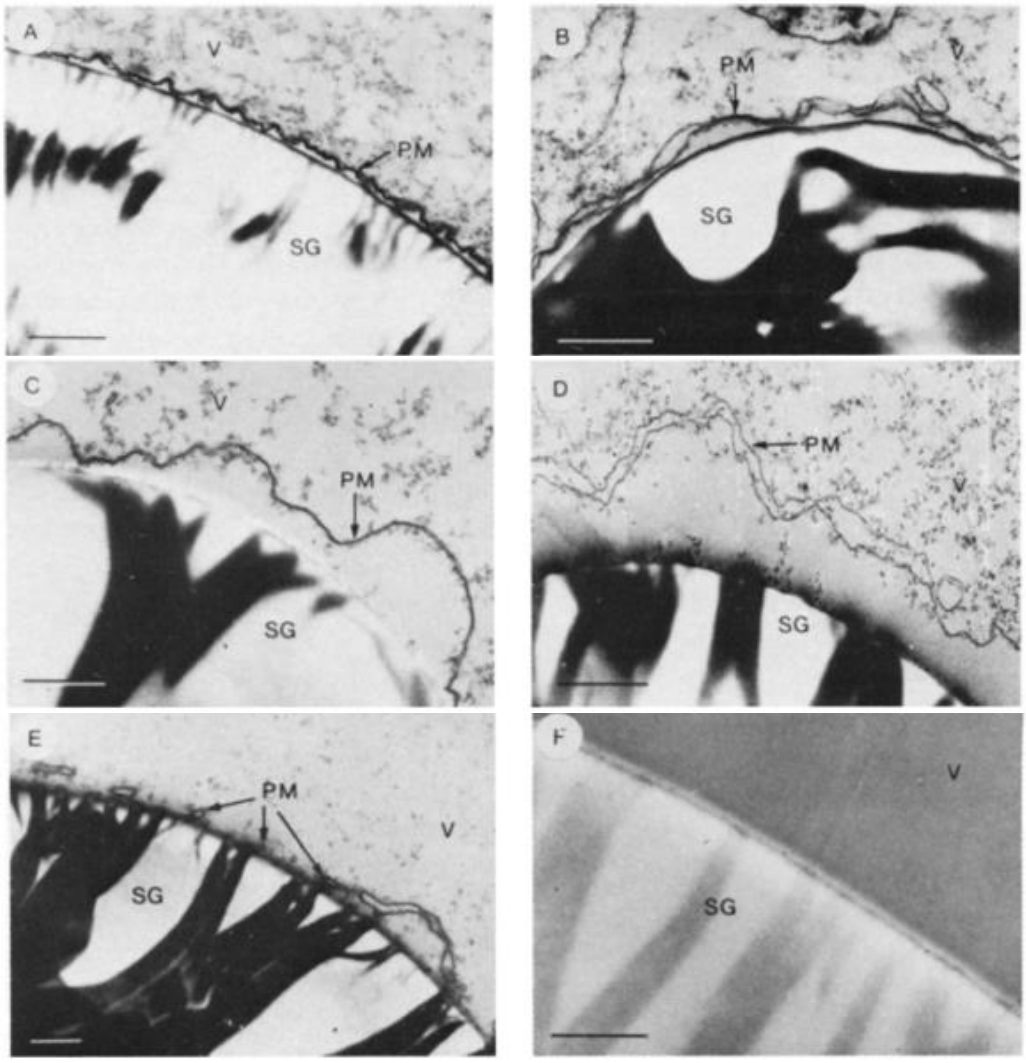


Figure 1-6. Electron micrographs of starch granules (SG) in the vacuole (V) of differentiated parenchymal cells of potatoes stored at 8.9°C. Frames A through F show the double-walled amyloplast membrane (PM = plastid membrane) representing six different physical states. Membrane integrity represented by each micrograph is: **(A)** bi-layers of the amyloplast membrane intact and closely associated with the granule, **(B)** bi-layers of the amyloplast membrane separating and closely associated with the granule, **(C)** bi-layers of the amyloplast membrane intact arid loosely associated with the granule, **(D)** bi-layers of the amyloplast membrane separating and loosely associated with the granule, **(E)** membrane fragmented, vesicles formed and **(F)** no membrane visible. Modified from Sowokinos *et al*. (1987).

1.9 Conclusions

Control of potato quality during storage represents a significant problem for the potato processing industry. Although a great deal of research has been conducted into the problem of cold sweetening yielding breeding tools, techniques for reconditioning, and improved storage methods, senescent sweetening has been much less examined and remains a problem in long-term tuber storage at moderate temperatures.

Literature associated with senescent sweetening is sparse, and little is known regarding its mechanisms. Potential mechanisms of senescent sweetening include enhanced starch degradation, reduced starch resynthesis, and reduced catabolism of sugars. The most accepted hypothesis seems to be senescent sweetening is produced by oxidative stress since it has been reported that long-term tuber storage leads to an increase of oxidative damage of cellular membranes and degradation of the amyloplast membrane. This membrane leakage could increase the exposure of starch granules to cytosolic amylolytic enzymes, and it has been postulated as a mechanism of senescent sweetening.

One of aim of this Ph.D. project entitled “Elucidating the mechanisms of senescent sweetening in stored potato tubers” was to test the hypothesis that senescent sweetening is caused by oxidative damage in both mitochondria and amyloplasts. Mitochondrial oxidative damage could promote sweetening as a result of a reduced capacity for respiration leading to an accumulation of sugars and reduced availability of ATP required for starch resynthesis while the increased oxidative damage of the amyloplast membrane has the potential to expose starch granules to cytosolic amylolytic enzymes as mentioned previously. A further objective to elucidate downstream biochemical and molecular responses to oxidative damage that may influence carbohydrate metabolism resulting in senescent sweetening.

However, to date, almost nothing is known regarding the mechanisms around senescent sweetening, and further research and approaches are necessary. The Ph.D. research project adopts physiological, biochemical, and molecular approaches to fully elucidate mechanisms of senescent sweetening and identify key factors controlling the trait.

Chapter 2: Materials & Methods

2.1 Plant Materials, Growing and Storage Conditions

For the 1st season (2016/2017) of this study, tubers of two different potato cultivars, Arsenal and VR 808, were obtained from PepsiCo. Tubers were either untreated or had been treated by misting with CIPC to inhibit sprouting. When treated, CIPC was applied post-curing at 13°C. Tubers were stored at 9°C in the dark in a cold storage unit (Porkka, UK) at The James Hutton Institute and sampled at the intervals described. For untreated tubers, sprouting buds were removed by hand every two weeks to avoid the development of carbon sinks. The 1st sampling point was in October 2016 and last sampling point in August 2017.

In season 2 (2017/2018) tubers of cultivars Arsenal and VR 808, were obtained from PepsiCo. All tubers had been treated with CIPC as described for season 1. Tubers were stored at The James Hutton Institute as previously described and sampled for tuber quality at the intervals described. The 1st sampling point was in November 2017 and last sampling point in October 2018.

In the final season (2018/2019) tubers of 9 different cultivars were obtained from PepsiCo, all of them CIPC-treated. The varieties used during this season are Pirol, SH C 909, VR 808, Lady Rosetta, Brooke, Arsenal and Shelford. An additional 'Unknown' variety was included. The name of this variety cannot be disclosed at the breeder's request. Tubers were stored at 9°C at The James Hutton Institute and sampled for tuber quality. The 1st sampling point was in December 2018 and last sampling point in September 2019.

During the experimental seasons all potato cultivars were grown in Shropshire, with the exception of the Shelford variety used during season 3 where tubers grown in two locations, Shropshire and Yorkshire were under study for this variety.

For all seasons of this study the growing conditions were different due to the weather. Cultivars were grown under drier weather during season 2 (2017/2018) and 3 (2018/2019) compared to season 1 (2016/2017). These differences in growing conditions may had an effect on the results observed.

Tubers were sampled following bisection transversally and twice longitudinally at a 90^{o} angle. Tuber samples were then taken from opposite eights (ends), and comprised periderm, cortex, vascular ring and outer core to capture the maximum range in sugars across the tuber. In the 1st season five tubers per variety and treatment were sampled and two sets of opposite eights were taken from each tuber and bulked by replicate for each sampling occasion. In the 2nd season five tubers per variety were sampled and bulked as in the previous season. An additional set was sampled, and tuber discs were extracted using a cork borer size N° 2 (5 mm) for the ^{13}C labelling experiments. In the last season three tubers were sampled per variety for each sampling occasion.

All the samples were snap frozen in liquid nitrogen and each set of replicates was either stored at -80°C or subject to 72 hours freeze drying at 0.700 mbar (ALPHA 1-4 LSC, CHRIST Freeze Dryers, Germany) before grinding to a fine powder using an electric grinder (DCG39, DE'LONGHI Electric Grinder, UK).

2.2 Fry Test Process

Tuber slices in a range 1.26 mm – 1.48 mm of thickness were fried at 177°C (L30PFS12, LOGIK Professional Deep Fryer, UK) for 3 minutes following the standard fry protocol developed by PepsiCo to test the fry colour of the crisps. Tubers were sliced using a mandoline (Bron Coucke Mandoline Vegetable Slicer, France) and slice thickness was measured using a digital caliper (RS PRO 150mm Digital Caliper, UK) (Figure 2-1). A long-life vegetable cooking oil (KTC (Edibles) Ltd, UK) was used for frying. Grade of darkening in fry colour was estimated using ImageJ based on the grey scale in each crisp. ImageJ displays images by linearly mapping pixel to display values in the range 0-255. Pixels with minimum value are displayed as black and those with maximum value are displayed as white (Schneider *et al.*, 2012). The calculated value was converted to percentage as an estimation of darkening in crisps.

Figure 2-1. Tools used to slice potato tubers and measure slice thickness during fry colour study.

2.3 Biochemical Analysis

2.3.1 Extraction and quantification of sugars

Sugars were extracted and analysed using an adaptation to the method described by Viola *et al*. (2007). Prepared lyophilised tuber powder (50 mg) was extracted in 1 ml of 80% (v/v) ethanol at 80°C for 1 hour with periodic vortexing. Samples were then centrifuged at 16,000 g for 10 minutes at 1°C, the supernatant was decanted, and the extraction was repeated. The two supernatant fractions were combined, reduced to the aqueous phase by evaporation at 40°C under reduced pressure in a centrifugal evaporator (miVac Duo Concentrator, Pump and Speed Trap, GeneVac, UK), The aqueous phase was then frozen, lyophilized and finally resuspend in 20 volumes of distilled H_2O. This fraction was subsequently used for analysis of glucose, fructose and sucrose by High Performance Anion Exchange Chromatography-Pulsed Amperometric Detection (HPAEC-PAD; Dionex) under the conditions described by Huang *et al*. (2016) (Table 2-1).

Table 2-1. Conditions used for sugars quantification by HPAEC-PAD (Dionex).

Conditions	
Column:	Thermo Scientific Dionex CarboPac PA20 analytical column (3x150 mm)
Eluent:	Sodium hydroxide
Isocratic:	100 mM NaOH from -15 min to -10.05 min only for column wash, 10 mM NaOH from -10.00 min to 0 min for reequilibration, 10 mM NaOH from 0-15 min
Flow Rate:	0.5 mL/min

2.3.2 H_2O_2 extraction and quantification

Five tubers of each variety were used for each sampling time (October and November 2016, January, March and May 2017). Tissue from opposite eights of cortex was snap frozen in liquid nitrogen individually and stored at -80°C. Tuber samples were ground to a powder in liquid nitrogen using a mortar and pestle and extracted in ice-cold 5% $HClO_4$ at a ratio of 1 ml 50 mg FW^{-1} using an adaptation of the method described by Queval *et al*. (2008). Following extraction, the homogenate was centrifuged at 14,000 g for 10 minutes at 4°C and the supernatant was neutralized with 5 M K_2CO_3. Insoluble $KClO_4$ was removed by centrifugation and H_2O_2 was immediately quantified by fluorimetry in a microplate reader (Varioskan™ LUX, Thermo Scientific™) using the commercially available Amplex Red Hydrogen Peroxide/Peroxidase assay kit according to the manufacturer's instructions (Invitrogen Ltd, Paisley, UK).

2.3.3 Malondialdehyde (MDA) determination

MDA content in five tubers per variety for each sampling time (October and November 2016, January, February, March, April, May, June, July and August 2017) were determined according to Hodges *et al*. (1999). Powdered freeze-dried samples (0.07 g) were homogenized and extracted in 20 volumes of 80% (v/v) ethanol. Samples were centrifuged for 10 minutes at 3,000 g at 1°C, and two aliquots were taken. A first 0.5 ml aliquot of extract was mixed in 0.5 ml 20% trichloroacetic acid, 0.65% thiobarbituric acid (TBA) containing 0.01% butylated hydroxytoluene (BHT). A second 0.5 ml aliquot of extract was mixed in 0.5 ml 20% trichloroacetic acid containing 0.01% BHT. Both aliquots were incubated for 25 minutes at

95°C and centrifuged as in the previous step. The absorbance of the supernatant was measured at 440, 532 and 600 nm for each sample using a spectrophotometer (Hitachi U-3010 UV-Visible) and MDA equivalents (nmol ml^{-1}) determined using the following calculation:

A) $(Abs\ 532_{+TBA}) - (Abs\ 600_{+TBA}) - (Abs\ 532_{-TBA} - Abs\ 600_{-TBA})$

B) $(Abs\ 440_{+TBA} - Abs\ 600_{+TBA}) \times 0.0571$

$MDA\ equivalents\ (nmol\ ml^{-1}) = (A - B/\ 157000) \times 1000000$

2.3.4 Total polyphenols extraction and quantification

Five tubers per variety were used for each sampling time (March, May and June 2017). Total polyphenols were estimated using a modification of the enzymatic method described by Stevanato *et al*. (2004). Powdered freeze-dried samples (0.1 g) were extracted in 10 volumes of 50% (v/v) methanol containing 0.1% formic acid. The homogenate was centrifuged at 4,000 g for 15 minutes. Total polyphenol content was determined in 10 µl of supernatant in a reaction mixture containing 145 µl potassium phosphate buffer pH 8.0, 20 µl 30 mM 4-aminophenazone, 20 µl 20 mM H_2O_2 and 5 µl 100 U ml^{-1} horse radish peroxidase (EC 1.11.1.7). Following 5 minutes incubation in the dark at 25°C, absorbance at 500 nm was recorded in a plate reader (Multiskan™ GO, Thermo Scientific™) and total polyphenols estimated by reference to a standard curve constructed using catechin.

2.3.5 Extraction and quantification of enzyme activities

2.3.5.1 Kinetic enzyme activity assays of plant ascorbate-glutathione cycle

Five tubers of each variety were used for each sampling time (October and November 2016, January, February, March, May and June 2017). Tissue from opposite eights of cortex was snap frozen in liquid nitrogen individually and stored at -80°C. Tuber samples were ground to a powder in liquid nitrogen using a mortar and pestle and extracted in ice-cold 50 mM MES-KOH buffer pH 6.0 containing 40 mM KCl, 2 mM $CaCl_2$ and 1 mM L-ascorbate (AsA) at a ratio of 4 ml g FW^{-1} following an adaptation to the method described by Murshed *et al*. (2008). The homogenate was clarified by centrifugation at 14,000 g for 10 minutes at 4°C and the supernatant was analysed immediately for enzyme activities. All kinetic enzyme activity assays were established in a total reaction volume of 0.2 ml and performed in flat bottom 96-well plates and absorbance measured using a spectrophotometer (Multiskan™ GO, Thermo

Scientific™). Samples and blanks were analysed in triplicate. Soluble protein content of the supernatants was quantified (Bio-Rad protein assay) using bovine serum albumin (BSA) as a standard in order to define activity as nanomole of substrate consumed or product formed per minute per milligram of protein. 800 µl of each standard and sample solution (dilution 1:100) were aliquoted in triplicate. A blank sample using distilled H_2O was included. 200 µl of Bio-Rad protein assay dye reagent concentrate were added to each sample, and the mixture was vortexed and incubated at room temperature for 15 minutes. Absorbance in samples were measured at 595 nm (Bradford, 1976).

Enzyme activity was quantified in microplate wells containing a mixture of buffer components and substrates depending on the enzyme assayed (Table 2-2):

i) Ascorbate peroxidase (APX, EC 1.11.1.11): The reaction mixture comprised 50 mM potassium phosphate buffer (pH 7.0), 0.25 mM AsA, and 10 µl of sample supernatant. The reaction was started by the addition of 5 µl of 200 mM H_2O_2 to give a final concentration of 5 mM. Enzyme activity was determined by measuring the decrease in the absorbance at 290 nm for 5 minutes to determine ascorbate oxidation. Specific activity was calculated using an extinction coefficient of 2.8 mM^{-1} cm^{-1}. A correction was carried out for the non-specific oxidation of ascorbate in the sample (first reading) and by H_2O_2 in the absence of the enzyme sample (blank).

ii) Dehydroascorbate reductase (DHAR, EC 1.8.5.1): The reaction mixture comprised 50 mM HEPES buffer (pH 7.0), 0.1 mM EDTA, 2.5 mM glutathione (GSH), and 10 µl of sample supernatant. The activity of DHAR was determined by monitoring the glutathione-dependent reduction of dehydroascorbate. The reaction was started by the addition of dehydroascorbate (DHA) (freshly prepared) to a final concentration of 0.2 mM. The activity was determined by measuring the increase in absorbance at 265 nm for 5 minutes. The specific activity was calculated using an extinction coefficient of 14 mM^{-1} cm^{-1}. A correction for the nonenzymatic reduction of DHA by GSH was carried out in the absence of the enzyme sample (blank).

iii) Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4): The reaction mixture comprised 50 mM HEPES buffer (pH 7.6), 2.5 mM AsA, 0.25 mM NADH, and 10 µl of sample supernatant. The reaction was started by adding of 0.4 units of ascorbate oxidase to generate the monodehydroascorbate radical. The activity was determined as oxidation of NADH by measuring the decrease in absorbance at 340 nm for 5 minutes. The specific activity was

calculated using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. The rate of non-specific NADH oxidation was subtracted using an enzyme blank.

iv) Glutathione Reductase (GR, EC 1.8.1.7): The reaction mixture comprised 50 mM HEPES buffer (pH 8.0), 0.5 mM EDTA, 0.25 mM NADPH, and 10 µl of sample supernatant. GR activity was measured spectrophotometrically as NADPH oxidation at 340 nm. The GR reaction was started by the addition of 5 µl of 20 mM glutathione disulfide (GSSG). The specific activity was calculated using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. Non-specific NADPH oxidation was determined before adding GSSG and subtracted from the GR specific activity.

Table 2-2. Simplified protocols for enzymatic assays. Adapted from Murshed *et al.* (2008).

	APX	DHAR	MDHAR	GR
Reaction buffer	50 mM potassium phosphate buffer (pH 7.0) 0.25 mM AsA	50 mM Hepes buffer (pH 7.0) 0.1 mM EDTA 2.5 mM GSH	50 mM Hepes buffer (pH 7.6) 2.5 mM AsA 0.25 mM NADH	50 mM Hepes buffer (pH 8.0) 0.5 mM EDTA 0.25 mM NADPH
Volume/well	200 µl	200 µl	200 µl	200 µl
Extract volume	10 µl	10 µl	10 µl	10 µl
Substrate (stock solution concentration)	200 mM H_2O_2	8 mM DHA	Ascorbate oxidase (40 U ml^{-1})	20 mM GSSG
Substrate volume (final concentration)	5 µl (5 mM)	5 µl (0.2 mM)	10 µl (0.4 U)	5 µl (0.5 mM)
OD	290 nm	265 nm	340 nm	340 nm
Extinction coefficient	2.8 mM^{-1} cm^{-1}	14 mM^{-1} cm^{-1}	6.22 mM^{-1} cm^{-1}	6.22 mM^{-1} cm^{-1}

2.3.5.2 Enzyme activity assays of carbohydrate metabolism

Three tubers of each variety were used for each sampling time (March, May and June 2017). Tissue from opposite eights of cortex was snap frozen in liquid nitrogen individually and stored at -80°C. 1 g of tuber samples were ground to a powder in liquid nitrogen using a mortar and pestle and extracted in 4 volumes of ice-cold 200 mM HEPES-NaOH buffer pH 7.5 containing 3 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 1 mM benzamidine, 5 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulphonyl (PMSF) and 5% polyvinylpolypyrrolidone (PVPP) following an adaptation to the method described by Jammer *et al*. (2015). DTT, PMSF and PVPP were added at least 1 hour before use. Extracts were clarified by centrifugation and then used either crude or following desalting using a PD-10 gel filtration column (Amersham Biosciences, UK)

depending on the enzymes assayed (Figure 2-2). Extraction buffer without PVPP added was used as desalting buffer for PD-10 columns.

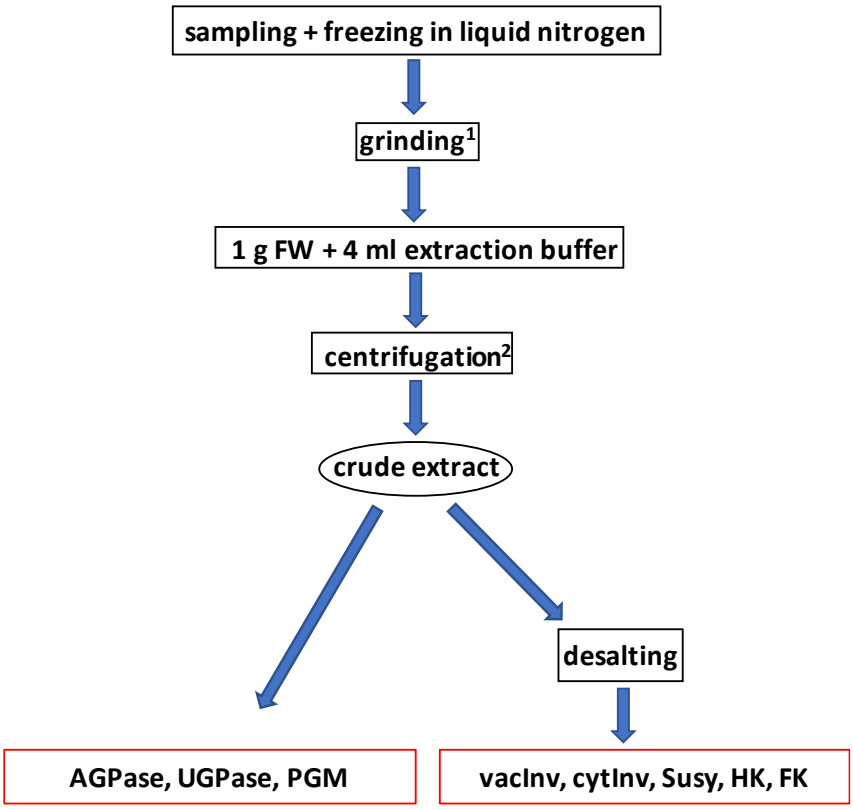


Figure 2-2. Flowchart of the universal protein extraction and desalting protocol. All steps were performed on ice. [1]In liquid nitrogen using a pre-cooled mortar and pestle. [2]Centrifugation at 20,000 g for 20 minutes at 4°C. AGPase, ADP-glucose pyrophosphorylase; UGPase, UDP-glucose pyrophosphorylase (EC 2.7.7.9); PGM, phosphoglucomutase (EC 5.4.2.2); vacInv, vacuolar invertase (EC 3.2.1.26); cytInv, cytoplasmic invertase (EC 3.2.1.26); Susy, sucrose synthase (EC 2.4.1.13); HK, hexokinase (EC 2.7.1.1); and FK, fructokinase (EC 2.7.1.4).

Kinetic enzyme activity and invertase activity assays were established in a total reaction volume of 0.16 ml or 1.2 ml, respectively. All enzyme activity assays were performed in flat bottom 96-well plates (Nunc™, MicroWell™, Thermo Scientific™), and absorbance measured using a spectrophotometer (Multiskan™ GO, Thermo Scientific™). Samples and controls were analysed in triplicate. Soluble protein content of the supernatants was quantified (Bio-Rad protein assay) using BSA as a standard in order to define activity as nanomole of substrate consumed or product formed per minute per milligram of protein. All specific activities were

calculated using the extinction coefficient of NADPH at 340 nm (6.22 mM^{-1} cm^{-1}). Reactions were performed with a mixture of buffer components, substrate(s), auxiliary substance(s), and auxiliary enzymes (Figure 2-3):

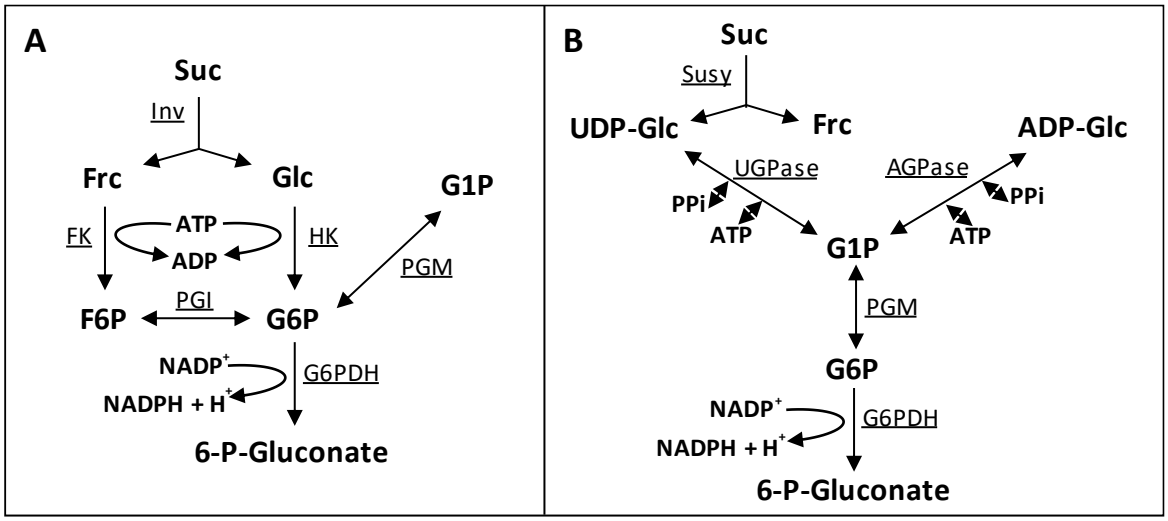


Figure 2-3. Reaction schemes to determine the selected enzyme activities. **A.** Inv, invertase; FK, fructokinase; HK, hexokinase; PGI, phosphoglucoisomerase (EC 5.3.1.9); PGM, phosphoglucomutase; and G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49). **B.** Susy, sucrose synthase; UGPase, UDP-glucose pyrophosphorylase; AGPase, ADP-glucose pyrophosphorylase; PGM, phosphoglucomutase; and G6PDH, glucose-6-phosphate dehydrogenase.

i) ADP-glucose pyrophosphorylase (AGPase): aliquots of untreated crude extract (25 µl) were incubated with 0.44 mM EDTA, 5 mM $MgCl_2$, 0.1% BSA, 2 mM ADP-Glc, 1.5 mM PPi, 1 mM NADP, 2 mM 3-PG, 0.432 U of PGM, and 1.28 U of G6PDH in 100 mM Tris-HCl at pH 8.0. For control reactions, ADP-Glc was omitted. The increase in absorbance at 340 nm due to conversion of NADP to NADPH was monitored.

ii) UDP-glucose pyrophosphorylase (UGPase): aliquots of untreated crude extract (25 µl) were incubated with 0.44 mM EDTA, 5 mM $MgCl_2$, 0.1% BSA, 2 mM UDP-Glc, 1.5 mM PPi, 1 mM NADP, 2 mM 3-PG, 0.432 U of PGM, and 1.28 U of G6PDH in 100 mM Tris-HCl at pH 8.0. For control reactions, UDP-Glc was omitted. The increase in absorbance at 340 nm due to conversion of NADP to NADPH was monitored.

iii) Phosphoglucomutase (PGM): aliquots of untreated crude extract (25 µl) were incubated with 10 mM $MgCl_2$, 4 mM DTT, 0.1 mM G1,6bisP, 1 mM G1P, 0.25 mM NADP, and 0.64 U of G6PDH in 20 mM Tris-HCl at pH 8.0. For control reactions, G1P was omitted. The increase in absorbance at 340 nm due to conversion of NADP to NADPH was monitored.

iv) Fructokinase (FK): aliquots of desalted crude extract (25 µl) were incubated with 5 mM $MgCl_2$, 5 mM fructose, 2.5 mM ATP, 1 mM NADP, 0.8 U of PGI, and 0.8 U of G6PDH in 50 mM BisTris at pH 8.0. For control reactions, fructose was omitted. The increase in absorbance at 340 nm due to conversion of NADP to NADPH was monitored.

v) Hexokinase (HK): aliquots of desalted crude extract (25 µl) were incubated with 5 mM $MgCl_2$, 5 mM glucose, 2.5 mM ATP, 1 mM NADP, and 0.8 U of G6PDH in 50 mM BisTris at pH 8.0. For control reactions, glucose was omitted. The increase in absorbance at 340 nm due to conversion of NADP to NADPH was monitored.

vi) Sucrose synthase (SuSy): for determination of SuSy activity, two reactions were performed, (A) including 1 mM UDP detecting both SuSy and cytInv background activity, and (B) without 1 mM UDP to detect the cytInv background activity only. SuSy activity was calculated by subtracting cytInv background activity (B) from total activity (A). For both reactions, aliquots of desalted crude extract (25 µl) were incubated with 1 mM EDTA, 2 mM $MgCl_2$, 5 mM DTT, 250 mM sucrose, 1 mM UDP (omitted for reaction B), 1.3 mM ATP, 0.5 mM NADP, 0.672 U of HK, 0.56 U of PGI, and 0.32 U of G6PDH in 50 mM Hepes-NaOH at pH 7.0. For control reactions, sucrose was omitted. The increase in absorbance at 340 nm due to conversion of NADP to NADPH was monitored.

vii) Invertase activity (Inv): invertase activity was assayed in an end-point assay modified from the method of Viola and Davies (1992). Aliquots (600 µl) of desalted crude extract were incubated at 30°C for 1 hour with 5 mM $MgCl_2$, 25 mM sucrose, 2.5 mM ATP, 1 mM NADP, 1.5 U of HK, 0.5 U of PGI, and 1.2 U of G6PDH in 25 mM acetate buffer at pH 5.2 or pH 6.8 for vacInv or cytInv, respectively, in a total reaction volume of 1.2 ml. For control reactions, sucrose was omitted. Aliquots (100 µl) were taken every 20 minutes and the increase in absorbance at 340 nm due to conversion of NADP to NADPH was monitored.

2.3.6 Metabolite profiling by gas chromatography/mass spectrometry

Gas chromatography/mass spectrometry (GC/MS) analysis was performed on extracts from five biological replicates per treatment and cultivar as described by Foito *et al.* (2013). Dried material (100 ± 1 mg) was weighed into glass tubes and extracted in 3 ml methanol for 30 minutes at 30°C with agitation (1,500 rpm). Polar (ribitol 2 mg ml^{-1}) and non-polar (nonadecanoic acid methyl ester 0.2 mg ml^{-1}) standards at 0.1 ml each and 0.75 ml distilled H_2O were added, and extraction continued for a further 30 minutes as described. 6 ml of

chloroform were added, and extraction continued for 30 minutes under increased agitation at 2,500 rpm. Phase separation was achieved by the addition of a further 1.5 ml of water and centrifugation at 1,000 g for 10 minutes. Polar and non-polar phases were separated and derivatized independently as follows. An aliquot (250 µl) of the polar fraction was dried and oximated with a methoxylamine hydrochloride solution (20 mg ml^{-1} in pyridine) for 4 hours at 50°C. Subsequently, extracts were derivatised with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) for 30 min at 30°C and a subsample (40 µl) was diluted in pyridine (1:1) prior to GC/MS analysis. For the non-polar fraction, a 4 ml aliquot was dried and trans-esterified at 50°C overnight by addition of 2 ml of 1% methanolic sulphuric acid solution and 1 ml of chloroform. For neutralisation and recovery of free fatty acids, 5 ml of 5% (w/v) aqueous sodium chloride and 3 ml of chloroform were added. Top aqueous layer was discarded and 3 ml of 2% (w/v) aqueous potassium hydrogen carbonate added to the lower chloroform:methanol layer. Water was removed by passage through a short column of anhydrous sodium sulphate and then evaporated to dryness. Samples were derivatised as described for the polar fractions and a subsample was diluted in pyridine (1:1) before injection onto the GC/MS. Metabolite profiles for the polar and non-polar fractions were acquired following separation of compounds on a DB5-MSTM column (15m×0.25mm×0.25µm; J&W, Folsom, CA, USA) using a Thermo Finnigan (San Jose, CA, USA) DSQII GC/MS system as described (Foito *et al*., 2013). The samples were analysed in a randomized order, while quality control samples as well as blanks were incorporated at the beginning and the end of the sequence. Data were then processed using the XCALIBUR software (Thermo Fisher Scientific, Waltham, MA, USA). Peak areas relative to internal standard (response ratios) were calculated following normalization to 100 mg extracted material.

2.3.7 ^{13}C labelling and metabolite flux analysis

Tuber discs (diameter 5 mm, thickness 1-2 mm) were cut from fresh potato tuber slices, washed three times in 50 mM methanesulphonic acid (MES)-KOH (pH 6.5) containing 300 mM mannitol and then incubated (500 mg in a volume of 1 ml in glass vials shaken at 100 rpm) for 2, 4 and 6 hours in 50 mM MES-KOH (pH 6.5) containing 300 mM mannitol and either (i) 25 mM [U-^{13}C]glucose or (ii) 25 mM unlabelled glucose. After the indicated time period the discs were washed three times in buffer (5 ml per vial) and patted dry with paper towels. Samples were rapidly frozen in liquid nitrogen, lyophilized and kept at -80°C until extraction.

Metabolite fluxes were analysed by GC/MS performed on extracts as previously described following the method by Foito *et al.* (2013). Total metabolic pool size and the relative abundance of each mass isotopomer for the metabolite in question was estimated by mass spectrometric analysis (Huege *et al*., 2007). The percentage of ^{13}C-label in each mass isotopomer was given from the fractional abundance of each mass isotopomer relative to total pool size. These two parameters (total pool size and percentage of label in each mass isotopomer) allowed the description of the mass-balance of mass isotopomers. The ^{13}C-enrichment data were normalized to the ^{13}C-enrichment of glucose within each sample so as to generate internally standardized 'relative ^{13}C enrichment' values. The '^{13}C pool size' value of metabolites was defined as the product of 'total pool size' and 'relative ^{13}C enrichment' (Baxter *et al*., 2007; Dethloff *et al*., 2017).

2.3.8 ^{14}C labelling and fractionation of labelled tissue extracts

Tuber discs (diameter 5 mm, thickness 1-2 mm) were cut from fresh potato tuber slices, washed three times in 50 mM MES-KOH (pH 6.5) containing 300 mM mannitol and then incubated (500 mg in a volume of 1 ml in glass vials shaken at 100 rpm) for 3 hours in 50 mM MES-KOH (pH 6.5) containing 300 mM mannitol and 0.148 MBq D-[U-^{14}C]glucose. Vials were sealed with a rubber stopper which held a paper filter moistened with 200 µl of 10% (w/v) KOH to trap $^{14}CO_2$. After incubation material was washed three times for 5 min in 5 ml of 50 mM MES-KOH (pH 6.5) containing 300 mM mannitol prior to successive extraction in 5 ml of 80% (v/v) ethanol. The supernatant was dried down under vacuum (miVac Duo Concentrator, Pump and Speed Trap, GeneVac, UK) and passed through both an anion-exchange cartridge (SAX; HPLC Technology, UK) and cation-exchange cartridge (SCX; Phenomenex® Strata, UK) as described by Souleyre *et al*. (2004). Material not retained by the ion exchange cartridges was considered as the neutral material while the separated anionic and cationic fractions were recovered from the cartridges by eluting with 5 M HCl and 5 M KOH, respectively. The insoluble plant material was separated into starch and non-starch components as described in Runquist and Kruger (1999). For starch analysis, the cell pellet from the insoluble fraction was washed with distilled water and the resuspended in 9 volumes of distilled water. Starch was gelatinized by incubating samples at 100°C for 2 hours with periodic vortexing. The suspension was cooled to room temperature; 1 volume of 1 M acetate buffer pH 4.5 containing 100 U ml^{-1} α-amyloglucosidase (EC 3.2.1.3) (Sigma-Aldrich, USA) was added

and incubation was continued at 40°C for 18 hours. After centrifugation for 10 minutes at 5,000 g supernatant containing glucose released from starch was collected. All fractions were analysed using scintillation counting.

Glucose, fructose and sucrose were separated by high-performance liquid chromatography (HPLC) using a Metacarb 87C 300x7.8 mm column (MetaChem Technologies Inc., Torrance, CA) with 0.6 ml min^{-1} ultrapure water as the mobile phase at a temperature of 70°C (Davies *et al*., 2005; Viola *et al*., 2007). Radiolabelled sugars were detected using a Radioflow detector LB 509. Radioactivity in the CO_2, anionic, starch, other insoluble and neutral fractions was determined by liquid scintillation counting (Tri-Carb 3100TR Packard) after dilution into ScintLogic HiCount cocktail (Lablogic Systems Ltd, Sheffield, UK).

2.3.9 Statistical analysis

Data analysis and graphical outputs were performed using Microsoft Excel 2013. Statistical analysis was undertaken by two-way ANalysis Of VAriance (ANOVA, parametric test) with potato cultivar and storage time as parameters in order to identify statistically significant differences between profiles using a significance level (P-value) ≤ 0.05 using GENSTAT (v. 18.1, VSN International Ltd, Hemel Hempstead, UK). When different treatments were present in the experiment, ANOVA was used with parameters cultivar, time of storage, and treatment (either untreated or CIPC-treated). Correlations were determined using Pearson tests ($P < 0.05$) and performed using GENSTAT (v. 18.1, VSN International Ltd, Hemel Hempstead, UK). Differences in the outputs of the 3 seasons of data were analysed, with correlations between changes in fry colour, sugar content and time of storage. For GC/MS data in season 3, a principal component analysis (PCA) was performed to observe differences in metabolic composition among the nine potato cultivars. In addition, Fisher's protected least significant difference (LSD) test was carried out for each cultivar independently in order to determine significant differences between time points in quantification of sugars, ascorbate-glutathione cycle enzymes activities and H_2O_2 as well as MDA content determination.

2.4 Molecular Protocols

2.4.1 RNA extraction

Four tubers of each variety were used for each sampling time (March and May 2017, May, July and October 2018, July and September 2019). Tissue from opposite eights of cortex was snap frozen in liquid nitrogen individually and stored at -80°C followed by freeze drying and grinding in an electric grinder. Freeze-dried tissue was kept at -80°C until RNA extraction.

RNA extraction was performed following the method of Ducreux *et al*. (2008). Approximately 1 g of freeze-dried tuber tissue was extracted with 14 ml of hot (80°C) extraction buffer (50 mM TRIS-HCl (pH 8.0), 50 mM LiCl, 5 mM EDTA, 0.5% SDS, 50% (v/v) phenol). Sterile distilled water (10 ml) was added and the samples were vortexed for 2 minutes. The samples were placed on ice and 16 ml of chloroform:isoamyl alcohol (24:1 v:v) was added and vortexed as before. Following centrifugation at 4°C at 14,000 g for 20 minutes, the upper aqueous layer was removed to a fresh, sterile 50 ml Sorval tube, containing an equal volume (16 ml) of 4 M LiCl. The samples were shaken and incubated overnight at -80°C, centrifuged at 4°C at 14,000 g for 40 minutes, the supernatant discarded, and the pellet resuspended in 5 ml sterile distilled water. One-tenth volume of 3 M sodium acetate buffer (pH 5.2) and 3 volumes of 100% ethanol were added, and the samples were incubated at -80°C for at least 1 hour. The precipitated RNA was pelleted by centrifugation at 4°C at 14,000 g for 40 minutes, washed with 10 ml of ice-cold 70% (v/v) ethanol, and centrifuged as in the previous step. The ethanol was removed, and the RNA pellet allowed to air-dry prior to resuspension in 300 µl sterile distilled water. RNA samples were purified, and genomic DNA contamination was removed using Qiagen RNeasy columns (Qiagen, UK) and DNase I, RNase-free (Thermo Scientific, UK) according to the manufacturer's protocol.

RNA quality was tested using an RNA 6000 nano chip on an Agilent 2100 Bioanalyzer (www.chem.agilent.com). RNA samples were aliquoted in 20 µg (1 µg μl^{-1}) batches and stored at -80°C.

2.4.2 Analysis of RNA

2.4.2.1 Quantification of RNA by spectrophotometry

Concentration of RNA was estimated using a NanoDrop® ND-1000 full-spectrum UV/Vis spectrophotometer (NanoDrop®, USA) measuring 1 µl aqueous RNA. The NanoDrop uses a modified Beer-Lambert equation to correlate the calculated absorbance with concentration. RNA samples were measured at 260 and 280 nm absorbance, with a ratio of 2.0 being accepted as 'pure'.

2.4.2.2 Gel electrophoresis

Agarose gels were prepared by mixing 0.4 to 1 g of agarose with 50 ml of 1X Tris-borate/EDTA (TBE) buffer (89 mM Tris-HCl pH 7.8, 89 mM borate, 2 mM EDTA). This mixture was heated in a microwave on low power for 1 minute at a time, before being mixed and re-heated until all agarose had dissolved. The resulting mixture was cooled to ca. 60°C before adding 1 µl of 10 mg ml^{-1} ethidium bromide. The gel was then cast in a gel tank with the required sized comb and allowed to set under a fume hood for 1 hour. Once set the comb was removed and sufficient TBE buffer added to ensure the gel was fully submerged. Samples were then loaded onto the gel, in the lanes formed by the comb, and separated for 40 to 50 minutes by electrophoresis at 40 V. Imaging of the gel was made under ultraviolet (UV) light, using the UVITech transilluminator (UVITech, Cambridge, UK).

2.4.2.3 RNA quality determination by gel electrophoresis

RNA was separated and analysed by electrophoresis on ethidium bromide stained agarose gels to check quality using the method described in section 2.4.2.2. To 2% (w/v) agarose gel stained with ethidium bromide, 5 µl of RNA samples were added to lanes and separated. Total RNA samples had 1 µl of RNA loading buffer (0.55 % (w/v) bromophenol blue, 0.8 mM EDTA, 0.23 M formaldehyde, 4% (v/v) glycerol, 6% (v/v) formamide) added. Gel was visualised under UV light as in section 2.4.2.2.

2.4.3 Enzymatic manipulation of nucleic acids

2.4.3.1 cDNA synthesis

cDNA was synthesised from purified RNA samples for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. TaKaRa CloneTech RNA to cDNA EcoDry^{TM} Premix (Double Primed) lyophilized master mix was used for this purpose. Once synthesis was complete all cDNA was diluted to 10 ng/µl through the addition of *dd*H_2O.

The method used for synthesising cDNA was using a TaKaRa CloneTech RNA to cDNA EcoDry^{TM} Premix (Double Primed) beads kit. 5 µg of DNase I treated total RNA in a final volume of 20 µl RNase-free H_2O was added to the lyophilized EcoDry^{TM} Premix. The mixture was then mixed by pipetting and centrifuged to remove any potential air bubbles before heating at 42°C for 60 minutes prior to deactivating the enzyme at 70°C for 10 minutes.

2.4.3.2 qRT-PCR (Universal Probe Library)

qRT-PCR was performed using cDNA prepared as described in section 2.4.3.1. Primers and probe sequences were designed using The Roche Universal Probe Library Assay Design Centre (https://lifescience.roche.com/en_gb/brands/universal-probe-library.html) (Table 2-3). All qRT-PCR reactions were performed using an Applied Biosystems StepOne Plus Real Time PCR system. 50 ng total cDNA was used as template in all reactions, which were composed of 12.5 µl 2x FastStart Universal Probe Master Mix (Rox) (Roche, UK), 10 µM Universal Probe, and 20 µM of both forward and reverse primer before being made up to a final volume of 25 µl with *dd*H_2O. Thermocycling conditions were as follows, denaturation stage of 95°C for 10 minutes, 40 cycles of 95°C for 15 sec and 60°C extension for 1 min. Samples were ran in triplicate, using *elongation factor-1-α* (*EF1α*) as an endogenous control. This gene can be used as a control as it encodes a ubiquitous protein which is utilised in protein synthesis by binding aminoacyl-transfer RNA to ribosomes (Stürzenbaum & Kille, 2001) and has been shown to be highly consistent between different tissue samples and growth stages (Nicot *et al.*, 2005). Calculations of relative expression levels for *glucose-6-phosphate/phosphate translocator 2* (*GPT2*) were performed using the Pfaffl method (Pfaffl, 2001).

Table 2-3. Primer/probe sequences used in this study.

Gene	PGSC ID	Forward	Reverse	Probe
StEF1α	PGSC0003DMT400059830	CTTGACGCTCTTGACCAGATT	GAAGACGGAGGGGTTTGTCT	AGCCCAAG
GPT2	PGSC0003DMT400013500	CACAATCGATACCAATCGACA	GAGTCCAATCTTGAGCTTCTGAG	CAGCAGCC

2.4.4 Microarray processing

A custom Agilent microarray was designed to the predicted transcripts from assembly v.3.4 of the DM potato genome as described (Hancock *et al*., 2014). A single-channel replicate block microarray design was utilised. RNA labelling and downstream microarray processing were as recommended in the One-Color Microarray-Based Gene Expression Analysis protocol (v.6.5; Agilent) using the Low Input Quick Amp Labelling Kit (Agilent).

Total RNA was spiked using One-Color RNA Spike-In Mix (Agilent) with *in vitro* synthesised polyadenylated transcripts to serve as positive controls for monitoring gene expression microarray flow from sample amplification and labelling to microarray processing. Total RNA (100 ng) was reverse transcribed into cDNA and then converted into Cyanine-3 labelled cRNA according to the manufacturer's instructions. Low Input Quick Amp Labelling Kit (Agilent) was used for reverse transcription and *in vitro* transcription (RT-IVT). The method uses T7 RNA polymerase blend for simultaneous amplification of target material and also incorporates Cyanine 3-CTP. Cyanine 3-labelled cRNA sample (600 ng) was fragmented and prepared for one-colour-based hybridization. Following manufacturer's protocol, samples were hybridized at 65°C for 17 hours. Microarrays were washed using Gene Expression wash buffers (Agilent) as recommended.

2.4.4.1 Microarray data analysis

Following microarray scanning using an Agilent G2505B scanner, data were extracted from images using Feature Extraction (FE) (v.10.7.3.1) software and aligned with the appropriate array grid template file (033033_D_F_20110315). Intensity data and QC metrics were extracted using the FE protocol (GE1_107_Sep09). Entire FE datasets for each array were loaded into GeneSpring (v.7.3) software and data were normalised using default one-colour Agilent settings: (i) intensity values less than 0.01 were set to 0.01; (ii) data from each array

was normalized to the 50th percentile of all measurements on the array and; (iii) the signal from each probe was subsequently normalized to the median of its value across the entire dataset). Quality control of the datasets were performed using Principal Components Analysis (PCA) to confirm that there were no outlying replicate samples and that dye labelling had no associated bias. Spot flags from FE (present or marginal) were used to remove probes with no consistent expression. Data were combined from replicate samples and for both cultivars Arsenal and VR 808 accessions in a new interpretation. Statistical filtering was performed using volcano analysis (*P*-value < 0.01, fold-change > 2x) for season 1 data. Data were visualised using PageMan (Usadel *et al*., 2006) and a gene tree heatmap in GeneSpring using default Pearson correlation. In addition, one-way ANalysis Of VAriance (ANOVA, parametric test) using storage time as parameter was used in both seasons to identify statistically significant expression profiles at a false discovery rate (P-value) ≤ 0.05. Strict multiple testing correction (Bonferroni) was applied to ensure low false discovery rates. Filtered gene lists were clustered into two groups using the K-means algorithm with default settings (100 iterations, Pearson correlation) in GeneSpring.

Chapter 3: Assessment of sugar accumulation and processing quality during storage

3.1 Introduction

Control of potato quality during storage represents a significant problem for the industry and a key issue remains the capacity to inhibit sprouting while preventing loss of processing quality as a result of sugar accumulation leading to problems of dark fry colour and acrylamide accumulation in processed products. Sugars accumulate in tubers when there is an imbalance between starch degradation, starch synthesis, and respiration of carbohydrate. Glucose, fructose and sucrose are the major sugars which accumulate in potato tubers (Burton, 1989).

Monitoring changes in sugar accumulation were used to chart changes in the aging of potatoes during storage that may help understand processes leading to senescent sweetening. This experiment also focused on the processing quality of tubers related to the sugar content.

The study was centred during the first and second seasons around the cultivars Arsenal, a variety 'susceptible' to senescent sweetening, and VR 808, considered to maintain stable sugar profiles (senescent sweetening resistant), with both of them used in the crisp industry (NIAB Pocket Guide, 2008, NIAB, Cambridge in Colgan *et al.*, 2012). During the third season and as additional material, seven new varieties were included in the experiment, providing contrasting profiles of sugar accumulation during storage.

3.2 Assessment of sugar accumulation during storage

3.2.1 Overall effect of varieties and seasons

A three-way ANOVA for season 1 (2016/2017) was carried out to determine whether there were significant differences in sugar content between samples using cultivar, time of storage and treatment (CIPC-treated or untreated) as factors. A two-way ANOVA was carried out for the rest of the seasons using factors cultivar and time of storage. For the three seasons, Fisher's

LSD test was carried out for each cultivar independently in order to determine significant differences between time points.

During the first season of this study, differences between cultivars were observed in glucose ($P < 0.001$), fructose ($P < 0.001$), and sucrose ($P = 0.01$). Moreover, cultivars exhibited significant changes dependent on time for glucose ($P < 0.05$), fructose ($P < 0.005$), and sucrose ($P < 0.001$) content. Arsenal exhibited an initial accumulation of glucose followed by a trough and then a second accumulation after 26 weeks of storage with similar behaviour observed for fructose. On the other hand, VR 808 had much lower levels of reducing sugars which fluctuated a little but with no clear pattern. On the contrary, patterns of sucrose accumulation were similar with both cultivars showing peak-trough-peak behaviour (Figure 3-1).

In the second season, cultivars Arsenal and VR 808 exhibited differences in both glucose and fructose content ($P < 0.001$), and sucrose ($P < 0.01$) related to cultivar and time of storage. Arsenal and VR 808 presented similar behaviour compared to the previous season with Arsenal exhibiting storage associated reducing sugar accumulation while both varieties exhibited accumulation of the non-reducing disaccharide sucrose. However, an overall lower content of sugars and a later onset of sweetening (43 weeks after storage) were exhibited during this season. Arsenal showed a small increase of glucose content at 5 weeks after storage followed by a decrease and no changes until the onset of sugar accumulation at 43 weeks after storage, with the exception of one significant increase at 27 weeks after storage, caused by a single replicate containing high levels of reducing sugars. Similar behaviour was observed for fructose. In contrast, VR 808 exhibited undetectable levels of glucose until 53 weeks after storage, when a little increase was observed. Regarding fructose content, VR 808 showed fluctuations with no clear pattern. Arsenal started accumulating reducing sugars after 43 weeks of storage whereas VR 808 had undetectable levels for glucose and much lower levels of fructose compared to Arsenal, showing no accumulation of reducing sugars. Nonetheless, both cultivars exhibited similar sucrose content and behaviour over time, with an accumulation occurring at 43 weeks of storage (Figure 3-2).

The later accumulation and lower content of reducing sugar during storage observed in season 2 (2017/2018) were also observed in all cultivars of season 3 (2018/2019). Glucose, fructose, and sucrose levels presented changes dependent on time ($P < 0.001$). In addition,

sugar profiles showed a significant effect of the variety in glucose ($P < 0.05$), fructose ($P < 0.001$) and sucrose ($P < 0.001$) content. All cultivars exhibited a decrease of glucose content at the beginning of the storage, then showed no clear pattern until 43 weeks after storage, when a significant increase was observed with the exception of Brooke. Lady Rosetta, Shelford (Shropshire), and Arsenal were the varieties with the highest levels of glucose. On the contrary, fructose accumulated in all cultivars at 43 weeks after storage, with Lady Rosetta and SH C 909 presenting the highest and lowest content, respectively. Although accumulation of sucrose was no observed in any case, SH C 909 and VR 808 had lower sucrose levels compared to the rest of the cultivars (Figure 3-3).

3.2.2 Effect of CIPC treatment

During season 1, CIPC-treated and untreated cultivars were under study. CIPC treatment had an effect on decreasing glucose ($P < 0.005$), fructose ($P < 0.001$), and sucrose ($P < 0.001$) content in tubers (Figure 3-1). In addition, fructose ($P < 0.05$) and starch ($P < 0.005$) content were dependent on CIPC treatment by time interaction. The initial fructose peak in Arsenal was higher in untreated tubers. CIPC-treated tubers had a marginally lower overall sugar content than untreated tubers in which buds were removed, might due to wound-induced catabolic response and/or sink demand in the untreated tubers. Although CIPC had statistically significant effects on sugar content, the overall patterns between CIPC-treated and untreated tubers were very similar. Therefore, it was decided to continue the study using only CIPC-treated cultivars in seasons 2 and 3.

3.2.3 Effect of growing location

In season 3, Shelford cultivars from two different locations were investigated. No differences ($P > 0.05$) in glucose content were observed between growing locations. However, changes dependent on growing location and time were reported for fructose ($P < 0.001$) and sucrose ($P < 0.05$) levels, highlighting the previously reported environmental influence (Kumar *et al*., 2004; Groves *et al*., 2005) on sugar accumulation in stored potato tubers.

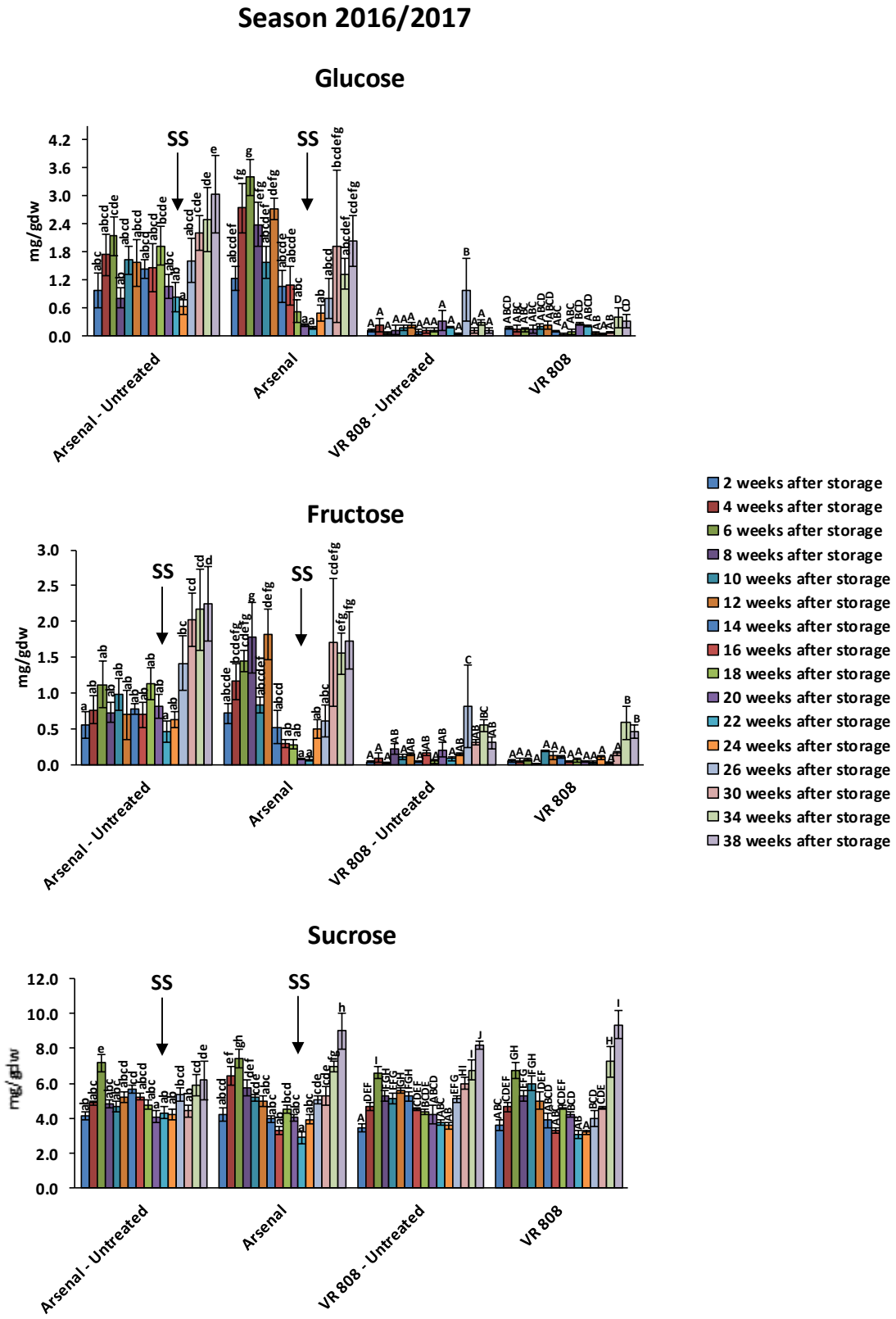


Figure 3-1. Sugar content in potato tubers during the season 1 (2016/2017) of storage at 9°C. Results showed an increase of sugar levels during long-term storage with remarkable differences for glucose and fructose concentration between two cultivars. Values are means ± SE (five biological replicates from one experiment). Mean values with different letters are significantly different according to the Fisher's LSD test. Abbreviations: SS, Onset of senescent sweetening as determined by sustained rise in reducing sugars and darkening of fry colour.

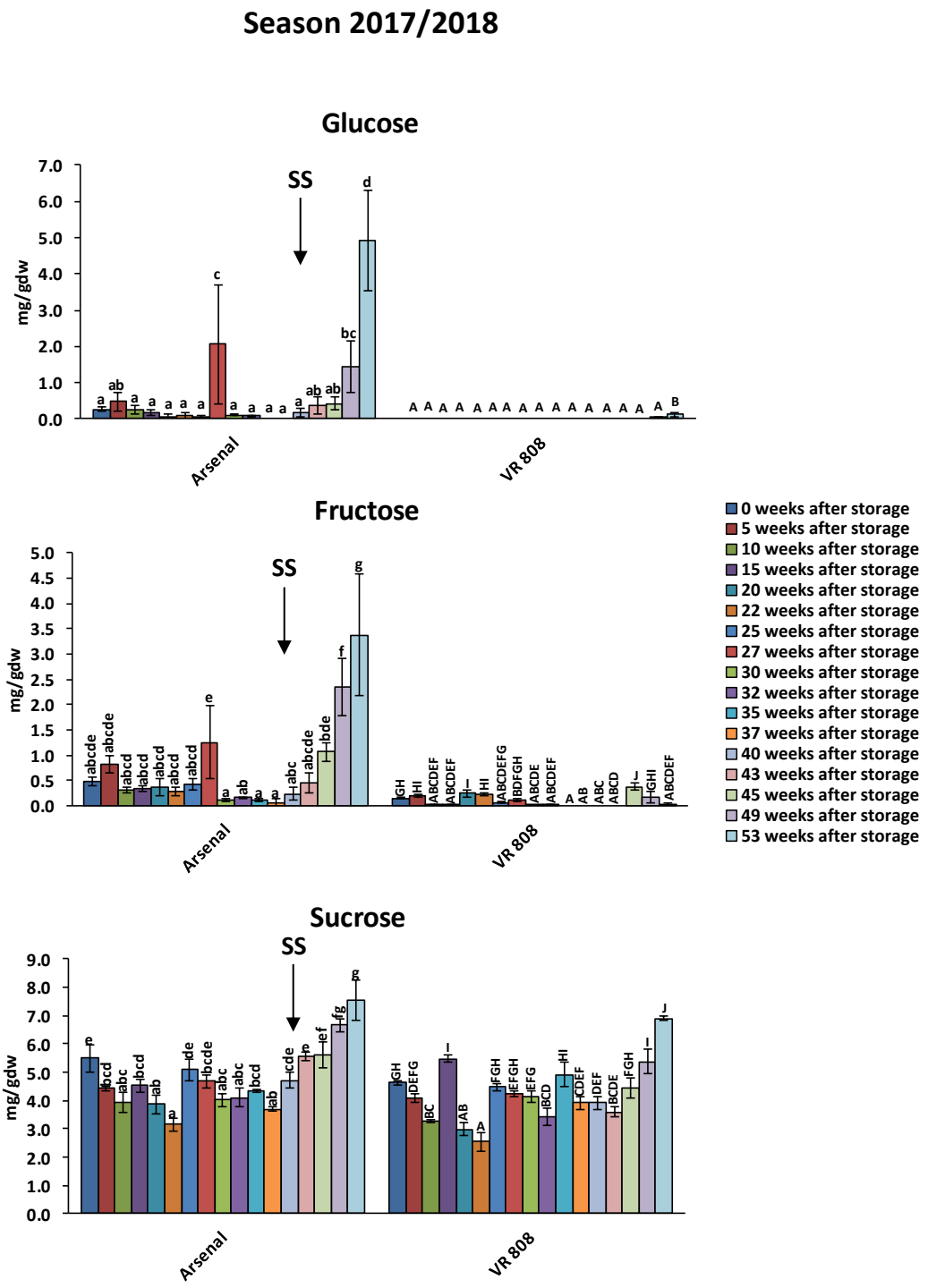


Figure 3-2. Sugar levels in potato tubers stored at 9°C over the season 2 (2017/2018) of study. Cultivars exhibited similar behaviour to the previous season. However, tubers presented a later onset of senescent sweetening. Values are means ± SE (five biological replicates from one experiment). Mean values with different letters are significantly different according to the Fisher's LSD test. Abbreviations: SS, Onset of senescent sweetening as determined by sustained rise in reducing sugars and darkening of fry colour.

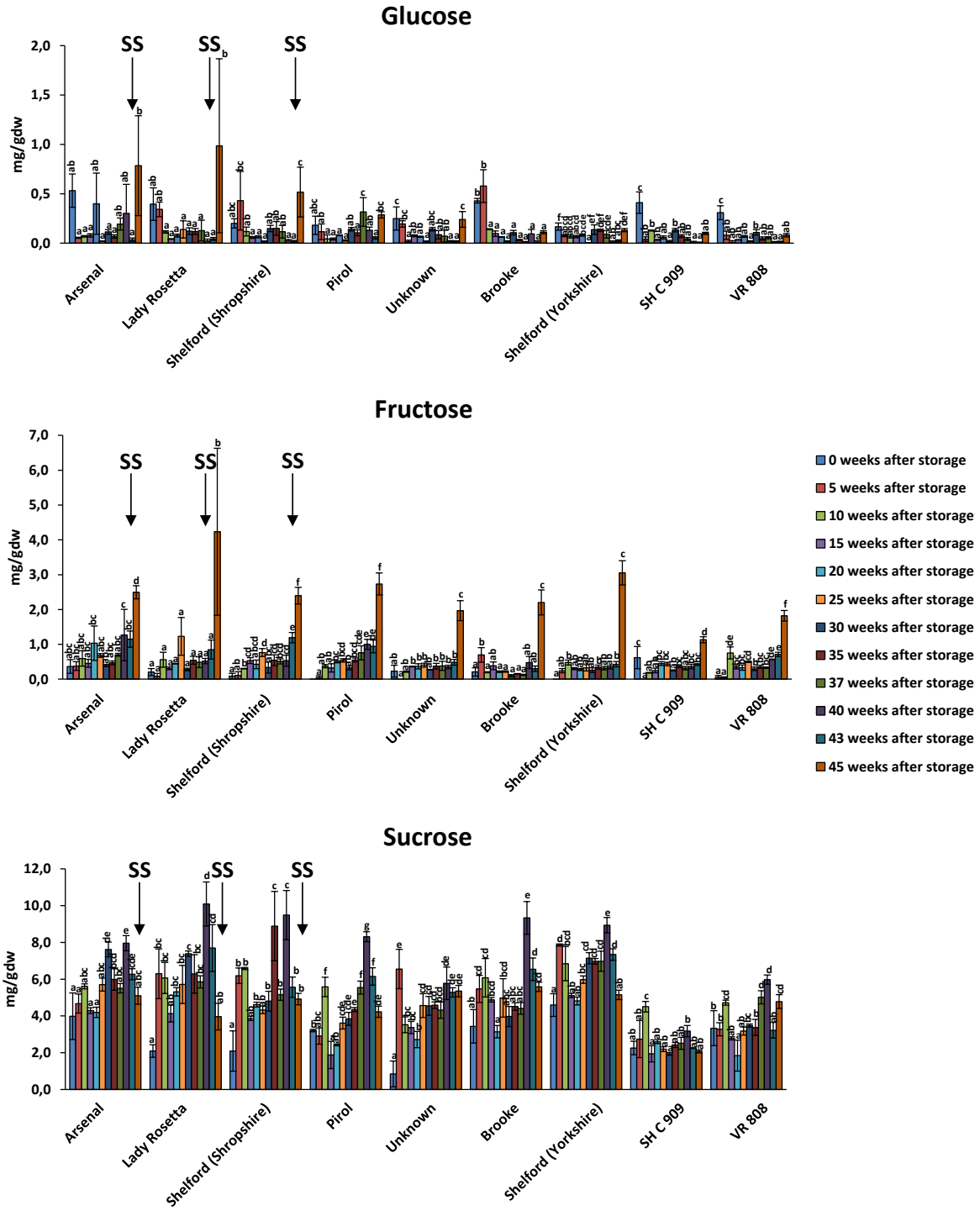


Figure 3-3. Sugar profiles of 9 different varieties during long-term storage for season 3 (2018/2019). Potato tubers showed variability of sugar content depending on the cultivar. Values are means ± SE (three biological replicates from one experiment). Mean values with different letters are significantly different according to the Fisher's LSD test. Abbreviations: SS, Onset of senescent sweetening as determined by sustained rise in reducing sugars and darkening of fry colour.

3.3 Assessment of crisping quality during storage

As sugar content is the key determinant of fry colour and ultimately affects quality of potato crisps, all varieties were subjected to fry quality tests over the 3 seasons of this study. Results are presented in Figure 3-4 (Season 1 2016/2017), Figure 3-5 (Season 2 2017/2018), and Figure 3-6 (Season 3 2018/2019). Crisps from senescent sweetening susceptible tubers presented darker fry colour than those which exhibited more stable sugar profiles during storage. Darkening increased over time following reducing sugars trend. This trend was consistent during the 3 seasons. In addition, untreated cultivars showed a darker fry colour, which was consistent with the sugar data. Quantification of darkening was estimated using ImageJ (Table 3-1). Grade of darkening in fry colour was estimated based on the grey scale in the isolated area of each individual crisp. ImageJ displays images by linearly mapping pixel to display values in the range 0-255. Pixels with minimum value are displayed as black and those with maximum value are displayed as white (Schneider *et al*., 2012). The calculated average grey value of each crisp was converted to percentage as an estimation of darkening. A three-way ANOVA for season 1 (2016/2017) was carried out to determine whether there were significant differences between crisp colour using cultivar, time of storage and treatment (CIPC-treated or untreated) as factors. Fisher's LSD test was carried out for each time point independently in order to determine significant differences between fry colour of crisps from cultivars Arsenal and VR 808. A two-way ANOVA was carried out for the rest of the seasons to determine whether there were significant differences between samples using cultivar and time of storage as factors. Fisher's LSD test was carried out independently for each cultivar (season 2 2017/2018) and time point (season 3 2018/2019) to determine significant differences between means over time and cultivars, respectively.

During season 1 (2016/2017) at 26 weeks after storage (post-sweetening early stage) no differences ($P > 0.05$) in fry colour related to cultivar or treatment (untreated or CIPC-treated) were observed. However, at 38 weeks after storage (post-sweetening late stage) crisps from Arsenal (susceptible to sweetening cultivar) untreated tubers exhibited a significantly darker fry colour related to cultivar ($P = 0.005$) and the interaction between cultivar and treatment ($P < 0.05$). In addition, an increase ($P < 0.05$) in darkening was observed over time depending on the cultivar.

For season 2 (2017/2018) significant changes ($P < 0.001$) in fry colour were reported over time. Differences ($P < 0.005$) in darkening between cultivars were observed. Although crisps from both cultivars exhibited an increase in dark fry colour post-sweetening at 43 weeks after storage, darkening in Arsenal crisps (susceptible to senescent sweetening) was higher than in crisps from VR 808 cultivar (senescent sweetening resistant).

During season 3 (2018/2019), differences ($P < 0.05$) in fry colour between cultivars were found as well as significant changes ($P < 0.001$) over time. Furthermore, each time point was analysed independently in order to find differences between cultivars. At 37 weeks after storage (prior to sweetening), crisps from Shelford (Shropshire) exhibited the highest level of dark fry colour whereas crisps from Shelford (Yorkshire) showed the lowest. This fact suggested growing location might affect fry colour. At 45 weeks after storage (post-sweetening early stage) no differences ($P > 0.05$) between cultivars were found, probably due to the early stage of sugar accumulation in tubers.

3.4 Correlations between fry colour, sugar content and storage

For season 1 (2016/2017) in Arsenal tubers fry colour was positively correlated with glucose, fructose and sucrose content, as well as storage (Figure 3-7). In VR 808 tubers, both reducing sugars and sucrose content were positively correlated with storage whilst negatively correlated with fry colour. This fact may be due to the low content of reducing sugars as well as low levels of darkening. During season 2 (2017/2018), Arsenal tubers exhibited positive correlations between fry colour and reducing sugars content (Figure 3-8). Fry colour was more strongly related to glucose rather than fructose and sucrose content. In addition, storage was positively correlated with the sugar content. VR 808 tubers showed a stronger positive correlation between fry colour, glucose and sucrose rather than total reducing sugars or fructose content. Storage was positively correlated with fry colour, glucose and sucrose content. For season 3 (2018/2019) in all cases fry colour was positively correlated with reducing sugars accumulation (Figure 3-9). In the varieties Pirol, VR 808, SH C 909, Shelford (Yorkshire) and Unknown, fry colour exhibited a higher positive correlation with fructose rather than glucose content. Moreover, glucose was negatively correlated with the sucrose content in Arsenal, Lady Rosetta, VR 808, SH C 909 and Shelford (Yorkshire).

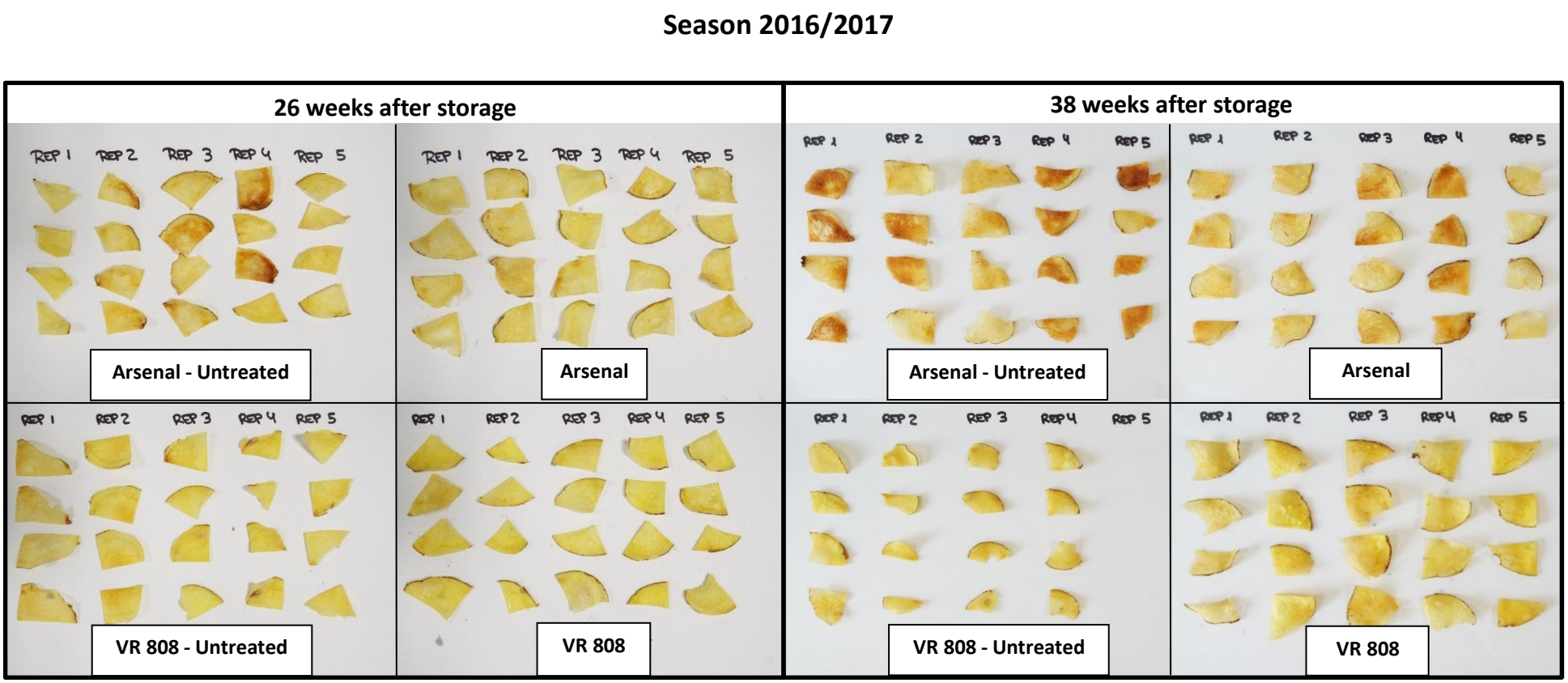


Figure 3-4. Appearance of crisps from long-term stored potato tubers during season 1 (2016/2017). Dark fry colour increased over time. Susceptible variety and untreated tubers showed higher darkening. Results of fry test colour at 26 and 38 weeks after storage, both time points considered after the onset of senescent sweetening.

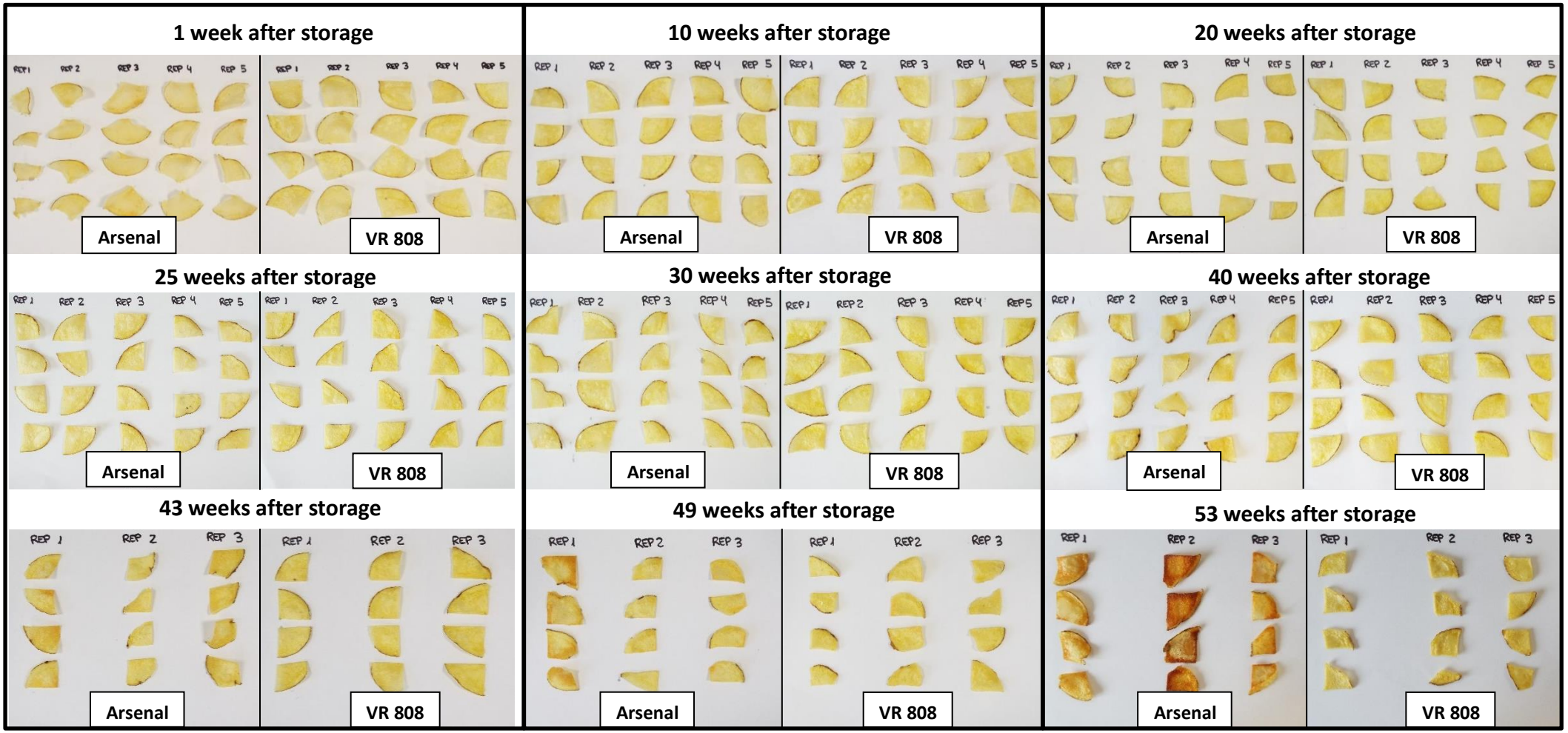


Figure 3-5. Appearance of crisps from long-term stored potato tubers during season 2 (2017/2018). Dark fry colour increased over time. Susceptible variety showed higher darkening. A later darkening of crisps was reported to be related to the later onset of accumulation of sugars for this season.

Season 2018/2019

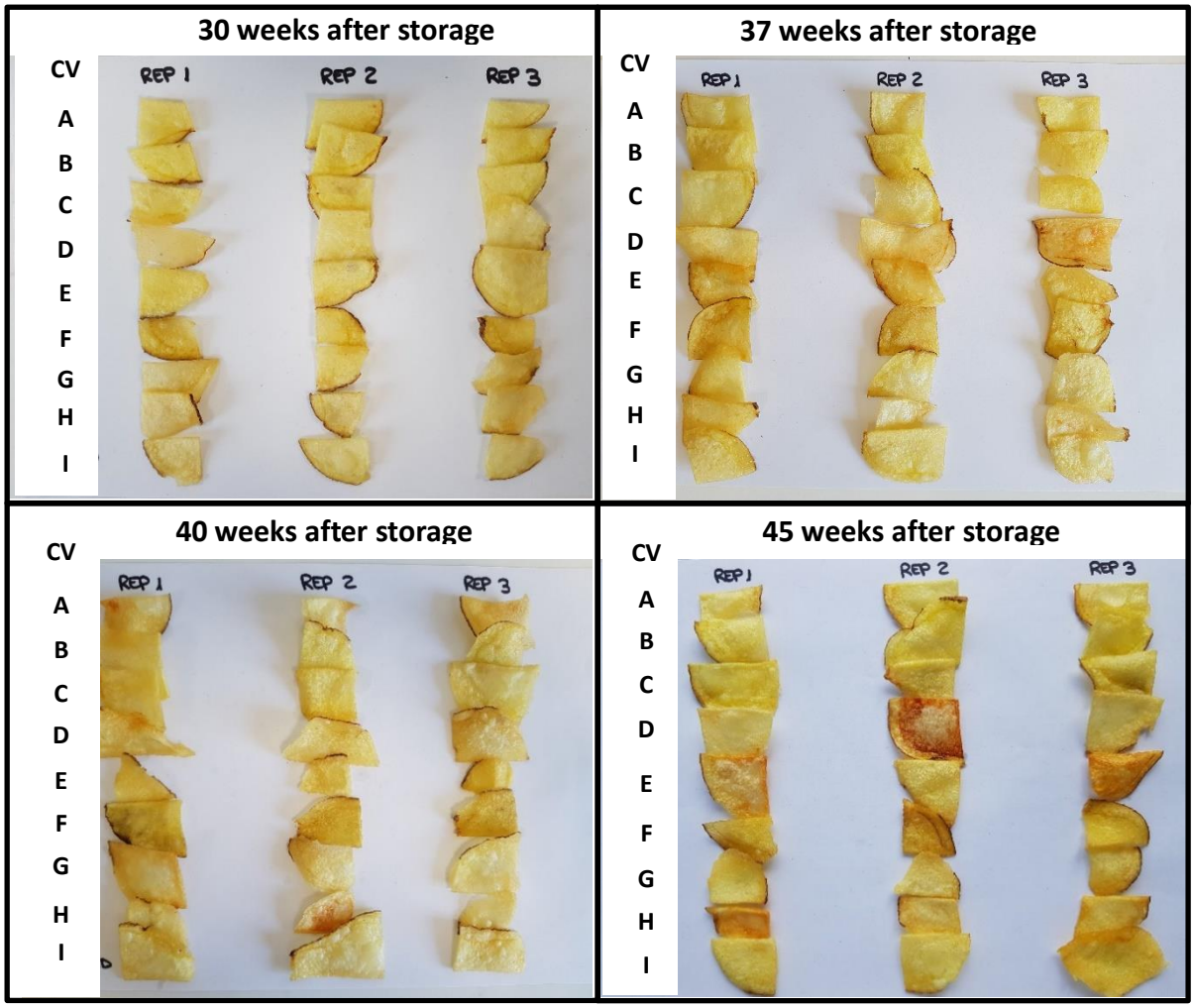


Figure 3-6. Appearance of crisps from long-term stored potato tubers during season 3 (2018/2019). Susceptible varieties showed higher darkening. Abbreviations: CV, cultivar; A, Pirol; B, SH C 909; C, VR 808; D, Lady Rosetta; E, Shelford (Shropshire); F, Unknown; G, Brooke; H, Arsenal; and I, Shelford (Yorkshire).

Table 3-1. Quantification of dark fry colour in crisps. Results are presented as percentage of darkening.

Grade of darkening (%) ± SE				
Season 1 (2016/2017)				
Weeks after storage	Arsenal - Untreated	Arsenal	VR 808 - Untreated	VR 808
26	41 ± 2^{a}	36 ± 2^{a}	38 ± 2^{a}	37 ± 2^{a}
38	51 ± 4^{a}	39 ± 3^{b}	35 ± 2^{b}	36 ± 1^{b}
Season 2 (2017/2018)				
Weeks after storage	Arsenal - Untreated	Arsenal	VR 808 - Untreated	VR 808
1	-	31 ± 1de	-	31 ± 1^{cd}
10	-	31 ± 1de	-	30 ± 1^{d}
20	-	33 ± 1^{cd}	-	34 ± 1^{bc}
25	-	27 ± 1^{e}	-	28 ± 1^{d}
30	-	28 ± 1^{e}	-	31 ± 1^{cd}
40	-	31 ± 2de	-	31 ± 2^{cd}
43	-	37 ± 2^{bc}	-	37 ± 1^{b}
49	-	40 ± 2^{b}	-	32 ± 3^{cd}
53	-	57 ± 4^{a}	-	43 ± 2^{a}
Season 3 (2018/2019)				
	Weeks after storage			
	30	37	40	45
Pirol	39 ± 1^{ab}	40 ± 2^{abc}	41 ± 5^{a}	44 ± 8^{a}
SH C 909	39 ± 1^{ab}	42 ± 3^{abc}	40 ± 4^{a}	44 ± 3^{a}
VR 808	38 ± 1^{ab}	37 ± 1^{abc}	42 ± 3^{a}	47 ± 1^{a}
Lady Rosetta	34 ± 3^{b}	39 ± 2^{abc}	39 ± 4^{a}	48 ± 4^{a}
Shelford (Shropshire)	37 ± 3^{ab}	45 ± 3^{a}	44 ± 2^{a}	50 ± 5^{a}
Unknown	45 ± 4^{a}	44 ± 3^{ab}	49 ± 3^{a}	53 ± 2^{a}
Brooke	40 ± 4^{ab}	36 ± 4^{abc}	38 ± 6^{a}	45 ± 4^{a}
Arsenal	39 ± 4^{ab}	35 ± 4^{bc}	41 ± 4^{a}	50 ± 4^{a}
Shelford (Yorkshire)	39 ± 2^{ab}	33 ± 3^{c}	38 ± 4^{a}	46 ± 3^{a}

Mean values with different letters are significantly different according to the Fisher's LSD test. Comparisons: Season 1, cultivars at individual time points; Season 2, time points in individual cultivars; Season 3, cultivars at individual time points.

Season 2016/2017

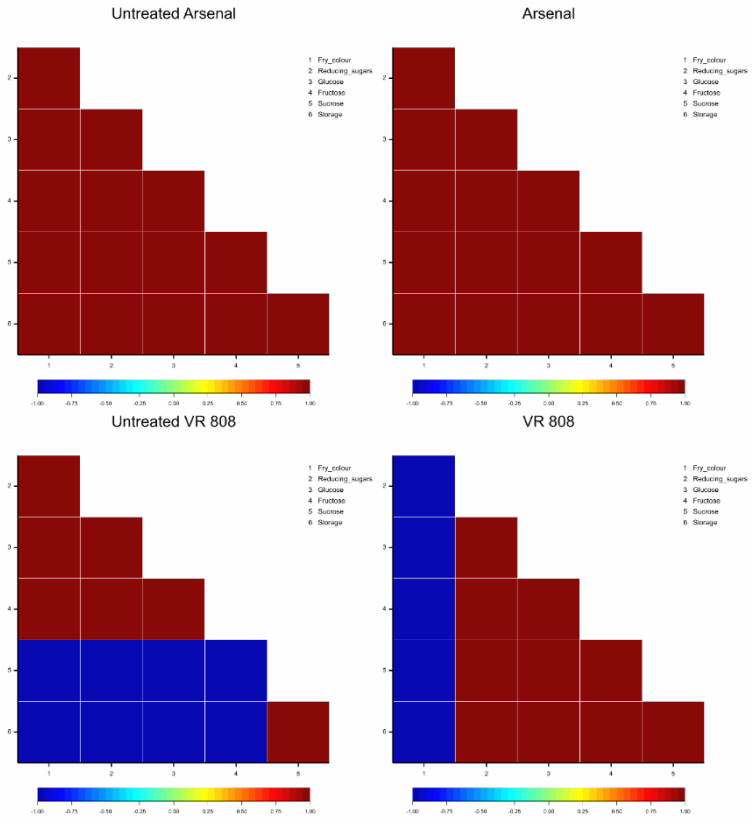


Figure 3-7. Correlations between fry colour, reducing sugars, glucose, fructose, sucrose and storage for season 1 (2016/2017). The colour represents the direction and strength of the correlation (Red, positive correlation and Blue, negative correlation).

Season 2017/2018

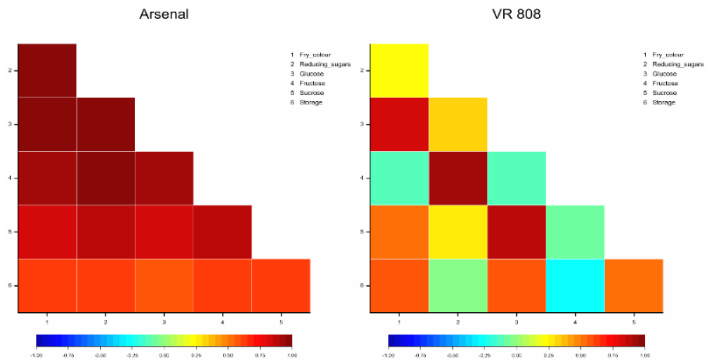


Figure 3-8. Correlations between fry colour, reducing sugars, glucose, fructose, sucrose and storage for season 2 (2017/2018). The colour represents the direction and strength of the correlation (Red, positive correlation and Blue, negative correlation).

Season 2018/2019

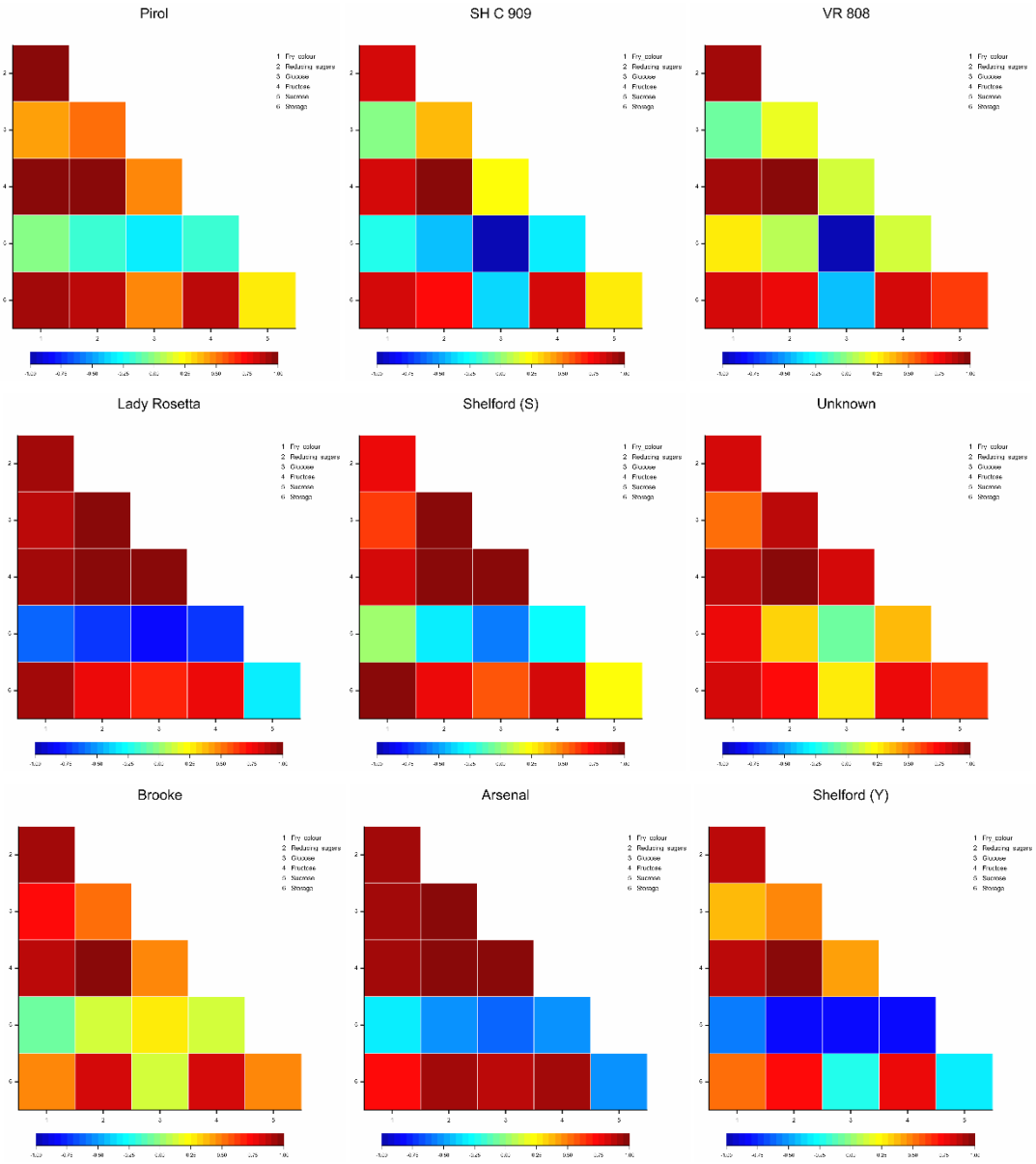


Figure 3-9. Correlations between fry colour, reducing sugars, glucose, fructose, sucrose and storage for season 3 (2018/2019). The colour represents the direction and strength of the correlation (Red, positive correlation and Blue, negative correlation).

3.5 Discussion

In general, Lady Rosetta, Shelford (Shropshire), and Arsenal, had the highest reducing sugars accumulation over the storage period and, SH C 909 and VR 808, showed no accumulation of glucose in any case as well as presented the lowest fructose accumulation. Although sucrose content was similar across all varieties during the three seasons of this study, SH C 909 and VR 808 exhibited an overall lower content in season 3. Moreover, sugar content showed differences within the same variety across seasons. Sugar content of potatoes and tuber maturity can be affected by factors such as genotype, environmental conditions and cultural practices during growth, and several post-harvest factors including storage (Kumar *et al*., 2004). Kumar *et al*. (2004) concluded that sugar was most affected by fertilization, temperature and soil moisture.

For all seasons of this study the growing conditions were different due to the weather. Crops are exposed to a wide range of environmental conditions, and heat and drought stress are two of the most serious and recurrent environmental stresses that affect crop quality (Iritani & Weller, 1978; Iritani, 1981; Ojala *et al*., 1990; Kincaid *et al*., 1993; Shock *et al*., 1992, 1993, 1998; Kumar *et al*., 2004). Even moderate environmental stress can impact the rate of tuber maturation and result in tubers that are either under- or over-mature at harvest. Immature or over-mature tubers can cause problems in storage including longer preconditioning periods, shorter times to senescent sweetening, increased skinning, increased water loss, and shrink (Nelson & Sowokinos, 1983; Lulai & Freeman, 2001; Sabba & Lulai, 2002; Sabba *et al*., 2007).

Physiological defects caused by environmental stress may not occur until weeks or months after the stress is experienced (Thompson *et al*., 2008). Defects resulting from transient stress during the growing season may be apparent at harvest, but often become more severe during storage (Iritani *et al*., 1973; Eldredge *et al*., 1996; Thompson *et al*., 2008). Periods of heat and drought stress, either alone or in combination, have been implicated in causing biochemical changes that result in sugar accumulation (Shock *et al*., 1993; Sowokinos *et al*., 2000). Accumulation of reducing sugars in maturing tubers may result from osmotic acclimation of the tuber during periods of water stress and subsequent conversion of accumulated sucrose into glucose and fructose.

Differences in reducing sugars accumulation during storage were observed between cultivars. However, most of cultivars accumulated sucrose to the same extent. The major sugars in potato tubers are glucose, fructose and sucrose (Burton, 1989). Sugar levels in tubers are conditioned by several factors, including genotype, environmental and growing conditions and different post-harvest factors such as storage. During storage, carbohydrates are converted from starch for respiration purposes and sugars start accumulating when their net production exceeds their use. The pattern of sucrose and reducing sugars might change depending on the mechanism of starch breakdown. Reducing sugars glucose and fructose have been observed to be present in equal equimolar amounts after cold induced and senescent sweetening (Hertog *et al*., 1997). However, differences between glucose and fructose contents were observed at the present work. This fact might be due to a differential turnover of glucose and fructose in the potato tubers under study. Regarding to biochemical pathways of sugar metabolism, degradation of transitory starch leads predominantly to the synthesis of neutral sugars such as glucose and maltose, and of triose phosphates, 3-phosphoglyceric acid (PGA) and CO_2 (Stitt & ap Rees, 1980; Stitt & Heldt, 1981; reviewed by Beck & Ziegler, 1989). Maltose and glucose are the two major forms of carbon exported from plastids during starch degradation (Servaites & Geiger, 2002; Ritte & Raschke, 2003; Weise *et al*., 2004). Glucose is then used to form fructose from which sucrose will be synthesised, and subsequently broken down. The differences observed between glucose and fructose content might be resulted from a differential turnover of glucose and fructose during these reactions. However, if there are several mechanisms of senescent sweetening, this observation might not always be true (Colgan *et al*., 2012).

Sweetening in tubers may be influenced by the pool of available sugars which are in constant flux, through changes in the rate of starch breakdown and sugar mobilisation. Fructose is the most responsive sugar to changes in storage temperatures (Smith, 1987). Some varieties, like White Rose, tend to accumulate fructose (Smith, 1987), as it was observed in the cultivars at the present study. Possibly by greater utilisation of glucose at high temperature storage. Considerable variations in sugar concentration may occur at the expense of the starch amount during storage. Sucrose content was reported to accumulate similarly in varieties Brintje and Désirée at 20°C in senescent tubers, both sprouting tubers and those treated with sprout suppressants (Fauconnier *et al*., 2002). Sucrose concentrations within tubers is dependent on tissue location (Carvalho, 2017). During sprouting, sucrose initially formed in parenchyma cells is translocated via phloem to the tuber apical region and the emerging sprouts, where

upon it is hydrolysed, into glucose and fructose (Burton *et al*., 1992; Hajirezaei *et al*., 2003; Viola *et al*., 2007; Sonnewald & Sonnewald, 2014). The relationship between sucrose and glucose and fructose content remains complex with the dynamics of starch breakdown and glucose and fructose utilisation under multiple feedback mechanisms. In the present study, differences in reducing sugars as well as similar levels of sucrose were observed between cultivars. Carvalho (2017) reported in Lady Rosetta a sucrose accumulation corresponded to a reduction in fructose content, suggesting the pool of glucose and fructose may be fully utilised during respiration. In addition, the author observed that in the varieties Lady Rosetta, VR 808, and Pentland Dell, glucose and fructose accumulation were positively correlated with the length of storage and respiration rates (CO_2 production and O_2 consumption), and sucrose accumulation. As sucrose is mobilised during sprouting, the differences observed between reducing sugars and sucrose accumulation might be due to sucrose being sequestered in certain organelles, such as the vacuole, preventing its breakdown.

The vacuole is an important plant specific organelle with functions in storage of solutes as nutrient reservoirs, but also with important roles in adaptation to stresses, such as cold stress (Wormit *et al*., 2006; Schulze *et al*., 2012), salt stress or drought (Rizhsky *et al*., 2004; Hedrich *et al*., 2015). Responses to a specific stress can vary with the genotype, but some general reactions occur in all genotypes. Abiotic stresses affect different cellular processes such as growth, photosynthesis, carbon partitioning, carbohydrate and lipid metabolism, osmotic homeostasis, protein synthesis and gene expression (Hasegawa *et al*., 2000; Munns, 2002; Rosa *et al*., 2004). Vacuolar sugar transporters are mainly membrane proteins belonging to the major facilitator superfamily whose subfamilies of sucrose transporters and monosaccharide transporters are well-studied under drought stress (Medici *et al*., 2014). There is evidence that in Arabidopsis various abiotic stresses, especially cold stress, lead to accumulation of sugars, particularly glucose and fructose in the vacuole (Wormit *et al*., 2006; Schulze *et al*., 2012). An increased accumulation of sugars upon drought and heat stress has also been observed (Rizhsky *et al*., 2004) suggesting a role of vacuolar sugar transporters also under these conditions. Expression of the putative sugar transporter ERD6 (early responsive to dehydration) is induced not only by dehydration but also by cold treatment (Kiyosue *et al*., 1998), and expression of an ERD6-like transporter (ESL1) is enhanced by drought, salt, and ABA treatment (Yamada *et al*., 2010). Osmotic stress and salt stress also affects vacuolar transporters, particularly the sucrose transporter SUC4 (Gong *et al*., 2015a) and the v-

ATPases (Kirsch *et al*., 1996). In this context, all cultivars at the present work might exhibit vacuolar accumulation of sucrose due to water stress following long-term storage.

The level of sugars in potato tubers is an important factor affecting quality in potatoes. The principal reason is the fact that the reducing sugars such as glucose and fructose react with free amino acids during frying to produce distasteful dark processed fries and chips via a non-enzymatic Maillard-type reaction (Shallenberger *et al*., 1959). This reaction has played an important role in the appearance and taste of foods since it is related to aroma, taste and colour. Moreover, acrylamide is present in different foods processed at high temperature and it is formed from asparagine in the presence of a carbonyl compound such a reducing sugar in the process of Maillard reactions (Mottram *et al*., 2002; Stadler *et al*., 2002). During the 3 seasons of this study, we reported a darkening increased over the storage period following reducing sugars trend. Susceptible cultivars exhibited darker fry colour than varieties with a stable sugar profile. Darker fry colours were reported to be more related to high glucose rather than fructose or sucrose content. Fry colour depends on the quantity of superficial reducing sugars and the temperature of frying oil as well as frying time (Pedreschi, 2009). A darker fry colour has been reported to be correlated to a higher glucose (Coleman *et al*., 1993; Pritchard & Adam, 1994) as well as higher acrylamide (Shepherd *et al*., 2010) contents.

The benefit of storing potatoes at 8-12°C is the minimum accumulation of sugars in tubers. This storage method keeps the stored potatoes suitable for table and processing purposes. However, the relatively high temperature favours sprouting and sprout growth once the natural dormancy period of potato is over. Hence, use of sprout suppressant becomes essential under these methods of potato storage. CIPC is considered as the most effective sprout suppressant for potatoes and it is usually applied as a post-harvest fogging treatment on stored potatoes (Paul *et al*., 2016). CIPC is a selective and systemic herbicide with an ability to translocate acropetally in plant system (Ashton & Crafts, 1981). CIPC acts as a mitotic inhibitor by interfering the process of spindle formation during the cell division (Vaughn & Lehnen, 1991). It is known to inhibit protein synthesis, RNA synthesis, activity of β-amylase along with suppression of transpiration and respiration and interfere with oxidative phosphorylation and photosynthesis (Vaughn & Lehnen, 1991). The CIPC treatment starts after the wound-healing period, since wound-healing requires the production of new cell layers resulting from cell division, and before dormancy break or sprout growth initiation

(Kleinkopf *et al*., 2003). In the present study, CIPC treatment had an effect on the fry colour of crisps. Within the same cultivar, crisps from untreated potato tubers showed a darker fry colour than CIPC-treated tubers. Higher sugar content and darker fry colour observed in untreated tubers might be due to wound-induced catabolic response and/or sink demand. With the onset of sprouting, tubers become a source organ for the growing sprout (Sonnewald, 2001). This is accompanied by structural and metabolic changes as well as by an altered level of gene expression (Ronning *et al*., 2003; Viola *et al*., 2007; Hartmann *et al*., 2011). Initial bud outgrowth does not require massive reserve mobilisation but is fed by sucrose-synthesising capacity that ensures rapid conversion of hexoses into sucrose that can be transported into growing buds to meet its energy demand. This was concluded from labelling experiments which revealed similar metabolic competence, but different metabolite pools in dormant and open tuber buds with respect to sugar metabolism (Viola *et al*., 2007). While resting buds contained only limited amounts of soluble sugars, there was a massive increase especially in the amount of sucrose at bud break indicating that sucrose unloading into the buds is a prerequisite for bud outgrowth.

Accumulation of reducing sugars must be avoided as it leads to both deterioration in processing quality and the risk of acrylamide production (Fuller & Hughes, 1984). The amount of free sugar tubers accumulate depends on the cultivar (van Vliet & Schriemer 1960; Burton 1969; Samotus *et al*., 1974; Coffin *et al*., 1987; Richardson *et al*., 1990; Zrenner *et al*., 1996). Although the cultivars that are most susceptible to senescent sweetening tend to have short dormancy there are important exceptions to this rule such as Maris Piper and Record (Colgan *et al*., 2012). In addition to the effect of cultivar, growing conditions that affect the maturity of tubers at harvest can impact on the timing of the onset of senescent sweetening and an effect of storage temperature is also evident (Groves *et al*., 2005).

Considerable variation between different potato genotypes have been observed (Amrein *et al*., 2003; Kumar *et al*., 2004; Elmore *et al*., 2007) and this affects processing properties. Moreover, sugar content is affected by environmental factors during potato cultivation. Pre- and post-harvest environmental and management factors are important, including temperature, mineral nutrition and water availability during cultivation, crop maturity at harvest, mechanical stress and storage conditions (Kumar *et al*., 2004). Temperature during cultivation is a major factor because the processes of photosynthesis, transpiration, translocation of carbohydrates and respiration are all temperature dependent. The optimum

temperature range for most varieties is quite narrow, between 15°C and 20°C (Kumar *et al*., 2004). Soil nitrogen levels also appear to be important: De Wilde *et al*. (2006) showed that the levels of tuber sugars rose in nitrogen-deprived potatoes by up to 100% compared with adequately fertilized potatoes, and Kumar *et al*. (2004) similarly reported that plants adequately fertilized with nitrogen had lower reducing-sugar concentrations at harvest. Sulphur deprivation also causes large increases in sugar concentrations (Elmore *et al*., 2007). In addition, Muttucumaru *et al*. (2015) observed that a lack of irrigation in the field-grown potatoes resulted in a lower reducing sugar concentration in four out of five varieties in the study (Lady Claire, Saturna, Ramos, and Hermes).

Senescent sweetening resistant and susceptible varieties showed similar behaviours in all seasons. This work allowed to identify the sweetening transition, and sampling of tubers for subsequent physiological, biochemical and molecular analysis.

Chapter 4: Investigation of oxidative stress during long-term storage

4.1 Introduction

A widely accepted hypothesis is that senescent sweetening is induced by oxidative stress during storage which leads to a breakdown of cellular function (Sowokinos *et al*., 1987; Kumar & Knowles, 1993; Colgan *et al*., 2012; Zommick *et al*., 2013; Carvalho, 2017).

Sugars accumulate in tubers when there is an imbalance between starch degradation, starch synthesis, and respiration of carbohydrates. Enzymes of potato tuber carbohydrate metabolism can be influenced by the concentration and compartmentation of substrates, products, ions, cofactors, hormones, allosteric modifiers, and pH (Mares *et al*., 1985). Several studies have reported reduced potato tuber membrane integrity during storage. Amyloplast membranes have been observed to physically deteriorate during extended long-term storage of potato tubers (Figure 4-1) (Ohad *et al*, 1971; Isherwood, 1976; Sowokinos *et al*., 1987). Reduced potato tuber membrane integrity measured as increased membrane electrolyte leakage has previously been implicated in the accumulation of sugars during low temperature storage (Shekhar *et al*., 1979; Workman *et al*., 1979) and long-term storage (Isherwood, 1976). In these instances, changes in either the physical status or chemical composition of the tuber membranes must account for the increased electrolyte leakage. Increased membrane lipid unsaturation is one chemical change that appears to confer resistance to increased electrolyte leakage in stored potato tubers (Knowles & Knowles, 1989).

Lipids represent approximately 0.1% of the fresh weight of a potato tuber (Galliard, 1968; Lepage, 1968) and the total fatty acid composition of potato tubers primarily reflect the composition of cellular membranes (Galliard, 1973). Fatty acids in the cell membranes can be affected by several chemical or physical changes. Spychalla and Desborough (1990) proposed that high or increased rates of tuber membrane permeability negatively influence the sugar status and processing quality of stored tubers. Furthermore, multilevel cellular responses may also affect ultimately the integrity of plastid membranes. For example, cold temperatures may initially act through hormone changes resulting in altered structure and function of cellular membranes (Isherwood, 1976; Ewing *et al.*, 1981).

The levels of lipoxygenase and lipolytic acyl hydrolase, affecting both starch granule breakdown and lipid metabolism are altered in the prematurely sweetened tissue (Sowokinos *et al*., 1985; Lulai *et al.*, 1986). Alterations in lipid metabolism may influence the structure and transport properties of cellular membranes. The double walled plastid membrane surrounding the amyloplast has been suggested to play an important function in the regulation of carbon partitioning between the granule and free sugars in the cytoplasm (Mares *et al*., 1985). Loss of cellular compartmentalization during senescence has been associated, in part, with gross physical changes affecting the physical integrity of the amyloplast membrane (Isherwood, 1976; Wetstein & Sterling, 1978).

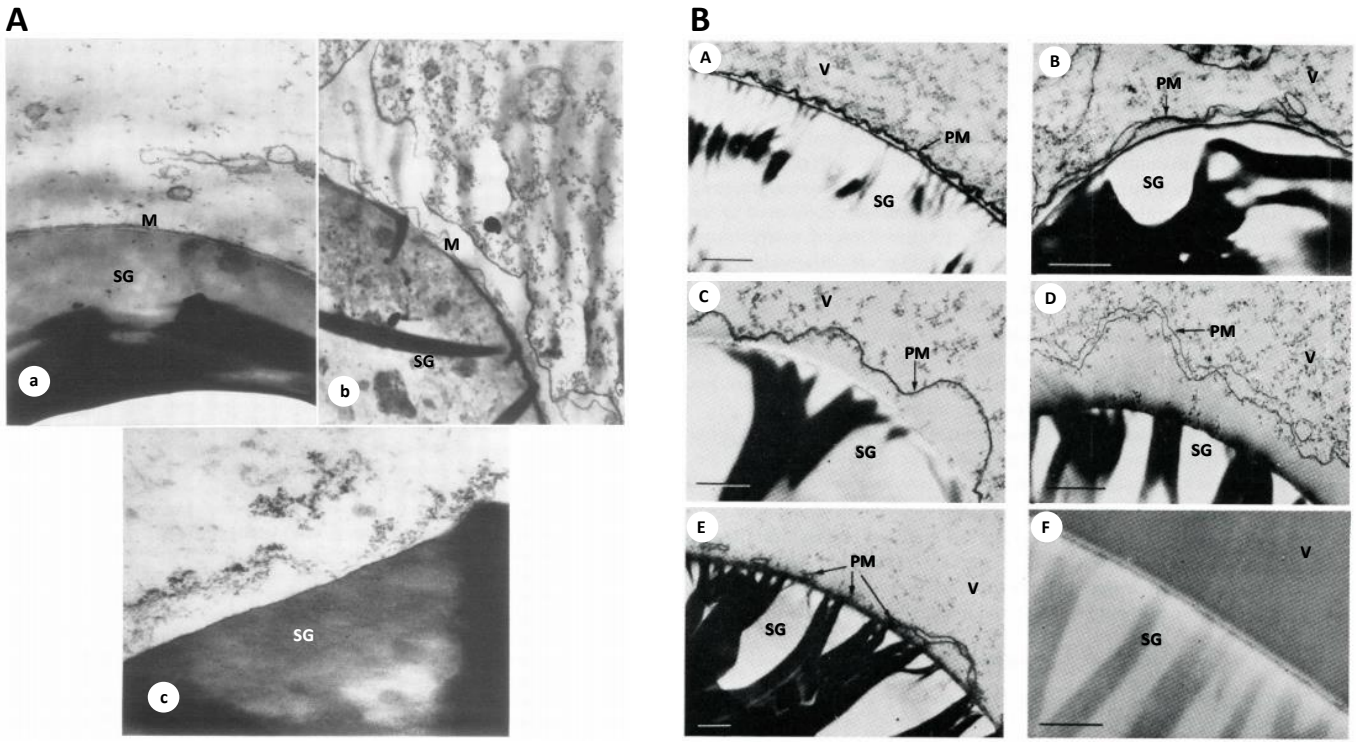


Figure 4-1. Electron micrographs of starch granules (SG) of stored potato tubers.
A. Sections through cells of mature potato tubers. Changes in the morphology of the starch granule membranes as a function of storage conditions. (a) Potato tuber 1 day after harvesting. The membranes (M) around the starch granule (SG) apparently intact. X 35,000. (b) Same material as part (a), but after 32 days of storage at 25ºC. The membranes (M) around the starch granule (SG) are apparently intact, although they are slightly removed from the granule. X 11,300. (c) Same material as part (a), but after 12 days of storage at 4ºC. Notice the disintegration of the membranes around the starch granule (SG). X 43,500. Modified from Ohad *et al*. (1971).

Fig. 4-1. Continuation. Electron micrographs of starch granules (SG) of stored potato tubers. **B.** Frames A through F show the double walled amyloplast membrane (PM=plastid membrane) representing 6 different physical states over time of tubers stored at 8.9ºC. Membrane integrity represented by each micrograph is: (A) bi-layers of the amyloplast membrane intact and closely associated with the granule, (B) bi-layers of the amyloplast membrane separating and closely associated with the granule, (C) bi-layers of the amyloplast membrane intact and loosely associated with the granule, (D) bi-layers of the amyloplast membrane separating and loosely associated with the granule, (E) membrane fragmented, vesicles formed and (F) no membrane visible. Marker inserts equal to 2 microns. Modified from Sowokinos *et al*. (1987).

Activation of starch degradative enzymes might play the main role in the degradation process, and much work concerning the activities of enzymes involved in the pathways of starch synthesis and degradation has accumulated (Badenhuizen, 1965; Pazur, 1965; Smith, 1967; Manners, 1968). Nevertheless, it is possible that the process of degradation of starch is not related to a significant increase in some enzymatic activity but rather to a change in distribution of different enzymes or substrates within the subcellular compartments of the potato tuber. Amyloplast membrane leakage could lead to a loss of cellular compartmentalisation and affects the transport of different effectors and intermediaries of starch metabolism (O'Donoghue *et al*., 1995). Premature disruption of the amyloplast membrane could increase the exposure of starch to amylolytic enzymes and the rate of phosphorylytic and/or hydrolytic breakdown of the starch granule in an irreversible manner.

Aging and senescence are the result of complex changes in basic plant metabolism and they share similarities at the biochemical level. A gradual disruption of membrane integrity, resulting in loss of compartmentation of cytoplasmic organelles and increased permeability of the plasma membrane, is a widely reported phenomenon common to both progressive aging and senescence of plant tissues (Thompson, 1988). The age-induced loss in membrane integrity is often caused by an increase in saturation of membrane phospholipids, which results in increased gel phase, decreased fluidity, and increased permeability (Pauls & Thompson, 1981). In potato tubers, a progressive loss of membrane integrity during aging was highly correlated with a decrease in double-bond-index, and, thus, an increase in the saturation of membrane lipids (Knowles & Knowles, 1989). However, we reported in *Chapter 5* a general decrease ($P < 0.001$) in saturated fatty acids in aged tubers suggesting lipid membranes might be intact.

Progressive loss of membrane integrity and increased lipid peroxidation are characteristics of aging tubers (Kumar & Knowles, 1993). Increased saturation of membrane lipids is known to cause organizational changes that disrupt membrane integrity and increase permeability (Barber & Thompson, 1980; Pauls & Thompson, 1981). Such changes in the membrane lipid microenvironment can also affect membrane protein mobility (Shinitzky & Inbar, 1976), potentially altering the activities and kinetic properties of membrane-bound enzymes, most notably transport proteins (Carruthers & Melchior, 1986). The progressive loss of membrane integrity associated with aging of potato seed-tubers is a function of increasing peroxidative damage caused by an accumulation of free-radicals over time (Kumar & Knowles, 1993).

An unavoidable consequence of aerobic metabolism is production of ROS. ROS include free radicals such as $O_2^{\bullet -}$ and $HO^{\bullet}$, as well as non-radical molecules like H_2O_2, and $^1O_2$. Stepwise reduction of O_2 by high-energy exposure or electron-transfer reactions leads to production of the highly reactive ROS. In plants, ROS are always formed by the inevitable leakage of electrons onto O_2 from the electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a by-product of various metabolic pathways localized in different cellular compartments (Foyer & Harbinson, 1994; Foyer *et al*., 1997; Del Río *et al*., 2006; Blokhina & Fagerstedt, 2010; Heyno *et al*., 2011).

Mitochondria are considered the powerhouses of the cell and contain two membranes. The outer membrane fully surrounds the inner membrane, with a small intermembrane space in between. The outer membrane has many protein-based pores that are big enough to allow the passage of ions and molecules as large as a small protein. In contrast, the inner membrane has much more restricted permeability, much like the plasma membrane of a cell. The inner membrane is also loaded with proteins involved in electron transport and ATP synthesis. This membrane surrounds the mitochondrial matrix, where the TCA cycle produces the electrons that travel from one protein complex to the next at the inner membrane. At the end of this ETC, the final electron acceptor is O_2, and this ultimately forms H_2O. At the same time, the ETC produces ATP in a process called oxidative phosphorylation. During electron transport, the participating protein complexes push protons from the matrix out to the intermembrane space. This creates a concentration gradient of protons that another protein complex, called ATP synthase, uses to power synthesis of the energy carrier molecule ATP (Figure 4-2).

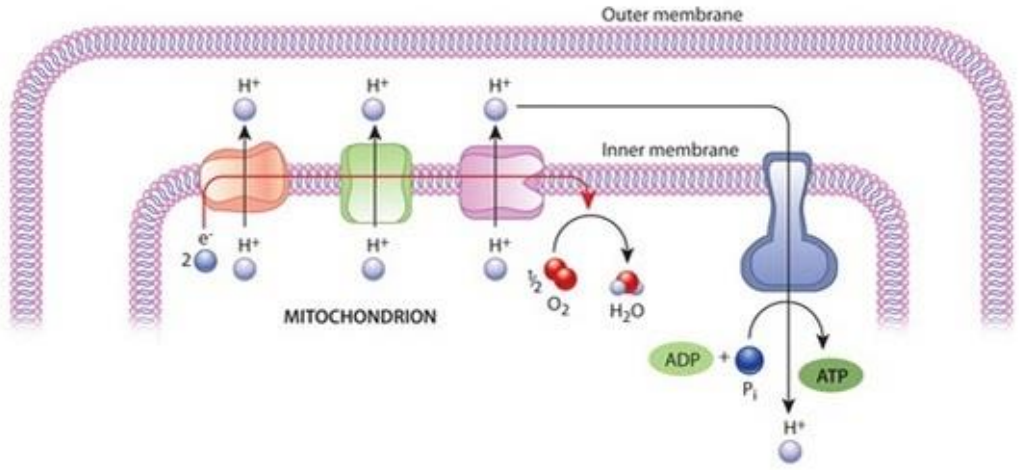


Figure 4-2. The electrochemical proton gradient and ATP synthase. At the inner mitochondrial membrane, a high energy electron is passed along an electron transport chain. The energy released pumps hydrogen out of the matrix space. The gradient created by this drives hydrogen back through the membrane, through ATP synthase. As this happens, the enzymatic activity of ATP synthase synthesises ATP from ADP. Taken from O'Connor and Adams (2010).

The mitochondrial ETC is the major site of ROS production in mammalian and non-photosynthesizing plant cells (Puntarulo *et al.*, 1991; Halliwell & Gutteridge, 2007). Depending on the mitochondrial respiratory states, a small portion of the consumable oxygen is partially reduced to generate ROS (Skulachev, 1996; Liu, 1997; Turrens, 1997; Møller, 2001; Considine *et al.*, 2003; Smith *et al.*, 2004). In plants, the monoelectronic reduction of oxygen by ETC leads to the production of $O_2^{\bullet -}$ that can be dismutated by superoxide dismutase (SOD), producing H_2O_2, and further decomposed by catalase and/or ascorbate-glutathione peroxidase cycles (Møller, 2001). An imbalance between the ROS production and antioxidant defences can lead to an oxidative stress condition.

Mitochondria can produce reactive oxygen species ROS at several sites of the ETC. In mitochondria direct reduction of O_2 to $O_2^{\bullet -}$ occurs in the flavoprotein region of NADH dehydrogenase segment (complex I) of the respiratory chain (Arora *et al.*, 2002). When NAD^+-linked substrates for complex I are limited, electron transport can occur from complex II to complex I (reverse electron flow). This process has been shown to increase ROS production at complex I and is regulated by ATP hydrolysis (Turrens, 2003). Ubiquinone-cytochrome region (complex III) of the ETC also produces $O_2^{\bullet -}$ from O_2. It is believed that

fully reduced ubiquinone donates an electron to cytochrome C1 and leaves an unstable highly reducing ubisemiquinone radical which is favourable for the electron leakage to O_2 and, hence, to $O_2^{\bullet-}$ formation (Murphy, 2009). In plants, under normal aerobic conditions, ETC and ATP synthases are tightly coupled; however, various stress factors lead to inhibition and modification of its component, leading to over reduction of electron carriers and, hence, formation of ROS (Noctor *et al.*, 2007; Blokhina & Fagerstedt, 2010).

Several enzymes present in the mitochondrial matrix can produce ROS. Some of them produce ROS directly, for example aconitase, whereas some others like L-galactono-γ-lactone dehydrogenase, are able to feed electrons to the ETC (Andreyev *et al.*, 2005; Rasmusson *et al.*, 2008). $O_2^{\bullet-}$ is the primary ROS formed by monovalent reduction in the ETC. It is converted quickly either by the MnSOD (mitochondrial form of SOD) or APX into the relatively stable and membrane-permeable H_2O_2. H_2O_2 can be further converted to the extremely active $HO^{\bullet}$ in the Fenton reaction.

Environmental stresses such as drought, salinity, chilling, metal toxicity, and UV-B radiation as well as pathogens attack lead to enhanced generation of ROS in plants due to disruption of cellular homeostasis (Shah *et al.*, 2001; Mittler, 2002; Sharma & Dubey, 2005; 2007; Hu *et al.*, 2008; Han *et al.*, 2009; Maheshwari & Dubey, 2009; Tanou *et al.*, 2009; Mishra *et al.*, 2011; Srivastava & Dubey, 2011). All ROS are extremely harmful to organisms at high concentrations. When the level of ROS exceeds the defence mechanisms, a cell is considered to be under oxidative stress. The enhanced production of ROS during environmental stresses can pose a threat to cells by causing peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death pathways and ultimately leading to death of the cells (Shah *et al.*, 2001; Mittler, 2002; Verma & Dubey, 2003; Meriga *et al.*, 2004; Sharma & Dubey, 2005; Maheshwari & Dubey, 2009; Mishra *et al.*, 2011; Srivastava & Dubey, 2011).

AGPase catalyses the conversion of G1P and ATP to ADP-Glc and PPi, which is the first committed step in the pathway of starch synthesis (Preiss, 1988; Martin & Smith, 1995; Smith *et al.*, 1997).

Whereas in chloroplasts the ATP necessary for starch synthesis can be readily provided through photosynthesis, potato tuber amyloplasts have to import ATP from the cytosol via an

ATP/ADP transport protein located on the inner-envelope membrane (Neuhaus & Emes, 2000). Tjaden *et al*. (1998) showed that a relatively small decrease in ATP/ADP transporter activity leads to a reduced level of total starch content and a lower amylose-to-amylopectin ratio. By contrast, increased transporter activity correlated with higher starch contents and a higher amylose-to-amylopectin ratio. These observations indicated that the rate of ATP import exerts considerable control on the rate of starch synthesis and affects the molecular composition of starch in potato tubers. (Hofius & Börnke, 2007).

Long-term tuber storage is associated with increased oxidative damage of the amyloplast membrane which has the potential to expose starch granules to cytosolic amylolytic enzymes (Kumar & Knowles, 1993). Starch hydrolysis may also be triggered by a requirement for increased respiration to provide ATP and reducing equivalents for membrane repair (Kumar & Knowles, 1996). The most prominent observed class of post-translational modifications in the potato tuber mitochondrial proteome is oxidative modifications (Salvato *et al*., 2014). This fact suggests that mitochondria presents a highly oxidative environment. Mitochondrial oxidative damage could drive the accumulation of reducing sugars as a result of a progressive loss of membrane integrity and malfunction of the ETC. Hence, senescent sweetening may be induced as a result of a reduced capacity for respiration leading to an accumulation of sugars and reduced availability of ATP required for starch re-synthesis. Furthermore, the final enzyme in the pathway of ascorbate biosynthesis, a key plant antioxidant, is intimately associated with the mitochondrial electron transport chain (Millar *et al*., 2003) suggesting that damage to plant mitochondria could further limit antioxidant capacity. To understand the relationship between senescent sweetening and oxidative damage in stored tubers several approaches were adopted. Oxidative load during storage was examined by measurement of H_2O_2 content. H_2O_2 is considered a marker of oxidative stress and the predominant ROS involved in cellular signalling (Bienert *et al*., 2006). Oxidative membrane damage was quantified as thiobarbituric acid reactive substances (TBARS) (Hodges *et al*., 1999). Tuber antioxidant capacity was estimated by quantification of key antioxidant enzyme activities (Murshed *et al*., 2008) using spectrophotometry. Taken together the work in this chapter directly tests the hypothesis that senescent sugar accumulation is associated with oxidative damage in aged potato tubers.

4.2 Results

4.2.1 Determination of H_2O_2 during long-term storage

In order to determine if an increase of oxidative stress was related to the accumulation of sugars in stored potato tubers over time, H_2O_2 was detected using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit, a one-step assay that uses 10-acetyl-3,7-dihydroxyphenoxazine in combination with horseradish peroxidase to detect H_2O_2 released from tuber samples following extraction in a buffer designed to quench enzyme activity and maintain H_2O_2 stability. Content of H_2O_2 was measured in tuber samples during storage over the first season. A two-way ANOVA was carried out to determine whether there were significant differences between samples using cultivar and time of storage as factors. To determine significant differences between means over time Fisher's LSD test was carried out for each cultivar independently. The content of H_2O_2 was different ($P < 0.001$) between cultivars during the storage period. Although H_2O_2 levels showed fluctuations over time, no significant differences were found. Hence, we reported no increase of H_2O_2 content related to the accumulation of reducing sugars (Figure 4-3).

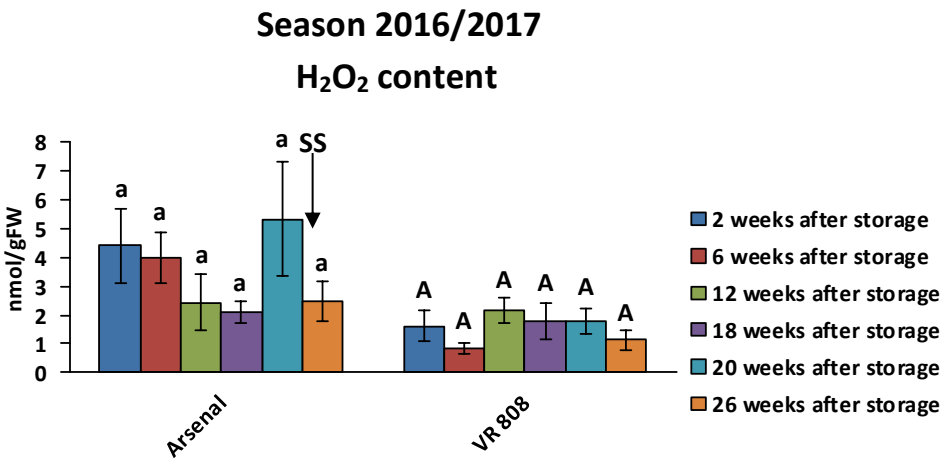


Figure 4-3. H_2O_2 content in sweetened and non-sweetened potato tubers during long-term storage as a marker of oxidative stress. Although differences in H_2O_2 content between cultivars were observed, no significant changes related to reducing sugars increase on onset of senescent sweetening were reported. Arrows indicate the onset of senescent sweetening (SS). Each value is the mean ± SE of measurements from five separate tubers. Mean values with different letters are significantly different according to the Fisher's LSD test, carried out independently for each cultivar and treatment. Differences within Arsenal and VR 808 cultivars are represented by lower- and upper-case letters, respectively.

4.2.2 Quantification of specific activity of ascorbate-glutathione cycle enzymes

Increase in levels of H_2O_2 were not observed in tubers during the storage. However, variations over time were reported. This suggests there may be temporary changes that could be mitigated by antioxidant systems. Therefore, the four enzymes of the ascorbate-glutathione cycle, considered to be the main antioxidant system in plants (Hancock, 2017), were quantified. Results showed fluctuations in the specific activity during storage. ANOVA was carried out to determine whether there were significant differences between samples with three factors (cultivar, time of storage and treatment). To determine significant differences between means over time Fisher's LSD test was carried out for each cultivar and treatment (CIPC-treated or untreated) independently.

Changes during the time of storage ($P < 0.001$) were observed in the specific activity from the set of four enzymes involved in the main antioxidant defence in plants. Cultivars exhibited no differences ($P > 0.05$) in the enzymatic antioxidant system. CIPC-treatment had no effect ($P > 0.05$) on the enzymatic activity of APX, MDHAR or GR. However, significant changes ($P < 0.05$) associated with CICP-treatment were observed in DHAR activity over time. Furthermore, all cultivars exhibited an overall decrease in APX, MDHAR and GR after the onset of senescent sweetening. Although we reported differences in H_2O_2 content between cultivars during long-term storage, Arsenal (susceptible to SS) and VR 808 (SS resistant) showed similar behaviours of antioxidant systems over time. Measurements of specific activities are presented in Figure 4-4.

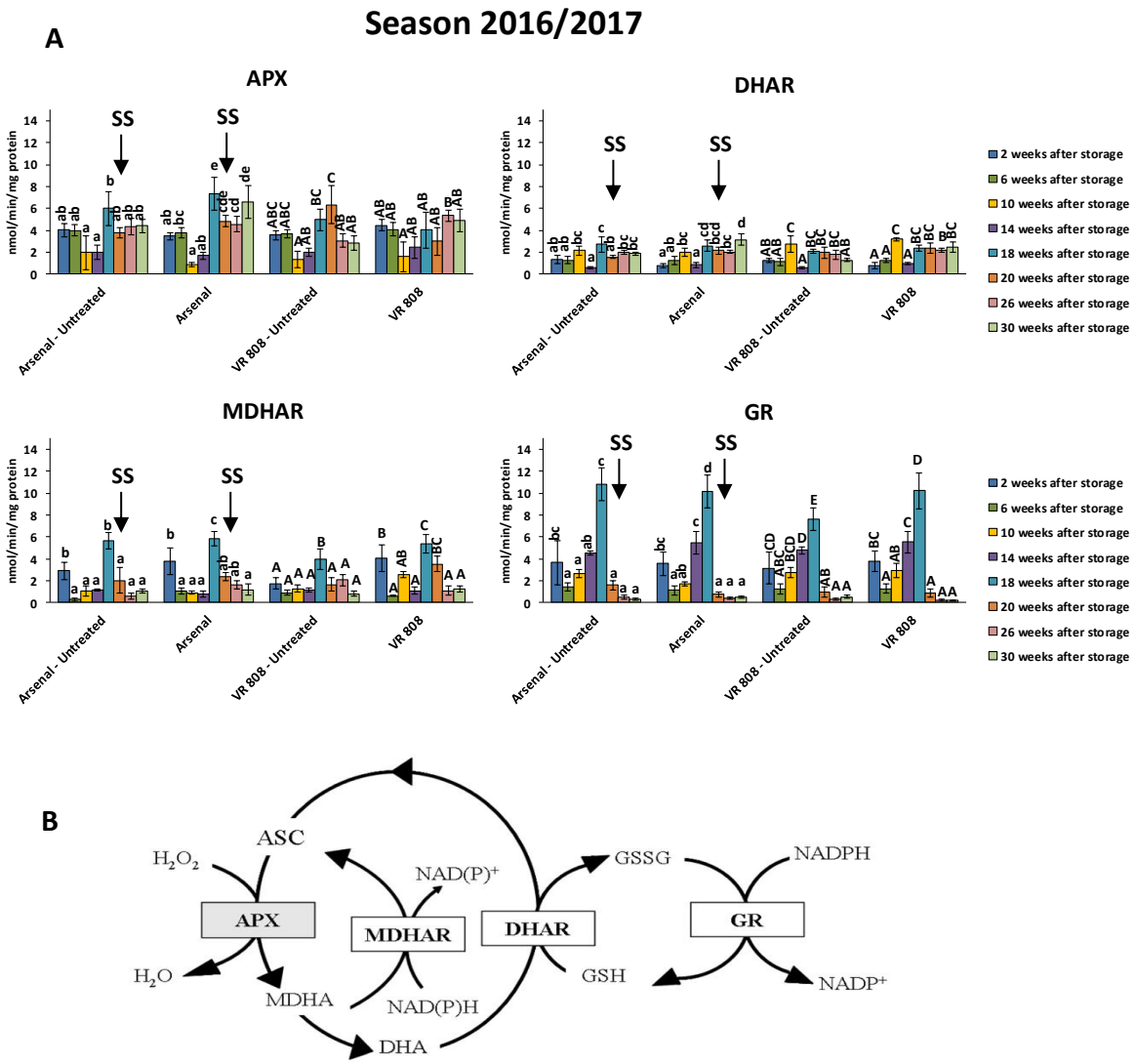


Figure 4-4. Impact of long-term storage on antioxidant systems in potato tubers. **A.** Specific activities of antioxidant enzymes in tubers. Arrows indicate the onset of senescent sweetening (SS). Each value is the mean ± SE of measurements from five separate tubers. Mean values with different letters are significantly different according to the Fisher's LSD test, carried out independently for each cultivar and treatment. Differences within Arsenal and VR 808 cultivars are represented by lower- and upper-case letters, respectively. **B.** Ascorbate-glutathione cycle pathway. Taken from Locato *et al*. (2013). An important role in the plant antioxidant defence mechanism has been attributed to this pathway. First, the hydrogen peroxide (H_2O_2) generated by oxidative stress is scavenged via the oxidation of ascorbate (ASC) by ascorbate peroxidase (APX). This enzyme is involved in the oxidation of ASC to monodehydroascorbate (MDHA), which can be converted back to ASC via monodehydroascorbate reductase (MDHAR). MDHA that escapes this recycling is converted rapidly to dehydroascorbate (DHA) which is converted back to ASC by the action of dehydroascorbate reductase (DHAR). DHAR utilizes glutathione (GSH), which is regenerated by glutathione reductase (GR) from its oxidized form, glutathione disulphide (GSSG).

4.2.3 Determination of lipid peroxidation by measurement of MDA levels

We observed no changes in average cellular levels of H_2O_2 over time relating to the onset of senescent sweetening. However, it is possible that oxidative damage may have occurred due to localised concentrations of this ROS in specific organelles or due to sudden spikes that were subsequently controlled. Hence, MDA was quantified to determine whether oxidative damage was occurring at cellular level.

MDA content in stored tubers were quantified as TBARS as an indicator of lipid peroxidation and overall biomarker of oxidative stress (Fletcher *et al*., 1973; Konze & Elstner, 1978; Dhindsa *et al*., 1981). A three-way ANOVA was carried out to determine whether there were significant differences between samples using cultivar, time of storage and treatment (CIPC-treated or untreated) as factors. Fisher's LSD test was carried out for each cultivar and treatment independently in order to determine significant differences between means over time. Levels of MDA in tubers were influenced by cultivar and time of storage ($P < 0.001$). We observed an increase during the beginning of the storage, which is potentially related to oxidative stress occurring due to harvest and manipulation of tubers. For this early accumulation of MDA, Arsenal showed higher levels ($P < 0.001$) in CIPC-treated tubers compared with the untreated tubers (Figure 4-5). Untreated and CIPC-treated tubers from Arsenal (susceptible to SS), and CIPC-treated VR 808 (SS resistant) tubers exhibited no increase of MDA levels related to the accumulation of sugars. MDA content in untreated VR 808 tubers showed a small increase around the time that senescent sweetening was first observed in Arsenal tubers.

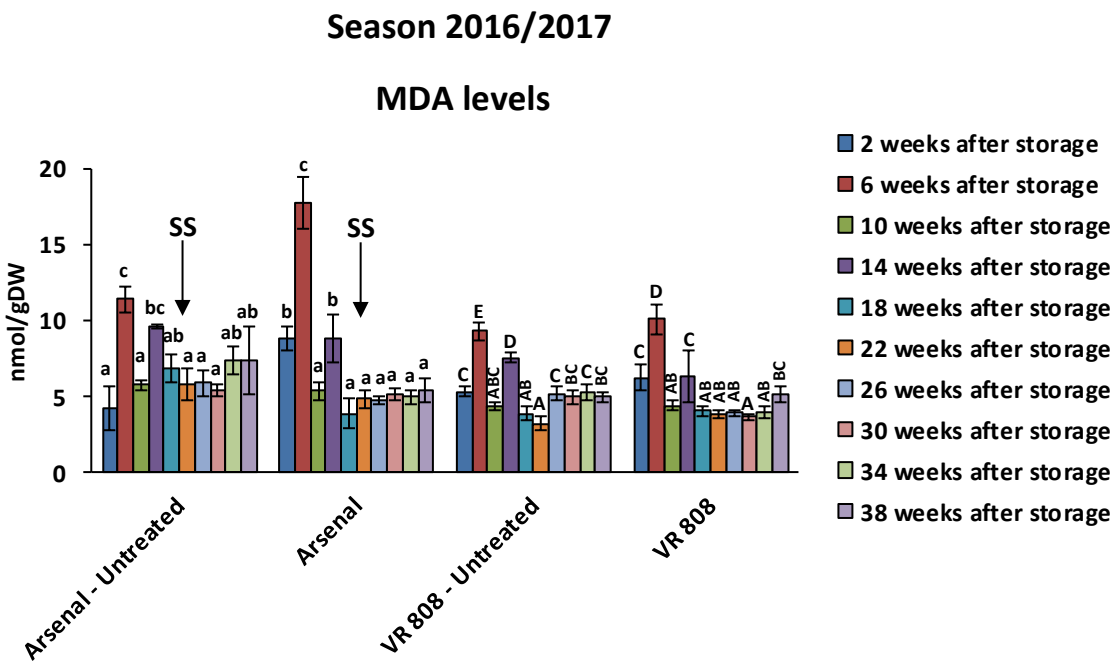


Figure 4-5. Quantification of malondialdehyde (MDA) content during long-term storage as an indirect measurement of lipid peroxidation and marker of oxidative damage. Similar behaviour between all cultivars and conditions were observed. Tubers presented no increase of MDA related to sugar accumulation suggesting senescent sweetening may not be linked to oxidative stress. Onset of senescent sweetening (SS) is indicated by arrows. Each value is the mean ± SE of measurements from five separate tubers. Mean values with different letters are significantly different according to the Fisher's LSD test, carried out independently for each cultivar and treatment. Differences within Arsenal and VR 808 cultivars are represented by lower- and upper-case letters, respectively.

4.3 Discussion

ROS are well recognized for playing a dual role as both deleterious and beneficial species depending on their concentration in plants. At high concentration ROS cause damage to biomolecules, whereas at low/moderate concentrations they act as second messengers in intracellular signalling cascades that mediate several responses in plant cells (Gechey & Hille, 2005). Among the ROS, H_2O_2 is the one which received most of the attention in the last seasons. H_2O_2 is the result of a two-step reduction molecular oxygen and has a relatively long lifespan in comparison to other ROS. The long half-life (1 ms) of H_2O_2 and its small size allow it to traverse cellular membranes and migrate in different compartments, which facilitates its signalling functions (Bienert *et al.*, 2006). It is well known that H_2O_2 is a regulator of a multitude of physiological processes like acquiring resistance, cell wall strengthening, senescence, phytoalexin production, photosynthesis, stomatal opening and the

cell cycle (Petrov & Van Breusegem, 2012) and is essential for suberization in potatoes (Razem & Bernards, 2002).The dual role played by ROS require the very strict control of H_2O_2 concentration in plant cells. The biological effect of H_2O_2 is mostly dependent on its concentration, but also on the site of production, the developmental stage of the plant and previous exposures to different kinds of stress.

H_2O_2 possesses some features typical for second messenger molecules and its production is up-regulated by many stimuli, mainly through NADPH-oxidases and peroxidases (Petrov & Van Breusegem, 2012). In addition, H_2O_2 is a small and relatively mobile molecule that has the potential to carry information between different cellular compartments. Moreover, H_2O_2 is able to modulate the activities of many other signalling components and intercalate in a number of signalling cascades with different biological outcomes, including the one that leads to its own synthesis. In the latter case, either a positive or a negative feedback is provided by inducing or inhibiting H_2O_2 modulating systems (Mittler, 2002; de Pinto *et al*., 2006; Van Breusegem & Dat, 2006). This is mainly dependent on the H_2O_2 concentration and the timing of its synthesis. The possibility of a positive feedback provides a way to amplify the initial signal, while the negative feedback option ensures that the system can be effectively switched off in order to prevent excessive damage. The most typical targets of H_2O_2 include effectors of calcium homeostasis, ion channels, protein kinases or phosphatases and transcription factors (TFs) (Petrov & Van Breusegem, 2012).

It is presumed that increased H_2O_2 levels could be perceived directly by redox-sensitive TFs that orchestrate downstream cascades (Miller *et al*., 2008). Good candidates for such TFs are class A heat shock factors, which are shown to be responsive to oxidative stress both in animals and plants (Miller & Mittler, 2006; Kotak *et al*., 2007).

Moreover, H_2O_2 has the ability to diffuse across membranes. H_2O_2 produced by the chloroplast electron transport chain can leak out of chloroplasts in a light-intensity-dependent manner (Mubarakshina *et al*., 2010). Nevertheless, H_2O_2 is a relatively neutral solute and native membranes present a significant barrier to its free diffusion (Bienert *et al.,* 2006). H_2O_2 can be transported through specific membrane aquaporin homologues of the tonoplast intrinsic protein (TIP) plasma membrane intrinsic protein (PIP) families. Bienert *et al*. (2007) showed that expression of *Arabidopsis thaliana AtTIP1;1* and *AtTIP1;2* genes in yeast cells decreased their survival rate in the presence of H_2O_2, while blocking this aquaporin-mediated

diffusion alleviated the effect of H_2O_2. Furthermore, Dynowski *et al*. (2008) suggested that the aromatic/arginine regions in PIP2 proteins are critical for their selectivity towards H_2O_2 and as all eight PIP2 proteins in Arabidopsis are conserved in these positions, presumably all of them are involved in the specific transport of H_2O_2.

As previously described, H_2O_2 has a potential role in cellular and membrane damage as a consequence of an imbalance between its production and antioxidant defences. This imbalance could lead to an oxidative stress condition, and subsequent damage of the mitochondria and amyloplast membrane. Malfunction in the mitochondrial machinery would result in a reduced capacity of respiration of reducing sugars, producing their accumulation. In the same way, damage of amyloplast membrane could expose the starch to amylolytic enzymes increasing its degradation and, therefore, accumulation of reducing sugars.

H_2O_2 is the longest living ROS and is considered as the predominant ROS involved in cellular signalling (Bienert *et al.*, 2006). H_2O_2 content was quantified as a marker of oxidative stress in tubers during storage. An increase of H_2O_2 content during senescent sweetening transition would suggest oxidative stress may be linked to the sugar accumulation in tubers. Both cultivars exhibited significant differences during the storage period. However, tubers from each did not exhibit a specific increase of H_2O_2 associated with the onset on sweetening.

Changes in absolute level of H_2O_2 were not observed during long-term storage. Nevertheless, oxidative damage may have occurred due to localised concentration in specific organelles or due to controlled sudden spikes. For that reason, an additional experiment was performed in order to determine if H_2O_2 produced lipid membrane damage at cellular level over time. MDA levels were quantified as an indirect measurement of lipid peroxidation in lipid membranes (Fletcher *et al*., 1973; Konze & Elstner, 1978; Dhindsa *et al*., 1981). Results in lipid peroxidation showed similar behaviours for both cultivars. An increase at the beginning of the storage period was observed which may be related to storage-induced stress suffered by the tuber. During the long-term storage MDA levels remained stable, suggesting no increase of oxidative damage in lipid membranes. In the present work, we report no increase of markers of oxidative stress during storage, suggesting senescent sweetening may not be linked to oxidative stress.

Aging and senescence are distinctly different but overlapping developmental processes. Aging encompasses the entire lifespan of an organism, whereas senescence can be thought of as the final developmental phase that culminates in death. Aging and senescence are the result of complex changes in basic plant metabolism and, although the two are distinguishable, they do share similarities at the biochemical level. For example, a gradual disruption of membrane integrity, resulting in loss of compartmentation of cytoplasmic organelles and increased permeability of the plasma membrane, is a widely reported phenomenon common to both progressive aging and senescence of plant tissues (Thompson, 1988). Membrane integrity declines with advancing age of potato seed-tubers (Knowles & Knowles, 1989). In senescing plant tissues, lipid peroxidation plays a role in the loss of membrane integrity (Leshem, 1987; Gidrol *et al*., 1989) and evidence of extensive lipid peroxidation during prolonged storage of potato seed-tubers has been reported (Kumar & Knowles, 1993). Moreover, recent studies using diaminobenzidine tetrahydrochloride (DAB) and nitroblue tetrazolium (NBT) staining indicated a relationship between the onset of senescent sweetening and an increase in ROS, suggesting senescent sweetening resistant varieties exhibit a delayed rise in ROS accumulation (Carvalho, 2017). These findings support the hypothesis that senescent sweetening may be produced by an increase of oxidative damage. However, since DAB stain is dependent not only on ROS but also on the presence of peroxidase, this might explain the observed difference. Moreover, membrane permeability may change over time leading to a better access of the stain into the tissue.

Loss of membrane integrity in the amyloplast due to an increase of lipid peroxidation could expose the starch to amylolytic enzymes leading to an accumulation of sugars. Nonetheless, MDA measurements reported in this chapter suggest that if there is a loss of membrane integrity is not caused by oxidative damage. We showed previously there was no evidence of oxidative damage related to accumulation of sugars during long-term storage. In addition, a general decrease in fatty acids ($P < 0.001$) associated with the senescent sweetening transition was observed (*Chapter 5*). However, Spychalla and Desborough (1990) proposed induced or initial high levels of membrane lipid unsaturation mitigated increases in tuber membrane permeability during storage, and alterations in the levels of fatty acids had little bearing upon tuber membrane permeability. Literature has also reported mixed evidence of how long-term stored affects amyloplast membrane integrity in potato tubers. Electron micrographs of potato tubers stored at 10°C for 8 months indicated that the amyloplast membrane was still intact and continuous around starch granules in both normal and prematurely sweetened tissue

(Sowokinos *et al*., 1985). Moreover, different authors reported fragmented or disintegrated starch granules membranes in potato tubers during storage (Ohad *et al*., 1971; Sowokinos *et al*., 1987).

Despite their destructive activity, ROS are well-described second messengers in a variety of cellular processes including tolerance to environmental stresses (Desikan *et al*., 2001; Neill *et al*., 2002; Yan *et al*., 2007). Whether ROS will act as damaging or signalling molecule depends on the delicate equilibrium between ROS production and scavenging. Increased levels of ROS may be a consequence of the action of plant hormones, environmental stress, pathogens, or high levels of sugars and fatty acids (Bolwell *et al*., 2002; Couée *et al*., 2006; Gechev *et al*., 2006; Liu *et al*., 2007; Rhoads & Subbaiah, 2007). These conditions may lead to storage deterioration or impairment of seedling growth decreasing on crop yield. Because of the multifunctional roles of ROS, it is necessary for the cells to control the level of ROS tightly to avoid any oxidative injury and not to eliminate them completely. Scavenging or detoxification of excess ROS is achieved by an efficient antioxidant defence system comprising of the non-enzymatic as well as enzymatic antioxidants (Schreck & Baeuerle, 1991; Noctor & Foyer, 1998; Møller, 2001). The enzymatic antioxidants include SOD, catalase (CAT), glutathione peroxidase (GPX), enzymes of ascorbate-glutathione (AsA-GSH) cycle such as APX, MDHAR, DHAR, and GR (Noctor & Foyer, 1998, Möller *et al*., 2001). AsA, GSH, carotenoids, tocopherols, and phenolics serve as potent non-enzymatic antioxidants within the cell. Maintenance of a high antioxidant capacity to scavenge the toxic ROS has been linked to increased tolerance of plants to these environmental stresses (Zaefyzadeh *et al*., 2009; Chen *et al*., 2010). Considerable progress has been made in improving stress-induced oxidative stress tolerance in crop plants by developing transgenic lines with altered levels of antioxidants (Allen *et al*., 1997; Faize *et al*., 2011). Simultaneous expression of multiple antioxidant enzymes has been shown to be more effective than single or double expression for developing transgenic plants with enhanced tolerance to multiple environmental stresses (Lee *et al*., 2007). Plants have developed antioxidant defence systems to minimize the concentrations of ROS and to protect plant cells from oxidative damage (Noctor & Foyer, 1998). Given the lack of any evidence for oxidative stress in stored tubers, the hypothesis that antioxidant systems were up-regulated to deal with an increased production of oxidants after prolonged storage was tested. An important role in the antioxidant defence system has been attributed to the ascorbate-glutathione pathway, which is catalysed by a set of four enzymes (Noctor & Foyer, 1998; Asada, 2006). The specific

activity of this set of enzymes from the ascorbate-glutathione cycle was quantified to monitor any change during storage that could be related to senescent sweetening. Results showed fluctuations in the specific activity of antioxidant enzymes during storage. However, there was no consistent change in antioxidant enzyme activity associated with the onset of senescent sweetening. These data indicate a lack of support for the hypothesis that senescent sweetening is associated with oxidative stress in stored potato tubers.

Chapter 5: Metabolome profiles of potato tubers during long-term storage

5.1 Introduction

Metabolites are the end products of cellular regulatory processes, and their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes. This work investigates the possible causes of senescent sweetening in potato by examining the impact of long-term storage on metabolism in potato tubers.

An analysis of the potato (*Solanum tuberosum* L.) tuber metabolome during long-term storage has been completed using a GC/MS based approach. Potato tubers stored at 9°C were examined during long-term storage in season 1 (2016/2017), including early storage and senescent sweetening transition. In season 2 (2017/2018), ^{13}C-labelled extracts from potato tuber discs were analysed in order to obtain a better understanding of glucose metabolism in stored potato tubers. For season 3 (2018/2019), an analysis of the potato tuber metabolome profiling was performed, including additional cultivars that provided contrasting profiles of metabolites during storage.

5.2 Metabolome profiling during long-term storage in season 1 (2016/2017)

The metabolite profiles of tubers from Arsenal (sweetening susceptible) and VR 808 (sweetening resistant) were compared in untreated and CIPC-treated tubers during long-term storage, at 2, 4, 6, 12, 20, and 26 weeks of storage. Five different biological replicates were used for each cultivar and time point. Polar extracts (mainly sugars, organic acids, and amino acids) and non-polar extracts (mainly fatty acids and fatty alcohols) were examined. In the tubers analysed in this study, a total of 123 metabolites were detectable (72 polar and 51 non-polar), of which 76% could be identified.

Metabolites, whose abundance was changed in a statistically significant manner over time, were identified using three-way ANOVA based on cultivar, treatment (CIPC-treated or untreated), and time of storage. Arsenal and VR 808 exhibited differences ($P < 0.05$) in 83 metabolites during the storage period. The levels of 11 metabolites were significantly different ($P < 0.05$) between CIPC-treated and untreated tubers. A total of 123 metabolites

were significantly ($P < 0.05$) influenced by the time of storage (Supporting Information Table S5-1). All metabolites analysed are presented in Supporting Information Figure S5-1. An overall 'U' pattern response during storage was observed in reducing sugars, amino acids, and organic acids, showing higher peaks at the beginning and the end of the storage period. Arsenal tubers exhibited a higher concentration of glucose and fructose than VR 808 tubers. In addition, CIPC-treated tubers from both cultivars had a lower concentration of reducing sugars during the onset of the senescent sweetening (at 20, and 26 weeks of storage). Sugar phosphates showed no clear pattern. Amino acids generally showed an initial high peak followed by a rapid decrease and then gradual increase until the end of the storage. Arsenal and VR 808 showed initial higher levels of valine, and urea, respectively. Moreover, a higher concentration of aspartic acid in VR 808 was observed over time. Glycerol and caffeic acid showed a declining concentration in both cultivars. On the contrary, both Arsenal and VR 808 exhibited an increase in galactinol and chlorogenic acid during storage. Arsenal had higher levels of quinic acid than VR 808 during the storage. VR 808 showed higher concentrations of galactaric acid and galactosyl glycerol than Arsenal.

Both cultivars showed a general decrease in saturated and unsaturated fatty acids as well as fatty alcohols during the onset of senescent sweetening (at 20, and 26 weeks of storage). Additionally, Arsenal exhibited higher levels of heneicosanol whereas VR 808 presented higher solanid-5-enol at the onset of sweetening.

5.3 Estimation of ^{13}C fluxes in glucose metabolism in season 2 (2017/2018)

Metabolite fluxes were analysed to obtain more profound knowledge about glucose metabolism in potato tubers during long-term storage. Potato tuber discs were prepared from three different biological replicates. Metabolism in tuber discs was monitored by determining the redistribution of label following incubation in [U-^{13}C] glucose for 2, 4, and 6 h. ^{13}C-labelled extracts were analysed by GC/MS. Time points under study were at 5, 10, 15, 43, and 53 weeks of storage. In the tubers analysed in this study, a total of 30 polar metabolites were observed to accumulate ^{13}C. Over the storage period, ^{13}C mainly accumulated in glycine, aspartic acid, glutamic acid, and glutamine (Figure 5-1A), citric acid, and malic acid (Figure 5-1B), and sucrose (Figure 5-1C). The rest of the metabolites analysed only accumulated ^{13}C at certain time points (Supporting Information Figure S5-2).

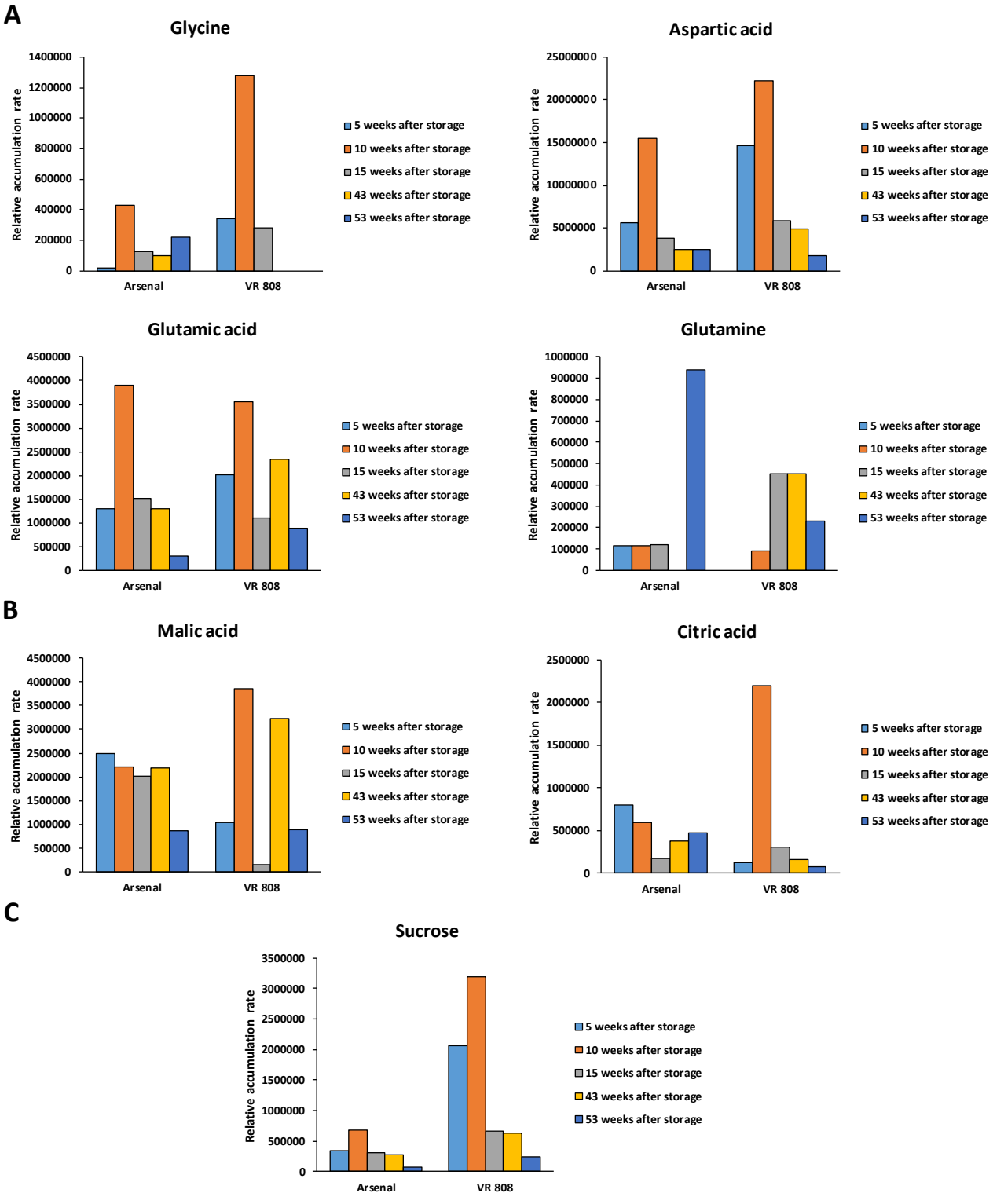


Figure 5-1. Metabolite fluxes on the metabolism of [U-^{13}C] glucose by potato tubers. Metabolites which mainly accumulated ^{13}C are shown. **A.** Amino acids. **B.** Organic acids. **C.** Sugars. Both cultivars generally showed similar behaviours. However, the non-sweetened cultivar exhibited a more rapid synthesis of sucrose from glucose, leading to a higher sucrose relative accumulation rate, suggesting sucrose metabolism might be involved in the mechanisms of senescent sweetening.

Results showed that ^{13}C accumulated in the amino acids glycine, aspartic acid, and glutamic acid at 5 and 10 weeks of storage in both cultivars. However, the increase in glycine and aspartic acid was higher in VR 808, the senescent sweetening resistant cultivar. Moreover, VR 808 presented a higher overall accumulation of label in glutamine during storage, while Arsenal exhibited increased accumulation of ^{13}C at 53 weeks of storage. Hence, the results suggested that although both cultivars exhibited similar behaviours during storage, glucose had greater net flux into the synthesis of amino acids in VR 808 tubers.

Malic acid synthesis from glucose was stable in Arsenal during storage, exhibiting a decrease after 43 of storage. On the contrary, VR 808 showed no clear pattern in ^{13}C accumulation in malic acid. In addition, both cultivars presented opposite behaviours regarding the synthesis of citric acid. At 10 weeks of storage, accumulation of ^{13}C in citric acid decreased in Arsenal while it increased in VR 808, showing the highest peak of accumulation. At 43, and 53 weeks after storage accumulation of label in citric acid increased in Arsenal and decreased in VR 808 tubers.

Interestingly, at the beginning of the storage period, VR 808, the senescent sweetening resistant cultivar, exhibited a quicker conversion of glucose into sucrose than Arsenal that was susceptible to senescent sweetening. After 10 weeks of storage, both cultivars decreased the sucrose accumulation followed by a second decrease at 53 weeks of storage. However, VR 808 showed higher accumulation of sucrose at all time points during the storage compared to Arsenal.

5.4 Metabolome profiling during long-term storage in season 3 (2018/2019)

Phytochemical diversity was examined by GC/MS in tubers of cultivars Arsenal, VR 808, Pirol, SH C 909, Lady Rosetta, Brooke, Shelford (both Shropshire and Yorkshire locations) as well as the unknown cultivar at 5, 30, 37, and 43 weeks of storage. Three different biological replicates were used for each cultivar and time point. Polar extracts (mainly sugars, organic acids, and amino acids) and non-polar extracts (mainly fatty acids and fatty alcohols) were examined. In the tubers analysed in this study, a total of 124 metabolites were detectable (74 polar and 50 non-polar), of which 77% could be identified. Metabolites, whose abundance was changed in a statistically significant manner over time, were identified using two-way ANOVA based on cultivar and time of storage. Cultivars showed differences ($P <$

0.05) in 94 metabolites during the storage period. In addition, a total of 106 metabolites were significantly ($P < 0.05$) influenced by the time of storage (Supporting Information Table S5-2).

PCA was used to summarise broad-scale variation among the cultivars and time points using all the metabolites simultaneously, and both polar and non-polar compounds independently. PCA was also used to identify which of the components accounted for specific differences among the cultivars and time points. All loadings scores are provided in Supporting Information Tables S5-3.1 to S5-3.6.

For all data, the first and third Principal Components (PC1 and PC3) together were able to distinguish between all time points analysed (Figure 5-2). The loadings for PC1 are dominated by amino acids, more specifically valine, leucine, and isoleucine. Analysis of the loadings for PC3 showed the separation of time points is driven by the levels of α-glycerophosphate, fucosterol, and galactose.

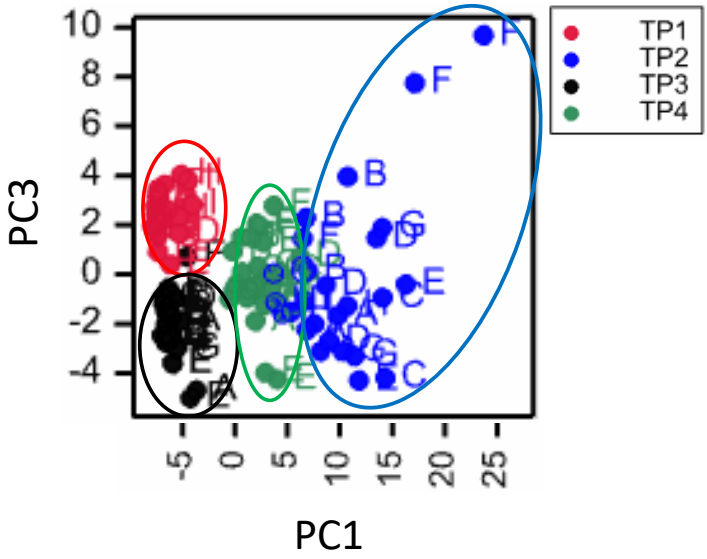


Figure 5-2. Selected score plot from PCA of all metabolites identified by GC/MS (polar/non-polar fraction) during long-term storage, with samples labelled according to time of storage. Plot of PC1 against PC3.Abbreviations: A, Pirol B, SH C 909; C, VR 808; D, Lady Rosetta; E, Shelford (Shropshire location); F, unknown cultivar; G, Brook; H, Arsenal; and I, Shelford (Yorkshire location). TP1, 5; TP2, 30; TP3, 37; and TP4, 43 weeks of storage.

For the polar compounds the first and fourth components separated all four time points into distinct groups (Figure 5-3A); 5, 30, 37, and 43 weeks of storage were separated. The separation of time points for PC1 was driven by glutamine, galactosyl glycerol, glycine, and β-alanine responses. The loading for PC4 are dominated by glutamine, α-glycerophosphate, galactosyl glycerol, galactose, and glycerol. In addition, the sixth and eighth separated the cultivars into two different groups (Figure 5-3B): cultivars from Shropshire location and cultivar from Yorkshire location. Analysis of the loadings showed the separation of cultivars is driven by galactose, α-glycerophosphate, and galactaric acid (for PC6), and galactose, and tyrosine (for PC8).

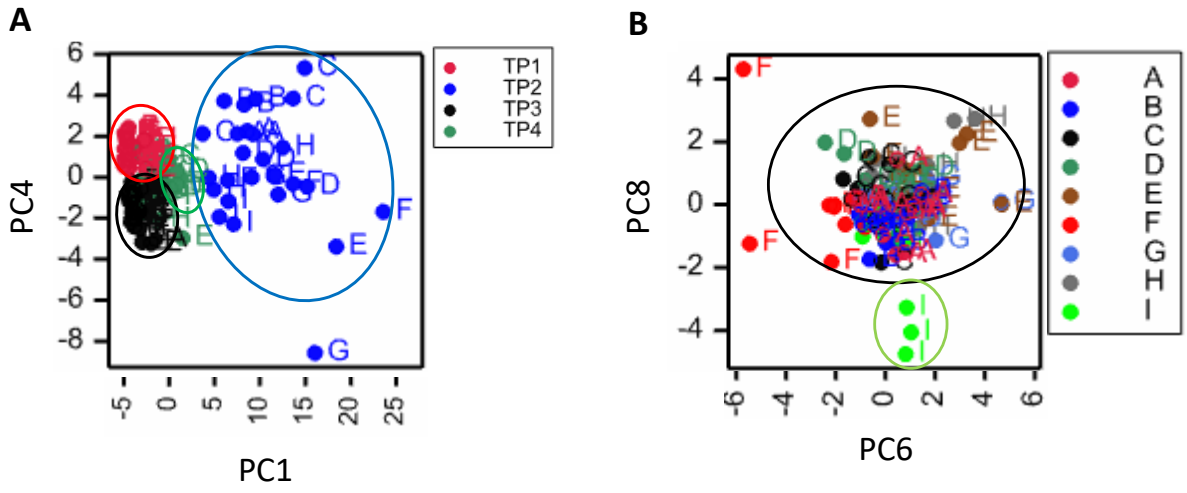


Figure 5-3. Selected score plots from PCA of polar metabolites identified by GC/MS, with samples labelled according to time of storage and cultivar. **A.** Plot of PC1 against PC4. **B.** Plot of PC6 against PC8. Abbreviations: TP1, 5; TP2, 30; TP3, 37; and TP4, 43 weeks of storage. A, Pirol B, SH C 909; C, VR 808; D, Lady Rosetta; E, Shelford (Shropshire location); F, unknown cultivar; G, Brook; H, Arsenal; and I, Shelford (Yorkshire location).

PCA of the non-polar data separate cultivars by location on PC3 vs. PC4 (Figure 5-4A) and time points on PC1 vs. PC2 (Figure 5-4B). The separation for PC3 is driven by fucosterol, and δ-5-avenasterol as well as unknown compounds. The compounds driving the separation by cultivar location for PC4 are fatty alcohols (heneicosanol, tetracosanol, and docosanol). The first and second components separate the time points into three distinct groups: 5 and 37 weeks of storage are separated from 30, and 43 weeks of storage. The loadings for PC1 are

dominated by saturated fatty acids, mainly eicosanoic, hexadecanoic, and heptadecanoic acids. The separation for PC2 is driven by unknown compounds.

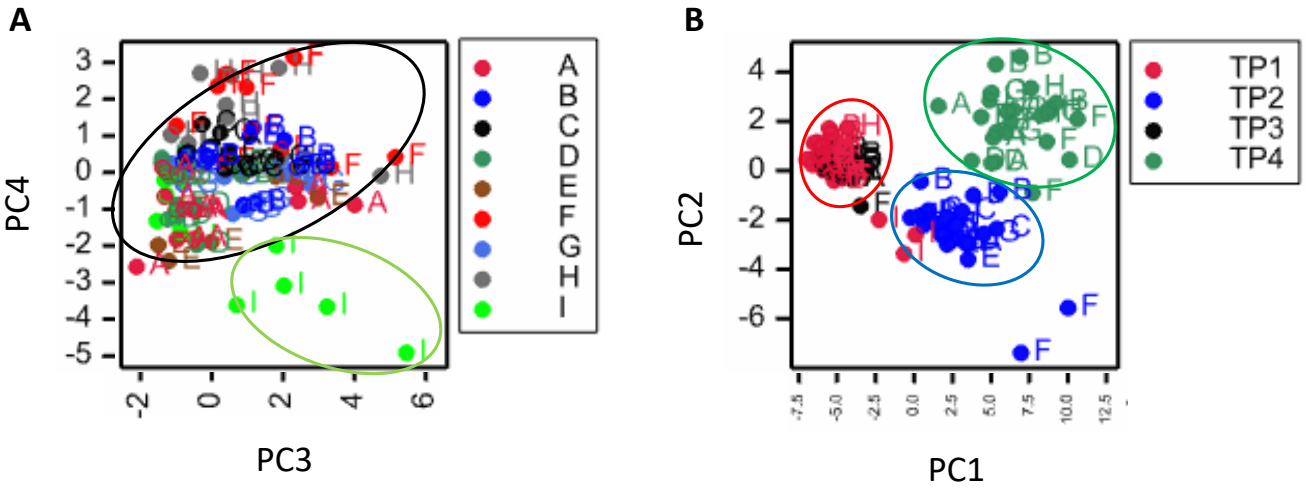


Figure 5-4. Selected score plots from PCA of non-polar metabolites identified by GC/MS, with samples labelled according to time of storage and cultivar. **A.** Plot of PC3 against PC4. **B.** Plot of PC1 against PC2. Abbreviations: A, Pirol B, SH C 909; C, VR 808; D, Lady Rosetta; E, Shelford (Shropshire location); F, unknown cultivar; G, Brook; H, Arsenal; and I, Shelford (Yorkshire location). TP1, 5; TP2, 30; TP3, 37; and TP4, 43 weeks of storage.

5.4.1 The influence of time of storage on metabolome profile

PCA were performed for each time point independently. Separation of cultivars was observed at 5 and 43 weeks of storage. PCA of 5 weeks after storage separate cultivars by location on PC1 vs. PC5 (Figure 5-5). Analysis of the loadings for PC1 showed the separation is driven by glutamine, galactosyl glycerol, glycine, and β-alanine. For PC5, the separation is driven by unknown compounds as well as pentadecanoic acid. Moreover, PCA of 43 weeks after storage separate cultivars by sugar profile (sweetening resistance or susceptibility (*Chapter 3*)) on PC1 vs PC3, and PC2 vs. PC3 (Figure 5-6A); and PC3 vs PC4, and PC3 vs PC5 (Figure 5-6B). The separation for PC1 is driven by amino acids: methionine, lysine, serine, histidine, isoleucine, tyrosine, tryptophan, glycine, valine, threonine, asparagine, and leucine as well as unknown compounds. The loadings for PC2 are dominated by fumaric and malic acids, hexacosanol, tetracosanol, phosphate, threonic acid, ethanolamine, and valine. Analysis

of the loadings for PC3 showed the separation is driven by inositol, sucrose, proline, fructose, glucose, phenylalanine, putrescine, and quinic acid as well as noctadecanoic and tetracosanoic acids, octacosanol, and β-sitosterol. The separation for PC4 is driven by unknown compounds, piperidinecarboxylic acid, cinnamic acid, hexadecanoic acid, and asparagine. The loadings for PC5 are dominated by unknown compounds, octacosanol, phenylalanine, galactosyl glycerol, β-sitosterol, octacosanoic acid, and γ-aminobutyric acid.

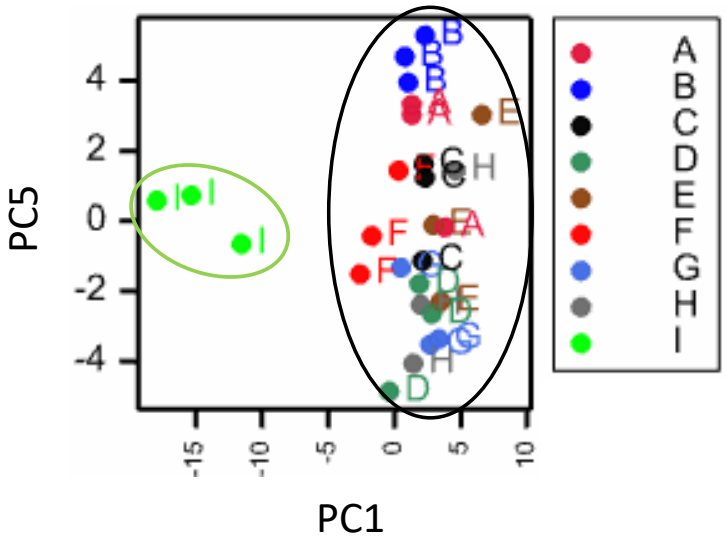


Figure 5-5. Selected score plot from PCA of all metabolites identified by GC/MS (polar/non-polar fraction) at 5 weeks of storage with samples labelled according to cultivar. Plot of PC1 against PC5. Abbreviations: A, Pirol B, SH C 909; C, VR 808; D, Lady Rosetta; E, Shelford (Shropshire location); F, unknown cultivar; G, Brook; H, Arsenal; and I, Shelford (Yorkshire location).

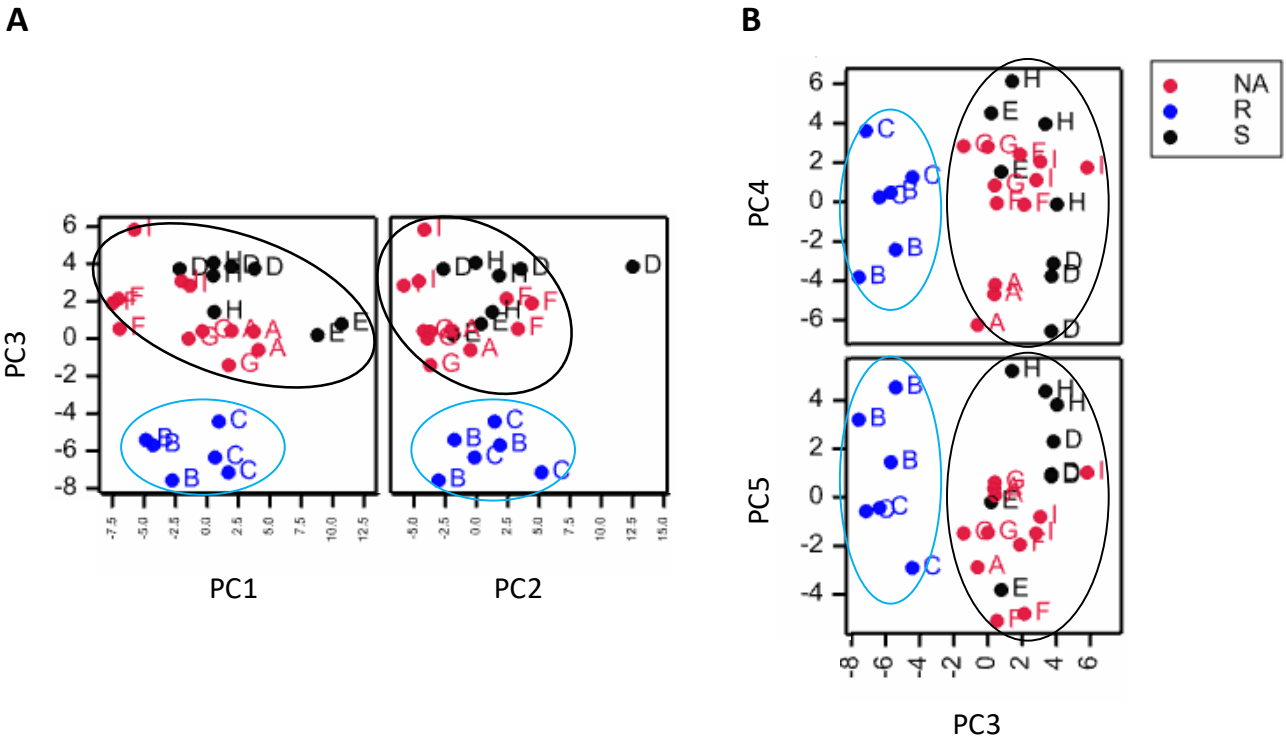


Figure 5-6. Selected score plots from PCA of all metabolites identified by GC/MS (polar/non-polar fraction) at 43 weeks of storage with samples labelled according to senescent sweetening susceptibility. **A.** Plot of PC1 against PC3; Plot of PC2 against PC3. **B.** Plots of PC3 against PC5; Plot of PC3 against PC4. Abbreviations: A, Pirol B, SH C 909; C, VR 808; D, Lady Rosetta; E, Shelford (Shropshire location); F, unknown cultivar; G, Brook; H, Arsenal; and I, Shelford (Yorkshire location). R, resistant to senescent sweetening; S, susceptible to senescent sweetening; and NA (not applicable), cultivars which showed an intermediate sugar content between R and S cultivars.

5.4.2 The influence of growing location on metabolome profile

Tubers of the Shelford variety grown either in Shropshire or Yorkshire are present in this study. In order to identify differences in propensity for sweetening, tuber metabolome profiles from both locations were compared by PCA. The first and fourth components separate the four time points into four different groups (Figure 5-7). The separation for PC1 is driven mainly by amino acids: β-alanine, valine, glycine, leucine, serine, isoleucine, ethanolamine, and aspartic acid. The compounds driving the separation for PC4 are nonacosanol, δ-5-avenasterol, solanid-5-enol, stigmastadienol, fucosterol, mannitol, α-linolenic acid, tetracosanoic, and octadecenoic acids. This result suggests that genotype and

storage are more significant than the growing environment in determining the potato tuber metabolome.

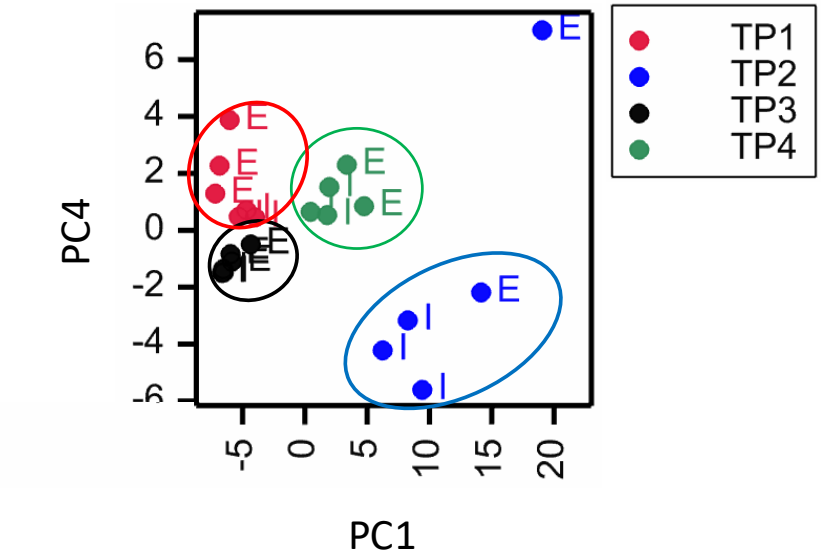


Figure 5-7. Selected score plot from PCA of all metabolites identified by GC/MS (polar/non-polar fraction), with samples labelled according to time of storage and cultivar. Plot of PC1 against PC4. Abbreviations: E, Shelford (Shropshire location); and I, Shelford (Yorkshire location). TP1, 5; TP2, 30; TP3, 37; and TP4, 43 weeks of storage.

Although within each time point group a minor separation of both cultivars is observed, PCA of each individual time point shows no separation based on location.

5.5 Discussion

In season 1 (2016/2017) and during the senescent sweetening transition, a general increase in amino acids was observed for both cultivars. During season 3 (2018/2019), differences in amino acids were observed between metabolite profiles of the cultivars. Results suggested that sugars profile may be related to the amino acids content since PCA separate resistant cultivars from the rest of the cultivars mainly by the amino acids content at 43 weeks after storage, during the senescent sweetening period. Variable trends in free amino acids contents in potato tubers during the storage have been previously reported (Talley *et al.*, 1984). Černá and Kracmar (2010) reported that the storage duration and cultivar have a significant effect on the total amino acids content. An increase in free amino acids content that occurred during

the latter part of long-term storage (for up to 40 weeks) has been related to an upturn of proteinase activity on the break of dormancy (Brierley *et al*., 1996).

As previously described, reducing sugars, such as glucose and fructose, react with free amino acids during high-temperature cooking and processing in the Maillard reaction (Nursten, 2005; Mottram, 2007; Halford *et al*., 2011). The relationship between reducing sugars, asparagine, and acrylamide formation in potato products during cooking and processing is complex. Asparagine is present approximately at one-third of the total free amino acid pool (Eppendorfer & Bille 1996; Oruna-Concha *et al*., 2001; Amrein *et al*., 2003; Elmore *et al*., 2007; Carillo *et al*., 2012; Halford *et al*., 2012; Muttucumaru *et al*., 2013). Due to this fact, sugar concentrations might be expected to be the limiting factor for acrylamide formation as asparagine is found in such a high concentration. However, the evidence is mixed. Some studies have reported sugar concentrations as the limiting factor (Amrein *et al*., 2003; Becalski *et al*., 2004; de Wilde *et al*., 2006), while others authors have observed asparagine concentration or asparagine concentration as a proportion of the total free amino acid pool to be also important (Elmore *et al*., 2007; Shepherd *et al*., 2010; Halford *et al*., 2012; Muttucumaru *et al*., 2014).

In addition, both cultivars exhibited an increase in organic acids after the onset of senescent sweetening. Wichrowska *et al*. (2009) described that the content of organic acids in potato tubers depends on cultivar and storage conditions. An increase in organic acids might be the result of reduced respiration as a consequence of mitochondrial damage produced by oxidative stress (Salvato *et al*., 2014).

On the contrary, total fatty acids decreased after 20 weeks of storage for both cultivars. Spychalla and Desborough (1990) revealed that potato cultivars with higher levels of fatty acid unsaturation had lower rates of membrane electrolyte leakage and lower sugar contents. However, at the present work both Arsenal and VR 808 exhibited similar unsaturated fatty acids while differences in reducing sugars content.

The fluxes of carbohydrate metabolism were measured by investigating the metabolism of [U-^{13}C] glucose in tuber discs in season 2 (2017/2018). During long-term storage, ^{13}C mainly accumulated in the amino acids glycine, aspartic acid, glutamic acid, and glutamine; the organic acids citric acid, and malic acid; and the sugar sucrose. Both cultivars exhibited

similar behaviours in the metabolism of [U-^{13}C] glucose. However, the senescent sweetening resistant cultivar VR 808 showed a higher synthesis of amino acids in some cases, suggesting this process as a mechanism to sink the glucose and avoid its accumulation. Besides, VR 808 exhibited an overall quicker accumulation of sucrose over time compared to Arsenal, a senescent sweetening susceptible cultivar. A progressive decrease in PGM activity was observed in VR 808 during storage in season 1 (2016/2017) (*Chapter 6*). In potato tubers, the reduction in the activity of plastidial PGM leads to both a reduction in starch accumulation and an increased sucrose accumulation (Fernie *et al.*, 2001). The accumulation of sucrose observed in VR 808 could be related to a reduction in the PGM activity, suggesting the accumulation of reducing sugars and sucrose observed in Arsenal might be due to different mechanisms.

Chapter 6: Changes in transcript levels associated with long-term storage in potato tubers

6.1 Introduction

The accumulation of reducing sugars in potato tubers during storage is a persistent and costly problem for the potato processing industry (Dale & Bradshaw, 2003). An unacceptable dark and bitter-tasting product is formed at high frying temperatures because of the Maillard reaction that takes place between the reducing sugars and amino acids (Shallenberger *et al*., 1959). Moreover, the Maillard reaction generates acrylamide, a neurotoxin and a potential carcinogen (Mottram *et al*., 2002; Stadler *et al*., 2002). In particular, potato crisps have high acrylamide contents (Rosen & Hellenäs, 2002; Tareke *et al*., 2002). Reducing sugars and asparagine are the two major substrates for acrylamide formation in processed potato products (Goekmen & Palazoglu, 2008). Developing methods to reduce acrylamide in fried potato products has become an important requirement for the potato processing industry. One effective way to decrease acrylamide content is to decrease the amount of reducing sugars in raw tubers (Matsuura-Endo *et al*., 2006; Muttucumaru *et al*., 2008). Hence, reducing sugar accumulation in stored potato tubers during CIS and SS is a critical factor influencing the quality of fried potato products.

Chen *et al*. (2001) published a potato molecular-function map for carbohydrate metabolism and transport, opening the way to find a candidate-gene approach to cold-sweetening (Menendez *et al*., 2002). This strategy aims to correlate allele variants at candidate loci with observed variations in fry colour and glucose and fructose content in potato germplasm. The use of molecular markers (e.g., restriction fragment length polymorphism, random amplified polymorphic DNA, simple sequence repeats, and amplified fragment length polymorphism) in linkage and QTL analyses has enabled increasingly detailed analyses of the potato genome. This has allowed the localization of many single gene and quantitative traits. In many cases, the use of molecular markers linked to genes controlling traits should enable a move away from conventional phenotypic selection to genotypic selection, not only for major genes but also for complex quantitative traits. However, QTL analysis is not precise, and the confidence interval can contain thousands of putative genes, which, by definition, could all be candidates for the target trait. Fine mapping within a QTL region can reduce the numbers to a few hundred candidate genes. A candidate-gene approach uses information relating to the

phenotype and its underlying biochemical or physiological basis, the genetic factors involved and the main genes likely to be involved in the trait.

Molecular physiological approaches have facilitated significant advances in the understanding of the processes that regulate sucrose to starch interconversion in the potato tuber (Fernie *et al*., 2002). The prospects are good for manipulating carbohydrate metabolism in potato tubers by conventional breeding and by transgenic approaches to achieve higher starch yields and to produce designer starches (Hamernik, 1998; Love *et al*., 1998; Hamernik *et al*., 2009; McCann *et al*., 2010). Therefore, thanks to the biotechnology techniques and breeding, besides the storage of potato tubers at higher temperatures with the use of sprout suppressants, the problem of CIS has been mostly solved. However, literature regarding SS is sparse and almost nothing is known about its mechanisms. In this study, a global analysis microarray technique was used to monitor the effect of long-term storage in potato tubers and indicate candidate genes associated with the trait.

6.2 Microarray analysis of gene expression

SS is genotype dependent, but the genetic basis remains uncertain. In order to gain further insights into the transcriptional networks associated with the accumulation of sugars in potato tubers during long-term storage observed in this study, microarray experiments were designed to identify genes that were differentially expressed during the SS transition for seasons 1 and 2. Four tubers of varieties Arsenal and VR 808 were used for each sampling time during season 1 (March and May 2017) and season 2 (May, July and October 2018) including SS transition. Spot flags from FE software (present or marginal) were used to remove probes with no consistent expression, leaving 20,634 and 20,321 probes for season 1 and 2, respectively. Statistical filtering was performed using volcano analysis (P-value < 0.01, fold-change > 2×) for season 1 data. In addition, one-way ANOVA using storage time as parameter was used in both seasons to identify statistically significant expression profiles at a false discovery rate (P-value) ≤ 0.05. Data were visualised using PageMan (Usadel *et al*., 2006) and a gene tree heat map in GeneSpring using default Pearson correlation. MapMan is a software tool that supports the visualization of profiling data sets in the context of gene ontologies and gene-by-gene basis on schematic diagrams of biological processes (Usadel *et al*., 2009). The PageMan module uses the same ontologies to statistically evaluate responses at the pathway or processes level.

6.2.1 Gene expression and biological processes influenced by long-term storage during season 1 (2016/2017)

In order to identify genes that may be related to the accumulation of sugars during long-term storage, two time points corresponding to the senescent sweetening transition were selected. The ratio of gene expression at 26 weeks relative to 20 weeks of storage were under study to identify genes that were significantly up- or down-regulated following senescent sweetening. During SS transition, between 20 and 26 weeks after storage, several processes were altered in Arsenal and VR 808 tubers (Figure 6-1). Significant changes in expression were observed for a total number of 329 genes in Arsenal and 224 genes in VR 808. Arsenal showed up-regulation in genes associated with photosynthesis. On the contrary, major carbohydrate metabolism, DOF transcription factors, and pseudouridine synthesis were down-regulated in Arsenal. On the other hand, over-expression of transcripts associated with secondary metabolism was observed in VR 808. Moreover, both cultivars exhibited an up-regulation in genes related to hormone metabolism and ethylene synthesis-degradation.

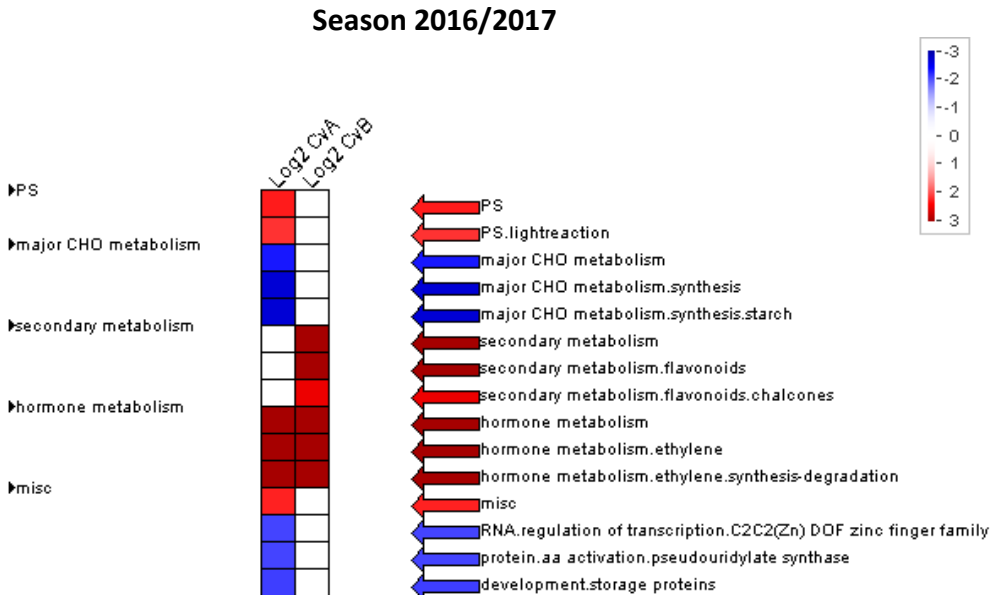


Figure 6-1. PageMan diagram representing changes in potato tubers during senescent sweetening transition for season 1 (2016/2017). Wilcoxon rank sum test (Benjamini-Hochberg corrected) was employed to identify BINs whose contents were differentially regulated.

Fig. 6-1. Continuation. Each coloured block represents an individual BIN/sub-BIN or gene in the PageMan analysis. The colour represents the direction and strength of the regulation (Logarithm to the base 2 colour scale; Red, highly up-regulated and Blue, highly down-regulated). Abbreviations: CvA, Arsenal; and CvB, VR 808.

Genes associated with photosynthesis, *PSBO2*, and *PETE2*, were up-regulated in Arsenal. *PSBO2* encodes a protein which is an extrinsic subunit of photosystem II (PSII) in Arabidopsis, and which has been proposed to play a central role in stabilization of the catalytic manganese cluster, which is the primary site of water splitting. In *Arabidopsis thaliana*, mutants defective in this gene have been shown to be affected in the dephosphorylation of the D1 protein of the photosystem II (Lundin *et al*., 2007). PETE2 is a recombination and DNA-damage resistance protein. One of two plastocyanin genes reported in Arabidopsis (Pesaresi *et al*., 2009). It is thought to be post-transcriptionally regulated via copper accumulation and is associated with copper homeostasis (Abdel-Ghany, 2009). Although these transcripts associated with photosynthesis have been observed to significantly change during the senescent sweetening transition, *PSBO2* and *PETE2* showed very low fluorescence levels, suggesting little relevance for potato tubers.

VR 808 exhibited up-regulation in transcripts associated with flavonoids synthesis. Chalcone synthase (CHS, EC 2.3.1.74) and flavanone 3-hydroxylase (F3H, EC 1.14.11.9) genes were over-expressed. CHS is a key enzyme of the flavonoid/isoflavonoid biosynthesis pathway (Dao *et al*., 2011). F3H plays important roles in flavonoid biosynthesis (Owens *et al*., 2008; Flachowsky *et al*., 2012).

Both cultivars exhibited up-regulation in jasmonic acid oxidase 2 (JAO2) and Acyl-CoA N-acyltransferases (NAT, EC2.3) genes. JAO2 plays a major role in repressing jasmonic acid (JA)-dependent defences in non-stimulated leaves (Smirnova *et al*., 2017). In cotton, NAT influences fertility by regulating lipid metabolism and JA biogenesis.

Major carbohydrate metabolism genes down-regulated in Arsenal during the onset of SS were associated with starch synthesis, including granule-bound starch synthase 1 (EC 2.4.1.242), 1,4-α-glucan branching enzyme (EC 2.4.1.18), *APL3* and *ADG1*. *APL3* and *ADG1* encode the large and the small subunits of AGPase 1, respectively. The large subunit catalyses the first, rate limiting step in starch biosynthesis whereas the small subunit is the catalytic

isoform responsible for AGPase activity. The presence of the small subunit is required for large subunit stability. Therefore, these changes observed in major carbohydrate metabolism are a potential reason for the sugars accumulation reported in Arsenal.

6.2.1.1 Effect of long-term storage on carbohydrate metabolism during season 1 (2016/2017)

As significant changes were found in carbohydrate metabolism during senescent sweetening transition for season 1 (2016/2017), different carbohydrate metabolism pathways were investigated. Changes associated with carbohydrate metabolism might underpin the transition to sweetening. Therefore, genes associated with sucrose-starch metabolism (involved in sugars recycling) and glycolysis pathways (related to turnover of sugars) were under study. A comparison of significant changes observed for Arsenal and VR 808 in sucrose-starch metabolism and glycolysis pathways is presented in Figure 6-2, and 6-3, respectively. In terms of sucrose-starch metabolism, Arsenal showed a strong down-regulation in AGPase (large subunit), and 1,4-α-glucan branching enzyme. Moreover, both cultivars exhibited down-regulation in α-amylase 2 (EC 3.2.1.1) as well as up-regulation in vacInv. Regarding the glycolysis pathways, similar behaviour for Arsenal and VR 808 were observed. However, Arsenal, which has a senescent sweetening susceptible profile, showed a strong down-regulation in GPT2. GPT2 is involved in the transport of glucose-6-phosphate into the plastids. The down-regulation observed in Arsenal could implicate that sugar phosphates are unable to be transported into the plastids, where they are used for starch synthesis. In addition, trehalose and T6P synthesis processes were down-regulated in Arsenal while up-regulated in VR 808. As previously described in *Chapter 1*, T6P is a signalling metabolite that regulates carbon metabolism, developmental processes, and growth in plants. More detailed information about differential gene expression for season 1, including carbohydrate metabolism and sugar transport genes, is shown in Figure 6-4.

6.2.1.2 Effect of long-term storage on cellular response during season 1 (2016/2017)

The main hypothesis of the present work is that senescent sweetening results from oxidative stress which leads to breakdown of amyloplast membranes leading to increased starch turnover and reduced capacity for respiration resulting in reduced sugar turnover. Although no differences in terms of markers of oxidative stress and/or oxidative damage were found

between Arsenal and VR 808 during long-term storage in season 1 (2016/2017), similar changes in antioxidant systems were observed in both cultivars (*Chapter 4*). Hence, genes associated with cellular response, including response to different stresses, were under study for this season (Figure 6-5). Arsenal and VR 808 exhibited comparable behaviours in genes associated with both biotic and abiotic stresses as well as redox during senescent sweetening transition, suggesting no differential expression in genes associated with stress and ROS detoxification. These data imply that differential stress responses are not associated with the onset of senescent sweetening.

6.2.1.3 Effect of long-term storage on SnRK genes during season 1 (2016/2017)

In addition to the differences previously observed between Arsenal (susceptible to sweetening) and VR 808 (sweetening resistant) cultivars, changes in sucrose non-fermenting-related protein kinase (SnRK) genes were observed (Figure 6-6). During the SS transition, SnRK3.15 was down-regulated while SnRK 2.6 was up-regulated. Both cultivars presented similar behaviours in SnRK genes expression. However, VR 808 had a stronger response within changes. Many studies have demonstrated that SnRK genes play various roles in the metabolism and development of plants (Halford & Hardie, 1998; Johnson *et al*., 2002; Mustilli *et al*., 2002; Boudsocq *et al*., 2004; Umezawa *et al*, 2004; Fujii & Zhu, 2009; Nakashima *et al*., 2009; Sun *et al*., 2010; Zheng *et al*., 2010; Fujii *et al*., 2011; Ghillebert *et al*., 2011).

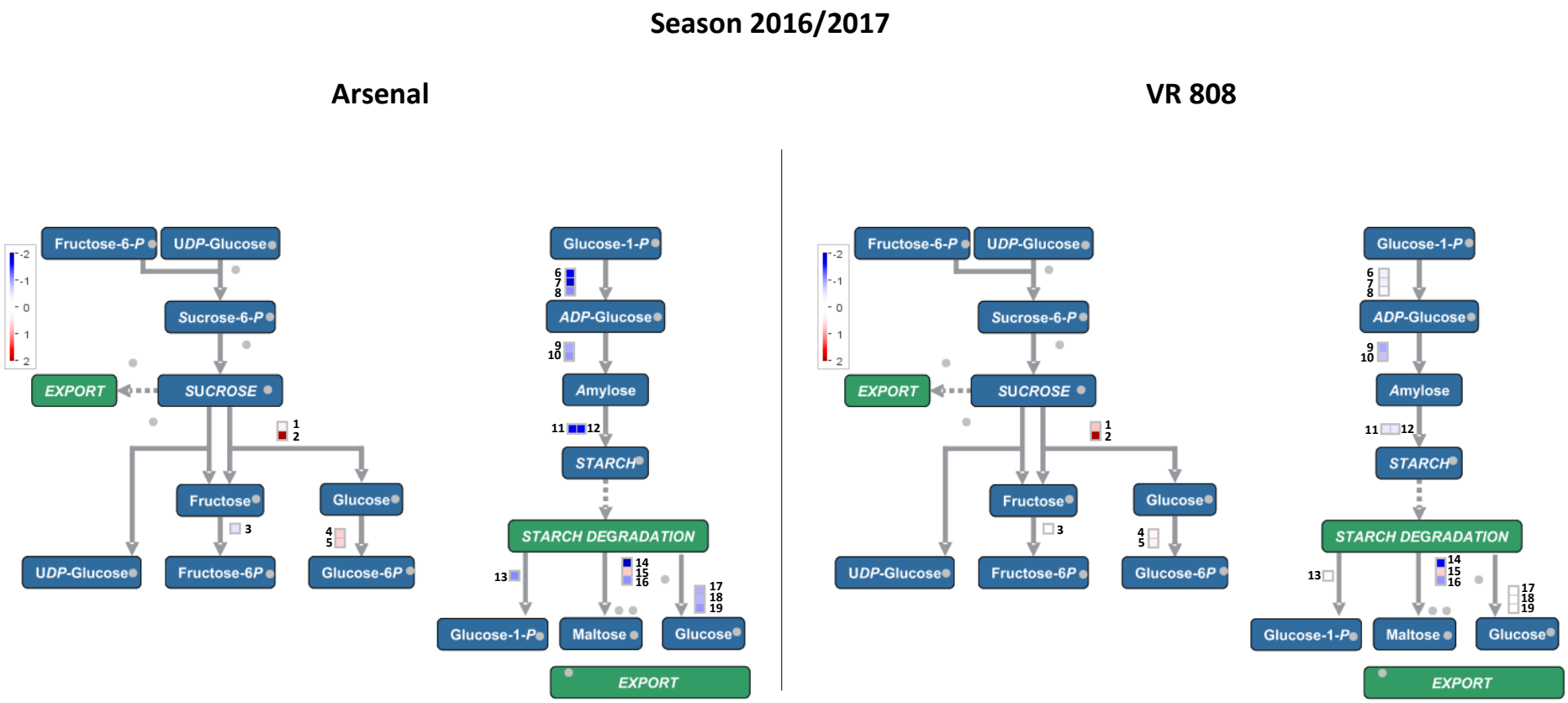
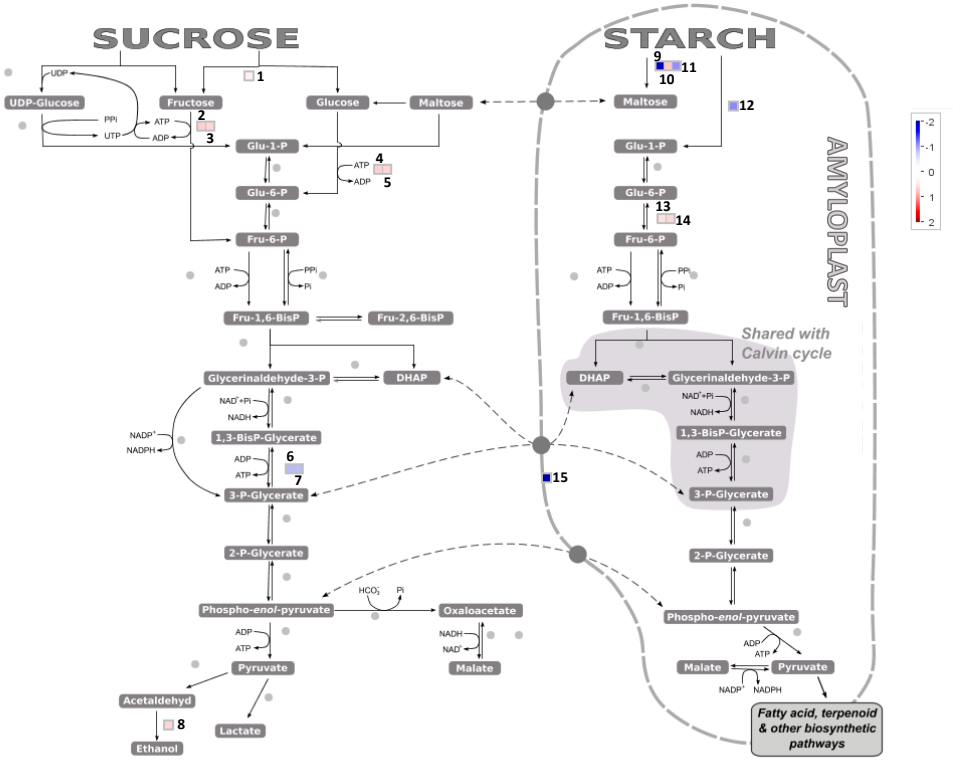


Figure 6-2. MapMan scheme representing sucrose-starch metabolism pathways gene expression for Arsenal and VR 808 tubers during senescent sweetening transition for season 1 (2016/2017). One-way ANOVA using time as factor was employed to identify significant changes during senescent sweetening transition. The colour represents the direction and strength of the regulation (Logarithm to the base 2 colour scale; Red, highly up-regulated and Blue, highly down-regulated). Abbreviations: 1, neutral invertase; 2, vacuolar invertase; 3, fructokinase; 4, hexokinase; 5, hexokinase; 6, ADP-glucose pyrophosphorylase (AGPase) (large subunit); 7, AGPase (large subunit); 8, AGPase (small subunit); 9, granule-bound starch synthase; 10, granule-bound starch synthase; 11, 1,4-α-glucan branching enzyme; 12, 1,4-α-glucan branching enzyme; 13, α-glucan phosphorylase; 14, α-amylase; 15, unknown protein; 16, α-amylase; 17, 4-α-glucanotransferase; 18, 4-α-glucanotransferase; 19, 4-α-glucanotransferase.

Season 2016/2017

Arsenal



VR 808

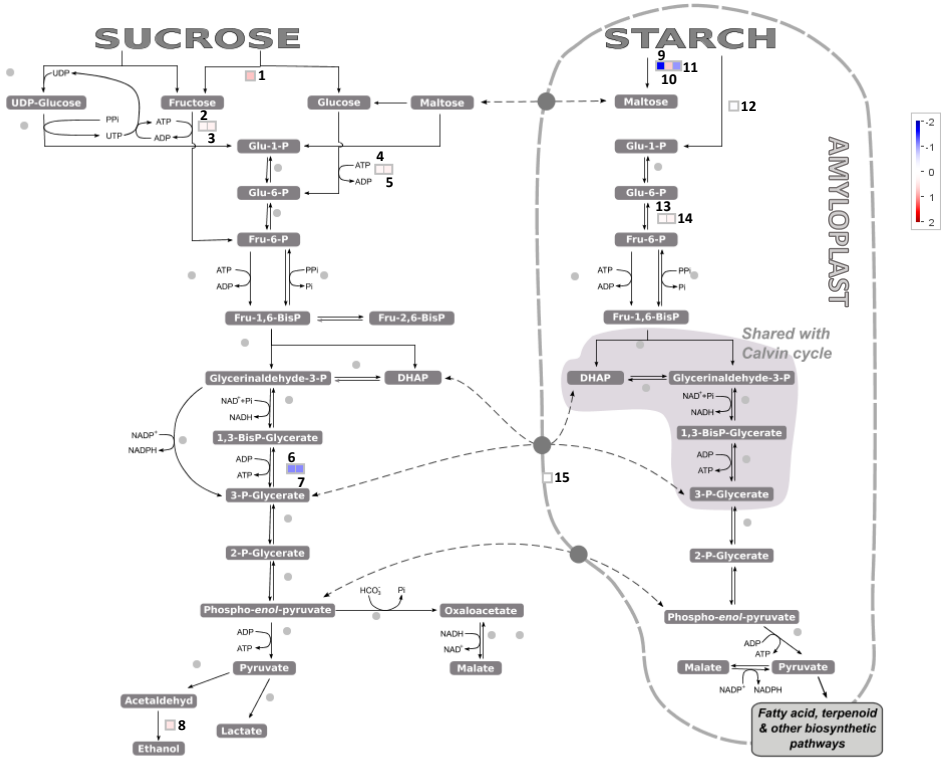


Figure 6-3. MapMan scheme representing glycolysis pathways gene expression for Arsenal and VR 808 tubers during senescent sweetening transition for season 1 (2016/2017). One-way ANOVA using time as factor was employed to identify significant changes during senescent sweetening transition. The colour represents the direction and strength of the regulation (Logarithm to the base 2 colour scale; Red, highly up-regulated and Blue, highly down-regulated). Abbreviations: 1, neutral invertase; 2, hexokinase; 3, hexokinase; 4, hexokinase; 5, hexokinase; 6, phosphatase; 7, phosphatase; 8, oxido-reductase; 9, α-amylase; 10, unknown protein; 11, α-amylase; 12, α-glucan phosphorylase; 13, phosphofructokinase; 14, phosphofructokinase; 15, glucose-6-phosphate translocator 2.

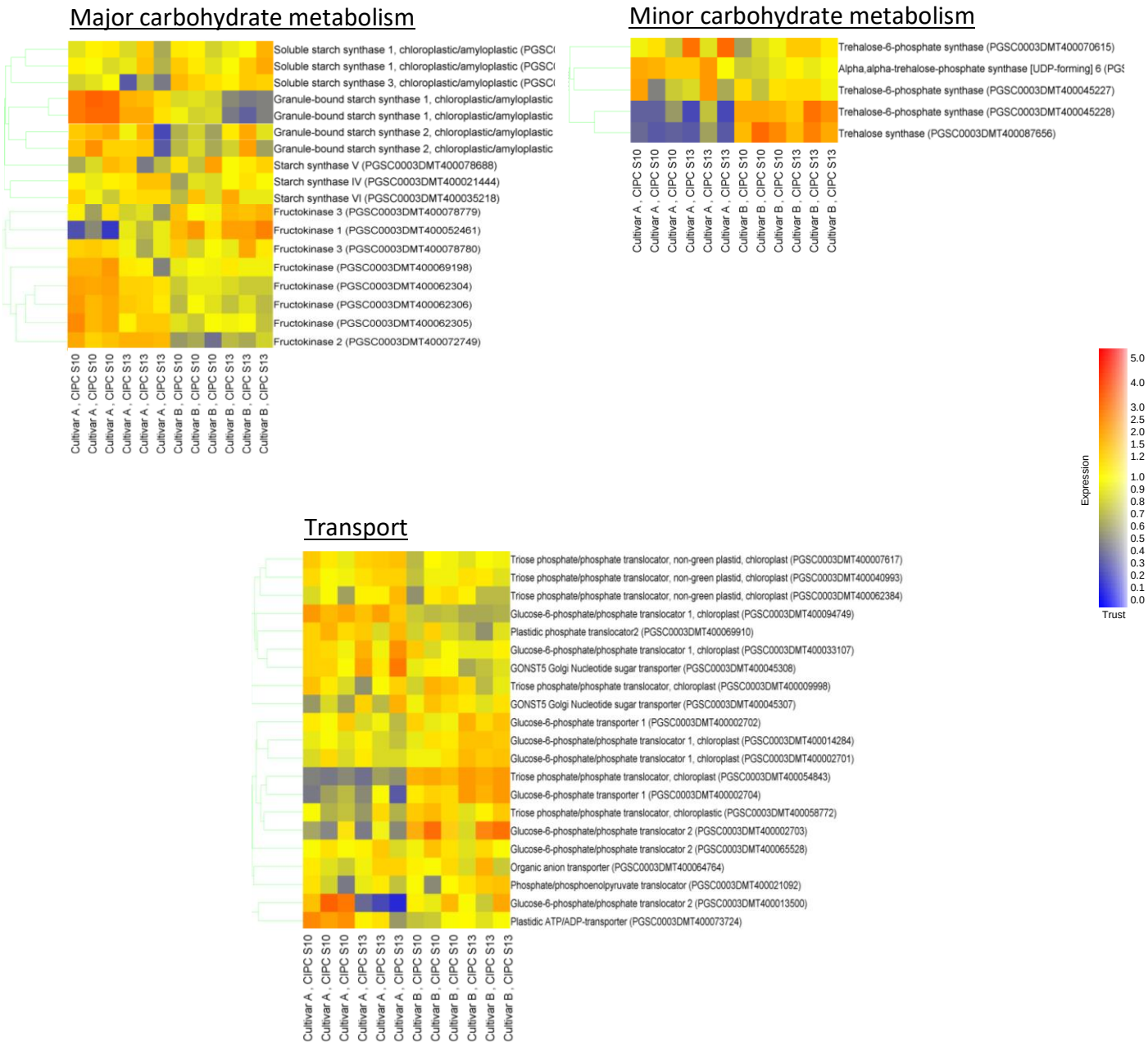


Figure 6-4. Carbohydrate metabolism and transport gene tree heat map. Comparison between susceptible to SS (Arsenal) and sugar stable (VR 808) profile cultivars during long-term for season 1 (2016/2017). Heat map was generated using GeneSpring GX software as described in Section *2.4.4.1* (Materials & Methods). The rows are labelled with individual gene function. The scale bar represents relative expression values. Red colour indicates genes that were up-regulated, and Blue colour indicates genes that were down-regulated according to the scale bar shown. Abbreviations: A, Arsenal; B, VR 808; S10, 20 weeks of storage; S13, 26 weeks of storage.

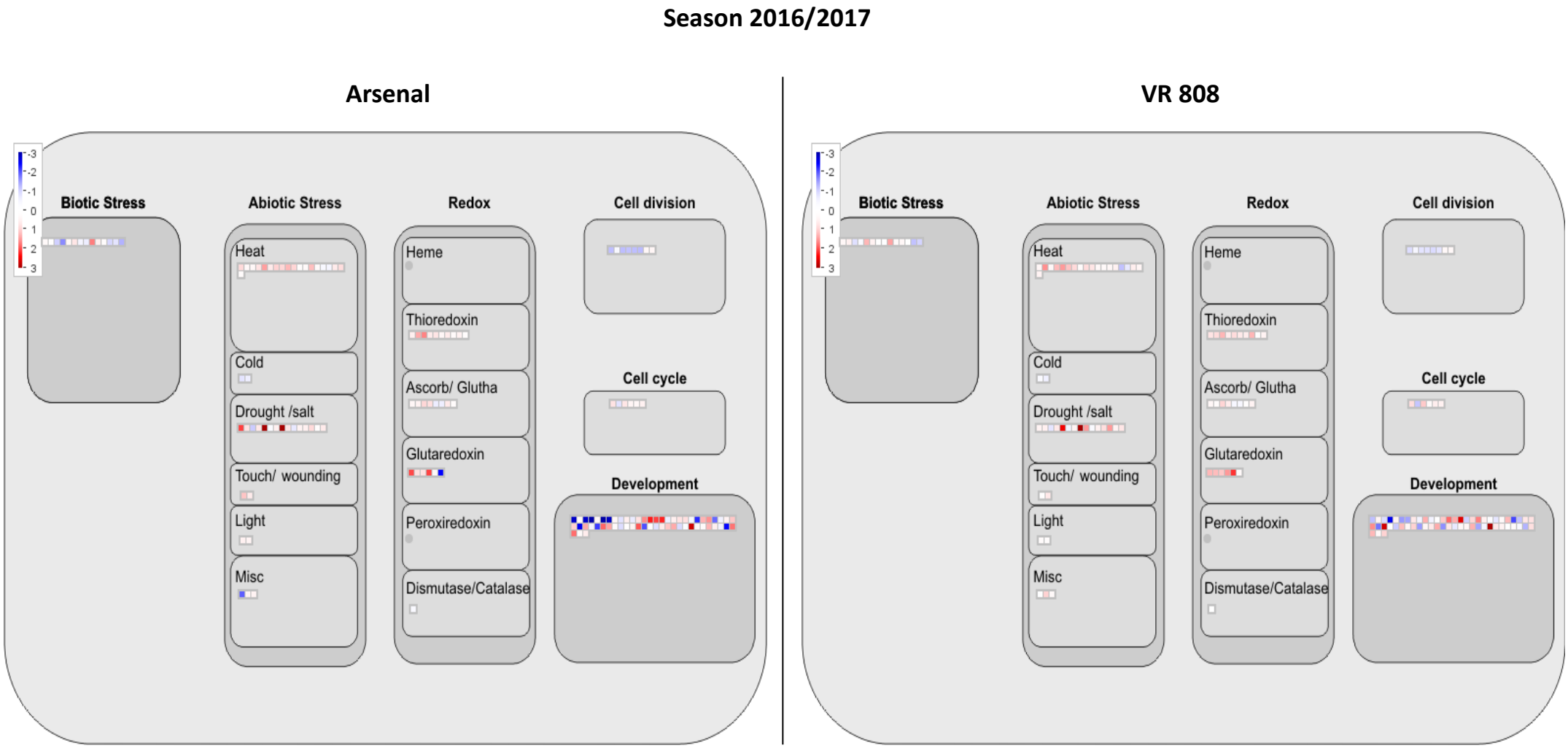


Figure 6-5. MapMan scheme representing cellular response gene expression for Arsenal and VR 808 tubers during senescent sweetening transition for season 1 (2016/2017). One-way ANOVA using time as factor was employed to identify significant changes during senescent sweetening transition. The colour represents the direction and strength of the regulation (Red, highly up-regulated and Blue, highly down-regulated as indicated on the logarithmic (base 2) scale bar shown).

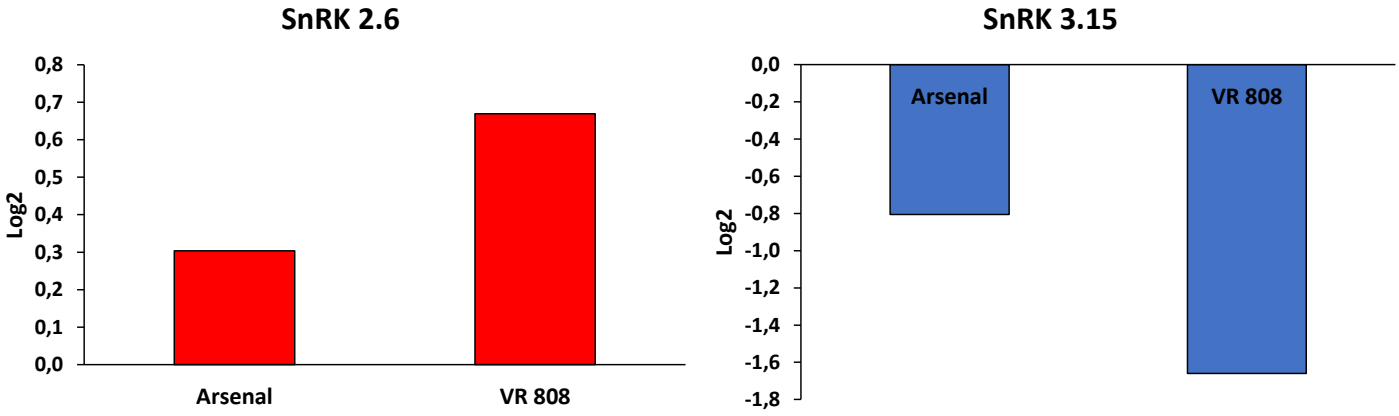


Figure 6-6. SnRK gene expression for Arsenal and VR 808 tubers during senescent sweetening transition for season 1 (2016/2017). One-way ANOVA using time as factor was employed to identify significant changes during senescent sweetening transition. The colour represents the direction of the regulation (Logarithm to the base 2 colour scale; Red, up-regulated and Blue, down-regulated).

6.2.2 Gene expression and biological processes influenced by long-term storage during season 2 (2017/2018)

During the second season, 4 different time points were under study. These time points were at 30, 37, 40 and 53 weeks after storage, corresponding to prior to sugar accumulation stage, senescent sweetening transition and late storage.

A total of 558 genes in Arsenal and 58 genes in VR 808 were differentially expressed over time. Significant changes were observed in genes associated with photosynthesis, major carbohydrate metabolism, lipid metabolism, metal handing, hormone metabolism, stress, nucleotide metabolism, regulation of transcription, DNA synthesis, signalling, development and transport (Figure 6-7).

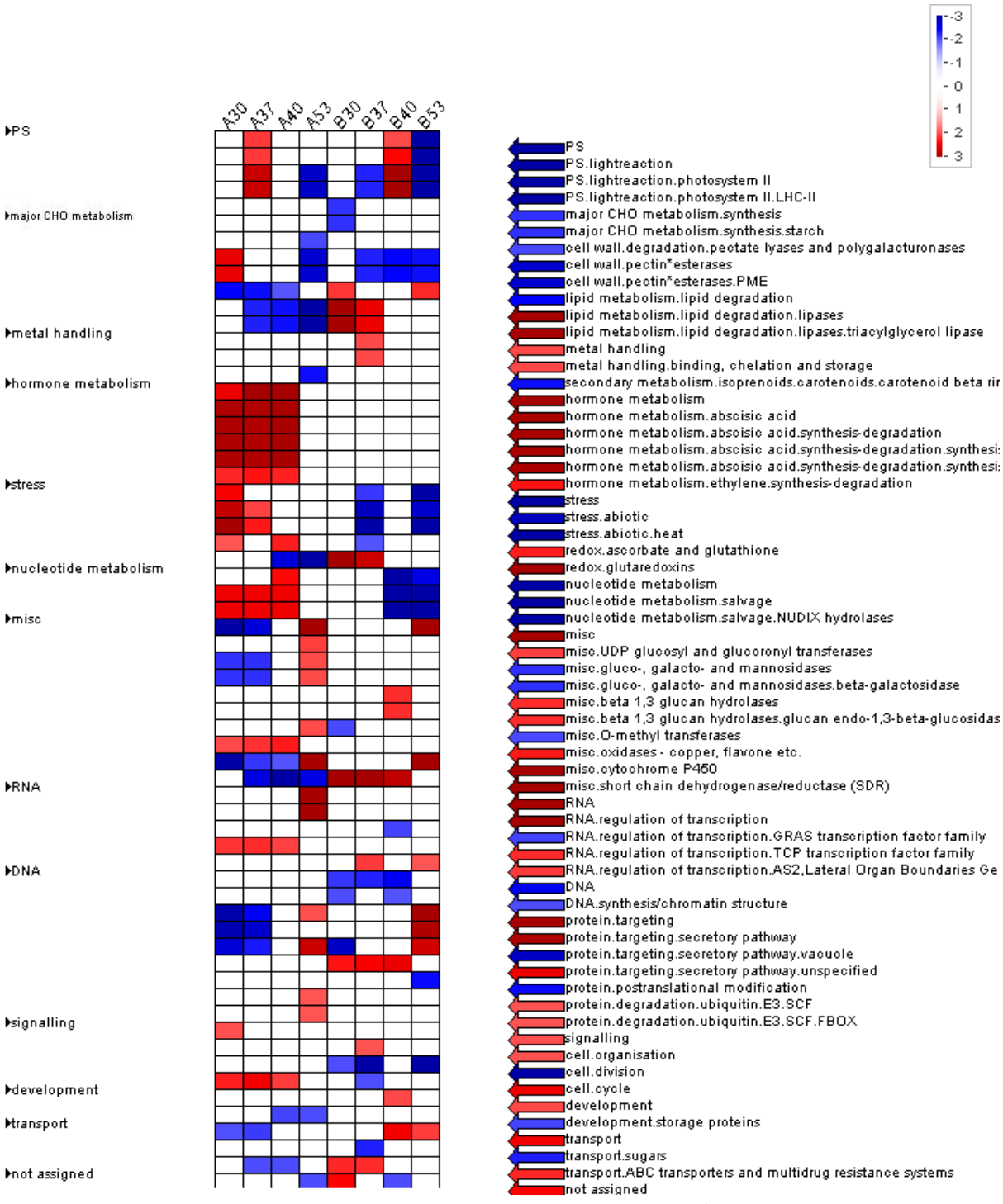


Figure 6-7. PageMan diagrams representing changes in potato tubers during long-term storage at 9°C. Differential gene expression of potato tubers during long-term storage in season 2 (2017/2018). A Wilcoxon rank sum test (Benjamini-Hochberg corrected) was employed to identify BINs whose contents were differentially regulated. Each coloured block represents an individual BIN/sub-BIN or gene in the PageMan analysis. The colour represents the direction and strength of the regulation (Red, up-regulated and Blue, down-regulated). Abbreviations: A, Arsenal; B, VR 808. 30, 37, 40, 53 represent weeks of storage.

6.2.2.1 Gene expression and biological processes influenced by long-term storage during season 2 (2017/2018) in Arsenal

In Arsenal, at 30, 37, and 40 weeks of storage, genes encoding the ABA biosynthetic enzymes 9-cis-epoxycarotenoid dioxygenase (NCED, EC 1.13.11.51) (Nambara & Marion-Poll, 2005) were up-regulated as well as gibberellin (GA) 20-oxidase (EC 1.14.11) genes. GA 20-oxidase activity is suggested to be one of the principal points of regulation in the GA-biosynthetic pathway (reviewed by Hedden & Kamiya, 1997). Moreover, teosinte branched1/cycloidea/proliferating cell factor (TCP) family of transcription factors genes were up-regulated. Endogenous ABA is involved in the regulation of wound-induced suberization and the processes that protect surface cells from water vapour loss and death by dehydration (Lulai *et al*., 2008). Both ABA and ethylene are required for dormancy induction, but only ABA is needed to maintain bud dormancy (reviewed by Suttle, 2004). TCP are involved in the regulation of cell growth and proliferation, performing diverse functions in plant growth and development and have been shown to be targets of pathogenic effectors and are likely to play a vital role in plant immunity (Bao *et al*., 2019). At 53 weeks after storage, protein degradation genes were up-regulated. Signalling genes encoding leucine-rich kinase family proteins were up-regulated at 30 weeks of storage. Wu *et al*. (2009) suggested these genes may participate in the responses against environmental stresses and disease resistance in potato. In addition, kip-related protein 3 (KRP3) genes, negative regulator of cell division (De Velder *et al*., 2001; Verkest *et al*., 2005; Weinl *et al*., 2005; Liu *et al*., 2008), were up-regulated at 30, 37, and 40 weeks after storage. Patatin group precursor genes involved in storage were down-regulated after the onset of senescent sweetening. Down-regulation was observed in genes encoding β-galactosidases (EC 3.2.1.23) at 30, and 37 weeks, followed by up-regulation at 53 weeks of storage. β-galactosidases are associated with fruit softening (Gross, 1984; Gross & Sams, 1984; Redgwell *et al*., 1992).

6.2.2.2 Gene expression and biological processes influenced by long-term storage during season 2 (2017/2018) in VR 808

VR 808 exhibited down-regulation in starch synthase (EC 2.4.1.21) and 1,4-α-glucan branching enzyme at 40 weeks of storage. Genes encoding pectin methyl esterase (PME, EC 3.1.1.11) were under-expressed at 37, 40, and 53 weeks of storage. Orthologous PME genes have been shown to impact on the texture of fruit from many species (reviewed in Fischer & Bennett, 1991). As pectin is a major component of the cell wall and the middle lamella, its structure is likely to be an important factor in texture in potato tubers as well as other plant tissues (Fischer & Bennett, 1991). Up-regulation in metal handing genes was observed at 37 weeks of storage. At 40 weeks of storage, GRAS family transcription factors were down-regulated. GRAS genes play diverse roles in root and shoot development, GAs signalling, and phytochrome A signal transduction (Bolle, 2004). At 30, and 40 weeks of storage, down-regulation in genes encoding WD-40 repeat family proteins, known to serve as platforms for the assembly of protein complexes or mediators of transient interplay among other proteins. In the model plant *Arabidopsis thaliana*, members of this superfamily are increasingly being recognized as key regulators of plant-specific developmental events (van Nocker & Ludwig, 2003).

At 30, 37 and 40 weeks after storage, genes encoding ADP-ribosylation factor (ARF) were up-regulated. ARF regulates metabolism and antioxidant capacity in transgenic potato tubers (Zuk *et al*., 2003). Transgenic plants resulted in the increase of soluble sugar-to-starch ratio parameter when compared to un-transformed plants (Zuk *et al*., 2003). Genes encoding protein phosphatase 2A (PP2A, EC 3.1.3.16) were down-regulated in VR 808 at 53 weeks of storage. PP2A may be involved in sucrose-phosphate synthesis (Reimholz *et al*., 1994). Genes encoding actin binding protein family associated with cellular organization were up-regulated at 37 weeks after storage. The actin cytoskeleton functions in the generation and maintenance of cell morphology and polarity, in endocytosis and intracellular trafficking, in contractility, motility and cell division (Winder & Ayscough, 2005). Cell division genes encoding regulator of chromosome condensation (RCC1) family protein were down-regulated at 30, 37, and 53 weeks after storage. In plants, RCC1 molecules act mainly as regulating factors for a series of downstream genes during biological processes such as the UV-B response and cold tolerance (Heijde & Ulm, 2012; Ji *et al*., 2015). Transport sugars

genes encoding major facilitator superfamily proteins were under-expressed at 37 weeks of storage. These proteins are involved in glucose transmembrane transport (Saier *et al*., 1999).

6.2.2.3 Differences between Arsenal and VR 808 in gene expression and biological processes influenced by long-term storage during season 2 (2017/2018)

Arsenal and VR 808 showed opposite behaviour in gene expression related to lipid degradation, abiotic stress, nucleotide metabolism, short chain dehydrogenase/reductase and transport. Gene expression was down-regulated for lipid metabolism, glutaredoxin, and transport in Arsenal and up-regulated in VR 808. On the contrary, stress abiotic and nucleotide metabolism were up-regulated in Arsenal and down-regulated in VR 808. The rest of significant changes in gene expression presented no clear pattern. For this season, no significant changes in SnRK genes were reported during the storage period.

6.2.2.4 Effect of long-term storage on carbohydrate metabolism during season 2 (2017/2018)

In terms of carbohydrate metabolism, differences in gene expression from the previous season were observed in the cultivars. Although no significant changes in gene expression were reported according to Wilcoxon rank sum test (Benjamini-Hochberg corrected), further details regarding sucrose-starch metabolism (Figure 6-8) and glycolysis pathways (Figure 6-9) were analysed. Arsenal showed a general down-regulation at 53 weeks of storage in AGPase (large and small subunits), starch synthase I, 1,4-α-glucan branching enzyme, and 4-alpha-glucanotransferase (EC 2.4.1.25). Furthermore, results in GPT2 expression were reproduced from season 1 (2016/2017). Arsenal exhibited a gradually and strong decrease in GPT2 transcripts whereas it had a little increase in VR 808 over the storage period (Figure 6-9). The GPT2 expression was progressively down-regulated over the storage period for the susceptible cultivar following the trend of reducing sugar accumulation. Additionally, down-regulation in triose-phosphate isomerase (TPI, EC 5.3.1.1) was observed in both cultivars after senescent sweetening transition, presenting VR 808 a higher response. Also for this season, trehalose and T6P synthesis processes were down-regulated in Arsenal while up-regulated in VR 808 (Figure 6-10).

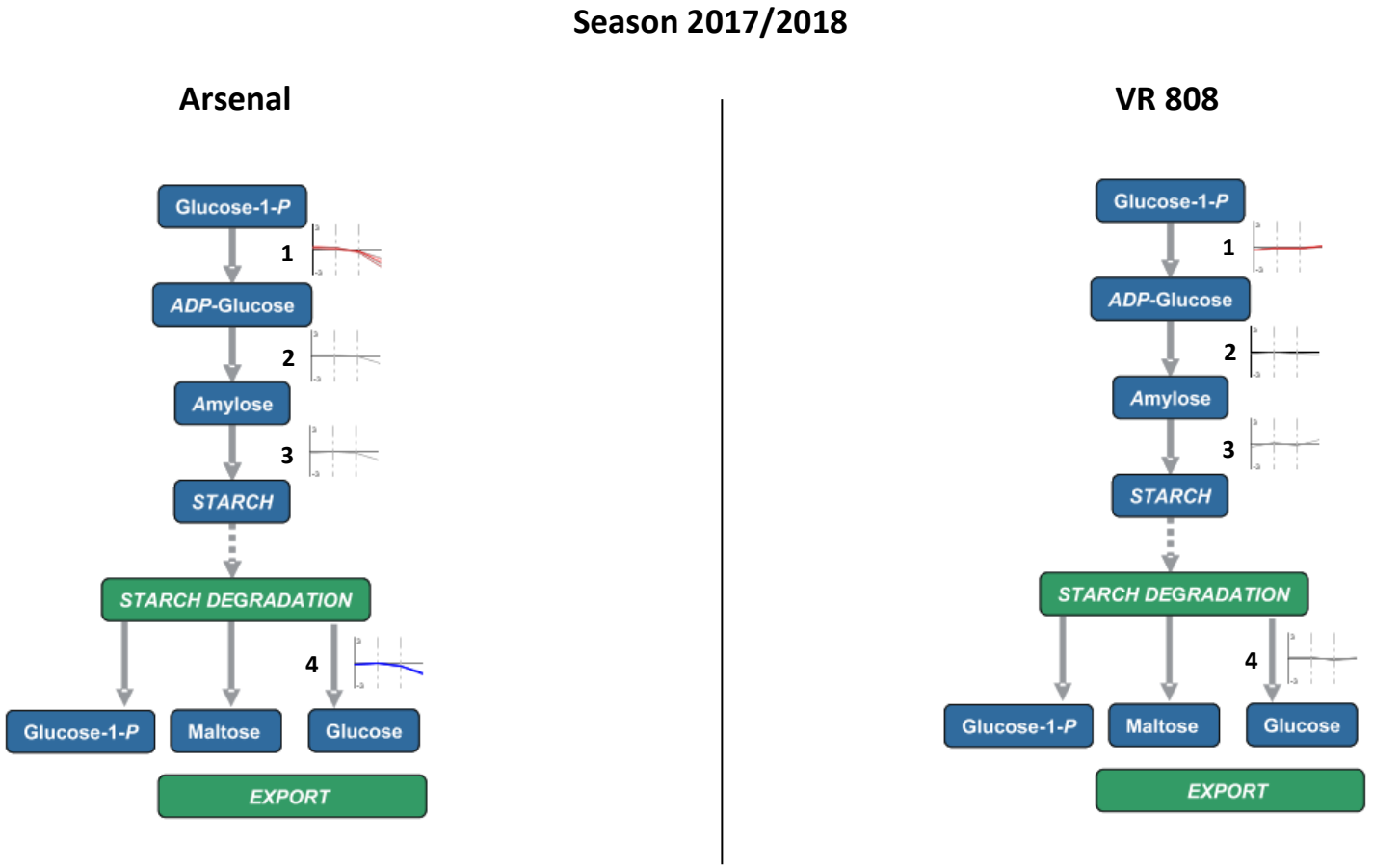


Figure 6-8. MapMan scheme representing starch metabolism pathway gene expression for Arsenal and VR 808 for season 2 (2017/2018). One-way ANOVA using time as factor (Benjamini-Hochberg corrected) was employed to identify significant changes over storage at 30, 37, 40, and 53 weeks of storage. The lines on the graphics represent the direction of the regulation. Abbreviations: 1, ADP-glucose pyrophosphorylase (AGPase, large and small subunits); 2, starch synthase; 3, 1,4-α-glucan branching enzyme; 4, 4-α-glucanotransferase.

Season 2017/2018

Arsenal **VR 808**

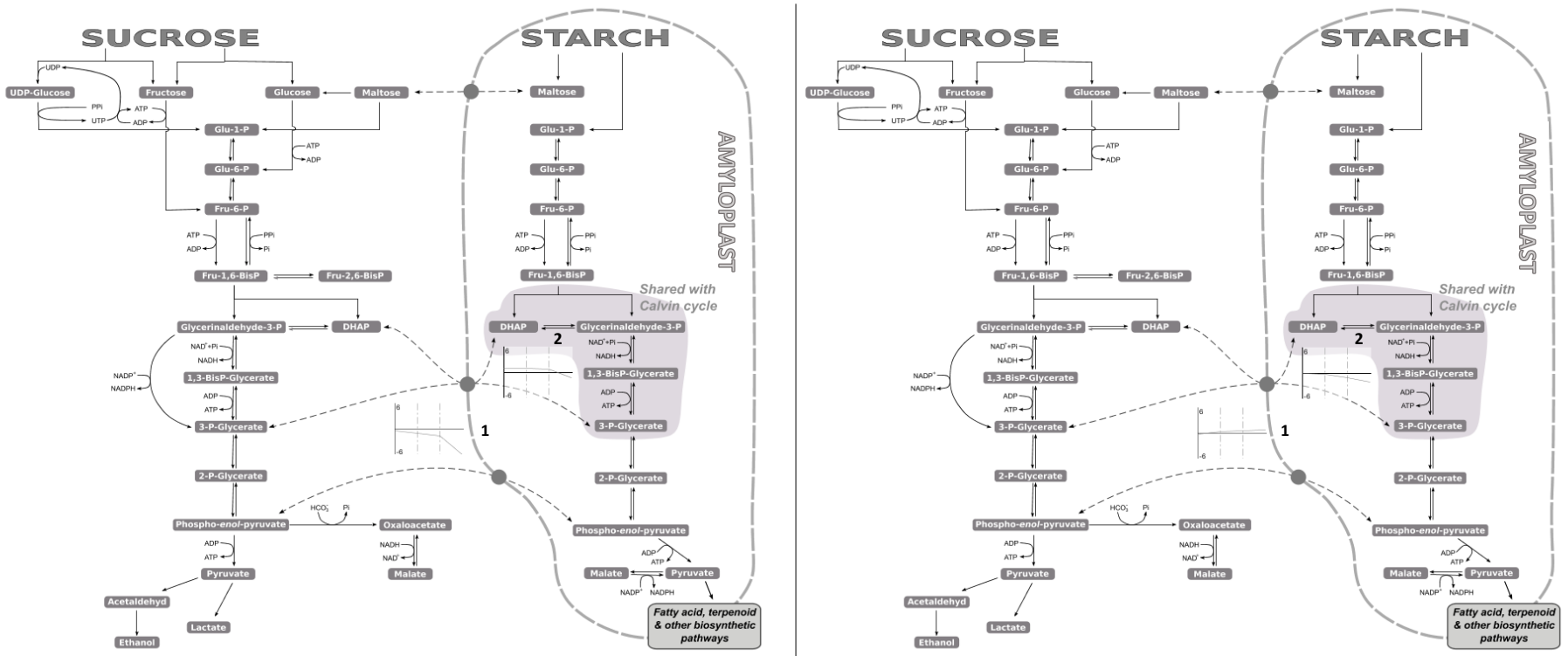


Figure 6-9. MapMan scheme representing glycolysis pathways gene expression for Arsenal and VR 808 for season 2 (2017/2018). One-way ANOVA using time as factor (Benjamini-Hochberg corrected) was employed to identify significant changes over storage at 30, 37, 40, and 53 weeks of storage. The lines on the graphics represent the direction of the regulation. Abbreviations: 1, glucose-6-phosphate translocator 2 (GPT2); 2, triose-phosphate isomerase (TPI).

Season 2017/2018

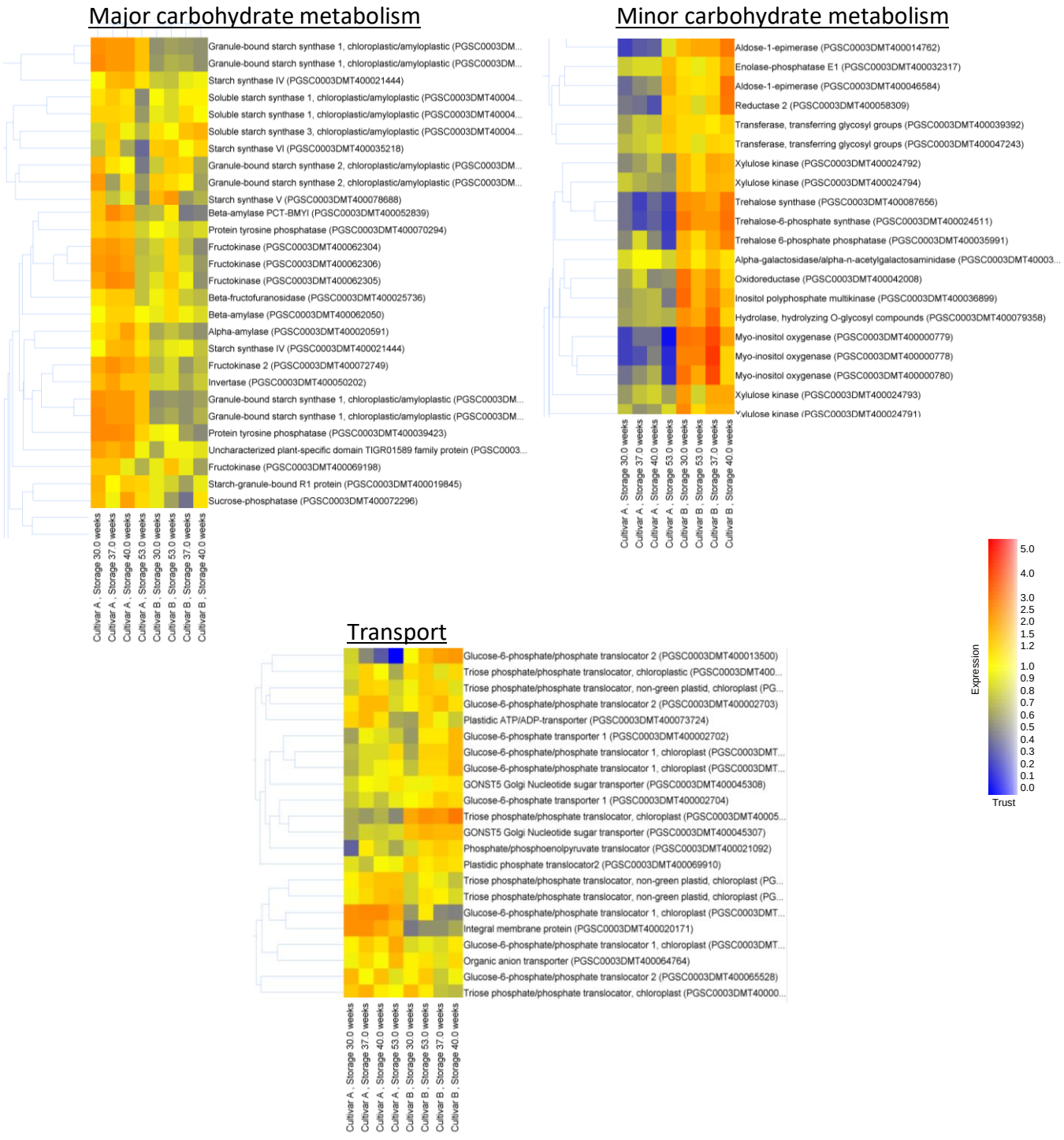


Figure 6-10. Carbohydrate metabolism and transport gene tree heat map. Comparison between susceptible to SS and sugar stable profile cultivars during long-term storage for season 2 (2017/2018). Heat map was generated using GeneSpring GX software as described in Section *2.4.4.1* (Materials & Methods). The rows are labelled with individual gene function. The scale bar represents relative expression values (fold change). Red colour indicates genes that were up-regulated, and Blue colour indicates genes that were down-regulated. Abbreviations: A, Arsenal; B, VR 808. 30, 37, 40, 53 represent weeks of storage.

GPT2 gene showed a gradual down-regulation over time in Arsenal, which accumulates reducing sugars. The decrease in gene expression was subsequently correlated to the reducing sugar accumulation during senescent sweetening transition. Hence, we suggested GPT2 to be a candidate gene involved in sugar accumulation during long-term storage. The initial identification of this GPT2 differential gene expression on the microarray was therefore checked and confirmed by qRT-PCR.

6.3 qRT-PCR analysis of GPT2

Analysis was performed using the same samples and time points from season 2 (2017/2018) microarray experiment. qRT-PCR data confirmed the results obtained by microarray analysis. GPT2 expression was decreasing over time in Arsenal (Figure 6-11A). Moreover, a further qRT-PCR analysis was performed using season 3 (2018/2019) material in order to identify GPT2 expression as a common mechanism associated to sugar accumulation during long-term storage. In this experiment, 2 susceptible to SS, and 2 with stable sugar profile cultivars, were selected and compared during the SS transition (Figure 6-11B). Results suggested down-regulation in GPT2 gene is a common mechanism in cultivar susceptible to senescent sweetening.

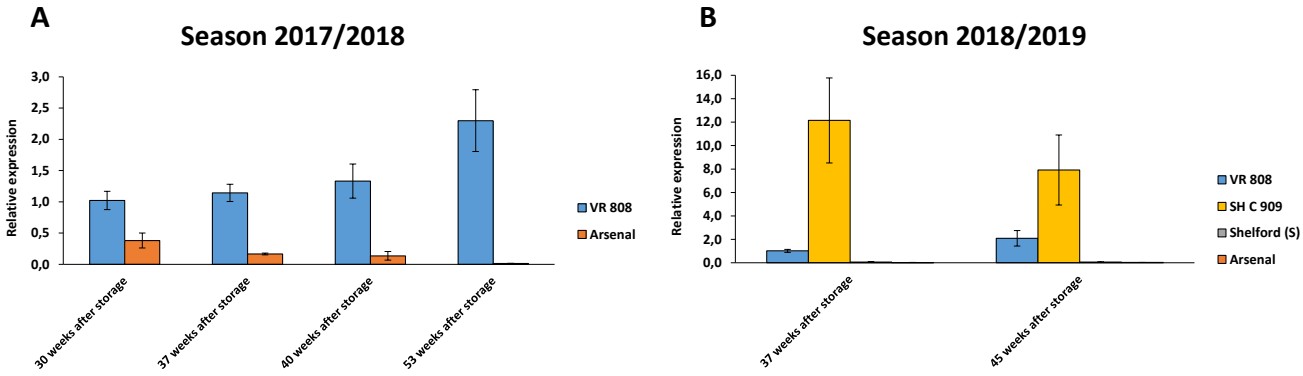


Figure 6-11. GPT2 expression in potato tubers during long-term storage at 9°C. All cultivars were from Shropshire location. **A.** Comparison between susceptible to SS and stable profile cultivars over time for season 2 (2017/2018). **B.** Comparison between 2 susceptible to SS and 2 stable profiles cultivars during SS for season 3 (2018/2019). Samples included the same cultivars studied previously. Results suggested GPT2 down-regulation is a common mechanism in susceptible to SS cultivars.

6.4 Analysis of the GPT2 promoter sequence

GPT2 promoter sequence was analysed using NSITE-PL (available through SoftBerry at http://www.softberry.com) to identify putative regulatory motifs. Inspection of the *GPT2* promoter sequence revealed the presence of a number of putative regulatory motifs which were used to identify similar promoters of other genes in order to obtain a better understanding of the regulatory mechanisms affecting GPT2 expression (Table 6-1).

Table 6-1. List of genes which exhibit similar regulatory motifs identified for *GPT2* promoter. Abbreviations: TFBS, transcription factor binding site; BF, transcription factor binding to TFBS; TFBS AC, TFBS accession number.

Organism	Gene	TFBS	BF	TFBS AC
Pisum sativum	*rbcS-3A*	G-box	GBF (CG-1)	RSP00313
Pisum sativum	*PSPAL2*	Box 1 homolog	Epicotyl-specific nuclear factor	RSP00739
Nicotiana tabacum	*RNP2*	CDE	Unknown nuclear factor	RSP00397
Zea mays	*Zc2*	Zc2 A/T-2	Unknown nuclear factor	RSP00492
Zea mays	*H3C4*	OCT	Unknown nuclear factor	RSP00845
Lycopersicon esculentum	*rbcS3C*	2' W1	Unknown nuclear factor	RSP00568
Solanum lycopersicum	*GAME4*	P box 2	JRE-4	RSP02845
Arabidopsis thaliana	*H4A748*	OCT	Unknown nuclear factor	RSP00839
Arabidopsis thaliana	*STK*	GA-6	BPC1	RSP00865
Arabidopsis thaliana	*C4H*	P-box 4	Unknown nuclear factor	RSP01229
Arabidopsis thaliana	*VDD*	CArG1	STK-SEP3	RSP02393
Petroselinum crispum	*CCoAOMT*	Box L	Unknown nuclear factor	RSP01044
Brassica oleracea	*BoCRC*	EM1 (CArG box 1)	MADS box proteins	RSP01214
Oryza sativa	*RSs1*	BoxII	RNFG2	RSP01435
Catharanthus roseus	*CrWRKY1*	CT-rich	Unknown nuclear factor	RSP02375

The five members of the rbcS gene family are rbcS1, rbcS2, rbcS3A, rbcS3B, and rbcS3C. This gene family encodes for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (3-phospho-D-glycerate carboxylyase (dimerizing) (Rubisco, EC 4.1.1.39), the key enzyme in photosynthetic carbon assimilation. The expression of this gene and other rbcS genes is regulated by at least three parameters: tissue type, light conditions, and stage of development (Tobin & Silverthorne, 1985; Kuhlemeier *et al*., 1987). The highest level of expression of this gene family, which is associated with photosynthesis, is found in

the leaf. Gene expression is of the rbcS gene family is turned off in non-photosynthetic tissues (Sugita & Gruissem, 1987).

Zhang *et al*. (2015) suggested that opaque2 (O2), O2 heterodimerizing proteins (OHPs), and prolamine-box binding factor (PBF) are master regulators of zein storage protein synthesis in maize (*Zea mays*), acting in an additive and synergistic mode.

PSPAL family (the genes encoding phenylalanine ammonia-lyase in Pisum sativum) have been shown to be activated by UV light while partially suppressed in response to a fungus pathogenic on pea suppressor (Yamada *et al*., 1992). Cinnamate-4-hydroxylase (C4H) is the first Cyt P450-dependent monooxygenase of the phenylpropanoid pathway. C4H expression is light-dependent, but it has been detectable in dark-grown seedlings. C4H is widely expressed in various Arabidopsis tissues, particularly in roots and cells undergoing lignification (Bell-Lelong *et al*., 1997). Ogawa *et al*. (2011) reported that the rice protein RSS1, whose stability is regulated depending on the cell cycle phases, is a key factor for the maintenance of meristematic activity under stressful conditions in rice. The transcription factor CrWRKY1 positively regulates the terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* (Suttipanta *et al*., 2011).

Steroidal glycoalkaloids (SGAs) are cholesterol-derived specialized metabolites produced by Solanaceous plant species. Wang *et al*. (2018) reported that that light-signalling transcription factors elongated hypocotyl 5 (HY5) and phytochrome interacting factor 3 (PIF3) regulate the abundance of steroidal SGAs by modulating the transcript levels of GAME genes, associated with glycoalkaloid metabolism.

6.5 Assessment of transcriptome profiling results

Results from transcriptome profiling during season 1 (2016/2017) indicated differences in carbohydrate metabolism and flavonoids biosynthesis between Arsenal (susceptible to sweetening profile) and VR 808 (resistant to sweetening profile). In order to obtain further insights of whether this differential gene expression had a latter effect in the accumulation of reducing sugars, metabolites fluxes, carbohydrate metabolism enzymes activities, and total polyphenols content were analysed.

6.5.1 Metabolites flux estimation of [U-^{14}C] glucose metabolism

During season 3 (2018/2019) flux estimates from metabolism of [U-^{14}C] glucose were determined at 33, and 43 weeks of storage, both time points were prior to senescent sweetening. Potato tuber discs were incubated with [U-^{14}C] glucose for three hours. [U-^{14}C]-labelled extracts were fractionated into CO_2, starch, cell wall and protein, neutral, anionic and cationic fractions as well as glucose, fructose, and sucrose. A two-way ANOVA was carried out using factors cultivar and time of storage. Percentages of metabolised [U-^{14}C] glucose in potato tuber discs are presented in Table 6-2. [U-^{14}C]-labelled CO_2 was significant different dependent on cultivar and time ($P < 0.05$). Percentage of metabolised [U-^{14}C] glucose into starch increased over time ($P = 0.001$) in both cultivars. However, no differences ($P > 0.05$) between cultivars were reported. No differences ($P > 0.05$) were observed for glucose, fructose, sucrose, cell wall and protein. Neutral fraction was significant different ($P < 0.01$) between cultivars at 33 weeks of storage. In addition, anionic and cationic fractions changed over time ($P < 0.05$).

Table 6-2. [U-^{14}C] in metabolic fraction in potato tuber discs at 33 and 43 weeks of storage for season 3 (2018/2019). Data presented is the percentage of [U-^{14}C] glucose metabolised per gram of fresh weight.

Metabolic fraction	14C in metabolic fraction in tuber discs (% of metabolised)			
	33 weeks of storage		43 weeks of storage	
	Arsenal	VR 808	Arsenal	VR 808
Glucose	0,96 ± 0,52	0,61 ± 0,09	2,62 ± 1,15	1,17 ± 0,10
Fructose	1,21 ± 0,60	0,66 ± 0,07	1,97 ± 0,65	1,14 ± 0,14
Sucrose	0,76 ± 0,18	0,76 ± 0,16	2,01 ± 0,76	2,63 ± 1,44
Starch	0,61 ± 0,08	1,04 ± 0,11	4,91 ± 1,40	4,72 ± 0,77
CO_2	64,77 ± 7,66	37,82 ± 3,18	28,55 ± 3,72	28,9 ± 4,55
Cell wall, Protein	1,59 ± 0,51	1,84 ± 0,04	1,49 ± 0,12	1,61 ± 0,03
Neutral fraction	7,02 ± 1,57	14,83 ± 0,65	26,39 ± 3,41	27,28 ± 3,41
Anionic fraction	11,60 ± 3,01	12,09 ± 1,27	16,12 ± 1,77	15,36 ± 0,29
Cationic fraction	2,28 ± 0,52	3,57 ± 0,18	6,05 ± 0,60	4,69 ± 0,91

6.5.2 Carbohydrate metabolism enzymes activity measurement

Enzymatic activities from carbohydrate metabolism were measured for season 1 (2016/2017). AGPase, UGPase, and PGM were analysed at 20, 26, and 30 weeks of storage (Figure 6-

12A). In addition, HK, FK, SuSy, vacInv, and cytInv were under study at 30 weeks after storage, a post-sweetening stage (Figure 6-12B). A two-ways ANOVA was performed using cultivar and time of storage as factors. HK, SuSy, vacInv and cytInv, AGPase, and UGPase activities exhibited no differences ($P > 0.05$) between cultivars or changes over time ($P > 0.05$). However, significant differences affected by cultivar for FK ($P < 0.05$) and by cultivar and time for PGM ($P < 0.005$) were observed. VR 808 exhibited a significant lower FK specific activity compared to Arsenal at 30 weeks of storage. Besides, VR 808 showed a progressive decrease in the specific activity of PGM after 20 weeks of storage.

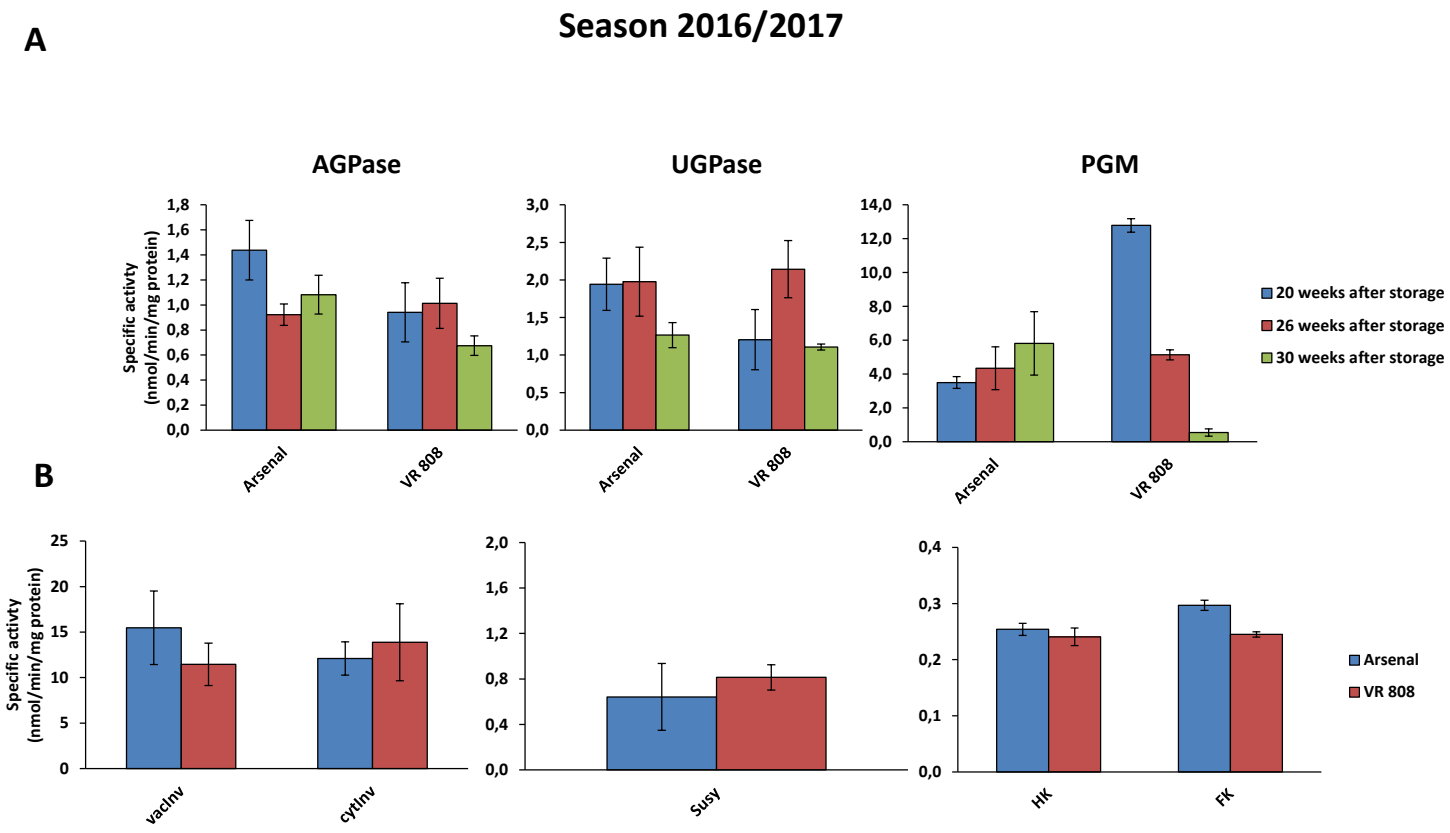


Figure 6-12. Measurement of specific activity of carbohydrate metabolism enzymes during senescent sweetening in season 1 (2016/2017). **A.** Enzyme activity of AGPase, UGPase, and PGM during senescent sweetening transition (20 and 26 weeks of storage) and post-sweetening at 30 weeks of storage. **B.** Enzyme activity of vacInv, cytInv, SuSy, HK, and FK. Values are means ± SE (three biological replicates from one experiment). Abbreviations: AGPase, ADP-glucose pyrophosphorylase; UGPase, UDP-glucose pyrophosphorylase; PGM, phosphoglucomutase; vacInv, vacuolar invertase; cytInv, cytosolic invertase; Susy, sucrose synthase; HK, hexokinase; FK, fructokinase.

6.5.3 Quantification of total polyphenols

Phenolic compounds are secondary metabolites produced in plants that have a common structure based on an aromatic ring with one or more hydroxyl substituents (Beckman, 2000; Parr & Bolwell, 2000; Valcarcel *et al*., 2015). These compounds can be divided according to their chemical structure into flavonoids, phenolic acids, tannins, stilbenes, coumarins, and lignans (Ignat *et al*., 2011; Lemos *et al*., 2015). Their presence affects the sensory qualities of plant-derived processed foods, including taste, colour, and texture (Kroon & Williamson, 1999; Alasalvar *et al*., 2001; Rytel *et al*., 2014). Differences in gene expression related to flavonoid biosynthesis between cultivars were reported during senescent sweetening for season 1 (2016/2017). It was hypothesized that flavonoid synthesis may be acting as a sink for reducing sugars in VR 808, resistant to sweetening cultivar, avoiding their accumulation. Therefore, total polyphenols were quantified at 20, 26 and 30 weeks after storage in season 1 (Figure 6-13). These time points were related to senescent sweetening transition and a post-sweetening stage. A two-ways ANOVA was performed using cultivar and time of storage as factors. Results showed no significant differences ($P > 0.05$) between cultivars as well as no significant changes ($P > 0.05$) influenced by time of storage.

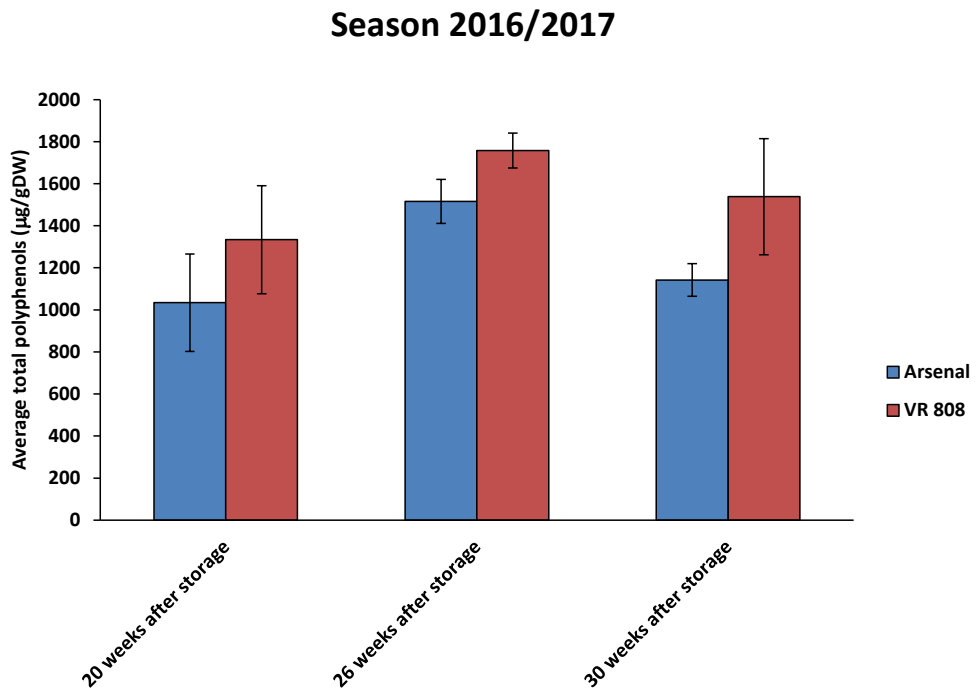


Figure 6-13. Quantification of total polyphenols content in Arsenal and VR 808 tubers during season 1 (2016/2017). Values are means ± SE (four biological replicates from one experiment).

6.6 Discussion

The aim of this work was to utilize transcriptome profiling to understand how long-term storage affects potato gene expression to promote senescent sweetening. A microarray experiment was carried out using two different time points representing senescent sweetening transition during the first season. For the second season, the experiment was conducted using 4 time points, including prior to sweetening stage, senescent sweetening transition and late storage stage. qRT-PCR was performed to verify the transcriptome results.

Carbohydrates provide energy and building blocks for plant growth and development. Furthermore, soluble sugars including glucose, fructose and sucrose are known to act as signal molecules to regulate the expression of many key genes involved in plant metabolic processes and defence responses, consequently regulating plant growth and development (Rolland *et al*., 2006; Mishra *et al*., 2009; Ruan *et al*., 2010; Cho & Yoo, 2011; Li *et al*., 2011). Carbohydrates are also central to quality and yield of crops. In fleshy fruits, the accumulation of soluble sugars during fruit development largely determines their sweetness at harvest. Plants have evolved an elaborate system for sugar metabolism and accumulation in sink cells (Li *et al*., 2012).

The results of both transcriptome analyses suggested that carbohydrate metabolism was altered during the storage period. In the major carbohydrate metabolism, genes associated with starch synthesis were down-regulated in both analyses for the susceptible cultivar, presenting these genes up-regulation for the stable profile cultivar. In addition, Arsenal exhibited up-regulation of FK genes as well as higher specific activity of this enzyme compared to VR 808. FK efficiently catalyses the phosphorylation of fructose to fructose 6-phosphate. However, it has been suggested that FK has little impact on glycolysis and starch synthesis (Davies *et al*., 2005).

For season 1 (2016/2017), genes encoding AGPase were down-regulated in Arsenal during the senescent sweetening transition. In season 2 (2017/2018), Arsenal also exhibited a general down-regulation in starch synthesis, including AGPase genes, at 53 weeks of storage. In heterotrophic storage organs such as potato tubers, most of the incoming sucrose is converted to starch as a long-term carbon store for reproductive growth. AGPase catalyses the first committed step of starch synthesis in the plastid, converting glucose 1-phosphate and ATP to

ADP-Glc and PPi. ADP-Glc is subsequently used by starch synthases and branching enzymes to elongate the glucan chains of the starch granule. AGPase is a heterotetramer that contains two large (AGPS, 51 kDa) and two slightly smaller subunits (AGPB, 50 kDa) (Morell *et al*., 1987, Okita *et al*., 1990). Work with Arabidopsis mutants (Neuhaus & Stitt, 1990) and potato tubers (Geigenberger *et al*., 2004) showed that the enzyme catalyses a near rate-limiting step in the pathway of starch synthesis. There is evidence for the in vivo role of posttranslational redox modulation of AGPase in regulating starch synthesis in heterotrophic potato tubers (Tiessen *et al*., 2002) and photosynthetic leaves of potato, pea, and Arabidopsis plants (Hendriks *et al*., 2003). Posttranslational redox activation of AGPase allows the rate of starch synthesis to be increased in response to external inputs and independently of any increase in the levels of glycolytic intermediates (Tiessen *et al*., 2002). More recent studies in potato tubers revealed that sucrose and glucose lead to redox activation of AGPase via two different signalling pathways involving SnRK1 and hexokinase, respectively (Tiessen *et al*., 2003). Hexokinase and SnRK1 are both implicated in a regulatory network that controls the expression and phosphorylation of cytosolic enzymes in response to sugars (Smeekens, 2000). How they are linked to reductive activation of AGPase and starch synthesis in the plastid remains unresolved. Trehalose metabolism has been implicated in the regulation of sugar utilization in yeast and plants (Thevelein & Hohmann, 1995; Eastmond & Graham, 2003; Gancedo & Flores, 2004).

Genes included in the minor carbohydrate biosynthesis suggested down-regulated production of T6P in Arsenal potato tubers. Trehalose and the metabolism associated with its synthesis have been proposed to be a component of the plant's sugar signalling system (Paul, 2007; Paul *et al*., 2008). T6P is an intermediate product of trehalose biosynthesis. T6P is a product of the reaction between UDP-Glc and G6P (Cabib & Leloir, 1958), which is catalysed by TPS. T6P is further metabolised to trehalose by TPP (Cabib & Leloir, 1958; O'Hara *et al*., 2013), which is eventually hydrolysed by trehalase into glucose (Elbein *et al*., 2003). In potato, T6P overproduction has been shown to cause the down-regulation of cell proliferation and delayed growth and sprouting (Debast *et al*., 2011). It has been reported that the addition of T6P to isolated chloroplasts leads to redox activation of AGPase (Kolbe *et al*., 2005). Lunn *et al*. (2006) reported that rising sugar levels in plants are accompanied by increases in the level of T6P, redox activation of AGPase and the stimulation of starch synthesis in vivo. These results indicate that T6P acts as a signalling metabolite of sugar status in plants and

support the proposal that T6P mediates sucrose-induced changes in the rate of starch synthesis (Lunn *et al*., 2006).

Debast *et al*. (2011) reported that transgenic potato plants with elevated T6P levels displayed reduced starch content, decreased ATP contents, and increased respiration rate diagnostic for high metabolic activity. On the contrary, lines with significantly reduced T6P showed accumulation of soluble carbohydrates, hexose phosphates, and ATP, no change in starch when calculated on a fresh weight basis, and a strongly reduced tuber yield. T6P-accumulating tubers were strongly delayed in sprouting, while those with reduced T6P sprouted earlier than the wild type (Debast *et al*., 2011). This observation may be related to the fact that potato cultivars that are most susceptible to SS tend to have short dormancy (Colgan *et al*., 2012).

T6P is considered a signal regulating plant sugar metabolism, growth and development, possibly due to its interaction with sucrose non-fermenting (SNF) kinases (Lunn *et al*., 2014). T6P acts as an intermediary, increasing the rate of starch synthesis via the redox activation of AGPase (Kolbe *et al*., 2005). T6P has no significant inhibitory effects on the hexokinase activities of spinach (Wiese *et al*., 1999), Arabidopsis (Eastmond *et al*., 2002), or tomato (Kandel-Kfir *et al*., 2006). However, T6P indirectly responds to glucose or fructose but is directly influenced by sucrose (Yadav *et al*., 2014).

In Arabidopsis, T6P signalling is partially mediated through inhibition of the SnRK1 (Debast *et al*., 2011). Protein phosphorylation is involved in regulation of various cellular activities in plants and one of the main signals mediating the responses to environmental stresses (Laurie & Halford, 2001; Yoshida *et al*., 2006; Fujii *et al*., 2007; Movahed *et al*., 2012; Hong *et al*.,2013). The SnRKs are a gene family coding for Ser/Thr protein kinases and play important roles in linking abiotic stress tolerance and the metabolic responses of plants (Qin *et al*., 2011; Bing *et al*., 2013; Tao & Lu, 2013). Based on sequence similarity, domain structure and metabolic roles, the plant SnRK family is divided into three subfamilies: SnRK1, SnRK2 and SnRK3. Several studies have demonstrated that these three subfamilies play various roles in the metabolism and development of plants. SnRK1 plays an important role in regulating carbon metabolism and energy conversion in plants (Halford & Hardie, 1998; Ghillebert *et al*., 2011), SnRK2 members are the major players in plant responses to osmotic stresses (Boudsocq *et al*., 2004; Umezawa *et al*, 2004; Fujii & Zhu, 2009; Fujii *et al*.,

2011), ABA dependent and independent stomatal closure-opening (Mustilli *et al*., 2002), fruit development (Sun *et al*., 2010), seed dormancy (Zheng *et al*., 2010) and germination (Johnson *et al*., 2002, Nakashima *et al*., 2009), while SnRK3 is involved in plant development, calcium-responsive regulatory loop and ABA sensitivity.

Differential expression of genes involved in ethylene and ABA pathways were observed during both seasons. After harvest, synthesis of endogenous hormones in tubers continue to perform their roles, participating in or even causing physiological events in the tuber during storage, ceasing their functions only with the death of the tuber, that they may be as well the precursors, since they may affect the rate of aging (Isenberg & Ludford, 1988; Coleman, 2000). Studies show that ethylene and ABA are associated with the onset and maintenance of tuber dormancy, and that genes associated with the anabolic and catabolic metabolism of ABA are correlated with dormancy in potato meristems and tubers (Teper-Bamnolker *et al*., 2012; Muthoni *et al*., 2014). During season 1, both cultivars exhibited up-regulation of genes involved in ethylene synthesis. Exposure to ethylene increases tuber respiration rate and accelerates the conversion of starch to sugars causing a dose-dependent effect in the darkening of potato fry colour (Daniels-Lake *et al*., 2005). The effects of elevated CO_2 concentrations, reduced O_2 concentrations and ethylene gas on the fry colour and sugar content in the variety Russet Burbank have been observed (Daniels-Lake *et al*., 2005). Tubers exposed to both elevated CO_2 concentrations and ethylene exhibited darker fry colour and higher reducing sugar concentrations, not only than the controls, but those treated just with ethylene, suggesting a synergistic negative effect of trace ethylene and elevated CO_2 on fry colour (Daniels-Lake *et al*., 2005). However, in the present work both cultivars showed an up-regulation of ethylene synthesis during storage but only the cultivar susceptible to senescent sweetening accumulated reducing sugars, suggesting ethylene may not be involved in the mechanisms of senescent sweetening.

Sugar signalling pathways and their interactions with each other and with hormonal signalling pathways have been reported (Rolland *et al*., 2006). Genetic approaches have demonstrated the importance of ABA in sugar response pathways, with both pathways using common signalling components (Cheng *et al*., 2002). Several studies have indicated that SnRK1 could be implicated in these interactions (Nemeth *et al*., 1998; Bradford *et al*., 2003; Thelander *et al*., 2004). Moreover, it has been suggested that SnRK1 plays a key role during germination, and could mediate ABA functions during seed maturation (Radchuk *et al*.,

2006; Lu *et al*., 2007). ABA plays important roles in plant response to drought stress by inducing the expression of TF-, heat shock protein-, transporter-, and osmotic regulator-encoding genes downstream of stress signalling pathways in both ABA-dependent and ABA-independent manners (Shinozaki & Yamaguchi-Shinozaki, 2007). ABA and anabolism related genes are also important regulators for drought-stress response in potato (Padmalatha *et al*., 2012; Gong *et al*., 2015b; Hayano-Kanashiro *et al*., 2019). Moreover, changes involved in ABA as well as starch synthesis genes have been reported in potato tubers under drought stress (Gong *et al*., 2015b). During season 2, genes related to ABA were up-regulated over time for the susceptible cultivar. In terms of senescent sweetening, Arsenal tubers could undergo a more severe drought stress response inducing ABA signalling followed by changes in carbohydrate metabolism.

In addition, we reported a GPT2 gene was progressively down-regulated over time in the susceptible cultivar. This decrease in expression during the senescent sweetening transition was related to the increase of reducing sugars previously observed. A number of genes have been identified as being up-regulated by exogenous increases in sugar, including *At1g61800*, encoding a GPT2 (Knappe *et al*., 2003). GPT2 is involved in the transport of G6P across plastid membranes in return for inorganic phosphate (Niewiadomski *et al*., 2005). Microarray analyses have shown that GPT2 expression has been associated with impaired carbon metabolism (Kunz *et al*., 2010), senescence (Pourtau *et al*., 2006), and increases in carbon fixation due to increased light (Athanasiou *et al*., 2010). Moreover, GPT2 has been suggested to be associated with sugar sensing by affecting the balance of metabolites between cellular compartments (Dyson *et al*., 2015).

Kunz *et al.* (2010) showed that GPT2 expression is up-regulated in mutants impaired in starch synthesis (Kunz et al., 2010). In Arabidopsis, whereas the glucose-6-phosphate translocator 1 (GPT1) is constitutively present in particular cells such as stomatal guard cells of leaves or cells of the root tip, GPT2 is induced when carbohydrate metabolism is impaired, e.g. at higher concentrations of soluble sugars (Kunz *et al*., 2010). In potato tubers, the under-expression of GPT2 gene could lead to a decrease in transport of G6P into the amyloplasts for the synthesis of ADP-Glc, which is the substrate for starch synthases and represents the first committed precursor for starch synthesis.

The key enzyme in starch biosynthesis, the stroma-localised AGPase, catalyses the ATP-dependent conversion of G1P to ADP-Glc, the substrate for starch synthases. A knockout mutation in the catalytic subunit of AGPase in Arabidopsis results in a lack of starch in all parts of the plants (Lin *et al*., 1988), as is the case for a mutant plant with a defect in the plastid-localised PGM, catalysing the reversible conversion of G6P to G1P as substrate for AGPase (Caspar *et al*., 1985; Kofler *et al*., 2000; Periappuram *et al*., 2000). During the season 1 (2016/2017) of this project, VR 808 exhibited a progressive decrease in PGM activity at 26 weeks of storage. In plastids of heterotrophic tissues, G6P can be imported from the cytosol via a glucose-6-phosphate/phosphate translocator (GPT) and converted to starch via PGM, AGPase and starch synthases. The proposed role of the GPT is delivery of G6P to non-green plastids as carbon skeletons for starch biosynthesis and/or to the oxidative pentose phosphate pathway (Kammerer *et al*., 1998; Rolletschek *et al*., 2007; Zhang *et al*., 2008).

Microarray analyses revealed that GPT2 was substantially up-regulated in a pgm mutant or in a wild type fed with glucose (Thimm *et al*., 2004; Bläsing *et al*., 2005; Pourtau *et al*., 2006). In Arabidopsis leaves, GPT2 is strongly induced by light and contributes significantly to the measurable G6P transport activity of mutants impaired in starch biosynthesis (Kunz *et al*., 2010, Weise *et al*., 2019). GPT2 has been suggested to be a safety valve under situations when carbohydrate metabolism is impaired or in the presence of increased soluble sugar concentrations (Kunz *et al*., 2010). Moreover, inverse correlation of GPT2 and cwInv gene expression has been reported (Ferreira *et al*., 2010).

Weise *et al*. (2019) reported that both redox responsive transcription factor 1 (RRTF1) and high amounts of cytosolic triose phosphate are required for induction of the expression of GPT2 in Arabidopsis leaves. In the present study, VR 808 exhibited increasing transcript levels of GPT2 as well as a gradual decrease of PGM activity at 26 weeks of storage during season 1. However, potato tubers from both cultivars showed similar expression in *RRTF1* genes. In mutants of Arabidopsis that are unable to synthesize starch due to a mutation in the gene encoding the plastid PGM, GPT2 transcripts amounts were more than two-fold higher than in the wild type (Weise *et al*., 2019). Potato lines with decreased activities of plastidial PGM exhibited a remarkable (up to 40%) decrease in the accumulation of starch, and significant increases in the levels of sucrose and hexose phosphates (Tauberger *et al*., 2000).

Cytosolic expression of yeast invertase in potato tubers leads to reduced starch content and increased respiration. Moreover, UDP-Glc production is associated with a reduced expression of cell wall biosynthetic genes (Ferreira & Sonnewald, 2012). In addition, Ferreira and Sonnewald (2012) observed the transgenic tubers are characterized by elevated expression of senescence-associated genes, coupled to reduced expression of genes related to photosynthesis and the cytoskeleton. Increased respiration, observed in Arsenal tubers, might be due to sugar signalling via released T6P inhibition of the SnRK1 complex. In Arsenal, expression of the GPT2 was significantly down-regulated during the storage for season 1 and 2. This could lead to a shift in the cytosolic to plastidic G6P ratio and hence might limit starch synthesis, but also the oxidative pentose phosphate pathway.

In Arabidopsis, GPT2 is rapidly induced by both glucose and sucrose, and thus is essential for leaf growth and acclimation of metabolism to daily environmental changes (Gonzali *et al*., 2006; Athanasiou *et al*., 2010; Dyson *et al*., 2014; Dyson *et* al., 2015; Van Dingenen *et al*., 2016). The induction of GPT2 by glucose is dependent on its concentration, and does not occur in response to light, ABA, or other indirect signalling pathways (Chen *et al*., 2019). Chen *et al*. (2019) suggested that when sugars are increased in the cytosol, the expression levels of sugar-responsive genes such as GPT2 increase by the coordinate actions of WRKY18, WRKY53, and HAC1. The increased cytosolic sugar content could then be lowered by more active sugar import into cellular compartments (e.g. amyloplast in potato tubers).

In the mature leaves of most plants, photosynthates formed during C3 photosynthesis are used in the formation of sucrose, which is allocated via the phloem to the heterotrophic plant organs, such as young leaves, roots, seeds, fruits, or tubers. In these sink tissues, sucrose serves as a source of carbon and energy and is cleaved by the action of invertases or sucrose synthase. Finally, the products of these reactions are converted into hexose phosphates.

Plastids of non-photosynthetic plant tissues depend metabolically on the supply of ATP and carbon compounds. In general, plastids are not able to generate hexose phosphates from C3 compounds due to the absence of fructose-1,6-bisphosphatase activity (Entwistle & ap Rees, 1988). Non-green plastids of heterotrophic tissues import carbon as a source of biosynthetic pathways and energy and, in the case of amyloplasts of storage tissues, the site of starch synthesis. Within plastids, carbon can be used in the biosynthesis of starch or as a substrate

for the oxidative pentose phosphate pathway. Several studies have reported that this transport in different plant tissues is mediated by a phosphate translocator that imports hexose phosphates in exchange with inorganic phosphate or C3 sugar phosphates (Borchert *et al.*, 1989, 1993; Hill & Smith, 1991, 1995; Neuhaus *et al*., 1993; Flügge & Weber, 1994; Schünemann & Borchert, 1994; Flügge, 1995; Schott *et al.*, 1995; Quick & Neuhaus, 1996). Although G6P has been reported to be the preferred hexose phosphate taken up by non-green plastids (Kammerer *et al*., 1998), in amyloplasts from wheat endosperm, G1P rather than G6P is the precursor of starch biosynthesis (Tyson & ap Rees, 1988; Tetlow *et al*., 1994). Amyloplasts from potato tubers showed to use G1P rather than G6P to support starch synthesis (Naeem *et al*., 1997), although previous studies reported that these plastids were able to transport G6P but not G1P (Schott *et al*., 1995).

In conclusion, long-term storage caused changes in carbohydrate metabolism and a progressive decrease in sugar transporters gene expression in susceptible cultivars. GPT2 was consistently down-regulated in sweetening tubers. This fact might limit sugar supply to the plastids, which could lead to a down-regulation of starch biosynthesis genes. These results suggest that down-regulation of sugar phosphate transport is a crucial factor that promotes senescent sweetening during long-term storage. Hence, we have identified GPT2 as a possible candidate gene involved in the mechanisms of senescent sweetening. Insight into the underlying mechanism that causes accumulation of sugars in stored potato tubers is needed to fully understand the senescent sweetening process.

Chapter 7: Concluding discussion

Control of potato quality during storage represents a significant problem for the potato processing industry and little is known regarding the mechanisms of senescent sweetening. The Ph.D. research project adopts physiological, biochemical, and molecular approaches to elucidate downstream biochemical and molecular responses to long-term storage that may influence carbohydrate metabolism resulting in senescent sweetening. Potential mechanisms include enhanced starch degradation, reduced starch resynthesis, and reduced catabolism of sugars.

7.1 Assessment of physiological changes of potato tubers during long-term storage

Long-term storage had a significant impact on sugar accumulation in potato tubers. Varieties showed differences on senescent sweetening susceptibility. Accumulation of sugars was higher in Arsenal, Lady Rosetta and Shelford (Shropshire location) than in VR 808 and SH C 909. Sugar content was higher in untreated tubers compared to CIPC-treated tubers in season 1. During this season, Arsenal showed senescent sweetening after 26 weeks of storage. However, for the second season Arsenal exhibited a later onset of senescent sweetening, after 37 weeks of storage. In season 3, susceptible cultivars were observed to accumulate sugars after 43 of storage. Furthermore, Shelford varieties showed a difference in sugar accumulation depending on growing location.

Despite changes in the timing of accumulation, the relative timing was consistent. In conclusion, senescent sweetening has a strong genetic component that is overlaid by environmental factors, as demonstrated by impact of season and growing location. This fact suggests that breeding for senescent sweetening resistance is a feasible objective. If breeding is a sensible objective then the work done in this thesis is validated and will make a valuable contribution towards the identification of markers.

7.2 Senescent sweetening and its relationship with oxidative stress

A relationship between the onset of senescent sweetening and oxidative stress was not observed during the storage period. Arsenal and VR 808 exhibited differences in H_2O_2 content, however, no increase was observed related to the onset of senescent sweetening. On the contrary, although no differences between cultivars were shown in the activity of antioxidant enzymes, fluctuations were observed during the storage. Moreover, MDA content did no increase during the accumulation of sugars.

In addition, metabolome profiles showed that both Arsenal and VR 808 had a general decrease of fatty acids while an increase in organic acids concentration. The decrease in fatty acids suggests that there is no reduction in membrane permeability. An increase in organic acids might be the result of reduced respiration as a consequence of mitochondrial damage. In conclusion, senescent sweetening was not related to oxidative stress. However, results suggest tubers undergo to different oxidative signalling affecting antioxidant systems during long-term storage. If oxidative stress is not the cause of sweetening then the implication is that is a normal physiological process associated with aging. The key question then becomes what are the triggers and signals for that aging process.

The data presented in this work is contrary to previous hypotheses and results suggesting that senescent sweetening is produced by oxidative stress during long-term storage. Nevertheless, the evidence it is based on ultrastructure and often the oxidative stress is assumed rather than directly quantified. Those facts could explain the discordances observed in this project in comparison with previous studies.

To test those hypothesis and to obtain a better understanding of the relationship between senescent sweetening and oxidative damage in stored tubers several approaches could be adopted in future works. Oxidative membrane damage can be quantified as TBARS (Hodges *et al*., 1999) in the isolated mitochondria (Considine *et al*., 2003). Oxidative damage to proteins can be measured in whole tissues and isolated mitochondria as protein carbonyl groups (Wehr & Levine, 2013). Direct damage to amyloplast membranes in unsweetened and sweetened tubers can be visualised by electron microscopy (Ohad *et al*., 1971). Tuber antioxidant capacity can be estimated by quantification of the major redox buffers (AsA-DHA, GSH-GSSG, NAD-NADH, and NADP-NADPH) (Queval & Noctor, 2007) using

spectrophotometry. Tuber energy status can be estimated by quantification of ATP/ADP ratios by HPLC (Collen *et al.*, 2004) providing a proxy for respiratory efficiency. Respiratory activity can be measured directly in mitochondria isolated from unsweetened and sweetened tubers using an oxygen electrode in both the presence and absence of KCN to estimate the contribution of the cytochrome c and alternative oxidase pathways (Bartoli *et al.*, 2000). Taken together, these experiments may directly test the hypothesis that senescent sugar accumulation is associated with oxidative damage in aged potato tubers. It could also directly address the hypotheses that sugar accumulation results from reduced mitochondrial respiratory capacity and/or damage to amyloplast membranes.

7.3 Potato tuber metabolome during long-term storage

Cultivars showed similar behaviours in terms of metabolome during the storage. The main difference observed was in the amino acids content, which it has been postulated to be cultivar-dependent. The amino acids content separate the cultivars into different groups based on their senescent sweetening susceptibility, suggesting a relationship between amino acids and sugar content. In addition, ^{13}C labelling experiment suggests an overall higher synthesis of amino acids in VR 808, a resistant cultivar. This experiment also showed a more rapid synthesis of sucrose in VR 808 compared to Arsenal, a susceptible cultivar, indicating an alteration in carbohydrate metabolism.

In conclusion, the metabolic processes are remarkably similar between cultivars. Moreover, and as evidenced from the data in this work, the metabolic adjustments leading to sweetening are minor. In this case, it might be expected that only a few key genes are significant in producing the sweetening effect. Therefore, a small number of QTL might be expected to have a large effect which means that marker assisted breeding may be a powerful tool in the creation of sweetening resistant cultivars.

7.4 Changes in transcript levels associated with long-term storage in potato tubers

Genes associated with carbohydrate metabolism exhibited differences between Arsenal and VR 808. Arsenal showed a general down-regulation in genes associated to both starch and trehalose-6-phosphate synthesis in season 1 and 2. Additionally, the plastid sugar transporter GPT2 gene was observed to be progressively down-regulated in Arsenal during the storage.

These genes associated with carbohydrate metabolism were down-regulated in Arsenal while up-regulated or with no changes in VR 808.

For future prospects, the study of the variations in 5' untranslated region (5' UTR) could lead to a better understanding of the gene regulation and what triggers the changes observed in gene expression.

7.5 Mechanisms of senescent sweetening

In the present work, results suggest that senescent sweetening may be the consequence of an altered carbohydrate metabolism. During the three seasons of study, all cultivars accumulated sucrose at similar levels. The accumulation of sucrose might result from a general water-deficit stress induced by long-term storage. As previously described, drought stress may affect vacuolar transporters. In this context, all cultivars under study might exhibit vacuolar accumulation of sucrose due to drought stress following long-term storage.

GPT2 expression was significantly lower in cultivars susceptible to senescent sweetening compared to resistant cultivars. GPT2 is involved in the transport of glucose-6-phosphate into the plastids. In potato tubers, the down-regulation observed in susceptible cultivars could implicate that sugar phosphates are unable to be transported into the amyloplasts, where they are used for starch synthesis. As a consequence, the decreased content in starch substrates in the amyloplasts might lead to the down-regulation in starch synthesis genes observed in Arsenal. Since reducing sugars could not been used for starch resynthesis, they would start to accumulate during long-term storage resulting in senescent sweetening.

On the contrary, VR 808 exhibited a higher synthesis of sucrose and some amino acids. The accumulation of sucrose in VR 808 could be related to the reduction in the PGM activity observed. These products might be acting as a sink for the reducing sugars, avoiding their accumulation during the storage in the resistant cultivars.

Although differences related to senescent sweetening were observed between cultivars, the reason for these differences and what are the molecular triggers of onset remain to be elucidated.

7.6 Implications of this work

The main contributions of the present research project are a better understanding of the physiological, biochemical, and molecular changes in potato tubers during long-term storage. A further understanding of the processes underlying senescent sweetening will enable strategies for control by optimising storage regimes and will underpin breeding programmes for the development of senescent sweetening-resistant varieties.

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Supporting information

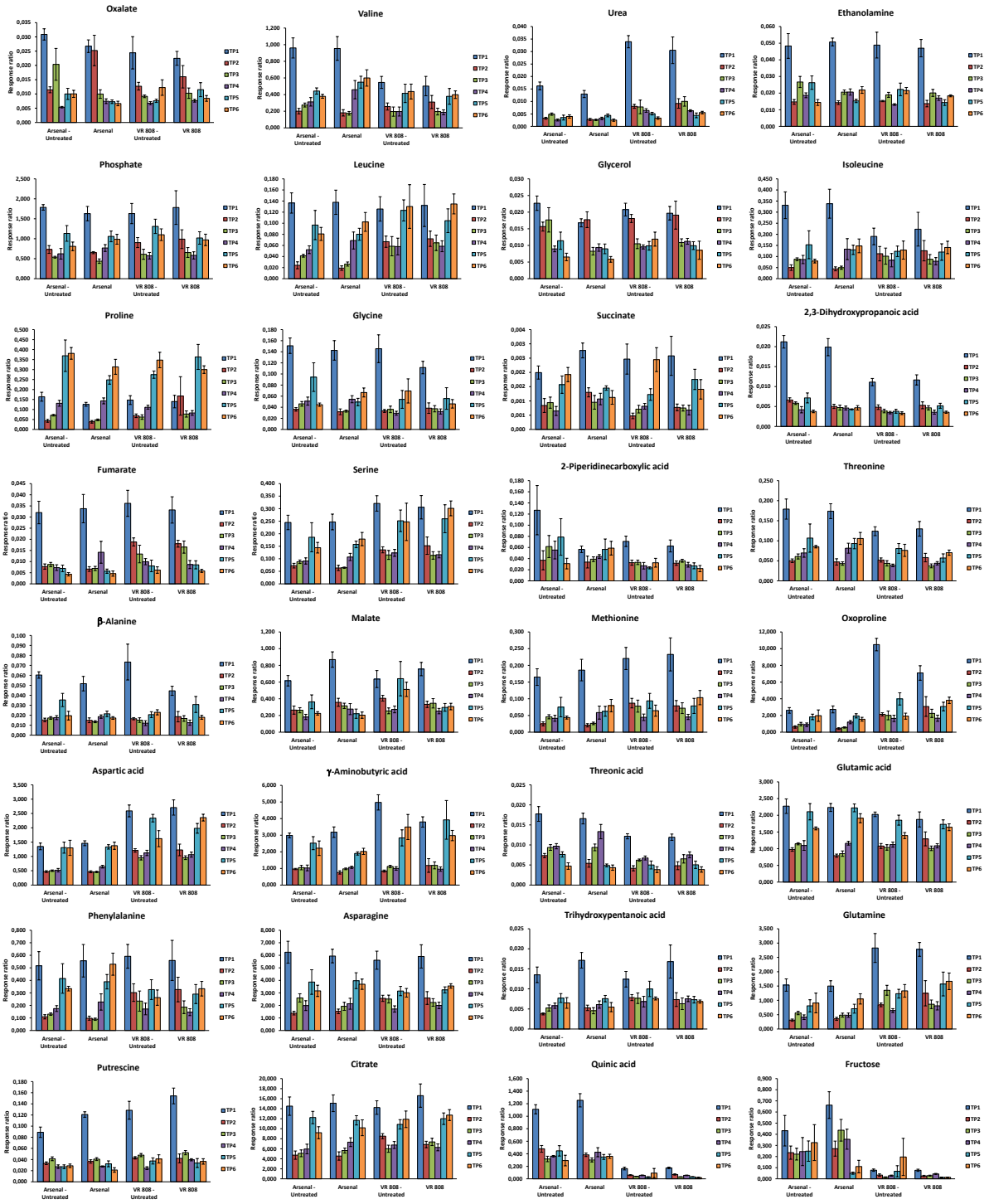

Figure S5-1. Influence of time of storage and CIPC-treatment on metabolites in potato tubers during season 1 (2016/2017). TP5 and TP6 represent senescent sweetening transition. Values are means ± SE (five biological replicates from one experiment). Abbreviations: TP1, 2 weeks of storage; TP2, 4 weeks of storage; TP3, 6 weeks of storage; TP4, 12 weeks of storage; TP5, 20 weeks of storage; and TP6, 26 weeks of storage.

Figure S5-1. Continuation (1).

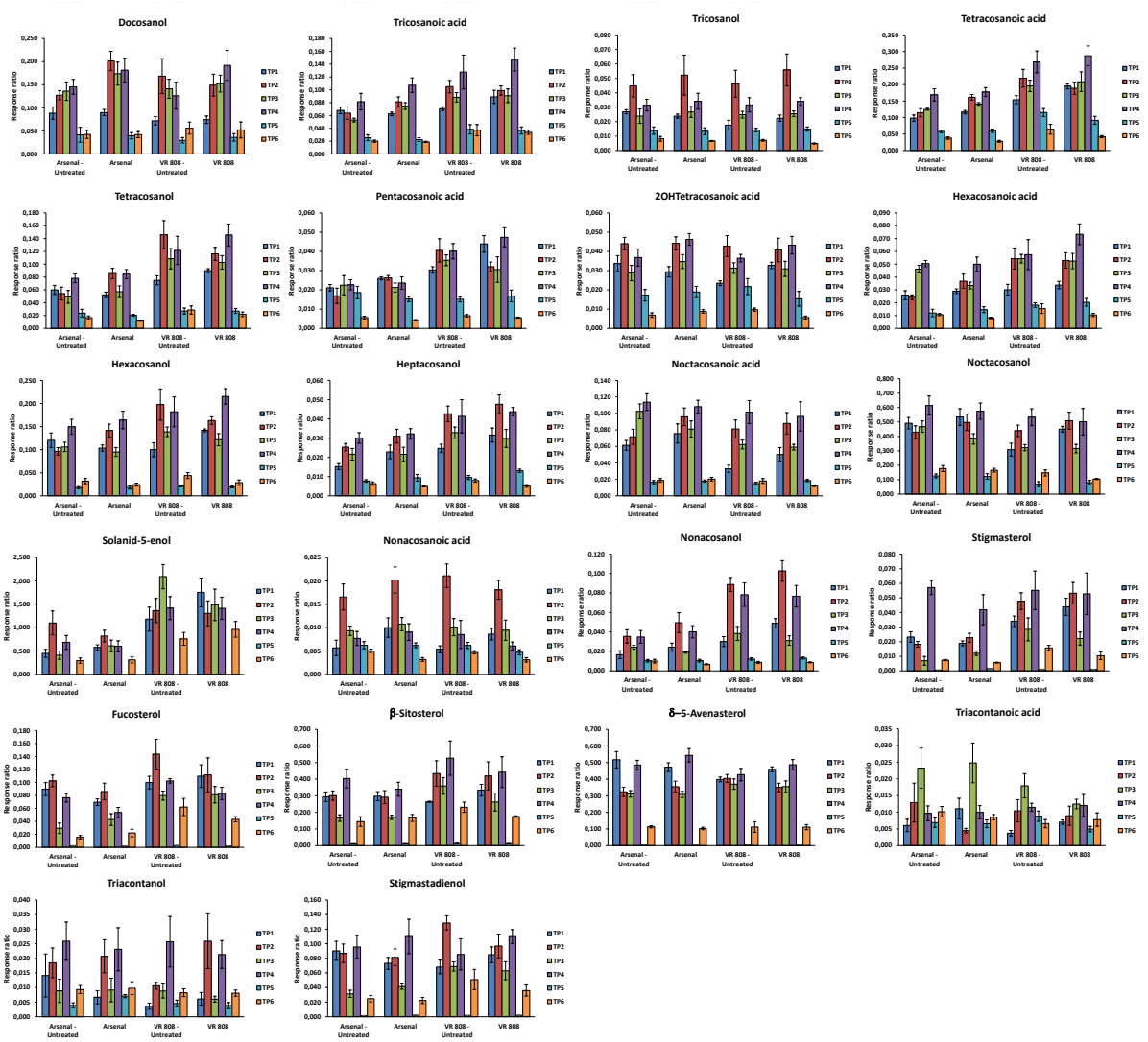

Figure S5-1. Continuation (2).

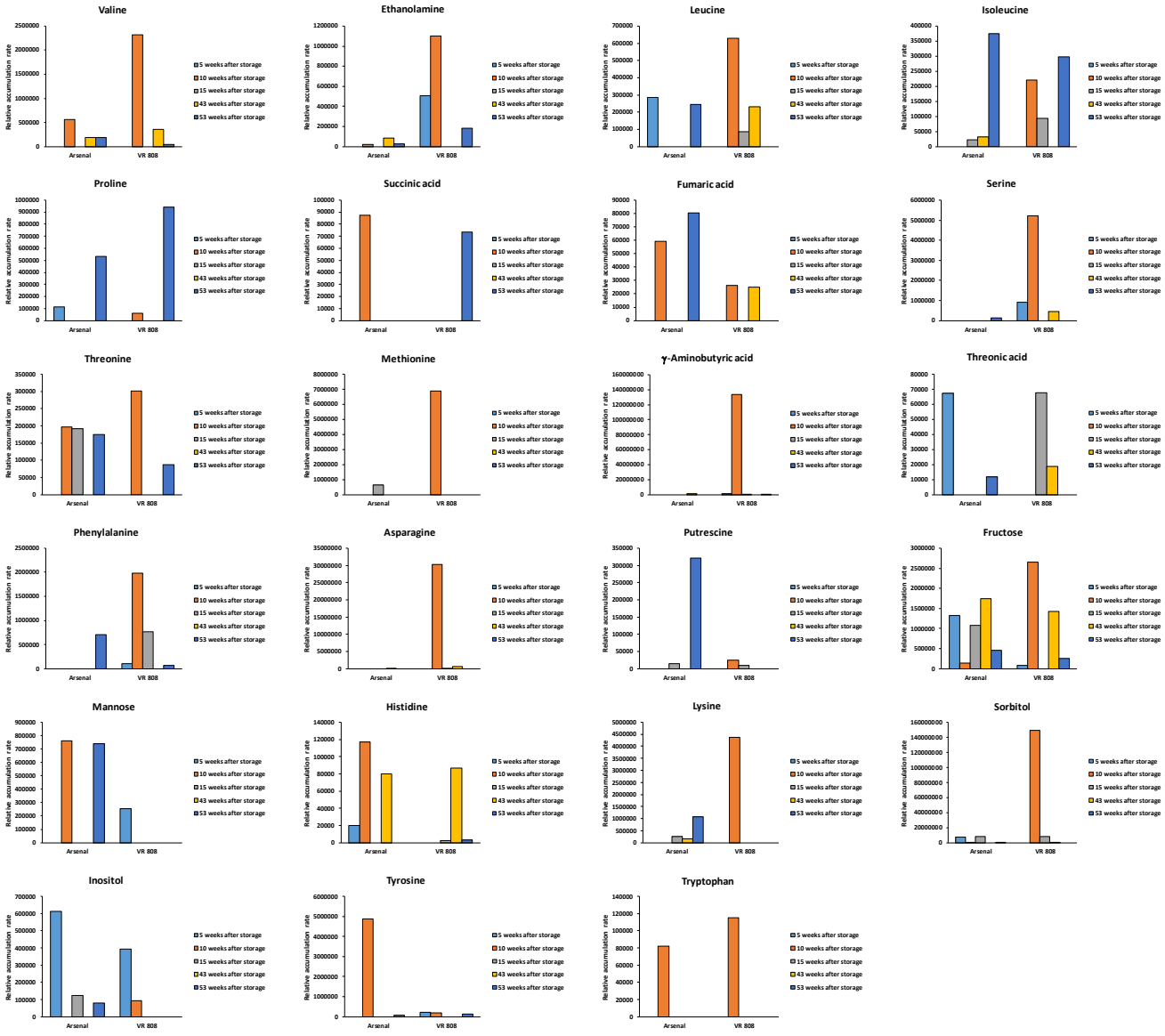

Figure S5-2. Metabolite fluxes on the metabolism of [U-^{13}C] glucose by potato tubers. Metabolites which accumulated ^{13}C at only certain time points during the storage are shown.

Table S5-1. Metabolites which exhibited significant changes in tuber metabolome during long-term storage in season 1 (2016/2017). Significant differences were estimated by three-ways analysis of variance (ANOVA) using cultivar, treatment (CIPC-treated or untreated), and time of storage as factors. Significant values ($P < 0.05$) are in bold. Abbreviations: Cv, cultivar; and Tr, treatment.

Metabolite (polar)	Cv	Time	Tr	Cv.Time	Cv.Tr	Time.Tr	Cv.Time.Tr
Oxalate	0,078	**<.001**	0,913	0,114	0,511	**0,004**	0,063
Valine	**<.001**	**<.001**	0,463	**<.001**	0,263	0,771	0,512
Urea	**<.001**	**<.001**	0,550	**<.001**	0,347	0,523	0,694
Ethanolamine	0,150	**<.001**	0,325	0,684	0,799	0,074	0,411
Phosphate	0,216	**<.001**	0,783	0,663	0,958	0,912	0,712
Leucine	**0,005**	**<.001**	0,936	0,196	0,988	0,886	0,975
Glycerol	0,280	**<.001**	0,068	0,298	0,121	0,171	0,253
Isoleucine	0,540	**<.001**	0,622	**0,009**	0,913	0,837	0,928
Proline	0,696	**<.001**	0,463	0,229	**0,033**	0,429	0,164
Glycine	0,067	**<.001**	0,129	0,557	0,990	0,539	0,138
Succinate	0,718	**<.001**	0,689	0,376	0,498	**0,012**	0,628
2,3-Dihydroxypropanoic acid	**<.001**	**<.001**	0,613	**<.001**	**0,026**	0,862	0,320
Fumarate	**0,003**	**<.001**	0,840	**0,049**	0,654	0,960	0,704
Serine	**<.001**	**<.001**	0,772	0,373	0,684	0,819	0,979
2-Piperidinecarboxylic acid	**<.001**	**<.001**	0,117	0,566	0,208	0,313	0,286
U1376_Unknown	**0,042**	**<.001**	0,329	0,080	0,529	0,773	0,204
Threonine	**<.001**	**<.001**	0,696	0,070	0,858	0,647	0,900
β-Alanine	0,964	**<.001**	0,064	0,815	0,735	0,075	0,101
Malate	**0,014**	**<.001**	0,766	0,107	**0,028**	**<.001**	0,854
U1509_Unknown	0,065	**<.001**	0,633	**0,005**	0,240	0,945	0,401
Methionine	**<.001**	**<.001**	0,552	0,249	0,888	0,458	0,997
Oxoproline	**<.001**	**<.001**	0,487	**<.001**	0,761	**0,021**	**0,002**
Aspartic acid	**<.001**	**<.001**	0,291	**0,003**	0,800	0,122	0,196
γ-Aminobutyric acid	**<.001**	**<.001**	0,552	**0,025**	0,742	0,760	0,120
Threonic acid	**<.001**	**<.001**	0,830	**0,004**	0,358	0,118	0,299
U1567_Unknown	**0,009**	**<.001**	0,504	**<.001**	0,734	0,886	0,159
U1586_Unknown	**<.001**	**<.001**	0,689	**<.001**	0,625	0,927	0,808
U1585_Unknown	**0,006**	**<.001**	0,338	**<.001**	0,511	0,848	0,051
U1598_Unknown	0,555	**<.001**	0,448	0,355	0,626	0,993	0,906
Glutamic acid	**0,050**	**<.001**	0,883	**0,002**	0,806	0,226	0,434
Phenylalanine	0,671	**<.001**	0,665	**0,020**	0,496	0,646	0,981
Asparagine	0,885	**<.001**	0,701	0,157	0,672	0,811	0,995
Trihydroxypentanoic acid	**0,029**	**<.001**	0,639	0,594	0,598	0,105	0,952
U1703_Unknown	**<.001**	**<.001**	0,474	**<.001**	0,172	0,969	0,906
Glutamine	**<.001**	**<.001**	0,504	0,063	0,554	0,598	0,812
Putrescine	**<.001**	**<.001**	**0,029**	**0,005**	0,911	**0,004**	0,797

Table S5-1. Continuation (1).

Metabolite (polar)	Cv	Time	Tr	Cv.Time	Cv.Tr	Time.Tr	Cv.Time.Tr
U1751_Unknown	**<.001**	**<.001**	0,112	**<.001**	0,393	0,216	0,766
U1755_Unknown	**<.001**	**<.001**	0,316	**<.001**	0,623	**0,029**	0,160
USA1768_Unknown	**<.001**	**<.001**	0,196	**<.001**	0,477	**0,035**	0,204
U1801_Unknown	0,131	**<.001**	0,422	0,092	0,473	0,779	0,916
Citric acid	**0,016**	**<.001**	0,270	0,182	0,884	0,772	0,808
Quinic acid	**<.001**	**<.001**	0,958	**<.001**	0,595	0,412	0,238
U1871_Unknown	**0,045**	**<.001**	0,290	**0,009**	0,924	0,715	0,992
Fructose	**<.001**	**0,007**	0,918	**0,016**	0,305	**0,021**	0,546
Allantoin	**<.001**	**<.001**	0,094	0,283	0,585	0,424	0,946
Mannose	**<.001**	**<.001**	0,855	0,183	0,378	**0,004**	0,927
Glucose	**<.001**	**0,013**	0,600	**0,013**	0,111	**0,026**	0,582
Histidine	**0,006**	**<.001**	0,568	0,213	0,474	0,458	0,991
Lysine	**0,044**	**<.001**	0,428	0,052	0,376	0,294	0,963
Mannitol	**<.001**	**<.001**	0,647	0,765	0,339	0,149	0,164
Sorbitol	0,851	**<.001**	0,339	0,991	0,127	0,103	0,612
Tyrosine	0,587	**<.001**	0,932	0,249	0,227	0,570	0,920
U1948_Unknown	0,345	**<.001**	0,908	0,632	0,760	0,570	1,000
UC2020_Unknown	**<.001**	**<.001**	0,053	**<.001**	**0,034**	**0,012**	0,314
Galactaric acid	**<.001**	**<.001**	**0,026**	0,384	0,253	0,195	0,832
Inositol	**<.001**	**<.001**	**0,044**	**0,013**	0,310	**<.001**	**0,008**
UC2105_Unknown	**<.001**	**<.001**	0,300	**0,015**	0,654	0,213	0,170
U2125_Unknown	**<.001**	**<.001**	0,082	0,078	0,283	**0,006**	**0,003**
Caffeic acid	**<.001**	**<.001**	0,372	**<.001**	0,236	0,130	0,347
U2190_Unknown	**<.001**	**0,020**	0,589	0,098	0,393	0,106	0,473
Tryptophan	**0,006**	**<.001**	0,322	**0,004**	0,371	0,343	0,983
Spermidine	0,495	**<.001**	0,428	0,697	0,503	0,306	0,413
Fructose-6-Phosphate	0,086	**0,008**	0,229	0,987	0,622	0,845	0,697
Galactosyl Glycerol	**<.001**	**<.001**	**0,017**	**<.001**	0,667	0,054	0,907
Glucose-6-Phosphate	0,259	**0,004**	0,118	0,998	0,822	0,862	0,871
U2367_Unknown	0,896	**<.001**	0,929	0,053	0,483	0,309	0,794
U2477b_Unknown	**<.001**	**<.001**	**0,001**	**0,003**	**0,048**	**0,050**	0,071
U2495_Unknown	**0,008**	**0,334**	0,420	0,458	0,394	0,615	0,539
U2502_Unknown	0,611	**<.001**	0,766	0,785	0,827	0,512	0,298
Sucrose	**<.001**	**<.001**	0,295	0,155	0,104	**<.001**	0,432
Galactinol	**<.001**	**<.001**	0,055	0,873	0,677	0,516	0,364
Chlorogenic acid	**0,009**	**<.001**	0,504	0,981	0,726	0,652	0,430

Table S5-1. Continuation (2).

Metabolite (non-polar)	Cv	Time	Tr	Cv.Time	Cv.Tr	Time.Tr	Cv.Time.Tr
U1595_Unknown	0,327	**<.001**	0,474	**0,007**	0,277	0,216	0,097
U1680_Unknown	0,634	**<.001**	0,448	0,665	0,360	0,410	0,855
Tetradecanoic acid	**<.001**	**<.001**	0,549	**<.001**	0,753	0,815	0,619
U1762_Unknown	0,525	**<.001**	0,913	**<.001**	0,945	0,582	0,910
Br-Pentadecanoic acid	0,989	**<.001**	0,237	0,844	0,517	0,916	0,933
Pentadecenoic acid	**0,008**	**<.001**	0,844	0,416	0,380	0,745	0,886
Cinnamic acid	**0,010**	**<.001**	**<.001**	0,399	0,930	**<.001**	0,713
Pentadecanoic acid	**<.001**	**<.001**	0,253	**<.001**	0,953	0,622	0,762
U1845_Unknown	**<.001**	**<.001**	**<.001**	**0,018**	0,480	**<.001**	0,717
Hexadecenoic acid	**<.001**	**<.001**	0,309	**0,002**	0,691	0,904	0,002
Hexadecanoic acid	0,435	**<.001**	0,413	**0,007**	0,476	0,092	0,475
Cinnamic acid	**0,027**	**<.001**	**0,047**	0,807	0,136	0,251	0,346
Me-Hexadecanoic acid	**<.001**	**0,004**	0,524	0,215	0,547	0,628	0,527
Heptadecanoic acid	**0,029**	**<.001**	0,093	0,128	0,412	0,610	0,255
Linoleic acid	0,405	**<.001**	0,358	0,157	0,807	0,253	0,747
α-linolenic acid	0,166	**<.001**	**0,048**	0,074	0,094	0,579	0,415
Octadecenoic acid	**<.001**	**<.001**	0,198	**0,007**	0,965	0,102	0,058
2OHHexadecanoic acid	**<.001**	**<.001**	0,776	**<.001**	0,303	0,393	0,873
Noctadecanoic acid	**0,022**	**<.001**	0,468	**0,006**	0,153	0,762	0,097
Nonadecenoic acid	**0,017**	**<.001**	0,091	0,060	0,795	0,533	0,260
U2263_Unknown	0,271	**<.001**	0,496	0,612	0,606	0,245	0,250
Tricosane	**<.001**	**<.001**	0,546	0,124	0,763	0,459	0,044
Eicosanoic acid	**<.001**	**<.001**	0,256	0,941	0,093	0,447	0,102
Heneicosanoic acid	0,258	**<.001**	0,535	0,180	**0,004**	0,831	0,447
Heneicosanol	**<.001**	**<.001**	0,905	**<.001**	0,343	0,148	0,336
U2457_Unknown	**<.001**	**<.001**	0,765	**<.001**	0,877	0,886	0,417
U2466_Unknown	0,264	**<.001**	0,133	0,179	0,861	0,723	0,887
U2510_Unknown	**<.001**	**<.001**	0,865	**<.001**	0,533	0,365	0,384
Docosanoic acid	0,410	**<.001**	**0,036**	0,148	**0,002**	**<.001**	**<.001**
Docosanol	0,509	**<.001**	**0,023**	0,941	0,354	0,296	0,253
Tricosanoic acid	**<.001**	**<.001**	0,074	0,225	0,576	0,441	0,569
Tricosanol	0,787	**<.001**	0,335	0,922	0,755	0,787	0,987
Tetracosanoic acid	**<.001**	**<.001**	0,323	**0,012**	0,275	0,343	0,310
Tetracosanol	**<.001**	**<.001**	0,589	**<.001**	0,508	0,755	**0,050**
Pentacosanoic acid	**<.001**	**<.001**	0,255	**<.001**	0,948	0,077	**0,040**
2OHTetracosanoic acid	0,346	**<.001**	0,273	0,965	0,473	0,265	0,231
Hexacosanoic acid	**<.001**	**<.001**	0,497	**0,027**	0,630	0,205	0,281
Hexacosanol	**<.001**	**<.001**	0,649	**0,021**	0,795	0,370	**0,027**
Heptacosanol	**<.001**	**<.001**	0,079	**0,007**	0,813	0,252	0,997
Octacosanoic acid	**<.001**	**<.001**	0,502	**0,026**	0,981	0,109	0,769
Octacosanol	**<.001**	**<.001**	0,586	0,224	0,414	0,109	0,887
Solanid-5-enol	**<.001**	**<.001**	0,915	**0,013**	0,913	0,284	0,226

Table S5-1. Continuation (3).

Metabolite (non-polar)	Cv	Time	Tr	Cv.Time	Cv.Tr	Time.Tr	Cv.Time.Tr
Nonacosanoic acid	0,639	**<.001**	0,721	0,942	0,075	0,280	0,756
Nonacosanol	**<.001**	**<.001**	0,137	**<.001**	0,838	0,103	0,936
Stigmasterol	**<.001**	**<.001**	0,748	**0,005**	0,671	0,609	0,596
Fucosterol	**<.001**	**<.001**	0,062	0,103	0,702	0,283	0,541
β-Sitosterol	**<.001**	**<.001**	0,320	0,096	0,536	0,632	0,836
δ–5-Avenasterol	0,664	**<.001**	0,531	**0,006**	0,892	0,392	0,233
Triacontanoic acid	0,087	**<.001**	0,474	0,058	0,860	0,248	0,505
Triacontanol	0,256	**<.001**	0,790	0,977	0,519	0,406	0,629
Stigmastadienol	**0,015**	**<.001**	0,844	0,060	0,844	0,284	0,463

Table S5-2. Metabolites which exhibited significant changes in tuber metabolome during long-term storage in season 3 (2018/2019). Significant differences were estimated by two-ways analysis of variance (ANOVA) using cultivar and time of storage as factors. Significant values ($P < 0.05$) are in bold.

Metabolite (polar)	Cultivar	Time	Cultivar.Time
Oxalate	**0.011**	**<.001**	**0.002**
Valine	**0.019**	**<.001**	0.092
Urea	**<.001**	**<.001**	**0.006**
Ethanolamine	**0.002**	**<.001**	**0.003**
Phosphate	0.305	**<.001**	0.792
Leucine	**<.001**	**<.001**	**0.018**
Glycerol	**<.001**	**<.001**	**0.006**
Isoleucine	**<.001**	**<.001**	**0.011**
Proline	**<.001**	**<.001**	**<.001**
Glycine	**<.001**	**<.001**	0.134
Succinic acid	**0.021**	**<.001**	**0.005**
2,3-Dihydroxypropanoic acid	0.606	**<.001**	0.723
Fumarate	0.209	**<.001**	0.766
Serine	**<.001**	**<.001**	0.132
2-Piperidinecarboxylic acid	**0.013**	**<.001**	0.630
U1376_Unknown	**0.012**	**<.001**	**0.021**
Threonine	**<.001**	**<.001**	0.414
β-Alanine	**0.016**	**<.001**	0.556
Malate	**<.001**	**<.001**	**0.003**
U1509_Unknown	**0.018**	**<.001**	0.877
Methionine	**<.001**	**<.001**	**<.001**
Oxoproline	**<.001**	**<.001**	**0.004**
Aspartic acid	**<.001**	**<.001**	**0.002**
γ-Aminobutyric acid	**<.001**	**<.001**	**<.001**
Threonic acid	**<.001**	**<.001**	**0.029**
U1567_Unknown	**<.001**	**<.001**	**<.001**
U1586_Unknown	**<.001**	**<.001**	0.087
U1585_Unknown	**<.001**	**<.001**	**<.001**
U1598_Unknown	**<.001**	**<.001**	0.442
Glutamic acid	**0.023**	**<.001**	0.184
Asparagine	**<.001**	**<.001**	**0.001**
Phenylalanine	**<.001**	**<.001**	0.440
Trihydroxypentanoic acid	**<.001**	**<.001**	**0.012**
U1703_Unknown	**0.049**	**<.001**	0.311
Glutamine	**<.001**	**<.001**	**<.001**
Putrescine	**0.001**	**<.001**	**0.002**
U1751_Unknown	**<.001**	**<.001**	**<.001**
U1755_Unknown	**<.001**	**<.001**	0.168
USA1768_Unknown	**0.012**	**<.001**	0.187

Table S5-2. Continuation (1).

Metabolite (polar)	Cultivar	Time	Cultivar.Time
α-glycerophosphate	0.084	-	-
U1801_Unknown	0.194	**<.001**	**0.006**
Citric acid	0.076	**<.001**	0.684
Quinic acid	**<.001**	**<.001**	**<.001**
U1871_Unknown	**<.001**	**<.001**	0.561
Fructose	**<.001**	**<.001**	0.174
Allantoin	**<.001**	**<.001**	**<.001**
Mannose	**0.006**	**<.001**	0.535
Galactose	0.208	-	-
Glucose	**<.001**	**<.001**	0.067
Histidine	**<.001**	**<.001**	**0.034**
Lysine	**<.001**	**<.001**	**0.001**
Mannitol	0.778	**<.001**	0.729
Sorbitol	0.491	**<.001**	0.521
Tyrosine	**<.001**	**<.001**	**<.001**
U1948_Unknown	**<.001**	**<.001**	0.364
UC2020_Unknown	**<.001**	**0.001**	0.530
Galactaric acid	**<.001**	**<.001**	**<.001**
Inositol	**<.001**	**<.001**	**<.001**
UC2105_Unknown	0.146	**<.001**	0.766
U2125_Unknown	**<.001**	**<.001**	**<.001**
Caffeic acid	**<.001**	**<.001**	**<.001**
U2190_Unknown	**<.001**	**<.001**	0.073
Tryptophan	**<.001**	**<.001**	**0.012**
Spermidine	0.074	**<.001**	**0.022**
Fructose-6-phosphate	0.188	**<.001**	0.172
Galactosyl Glycerol	**<.001**	**<.001**	**0.006**
Glucose-6-phosphate	0.617	**<.001**	0.790
U2367_Unknown	**<.001**	**<.001**	**0.004**
U2477b_Unknown	0.139	**<.001**	0.265
U2495_Unknown	0.520	**0.007**	0.921
U2502_Unknown	**0.050**	**<.001**	0.113
Sucrose	**<.001**	**<.001**	**<.001**
Galactinol	**0.019**	**<.001**	0.215
Chlorogenic acid	**<.001**	**<.001**	**<.001**

Table S5-2. Continuation (2).

Metabolite (non-polar)	Cultivar	Time	Cultivar.Time
U1595_Unknown	**0.008**	**<.001**	0.078
U1680_Unknown	**0.02**	**<.001**	0.165
Tetradecanoic acid	0.053	**<.001**	0.874
U1762_Unknown	**<.001**	**<.001**	**<.001**
Pentadecenoic acid	**<.001**	**<.001**	0.582
Cinnamic acid	**0.002**	**<.001**	0.072
Pentadecanoic acid	**<.001**	**<.001**	**0.032**
U1845_Unknown	**0.001**	**<.001**	0.070
Hexadecenoic acid	0.592	**<.001**	0.830
Hexadecanoic acid	**<.001**	**<.001**	**0.001**
Cinnamic acid	**<.001**	**<.001**	**<.001**
Me-Hexadecanoic acid	**<.001**	**<.001**	0.162
Heptadecanoic acid	**0.015**	**<.001**	**0.021**
Linoleic acid	**<.001**	**<.001**	0.496
α-linolenic acid	**<.001**	**<.001**	**<.001**
Octadecenoic acid	**<.001**	**<.001**	**<.001**
2OH-Hexadecanoic acid	**<.001**	**<.001**	**<.001**
Noctadecanoic acid	**<.001**	**<.001**	**<.001**
Nonadecenoic acid	**0.004**	**<.001**	0.163
U2263_Unknown	**<.001**	**<.001**	**<.001**
Tricosane	**<.001**	**<.001**	0.550
Eicosanoic acid	**<.001**	**<.001**	**<.001**
Heneicosanoic acid	**<.001**	**<.001**	**<.001**
Heneicosanol	**<.001**	**<.001**	**<.001**
U2457_Unknown	**0.025**	**<.001**	0.061
U2466_Unknown	**<.001**	**<.001**	**<.001**
U2510_Unknown	**<.001**	**<.001**	**<.001**
Docosanoic acid	**<.001**	**<.001**	**<.001**
Docosanol	**<.001**	**<.001**	**0.001**
Tricosanoic acid	**<.001**	**<.001**	**<.001**
Tricosanol	**0.011**	**<.001**	0.743
Tetracosanoic acid	**<.001**	**<.001**	**<.001**
Tetracosanol	**<.001**	**<.001**	**0.002**
Pentacosanoic acid	**0.001**	**<.001**	0.345
2OH-Tetracosanoic acid	**0.037**	**<.001**	0.184
Hexacosanoic acid	**<.001**	**<.001**	**<.001**
Hexacosanol	**<.001**	**<.001**	**<.001**
Heptacosanol	**<.001**	**<.001**	**0.011**
Octacosanoic acid	**<.001**	**<.001**	**<.001**

Table S5-2. Continuation (3).

Metabolite (non-polar)	Cultivar	Time	Cultivar.Time
Octacosanol	**<.001**	**<.001**	**0.002**
Solanid-5-enol	**0.016**	**<.001**	0.157
Nonacosanoic acid	**<.001**	**<.001**	**<.001**
Nonacosanol	**<.001**	**<.001**	**<.001**
Stigmasterol	**<.001**	**<.001**	**0.010**
Fucosterol	0.066	**<.001**	0.150
β-Sitosterol	**<.001**	**<.001**	**<.001**
δ-5-Avenasterol	**<.001**	**<.001**	**<.001**
Triacontanoic acid	**0.004**	**<.001**	0.251
Triacontanol	0.230	**<.001**	0.919
Stigmastadienol	**<.001**	**<.001**	**<.001**

Table S5-3.1. Loadings scores from PCA of all metabolites identified by GC/MS (polar/non-polar fraction) in season 3 (2018/2019).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
Oxalate	0.11051	0.08949	0.08248	0.03906	-0.07379	-0.02875	0.00933	0.00563	0.02915	0.09621
Valine	0.12707	0.04240	0.00149	0.01121	-0.00504	0.05085	0.01064	0.10821	0.06249	-0.08066
Urea	0.09939	0.06156	0.03632	0.05157	-0.11066	0.13775	0.06501	-0.03703	-0.19111	-0.07952
Ethanolamine	0.11807	0.06891	0.07846	0.00660	-0.08234	-0.00137	-0.04186	0.00554	0.08768	-0.01538
Phosphate	0.11378	0.07992	-0.00205	-0.06417	0.00679	-0.02708	-0.11812	-0.02539	0.09964	-0.03025
Leucine	0.12539	0.01663	-0.05786	-0.03252	0.00344	0.04235	-0.02199	0.02786	-0.00216	-0.03840
Glycerol	0.10254	0.06975	0.01096	-0.16640	0.00643	0.03325	0.08954	-0.00797	-0.09179	0.07832
Isoleucine	0.12304	0.03903	-0.03808	0.00045	-0.00948	0.13002	0.01499	0.08246	0.00298	-0.07878
Proline	0.11516	0.00986	0.00240	0.13062	0.03718	-0.00797	-0.07493	-0.08876	0.10083	0.01607
Glycine	0.12226	0.05597	-0.04775	0.00368	0.09025	0.02790	0.02611	0.07051	0.00408	-0.03000
Succinic acid	0.09649	0.03696	0.09360	0.03656	-0.07038	-0.14144	-0.13183	0.10180	-0.14656	0.11598
2,3-Dihydroxypropanoic acid	0.08993	0.08608	0.12246	0.07906	-0.10699	0.09306	0.01023	0.03833	0.20731	0.05409
Fumarate	0.09979	0.06779	0.07564	-0.03491	0.01069	-0.11353	0.03370	0.05532	-0.15966	0.09735
Serine	0.12180	0.03832	-0.07882	-0.06295	0.05043	0.01255	0.06566	-0.02011	0.10244	0.01572
2-Piperidinecarboxylic acid	0.09372	0.04851	-0.04602	0.04718	0.16506	0.08416	-0.02073	0.04162	-0.11122	-0.04158
U1376_Unknown	0.06241	0.06411	0.06606	0.16003	-0.13202	0.26100	0.21132	0.01362	-0.04511	-0.12551
Threonine	0.11106	0.05881	-0.03782	-0.03713	0.11892	-0.09835	-0.00423	0.04284	0.10867	-0.03689
β-Alanine	0.11402	0.02149	0.03500	-0.01843	0.03508	-0.04090	0.11076	0.16267	0.07753	-0.03591
Malate	0.09434	0.03086	0.16660	0.01015	-0.07832	-0.11575	-0.09455	0.08300	0.11784	-0.08930
U1509_Unknown	0.06518	0.11397	0.01332	0.04215	-0.14693	0.21541	0.09318	0.05896	0.04521	0.05407
Methionine	0.10636	0.06068	-0.10493	-0.12439	0.04055	0.11920	-0.04249	0.06720	-0.01750	0.02500
Oxoproline	0.11335	-0.00329	-0.06000	-0.01293	-0.01284	0.05107	-0.17066	0.10195	-0.03137	-0.05898
Aspartic acid	0.12187	0.04409	-0.00904	-0.12202	-0.01918	-0.05312	-0.04591	-0.01202	0.00324	-0.01771
γ-Aminobutyric acid	0.08723	0.04966	-0.07295	-0.05672	0.15565	-0.05116	0.08539	0.08005	-0.05297	-0.00940
Threonic acid	0.09643	0.09682	0.04495	0.04709	0.09550	-0.07389	-0.02603	0.10698	0.14492	-0.13231
U1567_Unknown	0.09937	0.06813	-0.13154	0.01952	-0.03465	0.13680	-0.13592	0.04846	-0.11289	0.05836
U1586_Unknown	0.06874	0.05174	-0.07219	-0.28216	0.01837	-0.03322	0.01143	-0.01682	0.19048	0.00353

Table S5-3.1. Continuation (1).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
U1585_Unknown	0.10633	0.04865	-0.08310	0.03121	0.02745	0.13376	-0.12221	0.03891	-0.20526	-0.00132
U1598_Unknown	0.01369	0.07697	-0.26303	-0.09174	-0.17677	0.01304	-0.11137	0.12270	0.04998	0.16832
Glutamic acid	0.11594	0.08151	-0.01644	-0.08314	0.06781	0.00243	-0.05026	-0.05141	-0.04182	-0.04346
Asparagine	0.10290	0.00721	-0.10629	-0.18766	0.08042	0.09643	-0.08736	0.04320	-0.05021	-0.02778
Phenylalanine	0.12064	0.01552	-0.07696	0.03200	0.11991	0.03346	-0.05423	0.01665	0.01815	-0.03455
Trihydroxypentanoic acid	0.11786	0.07617	0.04030	-0.06318	-0.01324	-0.07106	0.00793	-0.01316	0.01361	-0.00713
U1703_Unknown	0.09526	0.08279	-0.06327	-0.09080	0.05784	-0.09676	0.02122	0.01263	0.00402	0.04852
Glutamine	0.07510	0.02788	-0.02959	-0.27388	0.07290	-0.08511	0.10958	0.01139	0.07732	-0.10997
Putrescine	0.11239	0.05757	-0.03887	-0.03414	0.08856	-0.01152	-0.08134	-0.11696	-0.01725	0.01605
U1751_Unknown	0.09838	0.08052	-0.03244	0.09700	0.01162	0.14663	-0.04873	-0.15953	-0.10338	0.05015
U1755_Unknown	0.09841	0.05508	0.01896	-0.09570	-0.05345	0.10534	0.11156	-0.12933	0.01821	0.06729
USA1768_Unknown	0.09774	0.09226	0.06091	0.01245	0.04375	-0.02074	0.03626	-0.04229	-0.15163	0.19535
α-glycerophosphate	-0.06039	0.04722	0.19802	-0.09162	0.17350	0.10811	-0.00915	0.07440	0.01635	0.04944
U1801_Unknown	-0.01055	0.10137	-0.11374	0.03318	-0.23984	-0.14359	-0.00545	0.10820	0.07310	0.17445
Citric acid	0.11416	0.06737	-0.03090	-0.11934	0.03510	-0.05537	-0.06017	-0.00049	-0.02191	-0.06898
Quinic acid	0.07086	0.08325	0.01974	0.04337	0.13182	-0.19120	0.19826	-0.00156	0.08812	-0.13794
U1871_Unknown	-0.00153	0.07023	-0.26271	-0.05368	-0.21633	-0.07562	-0.11033	0.10666	0.05482	0.15655
Fructose	0.04444	0.02797	-0.06922	0.27963	0.19778	0.04271	0.03096	0.08226	0.01762	0.04541
Allantoin	0.11760	0.06701	-0.00723	-0.08968	0.00286	-0.01648	-0.12584	-0.04918	-0.02601	-0.01369
Mannose	0.09787	0.04985	-0.03434	0.13528	0.08664	-0.10023	0.04728	-0.03777	0.04417	0.12280
Galactose	-0.05732	0.04676	0.17077	-0.06488	0.18189	0.12034	-0.01005	0.08713	0.03563	0.12647
Glucose	0.07347	0.05263	-0.02960	0.25731	0.18674	-0.05832	-0.01530	0.03698	-0.01963	0.15022
Histidine	0.09128	-0.08252	-0.16342	-0.08804	0.03590	0.04622	-0.06355	0.07752	0.04860	-0.05376
Lysine	0.11548	0.02933	-0.12901	0.00054	0.06024	0.04438	-0.02515	0.06061	0.06896	0.04023
Mannitol	0.02730	0.10268	0.16567	-0.04540	0.20295	0.01919	-0.07436	0.10731	-0.02240	0.05547
Sorbitol	0.08401	0.10675	0.16018	0.01135	-0.04846	0.00265	-0.07843	0.06954	0.11600	0.07236
Tyrosine	0.10745	0.02211	-0.12516	0.07222	0.08171	0.05176	-0.05418	0.12437	0.11281	-0.01444

Table S5-3.1. Continuation (2).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
U1948_Unknown	0.03434	0.08874	-0.23303	-0.07611	-0.18846	-0.07453	-0.09721	0.05045	-0.03336	0.14385
UC2020_Unknown	-0.02501	-0.10400	-0.09450	0.16683	0.00758	0.02874	0.02405	0.14200	0.17328	0.12597
Galactaric acid	0.09344	0.08686	-0.06856	-0.02647	-0.02219	0.19851	0.14785	-0.09315	-0.14358	-0.02104
Inositol	0.09232	0.07513	-0.03359	0.14651	0.14735	-0.02610	-0.01765	-0.17023	-0.04251	0.06101
UC2105_Unknown	0.11167	0.09503	0.05949	-0.01234	-0.02129	-0.01462	0.06382	-0.05880	0.02856	-0.04009
U2125_Unknown	0.07087	0.06376	0.12628	-0.05666	-0.15894	-0.05492	0.14554	0.02592	-0.13539	0.15416
Caffeic acid	0.09159	0.07496	0.14718	0.03001	-0.07031	-0.14954	0.08078	-0.02630	0.06558	0.12974
U2190_Unknown	0.11082	0.02071	-0.07919	-0.01648	0.11907	0.00641	0.12672	0.02332	-0.04031	-0.06831
Tryptophan	0.09058	0.06097	-0.14070	0.01232	0.01267	0.12009	0.01709	0.08735	0.04079	-0.03909
Spermidine	0.09774	0.06761	0.11695	0.04831	-0.05850	-0.06754	-0.16885	0.09395	0.06786	0.15674
Fructose-6-phosphate	0.09257	0.07015	0.09380	0.10150	-0.06988	-0.16365	0.07884	-0.03624	-0.07634	0.21296
Galactosyl Glycerol	0.08944	0.09855	0.02998	-0.17603	0.02890	0.04070	0.12001	-0.04229	0.03737	0.10952
Glucose-6-phosphate	0.10174	0.08783	-0.02039	0.00660	-0.07951	0.11993	0.06020	-0.04836	0.02182	-0.21842
U2367_Unknown	0.11360	0.08616	0.02029	0.01853	0.00850	-0.07895	0.13599	0.03354	0.04145	-0.04746
U2477b_Unknown	0.07081	0.06184	0.13599	0.08069	-0.21618	0.18481	0.04281	0.05630	0.07460	-0.14346
U2495_Unknown	0.07964	0.08584	0.09636	0.15294	-0.11557	0.13020	0.13961	0.02001	0.10426	0.05144
U2502_Unknown	0.08623	0.08410	0.07696	0.09634	-0.11703	0.12033	0.21341	-0.00634	-0.06605	0.01394
Sucrose	0.08532	0.05485	-0.07751	0.17614	0.17927	-0.14118	0.06566	-0.01475	0.00968	-0.04026
Galactinol	0.09914	0.09489	0.02936	-0.04565	-0.03718	-0.06353	0.13721	-0.07723	0.08248	-0.04167
Chlorogenic acid	0.08100	0.06857	0.11437	0.10669	-0.11153	-0.11191	-0.00464	-0.00424	-0.06601	-0.10575
U1595_Unknown	-0.02597	-0.01032	0.15565	-0.14907	0.16946	0.16086	0.04953	0.16691	0.02490	0.23165
U1680_Unknown	0.02011	-0.10680	0.13864	-0.06910	0.09232	0.10818	0.01195	0.16600	0.01488	-0.02030
Tetradecanoic acid	0.08078	-0.13094	-0.03926	-0.03617	-0.01925	0.06658	0.04416	-0.01468	-0.08172	0.04433
U1762_Unknown	0.06762	-0.13362	0.04721	-0.16713	-0.03168	0.01321	0.15220	0.07816	0.02548	0.05753
Br-pentadecanoic acid	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Pentadecenoic acid	0.06731	-0.16587	0.06073	0.03570	-0.03039	-0.00336	-0.00639	0.01776	0.02015	0.03613
Cinnamic acid	0.12304	-0.01693	0.05006	-0.01981	0.04865	-0.02673	-0.00698	0.04582	-0.07175	-0.05926

Table S5-3.1. Continuation (3).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
Pentadecanoic acid	0.07993	-0.14441	-0.02334	-0.08064	-0.09566	0.01771	0.01100	0.02444	0.06336	0.01410
U1845_Unknown	0.10948	-0.10059	0.01988	-0.00591	0.04477	-0.02559	0.01654	0.08455	0.03984	-0.05467
Hexadecenoic acid	0.09463	-0.11513	-0.01438	-0.03033	0.03271	0.01722	-0.02870	-0.00849	-0.05953	0.00691
Hexadecanoic acid	0.09063	-0.13702	-0.04642	0.00467	0.02293	0.07090	0.00826	-0.07845	0.07119	0.05309
Cinnamic acid	0.10528	-0.10361	0.05799	0.01926	0.01480	-0.00494	-0.04601	0.09951	-0.02178	-0.05629
Me-Hexadecanoic acid	0.06369	-0.11410	-0.05415	0.06158	0.12174	0.00865	0.05981	-0.14943	0.21119	0.03223
Heptadecanoic acid	0.08277	-0.15460	0.01666	0.01316	-0.00504	-0.01935	-0.00055	0.01600	0.03338	-0.02290
Linoleic acid	0.11462	-0.04176	-0.11543	0.03478	-0.06793	-0.04735	0.00758	-0.07590	0.00454	-0.01067
α-linolenic acid	0.11007	-0.02586	0.04060	-0.00854	-0.06003	-0.02103	-0.10914	-0.18991	-0.07861	-0.08378
Octadecenoic acid	0.09362	-0.07128	-0.00023	0.01741	0.04247	0.01215	-0.05752	-0.14087	-0.03784	-0.06752
2OH-Hexadecanoic acid	0.10197	-0.12113	0.04480	-0.02177	-0.01867	0.07300	-0.05476	-0.00027	-0.04376	-0.02000
Noctadecanoic acid	0.08444	-0.14685	0.00593	0.08705	0.01826	0.06984	-0.05976	-0.06222	-0.00160	0.00077
Nonadecenoic acid	0.02885	-0.09606	-0.00434	0.01749	0.04545	0.01686	0.06682	-0.12806	0.15565	0.07408
U2263_Unknown	-0.01482	-0.00393	0.19664	-0.09688	0.12753	0.09627	-0.08561	0.10425	-0.07989	0.22999
Tricosane	0.06116	-0.15280	-0.06986	0.08831	0.02787	0.01388	0.03462	0.09964	0.03517	0.03206
Eicosanoic acid	0.09018	-0.14347	0.06113	0.01574	-0.04707	0.04283	-0.07669	-0.03896	-0.01918	0.00558
Heneicosanoic acid	0.07130	-0.16031	-0.02405	0.02205	-0.04532	0.07254	-0.03597	0.03439	-0.03656	-0.03295
Heneicosanol	0.06157	-0.08466	0.04506	0.08448	0.04525	-0.03681	-0.13088	0.26078	-0.16502	-0.12415
U2457_Unknown	0.02859	-0.19266	-0.00371	0.03407	0.00788	0.07073	0.06273	0.08855	0.07202	0.08536
U2466_Unknown	0.02751	-0.19216	-0.01212	0.03142	0.01878	0.08809	0.06259	0.10617	0.08068	0.08303
U2510_Unknown	0.02638	-0.12943	0.09142	-0.16001	0.01564	0.05689	0.13223	-0.00622	0.06625	0.20446
Docosanoic acid	0.08034	-0.15344	0.06418	-0.02188	-0.07791	0.00204	-0.02174	0.02706	-0.09663	0.01339
Docosanol	0.08977	-0.11634	0.05294	0.02147	-0.00897	-0.06178	-0.05516	0.10545	-0.17019	-0.05202
Tricosanoic acid	0.07640	-0.16004	0.00926	-0.03275	-0.08690	0.01704	-0.00660	0.00137	-0.05484	-0.01685
Tricosanol	0.10679	-0.10595	0.02118	0.02597	-0.00143	-0.07629	-0.00157	0.02038	-0.02281	-0.03098
Tetracosanoic acid	0.08194	-0.12810	0.08426	-0.12689	-0.06919	-0.02014	0.06668	0.02202	-0.09464	-0.05202
Tetracosanol	0.09178	-0.11554	0.04947	-0.06751	-0.07291	-0.12687	0.02061	0.08543	-0.05695	-0.12241

Table S5-3.1. Continuation (4).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
Pentacosanoic acid	0.07292	-0.13313	0.01060	-0.02480	-0.12217	-0.01945	0.02386	-0.00430	-0.04464	0.00650
2OH-Tetracosanoic acid	0.08349	-0.10605	0.03490	0.07977	-0.06176	0.11530	-0.14657	0.08443	0.22468	0.03613
Hexacosanoic acid	0.08558	-0.11457	-0.01182	-0.10250	-0.05999	-0.08987	0.13673	0.00045	-0.00617	-0.04601
Hexacosanol	0.09796	-0.09337	0.02792	0.02593	-0.00672	-0.18549	0.04815	-0.00020	0.07241	-0.14711
Heptacosanol	0.08580	-0.10623	-0.06386	0.04340	0.02548	0.07625	0.03980	-0.19432	0.05501	0.11764
Octacosanoic acid	0.07527	-0.14889	0.02742	-0.02557	-0.04665	-0.07782	0.07139	-0.05655	-0.02295	-0.02424
Octacosanol	0.10490	-0.09574	0.03001	0.07445	0.01692	-0.11871	0.01025	-0.09935	0.00138	0.03923
Solanid-5-enol	0.06599	0.04597	0.17155	0.04859	-0.13250	0.02424	-0.27272	0.01446	0.27301	-0.08150
Nonacosanoic acid	0.06795	-0.13962	-0.05748	0.02851	0.02166	0.03842	-0.01927	-0.11494	0.05876	0.15914
Nonacosanol	0.09049	-0.08532	-0.07360	-0.04377	0.01577	0.01763	0.05582	-0.26234	0.13071	0.14727
Stigmasterol	0.11262	-0.02154	0.06963	0.05003	-0.03481	-0.11224	-0.12051	-0.08099	-0.15288	0.12016
Fucosterol	0.02051	0.00833	0.17811	-0.05554	0.04309	0.06604	-0.24365	-0.19588	0.06783	-0.01797
β-Sitosterol	0.10911	-0.00025	-0.00697	0.04393	0.00582	0.15756	-0.13867	-0.17252	-0.09526	0.06391
δ-5-Avenasterol	-0.01705	0.04907	0.16894	-0.14941	0.03508	0.04128	-0.21794	-0.26121	0.09527	-0.07929
Triacontanoic acid	0.08197	-0.13195	0.04843	0.02099	-0.02146	-0.12375	0.00145	0.00862	-0.02927	0.09805
Triacontanol	0.11277	-0.06124	0.01702	0.04097	0.05650	-0.11682	-0.01009	-0.01050	-0.10230	0.08818
Stigmastadienol	-0.02748	-0.05800	0.18794	-0.02817	0.13065	0.09688	0.17855	-0.19015	0.06882	-0.11101

Table S5-3.2. Loadings scores from PCA of polar metabolites identified by GC/MS in season 3 (2018/2019).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
Oxalate	0.13984	0.12335	0.04607	-0.01556	0.04160	-0.06764	-0.05047	-0.02302	0.06948	-0.03559
Valine	0.14760	-0.01137	-0.03352	-0.02193	-0.07693	-0.06727	0.02297	0.15074	0.01324	-0.06271
Urea	0.12174	0.07525	0.03888	-0.11022	-0.20465	-0.00410	-0.00718	-0.13362	-0.19232	0.11008
Ethanolamine	0.14287	0.08719	0.03617	0.01152	-0.02759	-0.13887	-0.05889	0.01470	0.15681	0.00112
Phosphate	0.14154	-0.02138	0.02971	0.07969	0.04019	-0.05185	-0.11041	0.00336	0.16222	0.06552
Leucine	0.13943	-0.09290	-0.03309	0.00318	-0.09126	-0.09586	0.00334	0.03647	0.00300	0.02998
Glycerol	0.12841	-0.01481	0.08528	0.14676	-0.07748	0.06103	0.04163	-0.07646	-0.06931	0.03658
Isoleucine	0.14285	-0.04982	-0.01464	-0.05381	-0.14991	-0.01283	0.00941	0.11504	-0.02918	-0.03083
Proline	0.12527	0.00257	-0.14883	-0.07593	0.00818	-0.15130	-0.06741	-0.08807	0.21259	0.00732
Glycine	0.14624	-0.05737	-0.07254	0.00067	-0.00071	0.05968	0.01714	0.07233	-0.02715	-0.11768
Succinic acid	0.11082	0.07926	-0.00727	0.01816	0.09707	-0.24736	-0.13854	0.01777	-0.32367	0.10243
2,3-Dihydroxypropanoic acid	0.11743	0.17917	0.05039	-0.07240	-0.05233	-0.03445	-0.08930	0.15124	0.26091	-0.11220
Fumarate	0.12301	0.07408	0.01419	0.08489	0.09874	-0.03145	0.03376	0.02390	-0.37902	0.05032
Serine	0.14181	-0.10059	-0.02801	0.04505	-0.03650	0.00210	0.10424	0.06845	0.06310	0.01666
2-Piperidinecarboxylic acid	0.11368	-0.05284	-0.13864	-0.02584	-0.03240	0.17345	-0.12935	-0.02566	-0.01655	-0.15663
U1376_Unknown	0.08325	0.17123	0.04075	-0.24000	-0.26404	0.13455	0.14028	0.06300	-0.06737	-0.02057
Threonine	0.13487	-0.06256	-0.07967	0.09079	0.10941	0.01448	0.04139	0.08230	0.09968	-0.02560
β-Alanine	0.12885	0.01056	-0.06861	0.05588	0.00295	-0.06495	0.12016	0.16667	-0.03633	-0.19702
Malate	0.10683	0.13906	-0.01749	0.08392	0.02792	-0.27622	-0.03938	0.17073	0.06994	0.04725
U1509_Unknown	0.09874	0.10841	0.18995	-0.16250	-0.11820	0.19776	-0.01306	0.13090	0.06383	-0.00324
Methionine	0.13118	-0.14426	0.05189	0.04584	-0.09982	0.09705	-0.07595	0.11975	-0.09379	-0.00826
Oxoproline	0.12129	-0.12692	-0.05559	-0.03002	-0.09307	-0.17938	-0.20151	0.03269	-0.05679	-0.11564
Aspartic acid	0.14170	-0.05861	0.03769	0.11765	-0.01433	-0.12200	-0.03021	-0.06770	0.02699	0.04944
γ-Aminobutyric acid	0.10742	-0.09539	-0.07180	0.07525	0.09296	0.15623	0.07931	0.01748	-0.15667	-0.22747
Threonic acid	0.12753	0.06132	-0.06540	0.03050	0.14874	0.06925	-0.00152	0.17474	0.09729	-0.15499
U1567_Unknown	0.12427	-0.12250	0.05805	-0.14205	-0.04765	0.05692	-0.23377	-0.06309	-0.07328	-0.11893
U1586_Unknown	0.08795	-0.13502	0.16152	0.23998	0.01141	0.06083	0.09570	0.11240	0.20235	0.14930

Table S5-3.2. Continuation (1).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
U1585_Unknown	0.12671	-0.09679	-0.03399	-0.10022	-0.09161	0.04652	-0.23145	-0.10954	-0.18795	-0.11516
U1598_Unknown	0.03494	-0.21268	0.33237	-0.14476	0.15277	0.10128	-0.10168	0.10647	0.00675	0.06204
Glutamic acid	0.14464	-0.03932	0.00715	0.09815	0.00361	0.05461	-0.06484	-0.12501	0.06440	0.06028
Asparagine	0.11414	-0.20736	0.00193	0.13100	-0.14310	0.01759	-0.09250	0.02575	-0.09075	0.03157
Phenylalanine	0.13410	-0.11669	-0.13731	-0.02352	-0.01841	-0.01392	-0.03380	-0.01655	0.07900	-0.01670
Trihydroxypentanoic acid	0.14455	0.03465	0.04564	0.09131	0.04152	-0.05551	0.04999	0.00619	-0.02361	0.07813
U1703_Unknown	0.12368	-0.06690	0.04295	0.08777	0.14882	0.06136	0.03182	-0.00842	-0.05322	-0.13864
Glutamine	0.08935	-0.11087	0.05918	0.30089	-0.03720	0.02217	0.22400	0.07476	-0.02821	0.15386
Putrescine	0.13493	-0.06363	-0.06892	0.06912	0.00492	-0.02053	-0.09673	-0.14531	0.07496	0.21024
U1751_Unknown	0.12503	0.01623	-0.02214	-0.13387	-0.06934	0.10791	-0.15938	-0.26519	0.11494	0.07442
U1755_Unknown	0.11971	0.02338	0.07703	0.05303	-0.13919	0.03076	0.03363	-0.19528	0.17584	0.07544
USA1768_Unknown	0.12721	0.08970	-0.00504	0.03171	0.06142	0.09564	-0.07022	-0.14953	-0.12335	0.05706
α-glycerophosphate	-0.05236	0.17415	-0.04237	0.21231	-0.04309	0.25950	-0.17962	0.17452	-0.01795	0.11442
U1801_Unknown	0.01335	0.01012	0.31778	-0.18129	0.30960	0.02169	0.02502	0.10480	-0.01729	0.03403
Citric acid	0.13917	-0.06988	0.03651	0.12540	0.03361	-0.01268	-0.04012	-0.08965	0.05904	0.04392
Quinic acid	0.09689	0.06756	-0.07814	0.05173	0.23564	0.12642	0.30675	-0.00313	0.06099	-0.12978
U1871_Unknown	0.01628	-0.19340	0.33316	-0.17409	0.23046	0.01570	-0.06659	0.06117	0.04112	0.07292
Fructose	0.05600	-0.01313	-0.27379	-0.24729	0.09152	0.15159	0.01881	0.10096	-0.01145	0.36507
Allantoin	0.14242	-0.04479	0.02799	0.09428	-0.01561	-0.07242	-0.13067	-0.09268	0.04269	0.14780
Mannose	0.11777	0.00977	-0.14256	-0.08533	0.15946	-0.01709	0.04840	-0.03286	0.03844	0.16935
Galactose	-0.04914	0.15375	-0.05282	0.16554	-0.01816	0.29058	-0.17693	0.22632	-0.02507	0.28384
Glucose	0.09215	0.02412	-0.25533	-0.18588	0.19931	0.09364	-0.06073	0.00607	-0.03810	0.24743
Histidine	0.07940	-0.27693	-0.08437	0.01199	-0.14712	-0.18160	0.05809	0.12801	-0.09681	0.02391
Lysine	0.13278	-0.15391	-0.04843	-0.05279	-0.01543	0.00551	0.01189	0.11091	-0.01000	-0.02397
Mannitol	0.05515	0.15374	-0.08111	0.18319	0.09924	0.26452	-0.26056	0.19674	-0.13504	-0.05828
Sorbitol	0.11570	0.18704	0.04831	0.05211	0.04614	-0.03548	-0.19320	0.14504	0.05711	-0.05072
Tyrosine	0.12223	-0.14241	-0.09954	-0.11077	0.00050	-0.00315	0.00454	0.20919	0.02936	-0.07191

Table S5-3.2. Continuation (2).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
U1948_Unknown	0.05887	-0.17341	0.30946	-0.12227	0.19569	0.02644	-0.06582	-0.02553	-0.04994	0.03902
UC2020_Unknown	-0.05149	-0.10169	-0.15712	-0.21094	-0.01641	-0.15059	0.11729	0.30519	0.01888	0.31765
Galactaric acid	0.12293	-0.01975	0.08604	-0.07594	-0.17222	0.21656	0.08577	-0.14606	-0.08753	0.03720
Inositol	0.11715	0.01108	-0.15157	-0.09232	0.13848	0.14056	-0.06726	-0.27147	0.11009	-0.01507
UC2105_Unknown	0.14279	0.09086	0.03879	0.03666	0.00779	0.01478	0.08384	-0.04835	0.05841	0.11499
U2125_Unknown	0.09066	0.16232	0.15917	0.05707	-0.02357	-0.07630	0.14116	-0.00579	-0.33495	0.17239
Caffeic acid	0.11536	0.18921	0.03195	0.04955	0.13619	-0.11933	0.04814	-0.04344	0.06499	-0.06074
U2190_Unknown	0.12604	-0.10458	-0.11018	0.02552	-0.05640	0.04083	0.20586	0.02873	-0.14042	-0.04256
Tryptophan	0.11380	-0.12513	0.03861	-0.12387	-0.04493	0.10972	0.07482	0.13513	-0.00260	-0.01402
Spermidine	0.12015	0.11813	0.00826	0.00810	0.10389	-0.17763	-0.24107	0.12885	-0.02233	-0.04440
Fructose-6-phosphate	0.11501	0.15892	0.00817	-0.05014	0.17996	-0.11122	0.04572	-0.10875	-0.17369	0.00521
Galactosyl Glycerol	0.12176	0.02687	0.10776	0.17630	-0.03228	0.14427	0.04718	-0.03743	0.09785	-0.00113
Glucose-6-phosphate	0.13126	0.02138	0.07126	-0.06567	-0.15135	0.03305	0.09446	-0.05761	0.09722	0.11194
U2367_Unknown	0.14341	0.05888	0.00398	0.00498	0.09063	0.02945	0.15886	-0.00047	0.01707	-0.13325
U2477b_Unknown	0.09024	0.19645	0.11206	-0.12882	-0.23415	-0.08781	0.00887	0.11673	0.06134	0.06801
U2495_Unknown	0.10667	0.19877	0.03992	-0.18012	-0.07538	0.06136	0.04370	0.12094	0.08937	-0.13280
U2502_Unknown	0.11326	0.17068	0.07262	-0.14551	-0.10902	0.07638	0.15642	-0.01191	-0.09579	-0.06706
Sucrose	0.10558	-0.03286	-0.20416	-0.10650	0.22865	0.09075	0.14098	-0.07017	-0.00296	-0.04898
Galactinol	0.12984	0.06511	0.07640	0.05508	0.04102	0.01365	0.20977	-0.06220	0.07731	0.14321
Chlorogenic acid	0.10183	0.16620	0.01565	-0.05592	0.06146	-0.18259	0.02630	-0.08569	-0.08855	-0.01085

Table S5-3.3. Loadings scores from PCA of non-polar metabolites identified by GC/MS in season 3 (2018/2019).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
U1595_Unknown	-0.02083	0.18806	0.36194	0.00387	0.02780	-0.41851	-0.01441	0.25258	0.01364	-0.11028
U1680_Unknown	0.08179	0.17142	0.28564	0.12191	0.24246	-0.06970	0.12851	-0.01045	-0.28766	0.48525
Tetradecanoic acid	0.15561	0.07850	-0.07289	0.00761	-0.00485	-0.14651	-0.19947	0.04116	0.03768	0.15734
U1762_Unknown	0.14552	0.16027	0.08486	0.08769	-0.16498	-0.17989	0.05957	0.12308	-0.22255	0.12353
Br-pentadecanoic acid	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Pentadecenoic acid	0.16329	0.11027	0.03382	0.00053	-0.02259	0.12485	-0.07817	-0.01671	0.05438	-0.06387
Cinnamic acid	0.13807	-0.22256	0.05515	0.13475	0.07999	-0.24248	0.11828	0.14764	-0.08507	0.00742
Pentadecanoic acid	0.16331	0.07993	-0.04772	0.01133	-0.14216	0.00323	-0.20419	0.07419	-0.14548	-0.05250
U1845_Unknown	0.16936	-0.05374	0.02576	0.09192	0.09530	-0.08807	0.09482	0.01210	-0.10897	0.05841
Hexadecenoic acid	0.16192	-0.01861	-0.00609	-0.00126	0.04138	-0.13256	0.00306	-0.08698	0.08415	0.07028
Hexadecanoic acid	0.16968	0.02640	-0.06550	-0.16536	0.06240	-0.13181	-0.14692	-0.01580	-0.01139	0.04809
Cinnamic acid	0.16846	-0.07013	0.08034	0.11967	0.14222	0.03206	0.07045	0.04089	-0.05468	0.11418
Me-Hexadecanoic acid	0.12856	0.03934	-0.06914	-0.33313	0.12690	0.03255	0.31464	0.06047	-0.06406	0.16753
Heptadecanoic acid	0.17239	0.06330	0.01207	0.01125	0.02985	0.10109	0.06768	-0.01829	0.06715	0.06870
Linoleic acid	0.14134	-0.18464	-0.24146	-0.00620	0.00521	-0.18296	-0.11003	0.15121	0.00905	0.00089
α-linolenic acid	0.13193	-0.30092	0.02590	-0.02935	-0.11495	-0.08012	-0.14079	-0.07636	0.08020	0.13867
Octadecenoic acid	0.13800	-0.15672	-0.02812	-0.08132	0.09644	-0.18073	-0.19512	-0.30324	0.04654	0.04247
2OH-Hexadecanoic acid	0.17409	-0.03458	0.08714	-0.00308	-0.03586	0.07268	-0.08023	0.09724	0.05778	0.07566
Noctadecanoic acid	0.17040	0.00849	0.02142	-0.11615	0.13802	0.13102	-0.05856	-0.04787	0.10515	0.14558
Nonadecenoic acid	0.08324	0.10840	0.01874	-0.30536	-0.08847	0.16076	0.38912	0.20624	0.46508	-0.00316
U2263_Unknown	-0.01094	0.06287	0.43282	-0.00977	0.16112	-0.21830	0.09669	0.04536	0.32085	-0.31749
Tricosane	0.14652	0.13892	-0.12995	0.01362	0.20579	0.07258	0.02973	-0.14948	-0.12861	-0.25288
Eicosanoic acid	0.17539	0.00153	0.08751	-0.04412	-0.03371	0.14677	-0.11130	0.05831	0.07714	0.05959
Heneicosanoic acid	0.16320	0.09753	-0.03038	-0.00014	0.02750	0.09216	-0.21584	0.10393	0.13783	0.14553
Heneicosanol	0.11186	-0.02002	0.08685	0.34176	0.33169	0.08142	-0.08592	-0.23132	0.12282	-0.10887
U2457_Unknown	0.13576	0.30435	0.00543	-0.06966	0.13193	0.12422	-0.04346	-0.01100	0.01996	0.01369
U2466_Unknown	0.13391	0.31339	0.00595	-0.07232	0.15074	0.11733	-0.05150	0.00200	-0.01023	0.00254

Table S5-3.3. Continuation (1).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
U2510_Unknown	0.10089	0.23852	0.17924	-0.10396	-0.23354	-0.25664	-0.08138	-0.09020	-0.11475	-0.18508
Docosanoic acid	0.17131	0.05463	0.07684	0.06795	-0.10814	0.08517	-0.11201	-0.00104	0.16219	-0.02144
Docosanol	0.15982	-0.03295	0.05725	0.21737	0.05632	0.04668	0.00546	-0.09635	0.19045	-0.08464
Tricosanoic acid	0.16931	0.08648	0.00644	0.02759	-0.13979	0.06815	-0.16888	0.05936	0.16890	0.04569
Tricosanol	0.17085	-0.07211	-0.02694	0.09503	0.07048	-0.00921	0.09389	-0.00044	0.00199	-0.03738
Tetracosanoic acid	0.15854	0.04716	0.11397	0.11957	-0.29354	-0.01283	-0.00576	0.01363	0.04915	0.02260
Tetracosanol	0.16102	-0.02665	0.01645	0.22844	-0.19348	0.04832	0.09193	-0.01617	-0.00082	0.02008
Pentacosanoic acid	0.15084	0.05661	-0.04446	0.05108	-0.24371	0.09775	-0.18397	-0.00546	0.01463	-0.12828
2OH-Tetracosanoic acid	0.14635	-0.01343	0.07354	-0.10935	0.21948	0.22169	-0.11390	0.30763	-0.22655	-0.16807
Hexacosanoic acid	0.15264	0.03846	-0.04735	0.06999	-0.32479	-0.03076	0.20071	0.08420	-0.03153	-0.00939
Hexacosanol	0.15467	-0.09453	-0.06165	0.10408	-0.11104	0.10901	0.28670	0.02740	-0.01199	0.10656
Heptacosanol	0.14831	-0.01897	-0.10443	-0.29661	0.08659	-0.13433	0.03006	0.03954	0.00235	0.01315
Octacosanoic acid	0.16300	0.06266	-0.01938	0.05899	-0.13927	0.02099	0.09274	-0.18213	-0.12093	0.01010
Octacosanol	0.16443	-0.11643	-0.04715	-0.01905	0.04369	-0.01309	0.22221	-0.07262	-0.09087	-0.02639
Solanid-5-enol	0.04662	-0.28486	0.22330	-0.01801	0.00814	0.31743	-0.10096	0.40874	-0.30942	-0.25143
Nonacosanoic acid	0.14738	0.06968	-0.07569	-0.19830	0.08317	-0.01474	0.00941	-0.25506	-0.11960	-0.31967
Nonacosanol	0.14056	-0.05034	-0.13128	-0.31026	-0.09845	-0.19660	0.00795	-0.02922	-0.17868	-0.12917
Stigmasterol	0.13211	-0.27298	0.02657	0.07513	-0.01467	-0.03541	0.06549	0.06502	0.13620	-0.11271
Fucosterol	0.02112	-0.17823	0.40416	-0.22383	-0.05392	0.17799	-0.03506	-0.24876	-0.04387	0.07850
β-Sitosterol	0.11494	-0.28148	0.03613	-0.18652	0.14450	-0.13888	-0.17327	0.10330	0.13377	0.09131
δ-5-Avenasterol	-0.04049	-0.17071	0.37601	-0.23439	-0.21675	0.13295	0.00806	-0.32714	-0.10049	0.08991
Triacontanoic acid	0.16062	0.01401	0.00629	0.07681	-0.01336	0.05572	0.20339	-0.16719	-0.16255	-0.24176
Triacontanol	0.15174	-0.15191	-0.04019	0.07845	0.05981	-0.10855	0.21466	-0.04733	0.07454	-0.14237
Stigmastadienol	0.00616	0.05474	0.41724	-0.23588	-0.06654	0.25942	0.07167	-0.26879	0.01107	-0.12195

Table S5-3.4. Loadings scores from PCA of all metabolites identified by GC/MS (polar/non-polar fraction) at 5 weeks after storage in season 3 (2018/2019).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
Oxalate	0.04433	0.06187	0.07026	0.19513	-0.03240	-0.02383	0.05174	-0.06350	-0.01383	-0.06589
Valine	0.06751	0.18331	0.08789	-0.05123	-0.04023	-0.03854	0.05956	-0.00532	-0.01740	-0.04596
Urea	-0.00946	0.00251	0.07322	-0.02234	-0.11861	0.14764	0.17260	-0.13823	-0.15575	0.19939
Ethanolamine	0.07349	0.10520	0.11508	0.03383	0.06009	0.16482	-0.13379	0.06083	-0.04119	-0.01893
Phosphate	0.02976	0.16346	0.12300	-0.08717	0.02071	-0.09244	0.00485	-0.05852	-0.11049	-0.05402
Leucine	0.06983	0.18932	0.05056	-0.02809	-0.00126	0.05367	0.05745	0.01996	-0.04227	-0.10259
Glycerol	0.08995	0.09903	0.03993	-0.00985	0.04597	0.21753	-0.06759	0.02418	-0.07582	-0.11486
Isoleucine	0.07192	0.18487	0.03883	-0.03704	-0.05292	0.02709	0.08002	0.00624	-0.01829	-0.11761
Proline	-0.00614	0.09383	-0.00045	0.03777	0.01526	-0.23496	0.02740	-0.05154	-0.11077	-0.05920
Glycine	0.09665	0.13971	-0.01444	-0.07720	-0.00499	-0.13463	-0.00517	-0.09405	-0.09364	0.06647
Succinic acid	0.05087	0.11070	-0.07757	0.04445	-0.07765	0.26568	-0.06728	0.03914	0.10628	0.06799
2,3-Dihydroxypropanoic acid	0.07440	0.16918	0.02635	0.11482	-0.04220	0.04071	-0.04041	0.00448	-0.07176	0.07031
Fumarate	0.07214	0.16899	0.05513	0.09996	-0.01765	-0.08169	0.07046	-0.03032	0.00377	0.11343
Serine	0.08190	0.16294	-0.02383	0.01568	0.07166	-0.15891	0.02402	-0.02679	-0.06499	0.01328
2-Piperidinecarboxylic acid	0.02450	0.13117	-0.09824	0.04282	-0.06139	0.03834	0.01980	0.05435	-0.08911	0.09208
U1376_Unknown	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Threonine	0.06987	0.16847	0.05271	-0.02201	0.05526	-0.10397	-0.03701	0.04873	-0.05673	0.11464
β-Alanine	0.09212	0.11349	0.02685	0.01675	-0.02290	-0.12720	0.12353	-0.06771	-0.07279	0.13165
Malate	0.06728	-0.01159	0.19308	0.06606	0.02637	0.15017	0.04713	-0.12579	0.10992	0.19338
U1509_Unknown	0.06354	0.08261	-0.17464	0.16510	0.03976	0.07402	-0.07276	-0.02023	-0.02201	0.07506
Methionine	0.08884	0.16559	-0.08941	0.01893	-0.04575	0.02289	0.06924	0.04522	-0.00029	-0.08714
Oxoproline	0.03792	0.08372	-0.09274	-0.14886	-0.06590	0.09463	0.20848	-0.00909	-0.13219	0.02535
Aspartic acid	0.04745	0.00721	-0.00512	-0.01358	0.17798	0.22038	0.24435	-0.13841	-0.04745	0.05516
γ-Aminobutyric acid	0.09064	0.07801	-0.00194	-0.10584	0.02091	-0.11178	0.15106	0.09362	0.13249	0.19383
Threonic acid	0.06294	0.14937	0.11513	-0.09172	-0.08890	0.07662	-0.10676	-0.07937	0.04365	0.06277
U1567_Unknown	-0.03182	0.05433	-0.20427	-0.00882	-0.13450	0.16854	0.09659	-0.00898	-0.01900	-0.07716
U1586_Unknown	0.07501	0.04972	-0.09460	0.18604	0.06233	0.10090	-0.04559	-0.08690	-0.04792	0.15893

Table S5-3.4. Continuation (1).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
U1585_Unknown	-0.06444	0.03923	-0.13768	-0.08355	-0.15076	0.12847	0.18533	0.03056	-0.06361	-0.11539
U1598_Unknown	0.05519	0.08671	-0.17071	0.18871	0.01407	0.06776	-0.08879	0.07267	-0.01635	0.07435
Glutamic acid	0.03823	0.13864	0.07041	0.02017	0.05358	-0.16279	-0.07284	-0.19203	-0.19633	-0.09018
Asparagine	0.00941	0.14229	-0.12997	0.02410	-0.00029	-0.01560	0.17574	0.09514	-0.03217	-0.10022
Phenylalanine	0.02388	0.19182	0.03094	0.02949	-0.11573	0.02863	0.08264	-0.03502	-0.02424	-0.09345
Trihydroxypentanoic acid	0.08130	0.10231	0.09774	0.08564	0.09788	-0.13297	-0.01321	-0.11329	0.07710	-0.11608
U1703_Unknown	0.08085	0.04249	-0.12147	-0.04722	0.11315	0.01649	0.12794	-0.06850	-0.00702	0.14791
Glutamine	0.10858	0.05129	-0.04809	0.06450	0.10359	-0.09568	0.11868	0.00884	-0.08899	0.17295
Putrescine	0.05276	0.02021	-0.09179	-0.15287	0.07607	0.04810	0.15240	0.06091	0.07548	0.27479
U1751_Unknown	-0.07845	0.02771	-0.18479	0.02360	-0.02720	0.03029	-0.18717	-0.10073	-0.07324	0.01148
U1755_Unknown	-0.01134	-0.06560	-0.04624	0.00319	0.24720	0.02399	0.07936	0.03943	0.18445	-0.02453
USA1768_Unknown	0.03808	0.14894	0.01617	0.10808	-0.07309	0.18834	-0.08973	-0.03608	0.09535	-0.10138
α-glycerophosphate	0.06951	0.05372	0.14880	-0.12225	0.13422	0.11596	0.04243	0.16357	-0.06356	-0.07389
U1801_Unknown	0.04756	0.06895	-0.14343	0.16582	0.07277	0.10139	-0.15121	0.09791	-0.00605	0.13426
Citric acid	0.05384	0.09843	0.15832	0.13349	0.13355	0.01546	-0.02889	0.00382	0.00056	0.05673
Quinic acid	0.01639	-0.00217	0.15075	0.05354	-0.19135	-0.10927	-0.14503	0.05043	0.12879	0.02851
U1871_Unknown	0.05196	0.08547	-0.15012	0.16231	0.03621	0.09536	-0.14440	0.10754	0.00527	0.10989
Fructose	0.05342	0.08939	-0.00444	-0.21143	-0.15878	-0.00480	-0.06230	0.05110	0.10946	0.11397
Allantoin	-0.00532	0.17255	0.05211	0.01392	0.07132	-0.07488	0.02862	-0.15473	-0.12325	0.00421
Mannose	0.07343	0.09805	-0.01213	-0.20946	-0.01848	0.00487	-0.08446	0.02486	0.04054	0.11479
Galactose	0.07203	0.02490	-0.07028	-0.16859	-0.03439	-0.01852	-0.08654	0.00908	0.20406	0.18429
Glucose	0.03943	0.06482	-0.03243	-0.20713	-0.15711	0.01440	-0.06406	0.06721	0.15381	0.13974
Histidine	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Lysine	0.06182	0.17634	-0.03012	0.03340	-0.08471	0.03914	0.05907	0.09589	0.06374	-0.09416
Mannitol	0.03774	0.13659	-0.00775	0.09013	0.04933	-0.04320	0.15843	-0.05445	0.23526	-0.03182
Sorbitol	0.00399	0.01170	0.02426	-0.00049	-0.03907	0.02978	0.29970	0.14434	-0.19902	0.02922
Tyrosine	0.06338	0.16188	-0.03174	-0.02427	-0.07857	0.07466	0.07110	0.04369	0.03169	-0.17433

Table S5-3.4. Continuation (2).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
U1948_Unknown	0.03803	0.06538	-0.10896	0.18229	0.02297	0.01695	-0.13759	0.13506	0.02505	0.10180
UC2020_Unknown	0.03393	0.06533	-0.02808	-0.22720	-0.15855	-0.02648	-0.07561	0.06175	0.14574	0.11927
Galactaric acid	0.01821	0.03671	-0.23627	0.08262	0.02680	-0.02025	-0.09219	0.00639	0.10130	-0.14012
Inositol	-0.11811	0.08198	-0.03627	-0.09116	-0.05039	-0.08019	-0.11820	-0.00901	-0.06563	0.05309
UC2105_Unknown	0.02485	0.11666	0.09639	0.01898	0.17756	-0.00149	-0.20664	-0.01980	0.10266	0.03595
U2125_Unknown	0.07496	0.02333	-0.09461	0.19127	0.05492	-0.01722	0.01531	-0.16256	-0.08425	0.00525
Caffeic acid	-0.00496	-0.00651	0.22192	0.04482	0.10975	0.21145	0.01181	0.01380	0.00788	-0.02827
U2190_Unknown	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Tryptophan	0.06589	0.14371	0.00641	-0.02771	-0.06964	0.01934	0.05854	0.07243	0.06736	-0.20173
Spermidine	0.04179	0.08853	0.19611	0.01866	0.11738	0.07501	-0.04899	0.05701	-0.07867	-0.00971
Fructose-6-phosphate	0.01771	-0.00824	0.10123	-0.01039	0.09137	-0.07784	-0.08792	0.34474	-0.16438	0.07447
Galactosyl Glycerol	0.09910	0.01411	-0.13726	-0.11227	0.17522	0.07552	0.00839	-0.00059	-0.11419	-0.00882
Glucose-6-phosphate	-0.01158	-0.04657	0.12997	-0.07226	0.07425	-0.08786	-0.02358	0.30300	-0.21542	0.01819
U2367_Unknown	0.03491	0.01649	0.12195	0.09565	0.05717	0.02556	-0.11099	0.36963	-0.06967	0.12937
U2477b_Unknown	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
U2495_Unknown	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
U2502_Unknown	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Sucrose	-0.05196	0.13069	-0.10195	0.04514	-0.05220	-0.00797	0.09725	0.18347	0.10628	-0.01907
Galactinol	0.06706	0.02393	-0.10078	0.18706	0.09778	-0.02628	0.08832	0.03577	0.08800	0.17538
Chlorogenic acid	-0.00135	-0.03292	0.10789	0.00753	0.04071	0.19140	-0.07417	0.15907	-0.11912	-0.15409
U1595_Unknown	0.11370	0.06917	-0.02050	-0.16017	0.11681	0.00111	-0.07759	-0.02079	0.04519	-0.10129
U1680_Unknown	0.06473	0.05416	0.17620	-0.03692	-0.02264	0.05016	-0.07527	-0.16266	0.19513	0.00009
Tetradecanoic acid	0.05879	-0.01275	-0.01414	-0.12217	0.11660	0.15766	0.04573	-0.07205	0.14540	-0.00410
U1762_Unknown	0.07110	0.00126	0.11274	-0.04229	0.16452	-0.08261	0.10367	0.04771	0.20637	0.01724
Br-pentadecanoic acid	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Pentadecenoic acid	-0.12386	0.03304	0.06501	0.02641	0.05007	0.19197	-0.00443	-0.03162	-0.00326	-0.03770
Cinnamic acid	0.00576	0.05459	0.08536	0.14223	-0.01020	-0.18444	0.09048	0.12388	0.25143	-0.12566

Table S5-3.4. Continuation (3).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
Pentadecanoic acid	-0.07844	0.07161	0.10554	-0.02299	0.22537	-0.03809	0.04290	-0.07965	0.02141	0.05775
U1845_Unknown	-0.11759	0.08977	0.03313	0.08346	0.01853	-0.03226	0.01564	0.05857	0.22003	-0.09418
Hexadecenoic acid	-0.12275	0.10397	0.03082	0.01907	0.03587	-0.02612	-0.13731	-0.13228	-0.01190	0.05809
Hexadecanoic acid	-0.13112	0.10701	-0.05674	-0.09281	0.04163	-0.06247	-0.06837	-0.04231	-0.02499	-0.04090
Cinnamic acid	-0.11800	0.12377	0.04420	0.04888	-0.08475	-0.01694	0.03375	0.05285	0.13445	-0.03809
Me-Hexadecanoic acid	-0.13004	0.11134	-0.05660	-0.03899	0.00047	-0.12542	0.00498	0.05586	-0.02869	0.02518
Heptadecanoic acid	-0.14666	0.09382	0.02210	-0.04112	0.02709	0.03676	0.05162	-0.00549	0.00174	0.03232
Linoleic acid	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
α-linolenic acid	-0.16782	0.03526	-0.00931	0.00577	-0.00666	0.03887	-0.02211	-0.03982	-0.01675	0.01688
Octadecenoic acid	-0.15626	0.02412	-0.01751	-0.04407	0.01087	-0.01686	-0.05369	0.01478	-0.09568	-0.01301
2OH-Hexadecanoic acid	-0.13559	0.09973	0.06097	-0.04852	0.00069	0.02202	0.01627	-0.04286	0.02337	0.11567
Noctadecanoic acid	-0.15910	0.07338	-0.05157	-0.02560	-0.01751	0.01648	-0.01250	0.02174	0.02064	0.01942
Nonadecenoic acid	-0.11879	0.06973	-0.04700	0.02226	0.00577	-0.08695	-0.06950	0.00177	0.07858	0.02805
U2263_Unknown	0.04415	0.01785	-0.11997	-0.11871	0.24634	-0.03272	-0.11593	-0.03997	0.07581	-0.11328
Tricosane	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Eicosanoic acid	-0.16190	0.05277	-0.02160	-0.01543	0.03631	0.06041	-0.00542	-0.05008	0.03427	0.01964
Heneicosanoic acid	-0.13551	0.09478	0.00855	-0.06948	0.04727	0.09068	-0.02068	0.03065	-0.00364	-0.00464
Heneicosanol	-0.03625	0.08356	0.15272	-0.13043	-0.07412	0.12663	-0.14126	0.00158	-0.07219	0.07670
U2457_Unknown	0.01407	0.08070	-0.11486	-0.16426	0.19205	0.01311	-0.04683	0.05969	0.03102	-0.10317
U2466_Unknown	0.08124	0.06281	-0.06466	-0.14401	0.19067	0.00632	-0.08054	-0.00673	-0.01048	-0.15455
U2510_Unknown	-0.03634	0.04936	-0.09351	-0.11617	0.27859	0.00739	0.00696	0.02167	0.04316	-0.10851
Docosanoic acid	-0.15988	0.01500	-0.00228	-0.01879	0.06564	0.10669	0.05224	0.03056	0.01622	0.05169
Docosanol	-0.14751	0.05741	-0.00926	-0.02988	0.01818	0.09820	0.00325	0.11707	-0.01103	-0.00956
Tricosanoic acid	-0.15875	0.04235	-0.01803	-0.05446	0.05847	0.08150	-0.01898	-0.04818	0.00029	-0.00002
Tricosanol	-0.10545	0.06352	0.14174	0.04076	-0.04730	0.06542	0.00562	-0.11285	0.01365	0.02796
Tetracosanoic acid	-0.14035	-0.01935	-0.00031	0.01230	0.14376	0.08562	0.08810	-0.03887	0.11322	0.06617
Tetracosanol	-0.11784	-0.01164	0.14656	0.07609	-0.00711	0.10108	0.09293	0.02823	0.08535	-0.03690

Table S5-3.4. Continuation (4).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
Pentacosanoic acid	-0.13907	-0.03123	0.02684	-0.00525	0.11441	0.00351	0.06757	-0.06578	0.03681	0.10378
2OH-Tetracosanoic acid	-0.10255	0.13957	-0.03288	-0.09950	-0.01689	-0.03963	-0.10119	0.00798	-0.08627	0.07530
Hexacosanoic acid	-0.14582	0.02825	0.01735	0.02481	0.10831	-0.05913	0.11262	0.05897	0.03612	0.02216
Hexacosanol	-0.13445	0.05363	0.07449	0.12306	-0.07936	-0.03036	0.04247	0.05238	0.05644	-0.00921
Heptacosanol	-0.15013	0.03873	-0.10373	0.03085	0.02102	-0.05202	0.00408	0.09097	-0.00670	-0.06522
Octacosanoic acid	-0.15687	0.05942	0.00256	-0.02758	0.08122	-0.00976	0.01642	0.02688	0.02833	0.03861
Octacosanol	-0.15603	0.07126	0.01524	0.06540	-0.00480	-0.04112	0.02341	0.02838	0.07665	-0.00669
Solanid-5-enol	-0.13919	0.09638	0.03386	0.04160	-0.04208	-0.03053	-0.02958	-0.00166	-0.03846	-0.00390
Nonacosanoic acid	-0.15164	0.06162	-0.07963	-0.01633	0.06426	-0.05956	-0.00325	0.02950	-0.05306	0.02218
Nonacosanol	-0.15468	0.03116	-0.07548	0.02879	0.07663	-0.07639	0.02007	0.04537	-0.00361	-0.00347
Stigmasterol	-0.16371	0.05064	0.01756	0.01736	-0.00009	0.07844	0.02452	0.01397	-0.02532	0.02128
Fucosterol	-0.09106	0.05292	-0.05683	-0.00923	-0.00953	0.03965	-0.16362	-0.23292	-0.06696	-0.00212
β-Sitosterol	-0.14114	0.09521	-0.04384	-0.04920	-0.04244	-0.02684	-0.02180	-0.02776	-0.00194	-0.00614
δ-5-Avenasterol	-0.16437	0.03486	-0.02538	-0.00199	0.05657	-0.00173	0.01510	0.00160	-0.06456	0.05998
Triacontanoic acid	-0.13781	0.08023	0.08321	0.00260	0.03628	-0.02017	0.03119	-0.02203	-0.03357	0.14776
Triacontanol	-0.13968	0.08500	0.04457	0.08747	-0.02048	0.00210	0.07480	0.06195	0.03715	0.06234
Stigmastadienol	-0.03958	0.02038	0.01081	0.04412	0.02112	-0.00115	0.08064	0.05599	-0.00595	0.09486

Table S5-3.5. Loadings scores from PCA of all metabolites identified by GC/MS (polar/non-polar fraction) at 43 weeks after storage in season 3 (2018/2019).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
Oxalate	0.03784	0.09459	-0.09869	-0.18031	0.01480	-0.01866	-0.04470	-0.08242	0.06455	0.00984
Valine	0.15809	0.15224	0.03403	0.02388	0.00561	-0.00481	-0.00932	0.01277	-0.07649	-0.06543
Urea	-0.02094	0.05798	-0.08211	0.11534	-0.18059	-0.05531	0.03352	-0.20967	0.01813	-0.12407
Ethanolamine	-0.01292	0.16480	-0.01572	-0.07829	-0.15276	0.03139	-0.09214	0.11007	0.07369	-0.03943
Phosphate	0.09163	0.18340	0.01823	-0.07114	-0.00054	0.14189	0.05904	0.01452	0.01480	-0.02528
Leucine	0.14464	0.07919	-0.00464	0.02284	-0.11338	0.12936	-0.07328	-0.02399	-0.12494	-0.11431
Glycerol	0.01694	0.01031	-0.20107	-0.03932	0.03966	0.00186	0.01127	0.05738	0.11423	-0.04890
Isoleucine	0.17217	0.09957	0.03151	0.05614	-0.05510	0.06223	0.01982	0.07566	-0.09251	-0.06073
Proline	0.03395	0.01468	0.17385	0.01534	-0.06025	0.17467	-0.02232	-0.10915	-0.03551	0.02716
Glycine	0.16728	0.11936	0.00810	-0.03531	0.02699	0.01708	0.07542	0.06196	-0.07162	-0.10763
Succinic acid	0.01950	0.11653	-0.01080	0.06627	0.01946	-0.00828	0.15398	0.01488	-0.33679	-0.00827
2,3-Dihydroxypropanoic acid	0.07351	0.10298	-0.08918	-0.02281	-0.01700	0.13925	-0.00091	-0.21053	0.08111	0.02363
Fumarate	0.02253	0.20719	-0.02018	-0.00691	-0.02945	-0.02510	0.07990	0.08463	-0.05970	-0.04617
Serine	0.18489	0.08330	-0.02124	-0.04334	-0.02258	0.06613	0.01417	-0.13824	-0.02584	0.02963
2-Piperidinecarboxylic acid	0.10611	0.02521	0.05250	0.18292	0.09344	-0.03806	-0.12345	-0.09803	-0.05851	0.02704
U1376_Unknown	0.07174	0.09442	0.08677	0.01115	-0.04953	-0.10764	0.01388	-0.02008	0.07978	-0.17305
Threonine	0.15232	0.11933	0.02476	-0.00374	0.05949	-0.03002	-0.10705	-0.14081	0.00602	-0.04260
β-Alanine	0.04343	0.13798	-0.13224	-0.05792	0.08424	-0.05223	0.13536	-0.04046	-0.05267	-0.04423
Malate	-0.04641	0.19523	0.07319	-0.05911	-0.07953	-0.02006	0.01110	0.07596	-0.07927	0.00078
U1509_Unknown	0.15607	-0.02880	-0.08948	0.12462	-0.08824	-0.05771	0.03465	0.04255	0.05790	-0.01834
Methionine	0.19154	0.05801	-0.04301	0.07839	-0.03137	0.02443	-0.00325	0.05171	-0.01099	0.07151
Oxoproline	0.10703	0.09612	-0.08687	0.12746	-0.07589	-0.06871	0.13299	-0.05306	-0.06217	0.05730
Aspartic acid	0.07338	0.10976	-0.16953	0.00547	-0.06722	-0.01463	0.00081	-0.10691	0.05277	0.12635
γ-Aminobutyric acid	0.05308	0.05980	-0.11788	-0.05623	0.12886	-0.12884	0.17252	0.03559	0.02648	0.11454
Threonic acid	0.01898	0.16619	0.08593	-0.03104	0.09721	0.03607	0.11552	0.14307	-0.03833	-0.05902
U1567_Unknown	0.12878	-0.06771	-0.06079	0.17004	-0.09851	-0.05546	0.07935	0.03333	0.03359	-0.02456
U1586_Unknown	0.11428	0.03050	-0.12902	-0.02338	0.04671	0.04254	-0.04554	-0.04145	-0.06438	0.11277

Table S5-3.5. Continuation (1).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
U1585_Unknown	0.10955	-0.04759	-0.06753	0.20087	-0.07005	-0.04483	0.07727	-0.00064	-0.04397	-0.03527
U1598_Unknown	0.15994	-0.06634	-0.05357	0.12671	-0.05154	-0.02424	-0.01423	0.06725	0.09103	0.04225
Glutamic acid	0.09713	-0.02415	-0.03769	0.15786	0.05028	0.15205	-0.17616	-0.02501	0.00202	-0.14240
Asparagine	0.14629	-0.02859	-0.08647	0.17234	0.02296	0.00639	-0.06534	0.02025	-0.02348	0.02785
Phenylalanine	0.12481	0.07391	0.14541	0.11132	0.00809	-0.06214	-0.07552	0.06265	-0.01762	0.04809
Trihydroxypentanoic acid	0.03771	0.17283	0.01358	-0.09590	0.08506	0.07043	-0.00689	0.17892	-0.08733	0.03114
U1703_Unknown	0.10475	0.02222	-0.06848	0.00106	0.01370	-0.04033	0.04063	0.05225	-0.12785	0.12541
Glutamine	0.08728	0.06700	-0.16078	0.02750	0.10532	0.04040	-0.07785	-0.07438	-0.10660	0.10544
Putrescine	0.12725	-0.00905	0.11636	-0.13517	-0.12897	-0.03457	0.01976	-0.05033	-0.02547	-0.06211
U1751_Unknown	-0.01028	-0.12562	0.12204	0.11296	-0.12888	0.11684	0.10068	0.01078	-0.03836	-0.03576
U1755_Unknown	0.00260	0.00573	-0.17055	-0.06518	0.00558	0.08899	0.11122	0.04129	0.20030	0.02410
USA1768_Unknown	0.02131	0.05009	0.06974	0.18576	-0.02947	-0.04646	-0.02112	0.15371	0.02519	0.01924
α-glycerophosphate	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
U1801_Unknown	0.02947	0.00629	0.04064	-0.05992	-0.08150	-0.12837	-0.05720	-0.12469	-0.00434	-0.13921
Citric acid	-0.01176	0.10869	-0.10842	0.10924	0.16032	0.03108	-0.04639	0.13256	0.00912	-0.09104
Quinic acid	-0.00901	0.13257	0.11046	-0.05703	0.17339	-0.06420	0.04066	0.04436	0.07519	-0.12298
U1871_Unknown	0.17196	-0.03882	-0.03599	0.03593	-0.06790	-0.01516	-0.03795	0.07670	0.14327	0.03776
Fructose	0.07421	0.01423	0.15355	-0.03534	0.04662	-0.18249	0.03176	-0.05933	0.13898	-0.15155
Allantoin	0.07030	0.10071	-0.06178	0.03629	-0.10246	0.19102	-0.13214	-0.13049	0.01192	-0.13982
Mannose	0.11426	0.05734	0.10021	-0.13910	0.04013	-0.02567	-0.04513	0.04250	0.00011	-0.15092
Galactose	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Glucose	0.06574	-0.01594	0.15317	-0.04237	0.01365	-0.20171	0.07687	-0.07109	0.14230	-0.09655
Histidine	0.17820	0.05975	-0.00567	0.11937	-0.02306	-0.00778	-0.01477	-0.01342	0.00438	0.12293
Lysine	0.18675	0.02972	0.05969	0.09122	-0.02960	-0.04373	-0.01623	0.01421	0.06431	0.10966
Mannitol	-0.02842	-0.01246	-0.07895	0.02497	0.01643	-0.02610	0.13403	0.00792	0.04768	-0.02718
Sorbitol	0.08375	0.04079	-0.05817	0.00794	-0.17180	-0.08340	0.07654	0.03446	0.21226	-0.09181
Tyrosine	0.17093	0.07445	0.09110	0.06388	0.01071	-0.06666	-0.02394	0.05236	0.02215	0.13914

Table S5-3.5. Continuation (2).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
U1948_Unknown	0.14222	-0.02751	-0.13641	0.02582	-0.03113	0.01312	-0.02858	0.00384	0.12459	0.01294
UC2020_Unknown	0.06764	-0.00522	0.10852	-0.02935	-0.04514	-0.22629	0.06870	-0.04809	0.21747	-0.05133
Galactaric acid	0.07739	-0.12978	-0.05313	0.06358	-0.10826	0.11537	0.11942	0.19655	-0.03019	0.02492
Inositol	0.00183	-0.07020	0.19208	0.08663	0.00400	0.10013	-0.02319	-0.10612	0.00903	0.13026
UC2105_Unknown	0.08683	0.09997	0.02412	-0.14386	0.03681	0.10592	-0.19443	-0.00126	-0.00567	-0.09256
U2125_Unknown	0.01052	0.03626	-0.17841	-0.05017	0.03458	0.03651	-0.00391	-0.06656	0.09997	0.11158
Caffeic acid	-0.13576	0.12918	0.06510	-0.01079	-0.13697	-0.01226	-0.07776	-0.01607	0.06716	0.08141
U2190_Unknown	0.17727	0.02787	0.06402	0.08775	0.05624	-0.04533	-0.07614	-0.00308	0.02188	0.10426
Tryptophan	0.16934	0.01038	0.04158	0.07438	0.00378	-0.02361	-0.07569	0.09071	0.08258	0.13512
Spermidine	0.05769	0.11175	-0.00655	0.05310	0.02808	-0.02234	0.21464	0.09745	0.01365	-0.13105
Fructose-6-phosphate	-0.07039	-0.01713	0.06861	-0.11887	-0.08447	0.00182	0.07046	-0.07627	-0.05611	0.11910
Galactosyl Glycerol	0.06254	-0.04445	-0.20707	-0.01805	-0.01368	0.01087	0.06383	0.06378	0.08026	-0.08687
Glucose-6-phosphate	0.08522	0.04624	0.06447	-0.11713	-0.14999	0.04236	0.17990	0.07827	-0.04704	0.05810
U2367_Unknown	0.00914	0.11655	-0.00166	-0.04454	0.23859	-0.13770	0.03070	0.06467	-0.02546	0.09505
U2477b_Unknown	-0.05803	0.04443	0.02720	-0.02564	-0.19226	-0.14431	-0.07173	-0.06562	0.03067	-0.01225
U2495_Unknown	0.06757	0.09573	0.02562	-0.08295	0.09176	0.17766	0.07802	0.01143	0.13299	-0.08161
U2502_Unknown	-0.03779	-0.02963	-0.03542	-0.00146	-0.10498	-0.07290	-0.15062	0.06087	0.08615	0.04098
Sucrose	0.06271	-0.02566	0.17798	-0.08908	0.03100	-0.11395	0.09213	-0.05011	-0.01117	0.07597
Galactinol	0.04410	0.12676	0.02990	-0.12150	0.08380	-0.15937	0.15080	0.10859	0.02517	0.05632
Chlorogenic acid	-0.11024	0.10956	0.09072	-0.02529	-0.14747	-0.05087	-0.07502	0.01630	0.02042	0.13459
U1595	-0.06794	-0.06548	-0.08299	-0.04264	0.07099	-0.04840	0.02240	0.10927	0.15980	-0.09968
U1680	-0.09003	0.03649	0.06877	0.12298	0.13702	0.04968	0.03142	0.04179	-0.04931	0.10853
Tetradecanoic acid	-0.03423	0.06370	-0.04673	0.05908	-0.04571	0.17170	-0.04407	0.12154	-0.14211	-0.06551
U1762	-0.08877	0.02081	-0.13400	0.12955	0.11518	-0.02188	0.03294	0.05900	0.08820	0.06871
Br-pentadecanoic acid	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Pentadecenoic acid	-0.03290	0.12533	-0.02525	0.00465	-0.10979	0.00285	-0.02044	-0.06861	0.02624	-0.06456
Cinnamic acid	-0.12694	0.06173	0.03062	0.04181	0.10592	0.13037	-0.01762	0.04714	0.07878	0.08184

Table S5-3.5. Continuation (3).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
Pentadecanoic acid	-0.05979	0.08197	-0.09833	0.01851	-0.10529	0.06399	-0.11937	0.02449	0.20981	0.16660
U1845	-0.04070	0.11835	0.09123	0.09020	0.12221	-0.09920	-0.03439	0.03854	-0.01782	0.13592
Hexadecenoic acid	-0.01168	0.10687	-0.01313	0.02391	0.07230	0.14967	-0.13064	-0.04175	-0.10163	0.02228
Hexadecanoic acid	0.04682	0.06086	0.03085	-0.11295	0.00591	0.14878	0.02259	0.28727	0.08996	0.06165
Cinnamic acid	-0.07801	0.10602	0.09042	0.18144	0.02484	-0.03093	0.02035	0.02588	0.03407	-0.03078
Me-Hexadecanoic acid	0.04627	-0.01696	0.10892	-0.02010	0.13079	0.09723	0.24848	-0.04845	0.09132	0.09873
Heptadecanoic acid	-0.06111	0.12420	0.03919	0.10183	0.06736	-0.02099	-0.07062	-0.03006	-0.04822	-0.07872
Linoleic acid	0.01967	0.09864	0.02911	-0.14868	-0.12964	0.08127	-0.08799	0.13994	0.01419	0.06167
α-linolenic acid	-0.04601	0.13200	0.01660	-0.11259	-0.16443	-0.06252	-0.11722	0.10380	0.00521	0.10800
Octadecenoic acid	-0.00132	0.01349	0.09482	-0.12135	-0.07361	0.11975	-0.10451	0.13684	0.02646	-0.09099
2OH-Hexadecanoic acid	-0.08919	0.08493	-0.04279	0.17484	-0.07841	-0.10140	0.08917	-0.00220	0.12235	-0.07436
Noctadecanoic acid	-0.05665	0.05557	0.14789	0.15237	-0.04637	-0.06080	0.02638	0.12357	0.08182	0.08283
Nonadecenoic acid	-0.00163	-0.04363	-0.03809	-0.00032	0.08156	-0.02582	0.11208	-0.17940	-0.06957	-0.12971
U2263	-0.04203	-0.02316	0.06105	0.09804	0.00145	0.06861	0.10878	-0.08183	-0.17797	-0.05340
Tricosane	0.05673	0.06706	0.07765	0.08658	0.08607	0.11633	-0.00207	-0.08327	0.18713	-0.07703
Eicosanoic acid	-0.12096	0.08857	-0.03121	0.13917	-0.13756	0.01903	0.07905	0.04146	0.03533	0.01119
Heneicosanoic acid	-0.11251	0.04237	0.00650	0.09942	-0.18936	-0.00874	-0.06007	0.07224	0.04374	0.06891
Heneicosanol	-0.06856	0.03801	0.06657	0.13140	0.07552	-0.07394	-0.21910	0.05851	0.04067	-0.19493
U2457	-0.05740	-0.06315	0.03173	0.13275	0.15380	0.06352	0.02846	0.04953	-0.02767	-0.04457
U2466	-0.02983	-0.07423	0.02709	0.16922	0.16953	0.04084	0.03372	0.09569	0.05108	-0.01866
U2510	-0.02139	-0.03969	-0.16719	-0.07063	0.13813	0.04321	-0.06189	0.11021	0.09019	-0.04535
Docosanoic acid	-0.13885	0.08038	-0.09490	0.07679	-0.06549	0.00014	0.05795	0.04001	0.02605	-0.10406
Docosanol	-0.12793	0.07828	-0.01362	0.13740	0.00259	-0.01716	-0.07457	-0.02970	0.04636	-0.12359
Tricosanoic acid	-0.10758	0.08974	-0.13631	0.04728	-0.09472	0.02159	0.03986	0.08229	-0.07522	-0.04926
Tricosanol	-0.06333	0.12503	0.06224	0.10266	0.02714	0.09294	-0.01892	0.02920	0.15039	-0.07401
Tetracosanoic acid	-0.11211	0.08292	-0.18449	0.05024	0.00663	-0.00151	0.03207	-0.03737	-0.04135	0.03100
Tetracosanol	-0.08292	0.19106	-0.06502	0.02177	0.00241	0.00042	0.06098	-0.03061	-0.03544	-0.00586

Table S5-3.5. Continuation (4).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
Pentacosanoic acid	-0.01549	0.09413	-0.11809	-0.00867	-0.10154	-0.00107	0.11672	0.04899	0.04903	-0.16851
2OH-Tetracosanoic acid	0.00006	-0.02065	0.12763	0.14237	-0.00915	0.13917	0.12988	-0.02729	0.13617	0.11264
Hexacosanoic acid	-0.05042	0.08025	-0.15043	0.00705	0.07018	-0.10291	0.12571	-0.13595	0.03146	0.05477
Hexacosanol	-0.06066	0.19465	0.04650	-0.02452	-0.01912	-0.05701	0.04047	-0.09624	-0.05903	0.09996
Heptacosanol	0.01283	-0.06030	0.07324	-0.00919	-0.04218	0.20747	0.21551	-0.04690	0.12483	0.09236
Octacosanoic acid	-0.08945	0.13858	-0.03908	0.04842	0.06589	0.01131	-0.13026	-0.06590	0.09946	0.09161
Octacosanol	-0.07110	0.12325	0.12232	-0.01237	0.05288	0.05593	0.01962	-0.04348	0.16949	0.18743
Solanid-5-enol	-0.02961	0.12273	-0.00121	0.09733	-0.10952	0.00486	0.10672	-0.25558	-0.08775	0.07256
Nonacosanoic acid	0.10022	0.00382	0.07706	-0.00021	0.11810	0.13265	0.00868	-0.11858	0.14883	-0.19549
Nonacosanol	0.07540	-0.07827	-0.01833	-0.09613	-0.06314	0.23128	0.06157	-0.12215	0.12334	0.13570
Stigmasterol	-0.08854	0.13783	0.03945	0.06517	-0.11870	0.00433	0.11140	-0.06766	0.02105	0.01790
Fucosterol	-0.08848	0.08055	0.06099	0.02649	-0.03608	0.17844	0.18602	-0.01112	0.01336	-0.01732
β-Sitosterol	-0.01273	-0.05334	0.11473	0.11089	-0.10826	0.11398	0.09160	0.16029	0.02470	-0.08546
δ-5-Avenasterol	-0.06467	0.02729	0.06733	-0.04299	0.03804	0.17476	0.11746	-0.09711	0.05794	-0.14967
Triacontanoic acid	-0.03403	0.11120	0.00153	0.05489	0.13302	0.05741	-0.06945	-0.16179	0.18347	0.05782
Triacontanol	-0.03182	0.12880	0.04099	0.03913	0.10659	0.00314	-0.05100	-0.08076	0.04545	0.01798
Stigmastadienol	-0.04031	0.06742	0.05748	0.04235	0.02268	0.08133	0.03505	-0.05651	0.05782	-0.06784

Table S5-3.6. Loadings scores from PCA of all metabolites identified by GC/MS in Shelford varieties from two different locations in season 3 (2018/2019).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
Oxalate	0.10928	0.08592	0.03280	0.06226	-0.09254	0.04843	0.04971	-0.03158	0.00455	0.03368
Valine	0.12301	0.02566	-0.06978	0.06449	-0.02945	0.03594	-0.07706	0.00942	0.02051	0.01097
Urea	0.11526	0.04996	0.03212	-0.07474	-0.06518	-0.03482	-0.03694	0.10665	-0.01062	-0.05202
Ethanolamine	0.12073	0.06739	0.01851	0.02576	-0.05862	0.00918	0.02200	-0.01636	-0.00588	0.02446
Phosphate	0.10663	0.05478	0.01293	-0.11746	0.13903	-0.02403	0.06581	-0.08724	0.05707	0.02441
Leucine	0.12212	-0.00358	-0.07986	-0.00562	-0.08677	-0.00787	-0.06264	0.01603	-0.02045	0.02463
Glycerol	0.10464	0.08517	0.00421	0.14095	-0.00581	0.05018	0.01507	-0.02292	0.07799	0.01959
Isoleucine	0.12077	0.02241	-0.07178	0.08079	-0.05782	0.03086	-0.06539	0.01308	0.00468	0.01579
Proline	0.11370	-0.02827	-0.01347	-0.08031	-0.10252	-0.08127	0.07058	-0.05525	-0.14785	0.07464
Glycine	0.12214	0.05432	-0.03198	0.03525	0.04835	0.03203	0.00224	-0.06329	0.06162	0.01174
Succinic acid	0.10579	0.03234	-0.00491	-0.12977	0.12461	-0.08278	0.07460	0.02655	-0.02552	-0.01603
2,3-Dihydroxypropanoic acid	0.08766	0.07912	0.03577	0.18863	-0.11953	0.07218	0.04041	-0.01696	0.01058	0.01116
Fumarate	0.08639	0.05877	0.00896	-0.03997	0.26349	-0.07185	0.05235	-0.02505	0.09468	0.00786
Serine	0.12180	0.03308	-0.07655	0.05871	0.03991	0.04189	-0.05688	-0.03051	0.03322	0.00350
2-Piperidinecarboxylic acid	0.11342	0.04435	0.01834	0.09803	-0.07271	-0.00337	-0.08497	0.00893	-0.13226	-0.02079
U1376_Unknown	0.09159	0.07461	0.03495	0.16036	-0.14753	0.07698	0.02856	-0.04058	-0.00188	0.05923
Threonine	0.11587	0.05667	-0.03633	0.01485	0.12445	0.00442	-0.04013	-0.05310	0.04548	-0.00048
β-Alanine	0.12310	0.01987	-0.06268	0.05278	0.03885	0.00976	0.03843	-0.07045	0.08525	0.06345
Malate	0.10967	0.07790	0.03624	-0.01341	0.04816	-0.12416	-0.01927	0.09568	0.06627	-0.02284
U1509_Unknown	0.05317	0.12023	-0.04685	0.19236	0.03018	0.03835	0.08182	0.19234	-0.01737	-0.02672
Methionine	0.10379	0.06711	-0.06496	0.11804	0.11138	0.06341	-0.04999	-0.01105	0.03676	-0.03702
Oxoproline	0.11803	-0.00882	-0.07684	-0.10872	0.04697	-0.02803	-0.09631	0.01018	-0.01771	0.02535
Aspartic acid	0.12048	0.02689	-0.01541	-0.11051	0.01047	-0.05487	-0.08032	0.06571	-0.04933	-0.01238
γ-Aminobutyric acid	0.09536	0.06030	-0.02488	-0.00663	0.21962	0.03439	0.05524	-0.13100	0.14478	0.00990
Threonic acid	0.10023	0.09088	0.02968	0.03768	0.12847	0.00983	0.08848	-0.06516	0.12751	0.00877
U1567_Unknown	0.11692	0.06448	-0.05078	-0.02895	0.04271	0.07902	-0.00740	0.04897	0.03451	0.02818
U1586_Unknown	0.04225	0.08273	-0.07107	0.21304	0.09646	-0.08779	0.04553	0.23056	-0.11439	-0.00745

Table S5-3.6. Continuation (1).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
U1585_Unknown	0.12083	0.03310	-0.00778	-0.08922	0.05672	0.01075	-0.06252	-0.01412	-0.05691	0.00835
U1598_Unknown	-0.01370	0.08770	-0.21411	0.00912	0.13489	0.09578	0.06258	0.29895	-0.01777	-0.02366
Glutamic acid	0.11593	0.04959	0.04814	-0.10945	0.02869	-0.07463	0.00210	0.03092	-0.07028	-0.00397
Asparagine	0.10348	-0.01240	-0.06699	0.02312	0.13399	-0.02185	-0.22835	-0.05461	-0.19689	0.00419
Phenylalanine	0.11923	0.00578	-0.03412	-0.07883	-0.02013	-0.06458	-0.11474	0.11464	-0.07760	0.00823
Trihydroxypentanoic acid	0.11524	0.07888	0.02960	0.02215	0.04813	0.00134	0.05280	-0.07888	0.01953	0.01817
U1703_Unknown	0.10338	0.07116	-0.01146	-0.00208	0.16237	0.00958	0.06926	-0.13602	0.06387	0.01365
Glutamine	0.06981	0.03602	-0.08470	0.14105	0.17453	-0.15016	-0.11284	-0.01163	-0.26592	-0.02570
Putrescine	0.11130	0.03103	0.01405	-0.15415	0.02731	-0.10625	-0.06271	0.04971	0.03970	-0.00474
U1751_Unknown	0.10715	0.05521	0.07383	-0.08547	-0.13390	-0.05833	0.04872	0.07252	-0.07018	-0.00211
U1755_Unknown	0.11283	0.05498	0.03756	-0.07453	-0.03237	-0.05717	-0.02931	0.12666	0.03517	-0.05371
USA1768_Unknown	0.10579	0.09641	0.05549	0.06880	-0.05534	0.00516	0.05905	-0.01052	0.00229	0.02671
α-glycerophosphate	-0.05430	0.04812	0.08819	0.13446	0.08941	-0.29421	-0.11925	-0.04307	0.01406	-0.02861
U1801_Unknown	-0.06360	0.09031	-0.13283	-0.00393	0.03002	0.06051	0.14352	0.27836	-0.07757	-0.07602
Citric acid	0.11324	0.06048	0.01782	-0.10925	0.07528	-0.04332	0.05560	0.01320	-0.01124	0.01783
Quinic acid	0.09472	0.06879	0.11026	-0.07819	-0.12482	-0.06610	-0.00039	0.12907	-0.06847	-0.02163
U1871_Unknown	-0.02866	0.07899	-0.20538	-0.05500	0.09202	0.12413	0.07542	0.28204	0.00109	-0.05868
Fructose	0.07560	0.03162	-0.04730	-0.12546	-0.15992	-0.13273	-0.19944	0.08461	0.26032	0.01427
Allantoin	0.11682	0.05180	0.04556	-0.11173	0.01562	-0.06038	0.01054	0.00520	-0.04978	-0.00923
Mannose	0.10242	0.05918	0.02503	-0.07232	-0.15024	-0.06047	-0.12316	0.10029	-0.02287	-0.05021
Galactose	-0.04079	0.04134	0.05133	0.08639	0.03871	-0.28750	-0.15183	-0.08181	0.20426	0.04055
Glucose	0.09035	0.05929	0.00041	-0.06079	-0.18682	-0.11175	-0.13717	0.02145	0.19012	0.00980
Histidine	0.08459	-0.07260	-0.16999	-0.00860	0.10572	0.03495	-0.16899	-0.04800	-0.06803	0.01351
Lysine	0.10953	0.03656	-0.12320	0.08657	0.03787	0.08382	-0.09431	0.02297	0.01648	-0.00342
Mannitol	0.01248	0.08288	0.21270	0.13217	0.05715	-0.20456	-0.11787	0.05239	-0.03916	-0.01862
Sorbitol	0.08639	0.09445	0.11780	0.11661	-0.06147	0.04251	-0.06659	-0.01203	0.04464	-0.02905
Tyrosine	0.09863	0.03509	-0.14279	0.08898	0.03944	0.08326	-0.15823	0.00935	-0.04306	-0.02587

Table S5-3.6. Continuation (2).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
U1948_Unknown	0.03121	0.08726	-0.14484	-0.07591	0.19746	0.18050	0.16234	0.05867	0.15422	-0.03960
UC2020_Unknown	-0.03441	-0.05009	-0.19766	0.00514	-0.10620	0.04598	-0.19751	-0.03998	0.37379	0.06045
Galactaric acid	0.11402	0.08839	0.03006	0.01506	-0.04890	0.00993	0.05650	0.02334	-0.01230	-0.00241
Inositol	0.10966	0.04991	0.08845	-0.10182	-0.08208	-0.03985	0.02603	0.03375	-0.10329	-0.00551
UC2105_Unknown	0.10825	0.06883	0.07084	-0.11414	0.03709	-0.08552	-0.01839	0.03396	-0.06786	-0.00248
U2125_Unknown	0.05907	0.11048	-0.05024	0.18895	0.10948	-0.05477	0.11110	0.12672	-0.03300	-0.02878
Caffeic acid	0.09773	0.07474	0.06884	0.11170	-0.15478	0.03267	0.02418	0.05094	-0.02631	-0.02666
U2190_Unknown	0.11538	0.00607	-0.06477	0.09530	0.00921	0.05151	-0.15817	-0.07875	-0.05787	0.00160
Tryptophan	0.08647	0.05605	-0.15261	0.08368	0.09641	0.10180	-0.09436	0.00465	-0.01587	-0.03798
Spermidine	0.10472	0.07617	0.02321	0.12964	-0.06278	0.06753	0.07320	-0.05307	0.07162	0.04311
Fructose-6-phosphate	0.10700	0.07991	0.04614	0.03751	-0.12930	0.02342	0.06904	-0.05295	-0.03743	0.02213
Galactosyl Glycerol	0.08919	0.10216	0.03702	0.16550	-0.02308	0.00948	0.05220	-0.03845	0.08708	0.03432
Glucose-6-phosphate	0.10086	0.04812	0.01190	-0.20589	0.02754	-0.05150	0.03195	-0.02005	0.00210	-0.01653
U2367_Unknown	0.11149	0.08088	0.02073	0.06763	-0.07907	0.03468	0.03126	0.04586	-0.01962	-0.01794
U2477b_Unknown	0.07966	0.01870	-0.01067	-0.18247	0.22881	-0.04774	0.05322	-0.04815	0.13067	-0.04633
U2495_Unknown	0.08219	0.07727	0.03510	0.19677	-0.12635	0.09794	0.04199	-0.03060	0.02730	0.00197
U2502_Unknown	0.09330	0.08303	0.03209	0.16171	-0.09031	0.08930	0.06966	-0.04418	0.05303	0.04358
Sucrose	0.10552	0.06269	0.00864	-0.06976	-0.12141	0.00168	0.08268	-0.05767	0.01070	0.05342
Galactinol	0.08692	0.10723	0.05249	-0.03499	-0.08577	-0.03590	0.01516	-0.03261	-0.02650	0.05065
Chlorogenic acid	0.10277	0.06874	0.03214	-0.13218	-0.06527	-0.03076	0.09166	-0.01257	0.02256	-0.00520
U1595_Unknown	-0.01646	0.03755	-0.04692	0.12577	0.08942	-0.40831	0.00123	-0.01536	0.07620	0.11173
U1680_Unknown	0.02054	-0.10515	-0.01976	0.13311	-0.02354	-0.19939	0.24569	-0.10657	0.10782	-0.29365
Tetradecanoic acid	0.08761	-0.05976	-0.08523	-0.01331	-0.01080	-0.09022	0.04412	-0.21452	-0.26599	0.06673
U1762_Unknown	0.06080	-0.14254	-0.10502	0.05292	-0.00487	-0.13220	0.07207	0.02891	-0.02640	0.05355
Br-pentadecanoic acid	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Pentadecenoic acid	0.05525	-0.14537	-0.03835	0.04123	-0.01389	-0.05465	-0.06561	0.05210	0.09045	0.25604
Cinnamic acid	0.10698	-0.04624	0.04649	-0.05948	-0.02621	-0.14816	0.11901	0.05456	-0.00075	-0.21998

Table S5-3.6. Continuation (3).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
Pentadecanoic acid	0.06577	-0.13504	-0.13551	0.03797	0.01189	-0.00275	-0.08287	-0.11346	-0.10349	-0.05787
U1845_Unknown	0.09767	-0.11662	-0.02373	0.07702	-0.01623	-0.03280	-0.02540	0.05206	0.02921	-0.00427
Hexadecenoic acid	0.08679	-0.09457	0.00256	0.03237	0.02750	-0.02569	0.08971	0.14074	-0.05836	0.09999
Hexadecanoic acid	0.09972	-0.11583	-0.03477	0.01462	0.07525	0.02050	-0.05788	-0.04737	0.00449	0.05755
Cinnamic acid	0.08411	-0.12973	-0.00472	0.06500	0.05829	0.00566	0.04923	-0.03794	0.02573	0.03225
Me-Hexadecanoic acid	0.06471	-0.15498	0.02077	0.03184	0.03978	-0.01377	0.08239	-0.08315	0.03944	-0.15741
Heptadecanoic acid	0.08032	-0.14663	0.01869	0.00323	0.02912	0.01924	0.05000	0.01421	-0.04001	0.08624
Linoleic acid	0.11332	-0.00265	-0.11130	-0.11922	-0.01892	0.02737	0.02100	-0.04397	-0.05388	0.05894
α-linolenic acid	0.07344	-0.00760	0.21059	-0.04875	0.12040	0.16792	-0.14101	0.00941	0.02731	0.05896
Octadecenoic acid	0.06634	-0.09457	0.14360	0.09586	0.04156	0.14010	-0.05537	-0.12092	-0.01888	-0.21582
2OH-Hexadecanoic acid	0.09372	-0.12574	0.03418	-0.00594	0.05259	0.03365	-0.05702	0.01109	0.00419	0.11110
Noctadecanoic acid	0.07321	-0.15019	0.07460	0.00812	0.02671	0.05221	-0.05557	-0.00918	-0.03623	0.05028
Nonadecenoic acid	0.00849	-0.09845	0.08551	0.03252	-0.01105	-0.00770	0.19432	0.13432	-0.01421	0.53476
U2263_Unknown	-0.04726	-0.05299	0.12054	0.15215	0.06428	-0.24815	0.14861	0.20192	0.08251	0.05270
Tricosane	0.05933	-0.13281	-0.12274	0.04224	-0.10415	-0.04291	-0.00785	-0.03744	0.05209	-0.21304
Eicosanoic acid	0.07511	-0.14786	0.06830	0.02400	0.05964	0.03081	0.01962	-0.01701	-0.03224	0.01435
Heneicosanoic acid	0.06269	-0.15254	-0.09733	0.00678	-0.00396	-0.02072	0.02177	0.04884	-0.08924	0.12224
Heneicosanol	0.08185	-0.12440	-0.03625	0.00832	0.06468	0.02289	0.06928	0.00328	0.15308	0.17275
U2457_Unknown	0.02458	-0.16615	-0.11469	0.07607	-0.03666	-0.07949	0.08998	-0.00918	-0.04208	0.07009
U2466_Unknown	0.02659	-0.16326	-0.12754	0.08484	-0.03147	-0.07678	0.05305	-0.01948	-0.06173	0.04554
U2510_Unknown	0.00691	-0.10475	0.07748	0.16810	0.15057	-0.07963	-0.28189	0.14431	0.02445	0.02430
Docosanoic acid	0.06189	-0.15903	0.05955	0.03284	0.03262	0.01095	0.07256	0.05220	0.11402	-0.04812
Docosanol	0.07547	-0.12977	0.03233	0.02451	0.02102	0.03232	0.08332	-0.08199	0.08886	0.09238
Tricosanoic acid	0.07039	-0.15503	0.01462	-0.04744	0.00227	0.02292	0.03830	-0.01568	-0.06753	0.02332
Tricosanol	0.09543	-0.11392	-0.05029	0.00626	-0.04786	-0.02378	0.02261	-0.09842	-0.06684	-0.05298
Tetracosanoic acid	0.05821	-0.13147	0.14699	0.05362	0.08056	0.05032	0.04332	0.03692	-0.05943	-0.17062
Tetracosanol	0.08538	-0.11466	0.02264	0.02476	-0.05493	0.03384	0.05532	0.05452	0.13266	-0.24891

Table S5-3.6. Continuation (4).

Metabolite	Load 1	Load 2	Load 3	Load 4	Load 5	Load 6	Load 7	Load 8	Load 9	Load 10
Pentacosanoic acid	0.06375	-0.11053	-0.00082	-0.04660	-0.08980	0.07180	-0.13640	0.16081	0.23656	0.02426
2OH-Tetracosanoic acid	0.05713	-0.15767	-0.07640	0.02944	0.01506	-0.03962	0.10773	0.06897	0.05224	-0.08891
Hexacosanoic acid	0.05706	-0.13661	-0.00838	-0.06685	-0.07473	0.01395	-0.06583	0.28570	0.01425	-0.03175
Hexacosanol	0.08841	-0.12337	0.03677	0.01093	-0.06284	0.05040	0.06750	0.05478	-0.00530	0.13193
Heptacosanol	0.07752	-0.14841	-0.00776	0.01611	-0.02260	-0.01282	0.08684	0.06323	0.07144	0.00749
Octacosanoic acid	0.06443	-0.14347	0.05809	-0.00789	-0.04469	0.09127	-0.05972	0.01256	-0.15254	-0.06252
Octacosanol	0.10046	-0.11728	0.04881	-0.01588	-0.02055	0.00706	0.05970	0.02968	-0.03018	-0.06962
Solanid-5-enol	0.05500	-0.02601	0.26041	-0.08112	0.13361	0.07474	-0.03321	0.08022	-0.00122	-0.00996
Nonacosanoic acid	0.07263	-0.14572	0.00043	0.00443	-0.06124	0.00632	-0.11342	0.11797	0.06442	-0.07177
Nonacosanol	0.09607	-0.12601	0.08717	0.01650	-0.00822	0.01233	-0.01322	0.08611	0.00288	-0.05700
Stigmasterol	0.11747	-0.01390	0.07548	-0.06913	0.02567	0.00644	0.10842	-0.02924	0.10413	0.01502
Fucosterol	-0.01140	-0.03571	0.22987	0.06639	0.12150	0.03612	-0.02082	0.12096	-0.06714	-0.05606
β-Sitosterol	0.10854	-0.03907	0.12394	-0.06224	0.11056	0.06322	0.03941	-0.02224	0.02591	0.03172
δ-5-Avenasterol	-0.03561	-0.02419	0.27710	0.06727	0.11534	0.12837	-0.09350	0.03818	0.02876	-0.01587
Triacontanoic acid	0.07357	-0.14548	-0.00219	0.01741	-0.02420	-0.02811	0.03723	0.04631	0.08671	-0.21397
Triacontanol	0.11821	-0.03587	0.02777	-0.01206	-0.04030	-0.01397	0.06294	-0.10698	-0.02984	-0.03313
Stigmastadienol	-0.02691	-0.07729	0.24650	0.04842	0.08833	0.16388	-0.10314	0.06595	0.02966	0.06491