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**Sucrose Phosphate Synthase activity and
gene expression in relation to dehydration
induced sucrose accumulation in the
resurrection plant *Xerophyta humilis***

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List of abbreviations

ACN	acetonitrile
Acc	gene accession
BSA	bovine serine albumin
CE	collision energy
Con	control
DAF	days after flowering
DAP	dihydrodipicolinate
DEB	DNA extraction buffer
DM	dry mass
DW	dry weight
EST	expressed sequence tag
Exp	experimental
1,6-FBP	fructose-1,6-bisphosphate
2,6-FBP	fructose-2,6-bisphosphate
FA	formic acid
FD	freeze dried
FBPase	fructose-1,6-bisphosphatase
Fru-6-P	fructose-6-phosphate
Fructose1,6-BP	fructose-1,6-bisphosphate
FW	fresh weight
Grp	group
GC-TOF-MS	gas chromatography time-of-flight mass spectrometer
Glc-6-P	glucose-6-phosphate
Glc-1-P	glucose-1-phosphate
Glyc-P	glycerophosphoglycerol
Glyc-3-P	glycerol-3-phosphate
Glycerate-3-P	glycerate-3-phosphate
HSP	heat shock protein
ICA	independent component analysis
LC	liquid chromatography

LEA	late embryogenesis abundant
MRM	multiple reaction monitoring
MS	mass spectrometer
MS/MS	tandem mass spectrometer
MSTs	mass sequence tags
MW	molecular weight
NFD	non-freeze dried
NR	nitrate reductase
PEP	phosphoenolpyruvate
PEB	protein extraction buffer
PCA	principal component analysis
PCR	polymerase chain reaction
Pi	free phosphate
PGI	phosphoglucose isomerase
3-PGA	3-phosphoglycerate
PAM	pulse amplitude modulation
PPi	pyrophosphate
PSII	photosystem II
REB	RNA Extraction Buffer
RE	restriction enzyme
RTqPCR	real-time quantitative PCR
RT-PCR	reverse transcriptase PCR
RuBP	ribulose-1,5-bisphosphate
RWC	relative water content
ROS	reactive oxygen species
SD	standard deviation
SAM	S-adenosyl-l-methionine
dSAM	decarboxylated S-adenosyl-l-methionine
SPP	sucrose phosphate phosphatase
SPS	sucrose phosphate synthase
STD	standard

SuSy	sucrose synthase
Suc-6-P	sucrose-6 ^F -phosphate
TCA	tricarboxylic acid
TIC	total ion current
TPT	triose phosphate translocator
Triose-P	triose-phosphate
UDP	uridine diphosphate
UDP-Glucose	uridine diphosphate glucose
UGPase	UDP-Glucose pyrophosphorylase
UTP	uridine triphosphate
Xcorr	cross correlation

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ABSTRACT

In many resurrection plants, including *Xerophyta humilis*, severe water loss results in the accumulation of high levels of sucrose. The current work focuses on the role of the enzyme sucrose phosphate synthase (SPS) in dehydration-induced sucrose accumulation in the leaf tissues of *X. humilis*. The work investigated two central hypotheses: i) dehydration-induced sucrose accumulation is associated with increases in SPS activity, and ii) dehydration results in the upregulation of a specific form of SPS. In an attempt to identify possible sources of carbon supporting sucrose accumulation the study also investigated changes in a broad range of metabolites during tissue dehydration.

In response to tissue dehydration sucrose was found to accumulate in two phases, an early phase between 100 and 60 % relative water content (RWC) and a late phase between 40 and 5 % RWC. An overall four fold increase in tissue sucrose levels was observed from 100 to 5 % RWC, with the most rapid period of accumulation occurring between 20 and 5 % RWC. In the early phase of accumulation, photosynthesis, starch and hexose sugars may serve as carbon sources. However, in the late phase the starch and hexose sugar pools were depleted and photosynthesis had ceased. In this phase it is proposed that the amino acid pool may contribute carbon skeletons to sucrose synthesis. During tissue dehydration sucrose accumulation was accompanied by increases in other known osmoprotectants including, raffinose, glycerol, glycerophosphoglycerol and xylitol.

In response to water loss both total SPS total activity and activation state were altered. During dehydration increases in sucrose content were positively correlated with increases in SPS activity (Pearson correlation of 0.73 for total SPS activity). The correlation was strongest in the early stages of water loss (before 50 % RWC) where similar fold increases in sucrose content and SPS activity were observed. However, the peak sucrose accumulation rate - noted in the late stage of dehydration, did not coincide with the highest level of SPS activity. Thus, while increases in SPS activity appear to drive sucrose accumulation during early dehydration, in the later stages of dehydration (40 to 5 % RWC) other mechanisms may be more influential in determining leaf sucrose content.

A total of two SPS genes, named XhSPS1 and XhSPS2, were identified in leaf tissue. Gene expression was analysed at both the transcript and protein levels. XhSPS2 was the dominant transcript in fully hydrated tissue and both XhSPS1 and XhSPS2 transcript levels increased in a similar manner during early dehydration. Beyond the 50 % RWC mark, the XhSPS2 transcript was downregulated and XhSPS1 became the dominant transcript. During the early stages of dehydration, changes in SPS protein levels were similar to that observed for changes in transcript abundance. However, despite the switch in dominance at the transcript level XhSPS2 remained as the dominant SPS protein throughout dehydration. Thus, it was concluded that dehydration does not induce a specific form of SPS in *X. humilis* leaf.

A critical adaptation of any terrestrial plant is the ability to access and retain an adequate amount of water to support growth, repair and reproduction. Water is not only the medium in which all biochemical reactions occur, but its presence also provides the cellular structure and organization necessary for life supporting metabolism. In most higher plant orders the ability to tolerate tissue desiccation i.e. loss of more than 90% of cellular water content, is confined to a brief period during pollen or seed development (e.g. Bewley and Black, 1984). Beyond germination, a tissue desiccation event usually results in severe damage and consequent tissue death. Remarkably, a small group of higher plant species remain desiccation tolerant during most of their vegetative growth phase and are able to recover full metabolic activity after extended periods in the desiccated state. This group of species includes a number of fern and angiosperm species (Gaff, 1977; Gaff, 1987) but lacks representation in the gymnosperms (Gaff, 1980). Together with most lower order plant species, e.g. mosses and bryophytes, these species constitute a taxonomically diverse group known as resurrection plants (Gaff, 1971). In the study of plant water deficit stress resurrection plant species are unique as they allow the investigator to observe controlled responses to the full range of tissue water loss - from a slight reduction in water content to complete loss of tissue bulk water. Thus, knowledge obtained from their study not only provides insight into the phenomenon of desiccation tolerance but also significantly deepens our understanding of general water deficit stress tolerance.

Desiccation tolerance is a complex trait with tolerant species employing a suite of physiological and molecular mechanisms to ensure survival in the dried state and recovery upon rehydration (Vertucci and Farrant, 1995; Alpert and Oliver, 2002). Broadly, tolerant species can be grouped into two categories: i) species which are unable to control water loss and therefore equilibrate rapidly with the water potential of the environment, e.g. tolerant mosses and bryophytes, and ii) species which employ mechanisms to slow down water loss - described as 'modified' desiccation-tolerant plants, e.g. tolerant angiosperms (Oliver et al., 1997). Species in the first

category experience rapid cycles (in order of minutes) of wetting and drying and therefore rely extensively on constitutive protection and on repair mechanisms implemented upon rehydration (Oliver and Bewley, 1997; Oliver et al., 2000; Alpert and Oliver, 2002). In contrast, modified desiccation tolerant species usually experience much slower rates of water loss (in order of days) and hence rely more on induced protective mechanisms instead of innate protection (Bewley, 1979; Oliver et al., 1998; Oliver et al., 2000; Farrant, 2000; Farrant, 2007). Consequently most members of category two are not able to survive rapid drying and require time to lay down adequate protection. Repair mechanisms are likely to be important in both groups of species but they are considered less critical in modified desiccation tolerant species. The monocot resurrection plant *Xerophyta humilis* (Baker) T. Durand & Schinz is typical of a modified desiccation tolerant species requiring slow drying rates and taking at least 24 hours to recover after rehydration. *X. humilis* is a member of the Villoziaceae family and the species is indigenous to Southern Africa where it grows at high altitudes in summer rainfall regions. In its natural environment it experiences both short cycles of wetting and drying (in order of days) and extended periods in the dried state, usually during the dry winter months. Photographs of *X. humilis* in its fully hydrated and desiccated states are shown in Appendix A.

Dehydration-induced sucrose accumulation in resurrection plants

In angiosperms the trait of vegetative desiccation tolerance is shared by several phylogenetically unrelated species and it is postulated that tolerance evolved on at least eight independent occasions (Oliver et al., 2000). It is therefore improbable that all species will employ exactly the same strategies and mechanisms to survive desiccation. However, as all species will have to deal with a similar set of stresses during water loss it is expected that resurrection plants will share some common responses to dehydration. One such common response is the accumulation of sucrose in response to dehydration (Bianchi et al., 1991a; Bianchi et al., 1991b; Bianchi et al., 1993; Müller et al., 1997; Ghasempour et al., 1998; Whittaker et al., 2001; Whittaker et al., 2004; Peters et al., 2007 reviewed in Scott, 2000; Farrant, 2007). Furthermore, sucrose accumulation is also a widely noted response to abiotic stress in desiccation sensitive plant systems, with cold (Kaurin et al., 1981, Guy et al., 1992; Uemura et al., 2003), salt (Balibrea et al., 2000; Fernandes

et al., 2004) and water stress (Zrenner and Stitt, 1991; Keller and Ludlow, 1993; Pilon-Smits et al., 1995; Garcia et al., 1997; Geigenberger et al., 1997; Pelleschi et al., 1997; Iyer and Caplan, 1998) all known to elicit sugar, and predominantly sucrose, accumulation.

Naturally it could be argued that this widely observed sugar accumulation is more a consequence of stress, via growth inhibition, rather than a regulated response to mitigate stress damage. However, especially in the area of water deficit stress, a number of studies show a strong correlation between osmotic stress tolerance and sugar accumulation (Ramanjulu et al., 1994; Kerepesi and Galiba, 2000; Gilmour et al., 2000; Streeter et al., 2001; Taji et al., 2002; Buitink et al., 2002; Buitink et al., 2006; Berjak et al., 2007). In *X. humilis* leaf tissue dehydration is known to induce the accumulation of sucrose (up to 7 % dry mass) (Farrant et al., 2003; Bajic, 2006), and it is proposed that sucrose accumulation is of functional significance in conferring desiccation tolerance (Illing et al., 2005). While a complete understanding of the role of sugar accumulation in desiccation tolerance is yet to be realized, there is a growing body of evidence which implicates soluble sugars, e.g. trehalose in animal systems and sucrose in plant systems, in stabilizing macromolecules both during tissue dehydration and in the dried state.

Function of sucrose accumulation in desiccation tolerance

In fully hydrated plant tissue the hydrophobic effect associated with water is considered vital for the correct assembly of phospholipids into biological membranes and, in part, for the conformation of many proteins (Tanford, 1978). In the absence of water, membranes undergo structural changes, usually resulting in membrane fusion, and many proteins lose their native structure (Leopold and Vertucci, 1986; also reviewed by Hoekstra et al., 2001, inter alia). Thus a successful protective strategy would: i) maintain membranes and proteins in their hydrated state for as long as possible during tissue dehydration, and ii) would provide a mechanism to maintain structural integrity in the dried state. The survival of desiccation tolerant tissue despite severe water loss indicates that protective mechanisms exist which prevent macromolecular destabilization and thereby limit cellular damage during water loss. Much of the protection against macromolecular destabilization in resurrection plants has been attributed to the

accumulation of soluble sugars, and especially sucrose (reviewed in Scott, 2000; Hoekstra et al., 2001; Farrant, 2007). In addition to macromolecular destabilization, the other main stresses associated with water loss include oxidative stress due to metabolic imbalances and mechanical stress due to loss of water support (Vertucci and Farrant, 1995; Walters et al., 2002; Muller et al., 1997; Hoekstra et al., 2001). The current review focuses on the role of sugar accumulation in preventing macromolecular destabilization but for detailed reviews on all protective mechanisms the reader is referred to the following reviews: Oliver and Bewley (1997); Gaff (1997); Alpert (2000); Farrant (2000); (2007); Hoekstra et al. (2001); Vire et al. (2003); Vire et al. (2004); Berjak (2007).

Prior to the discussion of the proposed mechanisms of macromolecular stabilization by sugars it is necessary to define the terms used to describe the hydration state of tissue. Plant tissue which is in the process of losing water and has not reached equilibrium with its environment is described as 'dehydrating' tissue or as 'dehydrated' tissue. The term 'desiccated' is used to describe tissue that has lost all its bulk water and has reached equilibrium with its environment. The loss of bulk water in plant tissue is believed to occur at a water content of approximately 0.3 g H₂O g⁻¹ dry mass (Hoekstra et al., 2001). During tissue dehydration (prior to reaching desiccated state) the accumulation of sugars, including sucrose, is believed to be an important osmoregulatory mechanism. The accumulation of sugars and other osmotically active compounds will promote the influx of water and retard water efflux, thereby assisting in the maintenance of cell turgor despite water loss. It is also proposed that the accumulation of sugars results in the preferential hydration of proteins and membranes and hence protects cellular components from the destabilizing effects of water loss (e.g. Leopold, 1986; Arakawa and Timasheff, 1991; Yancey, 2005; Gagneul et al., 2007). Sugars commonly perform these proposed functions in conjunction with a range of other organic osmolytes termed 'compatible solutes' (Brown and Simpson, 1972; Yancey et al., 1982; Bohnert and Jensen, 1996; Hoekstra et al., 2001; Golovina and Hoekstra, 2002). Compatible solutes can be divided into polyhydroxylic compounds, which include sugars and polyols, and zwitterionic alkylamines, which include amino acids and quaternary ammonium compounds (Hare et al., 1998; Ortbauer and Popp, 2008; Groppa and Benavides, 2008). In both desiccation tolerant and desiccation sensitive species

dehydration is known to result in the increased abundance of compatible solutes (Gaff and McGregor, 1979; Tymms and Gaff, 1979; Ishitani et al., 1995; Le Rudulier et al., 1984; Yoshida et al., 1995; Ingram et al., 1997; Bohnert and Jensen, 1996; Chen and Murata, 2008). These solutes are described as 'compatible' as their accumulation usually does not adversely affect metabolism. Due to its chemically unreactive nature sucrose (a non-reducing sugar) in particular can accumulate to high concentrations without affecting normal metabolism. The osmotic adjustment provided by the accumulation of sugars and other compatible solutes is effective at moderate water loss but becomes ineffective upon loss of bulk water ($< \sim 0.3 \text{ g H}_2\text{O g}^{-1}$ dry mass) (Hoekstra et al., 2001). At this stage the lack of free water precludes the possibility of osmotic adjustment or preferential hydration and thus cellular protection must be afforded by another mechanism.

To explain the mechanism of protection provided by sugars in the desiccated tissue there are currently three main hypotheses. One proposed hypothesis, known as the 'water replacement hypothesis' (Clegg et al., 1982) suggests that sugars may effectively replace lost water by forming hydrogen bonds with membrane and protein surfaces. *In vitro* support of this proposed mechanism has accumulated (see Gaber et al., 1986; Caffrey, 1986; Allison et al., 1999; Billi et al., 2000; Buitink et al., 2002; van den Bogaart et al., 2007), but conclusive demonstration *in vivo* is difficult and its occurrence has become the subject of debate (see Berjak, 2007). Recently it has been suggested that sugars may still be able to perform their protective function without the need for the formation of hydrogen bonds. In this second hypothesis, described as the 'hydration forces explanation', it is postulated that sugars may serve as intra molecular spacers in membranes during water loss and in dried state and hence their presence alone can prevent membrane fusion (Bryant et al., 2001; Koster and Bryant, 2005; Berjak, 2007). The third proposed mechanism of protection by sugars, and one which is less debated, is that sugars protect macromolecules by promoting the formation of stable intracellular glasses in desiccated tissue (described as cellular vitrification) (e.g. Burke, 1986; Koster and Leopold, 1988; Sun and Leopold, 1997; Crowe et al., 1998). In plant tissue sugars considered important in the formation of these stable glasses include sucrose and the raffinose series oligosaccharides. It is also widely accepted that the presence of additional components, including late embryogenesis abundant

(LEAs) and heat shock proteins (HSPs), are important for the stabilization of the vitrified cytoplasm (refer to Hoekstra et al., 2001). Vitrification is proposed to offer protection by both supporting cellular components and by limiting deleterious inter molecular interactions which may occur due to water loss. Although described as two separate hypotheses it appears likely that a combination of vitrification and some form of direct molecular interaction (between sugars and macromolecules) will contribute to the stabilization of membranes and proteins in the desiccated state.

Role of SPS in regulating sucrose accumulation

While all the factors affecting dehydration induced sucrose accumulation in plant tissue are likely to be numerous and complex, on a very simplified level, sucrose accumulation will occur when sucrose synthesis is in excess of sucrose utilization/breakdown. Clearly both an up regulation of sucrose biosynthesis and/or a down regulation of sucrose degradation could account for an increase in sucrose concentration. However, as the central goal of the current study was to characterize the role SPS plays in the dehydration induced sucrose accumulation, sucrose synthesis forms the main focus of the work. The two enzymes known to catalyse sucrose synthesis in plant tissues are sucrose phosphate synthase (SPS) and sucrose synthase (SuSy). Of the two enzymes (SuSy and SPS), it is generally accepted that SPS rather than SuSy is the main enzyme responsible for sucrose biosynthesis (Stitt et al., 1987; Huber and Huber, 1996; Stitt et al., 1997; Winter and Huber, 2000).

Both SPS and SuSy catalyze reversible reactions, however in the case of SPS the rapid removal of its product sucrose-6^F-phosphate (Suc-6-P) by a specific phosphatase, Sucrose Phosphate Phosphatase (SPP), displaces the reaction far from equilibrium *in vivo* making the reaction highly irreversible. Thus, in sucrose accumulating tissues the combined action of SPS and SPP will allow sucrose synthesis to continue despite increasing sucrose levels. Physiological evidence supporting the SPS path of sucrose synthesis is to be found in radio labelling studies where the pattern of intermediate metabolite labelling is more consistent with the SPS mediated pathway than that of SuSy (Ap Rees, 1984). Furthermore, in most tissues where sucrose

synthesis is occurring SPS activity alone is sufficient to account for the rate of sucrose synthesis (ap Rees, 1988; Winter and Huber, 2000). The proposed role of SPS in sucrose metabolism has also been confirmed by studies on transgenic plants. Over expression of maize SPS in tomato resulted in increased sucrose synthesis and increased sucrose/starch ratio in tomato leaves (Galtier et al., 1993; Galtier et al., 1995; Micallef et al., 1995), while reduced expression, via antisense repression, resulted in the increased flow of carbon into starch and amino acids in transgenic potato plants (Krause, 1994). Contrary to its name, much of the work conducted on SuSy points to a role in sucrose utilisation rather than sucrose production. SuSy activity has been correlated with starch synthesis (Dejardin et al., 1997), cell wall synthesis (Chourey et al., 1998; Nakai et al., 1999), and overall sink strength (Sun et al., 1992; Zrenner et al., 1995). SuSy together with invertases are considered to be the principal sucrose degrading enzymes in plant tissues.

In photosynthetic tissues, SPS and an enzyme positioned upstream from it, fructose biphosphate phosphatase (FBPase), are considered to be the main regulators of sucrose biosynthesis (Stitt et al., 1987). Figure 1.1.1 illustrates the key metabolites and enzymes known to be involved in sucrose synthesis in photosynthesizing leaf tissue. Figure 1.1.2 displays the main reactions, and the Gibbs free energy of each reaction, involved in the synthesis of sucrose from hexose sugars via the combined activities of UDP-Glucose pyrophosphorylase (UGPase), SPS and SPP. SPS is considered to catalyze the first committed step in the sucrose biosynthesis pathway. As is to be expected, there is a tight coupling of sucrose synthesis in the cytosol to the production of photosynthate in the chloroplast. A critical component of this inter organelle communication is the exchange of triose-P/glycerate 3-P for P_i via the triose-phosphate translocator (TPT) during active photosynthesis (Lilley et al., 1977; Edwards and Walker, 1983; Flugge and Heldt, 1991). As photosynthetic rates increase, increased amounts of triose phosphate will be exported in exchange for P_i , resulting in a higher triose phosphate/glycerate-3-P: P_i ratio within the cytosol. The signal metabolite 2,6-FBP is known to be a strong inhibitor of FBPase activity (Stitt, 1990). The enzyme responsible for the formation of 2,6-FBP (6-phosphofructo-2-kinase) is inhibited by 3-PGA and activated by fructose-6-P and P_i , while the enzyme responsible for the degradation of 2,6-FBP (2,6-FBP phosphatase) is regulated in a converse manner. Thus, when the triose: P_i ratio

in the cytoplasm is increased (during active photosynthesis), 2,6-FBP levels are decreased and the inhibition on FBPase is relieved. Similarly the regulation of SPS activity has been linked to changes in cytosolic Pi levels.

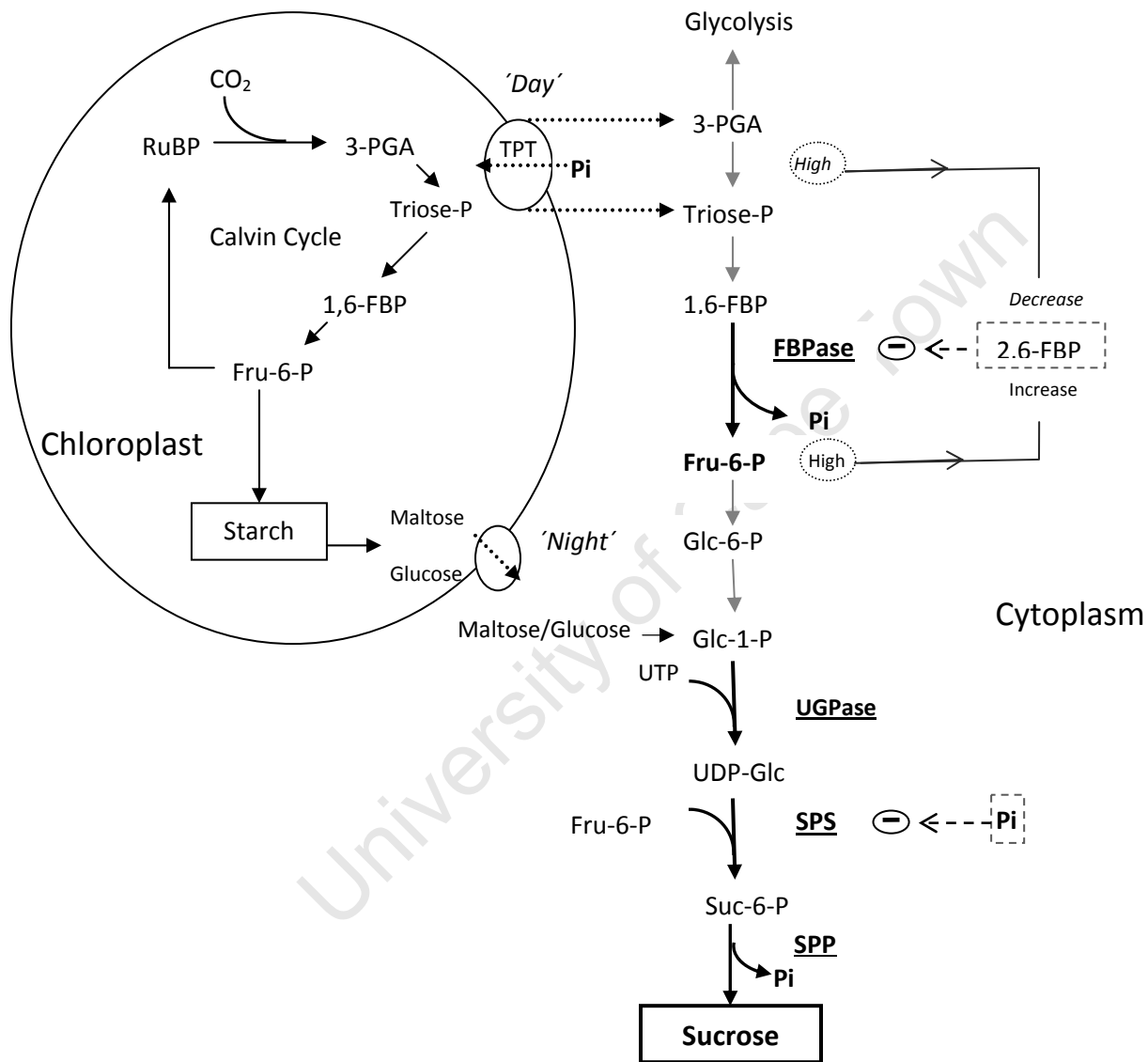


Figure 1.1.1 Illustration of carbon export from chloroplast and sucrose synthesis in cytosol of leaf tissue. 'Day' indicates mechanism of carbon exchange during active photosynthesis and 'Night' indicates exchange under dark conditions. Please refer to 'List of abbreviations' for full names.

UGPase

$$\Delta G^{\circ} = -2.88 \text{ kJ mol}^{-1}$$

SPS

$$\Delta G^{\circ} = -5.70 \text{ kJ mol}^{-1}$$

SPP

$$\Delta G^{\circ} = -16.5 \text{ kJ mol}^{-1}$$

Figure 1.1.2 Synthesis of sucrose from hexose sugars through the combined activities of UGPase, SPS and SPP. The standard free energy change (ΔG°) of each reaction is shown. Please refer to 'List of abbreviations' for full names

Regulation of SPS in photosynthetic tissue

The regulation of SPS has understandably generated much research interest as it catalyzes the first committed step in towards sucrose synthesis and therefore occupies a key position in the intracellular carbon allocation. In the well studied spinach system, SPS activity is known to be regulated by the allosteric effectors, Glc-6-P (activator) and Pi (inhibitor) (Amir and Preiss, 1982; Doehlert and Huber, 1984), with sensitivity to these metabolites being altered by multi-site phosphorylation/dephosphorylation (Huber et al., 1989; Siegl et al., 1990; McMichael et al., 1993). In the context of photosynthesis, regulation by allosteric effectors and SPS protein phosphorylation appear to be the mechanisms by which SPS activity is linked to photosynthesis. During active photosynthesis the increased Glc-6-P/Pi ratio (due to increased Pi import into chloroplast) will relieve SPS inhibition and promote sucrose synthesis. In addition, while it is not true for all species, the inhibitory effect of Pi has been found to be reduced by light activation of SPS via dephosphorylation of a specific Serine residue. This activation further amplifies the response of SPS to increases in photosynthesis.

The coupling of sucrose biosynthesis to photosynthesis is relevant to the current study as photosynthesis is down regulated at a relatively early stage of tissue dehydration in many studied resurrection plant species (Farrant, 2000; 2007). Based on the mechanism of down regulation, angiosperm resurrection plants can be divided into two sub-groups, namely poikilochlorophyllous species and homoiochlorophyllous species (Gaff, 1989; Smirnoff, 1993; Tuba et al., 1993a; Tuba et al., 1994; Sherwin and Farrant, 1996; Farrant, 2000). Poikilochlorophyllous species break down chlorophyll and dismantle thylakoid membranes during dehydration (Tuba et al., 1993b; Sherwin and Farrant, 1996; Farrant, 2000), while homoiochlorophyllous species retain their chlorophyll and thylakoid membranes and rely on alternative mechanisms to down regulate photosynthetic activity. These mechanisms include leaf folding and shading and the accumulation of 'sun-screening' compounds such as anthocyanins (Smirnoff, 1993; Sherwin and Farrant, 1996; Farrant, 2000). It is believed that the main purpose of this down regulation is to limit the production of damaging reactive oxygen species due to dysfunctional photosynthesis (Farrant, 2000).

X. humilis falls in the poikilochlorophyllous sub group as it is known to inhibit photosynthetic activity by degrading its chlorophyll and dismantling its thylakoid membranes (Farrant et al., 1999). In *X. humilis* photosynthesis effectively ceases when approximately 45 % of its tissue water content has been lost (Farrant et al., 1999). However, despite the drop in supply of photosynthate, sucrose continues to accumulate after the shutdown of photosynthesis (Farrant et al., 1999). This situation is very similar to that which occurs during night time synthesis of sucrose in many C3 species. The carbon substrate for this continued synthesis is not known, but as in the case of night time sucrose synthesis, the substrate may be maltose/glucose which recent reports have identified as the main products of starch breakdown at night (see Figure 1.1.1) (Schleucher et al., 1998; Weise et al., 2004). While both FBPase activity and SPS activities are modulated by photosynthetic rate, from the available literature it appears that FBPase activity is severely curtailed at night when fructose-2,6-bisphosphate levels increase dramatically (Stitt et al., 1985; Usuda et al., 1987; Servaites et al., 1989; Stitt, 1990; Scott and Kruger, 1994). Further, it has also been reported that fructose-2,6-bisphosphate levels increase in many species in response to drought and osmotic stress (Reddy, 1996; Reddy, 2000; Banzai et al., 2003). These

observations make it likely that after the downregulation of photosynthesis in *X. humilis*, the part of the sucrose biosynthesis pathway involving triose phosphate and FBPase will be bypassed and the regulation of sucrose synthesis will fall primarily to SPS. Thus, while both FBPase and SPS may be important regulators of sucrose synthesis in photosynthetically active tissues, SPS may play a more significant role during dehydration induced sucrose accumulation.

While the intricacies of SPS regulation have yet to be fully unravelled (discussed in more detail in Chapter 3), of particular interest is the finding that most plant species contain one or more SPS isoforms. Multiple SPS isoforms exhibiting distinct tissue, developmental and temporal expression patterns have been described in a wide range of plant systems including citrus (Komatsu et al., 1996), sugar cane (Sugiharto et al., 1997) potato (Reimholz et al., 1997) and more recently in rice (Pagnussat et al., 2000). A comprehensive phylogenetic analysis of known SPS proteins concluded that four families of SPS genes are present in higher plants (Langenkamper et al., 2002, Castleden et al., 2004) and it is postulated that most species will contain a representative in each of the A, B and C families. The regulation of SPS activity by multiple isoform expression is intriguing as it suggests that within each species different forms of SPS may be dominant in different tissues, at different stages of development and under different environmental conditions. Notably, a recent report on SPS isoform expression in tobacco demonstrated that a specific isoform was upregulated under dark conditions in leaf tissues (Chen et al., 2005) suggesting that there may be a need for a new form of SPS to support sucrose synthesis after the cessation of photosynthesis.

SPS activity and desiccation tolerance

To date the relationship between changes in SPS activity and dehydration induced sucrose accumulation in leaf tissues has been investigated in the dicot resurrection plant, *Craterostigma plantagineum* Hoecht (Ingram et al., 1997) and more recently in the monocot *Sporobolus stapfianus* (Whittaker et al., 2007). SPS activity was found to increase in response to tissue dehydration in both studies. Notably, the results from the study on *C. plantagineum* indicate the presence of two SPS isoforms in leaf tissue. The authors suggest that while one isoform may

represent a constitutively expressed form of SPS, the other SPS isoform may be involved in stress related metabolism (Ingram et al., 1997). These observations have led to the two central hypotheses of the current project: i) dehydration-induced sucrose accumulation is due to increases in SPS activity in *X. humilis* leaf tissue and ii) dehydration results in the upregulation of a specific form of SPS.

Aims

X. humilis leaf tissue is known to accumulate relatively high amounts of sucrose in response to dehydration (Farrant et al., 2003; Bajic, 2006). The current work represents the first attempt to link the observed sucrose accumulation in *X. humilis* leaf tissue to a regulated molecular mechanism. In previous work on desiccation sensitive and tolerant systems dehydration induced sucrose accumulation has been associated with increases in SPS activity. To establish if sucrose accumulation can also be linked to increases in SPS activity in *X. humilis* leaf, changes in SPS activity and gene expression during dehydration were characterized. Particular attention was paid to the possible up-regulation of a dehydration specific form of SPS in *X. humilis* leaf tissue.

The first aim of the study was to characterize changes in sucrose content during tissue water loss in leaf tissue. In addition to monitoring sucrose levels, changes in a broad range of metabolites were tracked to identify possible sources of carbon supporting the observed sucrose accumulation. Following on from describing the pattern of sucrose accumulation, the second aim was to determine if, and to what extent, SPS activity was modulated by dehydration. The pattern of sucrose accumulation was compared to that of SPS activity to establish a possible correlation between the two responses. To identify a dehydration specific form of SPS the strategy adopted was to isolate all SPS genes in *X. humilis* and to analyse their expression in response to dehydration. Thus, the third aim of the work was to identify all SPS genes in *X. humilis* and to characterize their expression patterns. To guide the search for the SPS genes a phylogenetic analysis of all known SPS genes was conducted. SPS gene expression was considered at both the transcript and protein levels.

Chapter 2 **Sucrose accumulation and metabolite changes in response to dehydration**

Introduction

A common characteristic of desiccation tolerant plant systems is the presence of high levels of soluble sugars, and in particular sucrose, in the dried state. In desiccation tolerant bryophytes, sucrose levels are maintained constitutively high at approximately 10% dry mass (e.g. Smirnoff, 1992), in seeds sucrose accumulation coincides with the onset of desiccation tolerance (e.g. Sun et al., 1994; Brenac et al., 1997; Greggains et al., 2000; Buitink et al., 2003; Avelange-Macherel et al., 2006 and reviewed in Berjak, 2007) and in many resurrection plants, including *Xerophyta humilis* (Cooper and Farrant, 2002; Farrant et al., 2003) dehydration is known to induce high levels of sucrose accumulation (e.g. Ghasempour et al., 1998; reviewed in Farrant, 2007). The primary goal of the current section of work was to establish the timing and extent of dehydration-induced sucrose accumulation in *X. humilis* leaf tissue. In addition, based on changes in a broad range of metabolites including soluble sugars, starch, organic acids and amino acids the possible carbon sources supporting sucrose accumulation are proposed. To provide the background to this study, a brief review on the occurrence and possible function of dehydration induced soluble sugar accumulation in desiccation tolerant plant tissue is given.

Sugar accumulation in orthodox seeds

In orthodox seeds (desiccation tolerant seeds) the acquisition of desiccation tolerance is associated with increased levels of non-reducing sugars, including sucrose and oligosaccharides (e.g. raffinose and stachyose). Most notable is the accumulation of sucrose but significant increases in oligosaccharides are also observed (e.g. Black et al., 1996; Brenac et al., 1997; Steadman et al., 1996; Corbineau et al., 2000; Bailly et al., 2001; see reviews by Buitink et al., 2002 and Kermode et al., 2002). In many seeds, longevity in the dried state has been linked with low sucrose to soluble oligosaccharide mass ratios (Horbowicz and Obendorf, 1994; Lin and Huang, 1994), suggesting that it is the ratio of sucrose to oligosaccharides which is important in

desiccation tolerance rather than actual total amounts of sugars . Relating sugar accumulation to desiccation tolerance in seeds is complicated by the many developmental changes which may precede or accompany the acquisition of tolerance. In both orthodox and recalcitrant seeds (desiccation sensitive) sugar accumulation is a significant feature of seed development and in particular instances, the level of sugar accumulation in certain recalcitrant seeds and orthodox seeds is comparable. The inability of recalcitrant seeds to survive desiccation suggests that sugar accumulation is not the sole component of desiccation tolerance in orthodox seeds. However, the lack of tolerance despite increased sugar contents does not necessarily reduce the importance of sugar accumulation in desiccation tolerance. In seeds protective sugar glasses tend to form at the threshold of 10% water content (Walters et al., 2005). Berjak et al., (2007) note that in the case of recalcitrant seeds, seed viability is lost at tissue water contents considerably above this threshold. Thus, while in orthodox seeds sugar accumulation is important in protecting cellular structure in the dried state, in recalcitrant seeds this ‘protection’ is not utilized as lethal damage has already occurred prior to cellular vitrification.

Sugar accumulation in angiosperm resurrection plants

In desiccation tolerant angiosperms the accumulation of sugars in response to dehydration is more pronounced when compared to sensitive plant species. Schwab and Heber, 1984 noted that in the dried state, the leaves of the resurrection species *Craterostigma plantagineum* and *Ceterach officinarum* contained more than 7 times the amount of soluble sugar than dried spinach leaves. In resurrection angiosperms both sucrose and oligosaccharides are accumulated while monosaccharide contents generally decline significantly during dehydration (Vertucci and Farrant, 1995; Walters et al., 2002). In a comparison between a desiccation tolerant grass *Sporobolus stapfianus* and a closely related desiccation sensitive grass *Sporobolus pyramidalis* Ghasempour et al., (1998) reported that sugar accumulation (particularly sucrose accumulation) was considerably higher in the more tolerant grass. In addition, in a study on *Eragrostis* spp., only the desiccation-tolerant species *E. nindensis* was observed to accumulate sucrose in its vegetative tissues in response to drying (Illing et al., 2005).

In a wide range of angiosperm resurrection plants, sucrose accumulation has been found to be the predominant accumulated sugar. Significant increases in sucrose content from hydrated to dry tissue have been noted in many angiosperm resurrection species, examples include the grasses *Eragrostis nindensis* (Illing et al., 2005) and *S. stapfianus* (Albini et al., 1994; Whittaker et al., 2001), the herbaceous *Craterostigma* species (Bartels and Salamini, 2001; Cooper and Farrant, 2002) the woody shrub *Myrothamnus flabellifolius* (Moore et al., 2007) and the monocotyledonous *Xerophyta* species (Ghasempour et al., 1998; Whittaker et al., 2001; Cooper and Farrant, 2002). In most monocotyledonous resurrection plants levels of sucrose accumulation in dehydrated leaf tissue vary between 65 $\mu\text{mol g}^{-1}$ DW and 400 $\mu\text{mol g}^{-1}$ DW (Albini et al., 1994; Ghasempour et al., 1998; Whittaker et al., 2001; Illing et al., 2005), and in most dicotyledonous species between 150 $\mu\text{mol g}^{-1}$ DW and 463 $\mu\text{mol g}^{-1}$ DW (Bianchi et al., 1993; Müller et al., 1997). However, there are exceptions on either side of the range with very low levels of sucrose accumulation (11 $\mu\text{mol g}^{-1}$ DW) reported in the tolerant *Eragrostiella nardoides* (Ghasempour et al., 1998) and extremely high levels (2000 $\mu\text{mol g}^{-1}$ DW) recorded in *Craterostigma plantagineum* (Bianchi et al., 1991a). In the former species it appears that other cellular components may supplement the low levels of sucrose while in the latter species sucrose accumulation appears to occur in excess of what is needed.

Aside from sucrose, the oligosaccharides raffinose and stachyose are the next most highly accumulated sugars in the desiccation tolerant angiosperms (Ghasempour et al., 1998; Peters et al., 2007). However, the extent and type of oligosaccharide accumulation varies significantly, and in some species oligosaccharide content actually declines in response to dehydration. The inconsistent accumulation of oligosaccharides could suggest that oligosaccharide accumulation is not essential for desiccation tolerance but it should be noted that in many angiosperm resurrection species the raffinose to sucrose mass ratio is not less than 0.2 in dried leaf tissue (Bianchi et al., 1991a; Müller et al., 1997; Ghasempour et al., 1998; Živkovic et al., 2005). This ratio is close to the estimated ratio of 0.175 which has been reported in the dried state of desiccation tolerant maize seeds (Koster, 1991; Koster and Bryant, 2005). Thus, with or without active accumulation, oligosaccharides may still be present at high enough concentrations to contribute to desiccation tolerance.

Significant accumulation of the sugar trehalose is common in desiccation tolerant animal species, e.g. *Artemia* species ‘brine shrimp’ (Clegg, 1986) and tardigrades (Westh and Ramløv, 1991) but it is only rarely noted in angiosperm resurrection species (Ghasempour et al., 1998). This lack of trehalose accumulation is surprising as i) in model systems trehalose has been found to be more effective than sucrose in protecting proteins and membranes from drying damage (Kaushik and Bhat, 2003) and ii) many angiosperms possess the enzymatic potential to synthesize trehalose (Paul, 2007). To date, substantial trehalose accumulation has only been reported in *Myrothamnus flabellifolius* (Drennan et al., 1993; Moore et al., 2007). Low levels of trehalose are detectable in dried tissues of a number of resurrection species (Albini, 1994; Ghasempour et al., 1998) but the physiological significance of this accumulation is not immediately clear. Interestingly, recent work has demonstrated that trehalose-6-phosphate is an important signal molecule in primary metabolism with both sugar utilization and starch metabolism being sensitive to changing levels of this metabolite (see review by Paul, 2007). Hence the accumulation of low levels of trehalose may provide a mechanism to alter trehalose-6-phosphate and thereby affect the partitioning of carbon between sucrose and starch during dehydration.

Function of sugar accumulation in desiccation tolerance

As discussed in Chapter 1, sucrose in combination with other sugars are proposed to perform their protective function in desiccation tolerant plant tissue by: i) serving as compatible solutes to slow water loss in early stages of water loss (e.g. Ishitani et al., 1995; Yoshida et al., 1995), ii) replacing hydrogen bonding function of water in the dried state (‘water replacement’) (Clegg, 1986; Crowe and Crowe, 1992) and iii) by promoting stable glass formation (vitrification) after loss of bulk tissue water (Koster and Leopold, 1988; Williams and Leopold, 1989). While these proposed functions have become entrenched in the literature certain aspects still need further investigation and clarification. The main areas of contention are the proposed ‘water replacement’ role of sugars (see Chapter 1 for full discussion) and the specific role of oligosaccharide (e.g. raffinose) accumulation in conferring desiccation tolerance (Bohicchio et al., 1997; Lin et al., 1998 and highlighted in review by Berjak et al., 2007). In the available literature a well cited role for oligosaccharide accumulation is the prevention of sucrose crystallization upon drying (e.g. Leopold et al., 1986; Vertucci and Farrant, 1995; Peterbauer and

Richter, 2007; Bailly et al., 2001). Evidence for this proposed role is derived from early work on model sugar systems (Caffrey, 1988) but more recent *in vivo* work has demonstrated that the presence of oligosaccharides is not essential for stable glass formation (Buitink et al., 2000). Rather, it is suggested that sucrose crystallization can be prevented by other cellular components and not necessarily by the presence of oligosaccharides alone (Buitink et al., 2002). Nevertheless, while oligosaccharides may or may not be essential in preventing sucrose crystallization *in vivo*, oligosaccharide accumulation appears unlikely to be insignificant in conferring desiccation tolerance. Oligosaccharide synthesis is widely noted in both desiccation tolerant seeds and resurrection plant species and, in particular seeds, e.g. maize, oligosaccharide accumulation has been found to be essential for the acquisition of desiccation tolerance (Brenac et al., 1997). Thus, even though oligosaccharides may not be essential for the prevention of sucrose crystallization, the accumulation of these sugars may still serve an equally important function in desiccation tolerance.

In addition to their classic roles of compatible solutes and agents of intracellular vitrification, sucrose and oligosaccharides have also been implicated in the removal/detoxification of potentially destabilizing compounds. One proposed function is the removal of reducing sugars (e.g. glucose) by their conversion to non-reducing sucrose and oligosaccharides. The removal of reducing sugars contributes to the stability of the dried state by reducing possible cross linking reactions between monosaccharides and proteins by Maillard reactions (Koster and Leopold, 1988) and by reducing respiration and other undesirable reactive oxygen species (ROS) yielding reactions which have reducing sugars as their substrates (Farrant et al., 1993; Leprince et al., 1993; Vertucci and Farrant, 1995). Thus, timely removal of these more reactive sugars will allow for the creation of a more stable and more long lived dried state. Indeed, monosaccharide levels have been found to be very low in most desiccation tolerant tissues (e.g. Blackman et al., 1992; Ghasempour et al., 1998) and it appears that the conversion of these sugars into non-reducing sucrose and oligosaccharides is one method utilized to reduce their content.

Sugar accumulation in desiccation sensitive plants

The accumulation of soluble sugars in response to dehydration has also been reported in a wide variety of desiccation sensitive species including *Arabidopsis thaliana* (Rizhsky et al., 2004; Taji et al., 2002), spinach (Quick et al., 1989; Zrenner and Stitt, 1991), maize (Pelleschi et al., 1997) soybean (Cheikh and Brenner, 1992) and aspen (Pelah et al., 1997). Accumulated sugars include sucrose and raffinose series oligosaccharides, but, in contrast to desiccation tolerant tissue, monosaccharides such as fructose and glucose are also commonly accumulated. In *A. thaliana* increases in sucrose are noted after imposition of water deficit stress (Rizhsky et al., 2004) while in maize water deficit results in only a slight increase in sucrose content but a much greater increase in hexose content of tissues (Pelleschi, 1997). It has also been observed that in some species either only sucrose, e.g. sugar beet (Fox and Geiger, 1986) and sugar cane (Moore, 1995; Moore, 2005; Pammenter and Allison, 2002) or only hexoses, e.g. barley (Wingler et al., 1999) increase due to water deficit. Overall the relative contributions of sucrose and hexoses to the change in sugars in response to water deficit varies considerably between species. In addition to sucrose and hexoses, dehydration is also known to induce accumulation of other soluble sugars including raffinose and other oligosaccharides (Taji et al., 2002). In most desiccation sensitive plant species, the primary role of soluble sugar accumulation is proposed to be the maintenance of tissue turgor during mild water stress. In conjunction with other compatible solutes, soluble sugar accumulation helps maintain turgor by decreasing the osmotic potential of cellular water and thereby slowing the rate of water loss (Yancey et al., 1982).

As mentioned previously (Chapter 1), it can be argued that dehydration induced sugar accumulation in leaves is more an indirect result of dehydration, possibly via growth inhibition, rather than a regulated adaptive response to dehydration. Indeed the carbon imbalances caused by stress are likely to result in changes in sugar levels. Nevertheless, there is also strong cause to believe that sugar accumulation is of functional significance in dehydration tolerance. In many species, including wheat (Kameli and Loseld, 1993; Kerepesi and Galiba, 2000), maize (Premachandra et al., 1989), sorghum (Premachandra et al., 1995) and pea (Sánchez et al., 1998), more stress tolerant cultivars are usually able to accumulate more sugar in response to dehydration. The proposal that sugar accumulation is an important component of dehydration

tolerance is also strengthened by results from work on transgenic plants. In *A. thaliana* the over expression of a key enzyme involved in the synthesis of raffinose and galactinol caused both an accumulation of these sugars and an improvement in dehydration tolerance (Taji et al., 2002). Gilmour et al., (2000) also observed that over-expression of a transcription factor (*CBF3*), known to be involved in cold (same paper) and dehydration response (Liu et al., 1998; Kasuga et al., 1999) in *A. thaliana*, led to increases in oligosaccharide content and an increase in stress tolerance. Further, dehydration induced sucrose accumulation in potato is significantly reduced when sucrose phosphate synthase expression is repressed (Geigenberger, 1999), suggesting that the observed sucrose accumulation is a regulated response to dehydration and not only a consequence of growth inhibition.

Carbon sources for sugar accumulation

Sugar accumulation in desiccation tolerant seeds and vegetative tissue would not be possible without access to a supply of carbon skeletons. In seeds it appears likely that the observed sugar accumulation will be mainly fuelled by either starch or lipid reserves (Chia et al., 2005) but carbon sources located in maternal tissues may also contribute. In desiccation tolerant leaf tissue, carbon sources may include i) carbon fixed by photosynthesis, ii) breakdown of storage reserves (e.g. starch and fructans) and iii) translocation from other parts of the plant, such as roots. Interestingly, in many resurrection plant species a large proportion of the sugar accumulation, and especially sucrose accumulation, occurs after photosynthesis is down regulated (usually below 60% RWC) (Whittaker et al., 2001; 2004; Mundree et al., 2002; Illing et al., 2005; Farrant, 2007). Thus, while photosynthesis may contribute to sucrose accumulation in the early stages of water loss, alternative sources of carbon will be required as water loss progresses. In the species *C. plantagineum* the rare sugar octulose is easily identified as the alternative source of carbon. In fully hydrated leaf tissue, 2-octulose levels are maintained high, but upon drying octulose levels drop as sucrose levels increase (Bianchi et al., 1991; Ingram et al., 1997). In drying leaves of a number of species, e.g. *Borya constricta* and *Coleochloa setifera* sucrose accumulates at the expense of the hexoses fructose and glucose (Ghasempour et al., 1998). As sucrose accumulates in *Ramonda nathaliae* the starch content of the leaf declines, but starch breakdown can only account for less than 20% of the accumulated sugar (Müller et al., 1997). In

Xerophyta villosa, the decrease in raffinose and stachyose during dehydration suggests that these oligosaccharides may contribute carbon skeletons to sucrose accumulation in this species (Ghasempour et al., 1998). However, in a closely related species, *Xerophyta viscosa*, all three sugars - sucrose, stachyose and raffinose, accumulate during dehydration (Peters et al., 2007) indicating that an alternative carbon source is utilized in this species. Thus, from the available literature it appears that a number of different carbon sources may be utilized in different species to support the observed sugar accumulation.

Aims

The aim of this section of work was to describe the pattern of sucrose accumulation in *X. humilis* leaf tissue during dehydration. In an attempt to identify the possible sources of carbon skeletons supporting sucrose accumulation, changes in a broad range of metabolites were also measured. Sucrose, glucose and starch content of leaf tissue at varying stages of water loss were measured by enzymatic assay. All other metabolites, which included soluble sugars, organic acids and amino acids, were quantified using a GC-TOF-MS based approach.

Methods

Plant material

Xerophyta humilis (Baker) T. Durand & Schinz plants were collected from Barakalalo National Park (Limpopo Province, South Africa). Two groups of plants, an experimental and a control were maintained in a greenhouse under ambient light and temperature conditions. Irradiance averaged $520 \mu\text{mol m}^{-2} \text{sec}^{-1}$ and ranged from $200 \mu\text{mol m}^{-2} \text{sec}^{-1}$ to $1000 \mu\text{mol m}^{-2} \text{sec}^{-1}$, temperatures averaged 18°C and ranged from 16°C to 25°C . Plants were grown in large trays (60x40x15 cm) with multiple plants occurring in one tray. The experimental group was dried by withholding water while the control group was kept in a hydrated state. At various points during drying, leaves of similar chlorophyll content were harvested from plants in the experimental group. As chlorophyll is progressively lost during dehydration, changes in the colour of the leaves provided a good indicator of tissue water loss (see Ingle et al., 2008). At each leaf harvest from the experimental group leaves were also removed from plants in the fully hydrated control group.

Determination of relative water content (RWC)

The relative water content (RWC) was calculated according to the formula:

$$\text{RWC} = (\text{wet mass} - \text{dry mass}) / (\text{full turgor mass} - \text{dry mass})$$

Full turgor mass was determined following a 12 h incubation of plant trays in sealed transparent plastic bags. Actual water content of leaf tissue was determined gravimetrically by weighing leaf tissue before and after oven drying for 48 h at 70°C , or by weighing tissue before and after freeze drying.

Efficiency of photosystem II electron transport (Φ PSII)

Photosystem II (PS II) operating efficiency was defined according to Genty et al., 1989

Φ PSII = $(F_m' - F_s) / F_m'$. Where F_m' is the maximum fluorescence yield obtained during a saturating flash and F_s is the steady-state fluorescence yield.

Fluorescence was determined by pulse amplitude modulation (PAM) (Schreiber et al., 1995) fluorescence measurements using the PAM101/102/103 system (Walz, Effeltrich, Germany) with a photomultiplier tube accessory. Measurements were done in triplicate (3 individual leaves from 3 three plants). The digital signal was recorded using Labview 4.0 (National Instruments, Austin, TX). Minimal fluorescence (F_s) was measured using a light-emitting diode delivering a modulated measuring light beam ($\lambda = 650$ nm), too weak to induce reaction center closure. For the measurement of maximal fluorescence (F_m), a minimum of 16 saturating pulses (Schott KL1500-E; $E > 5000 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) each of 600 ms duration were delivered at 30-s intervals. Φ PSII has been demonstrated to provide an estimate of the quantum yield of PSII phytochemistry (Genty et al., 1989). The parameter has been shown to be directly related to the rate at which CO_2 is assimilated in leaf (e.g. Genty et al., 1989; Edwards and Baker, 1993; Siebke et al., 1997) and hence has been widely used to detect perturbations in photosynthetic performance.

Leaf sampling strategy

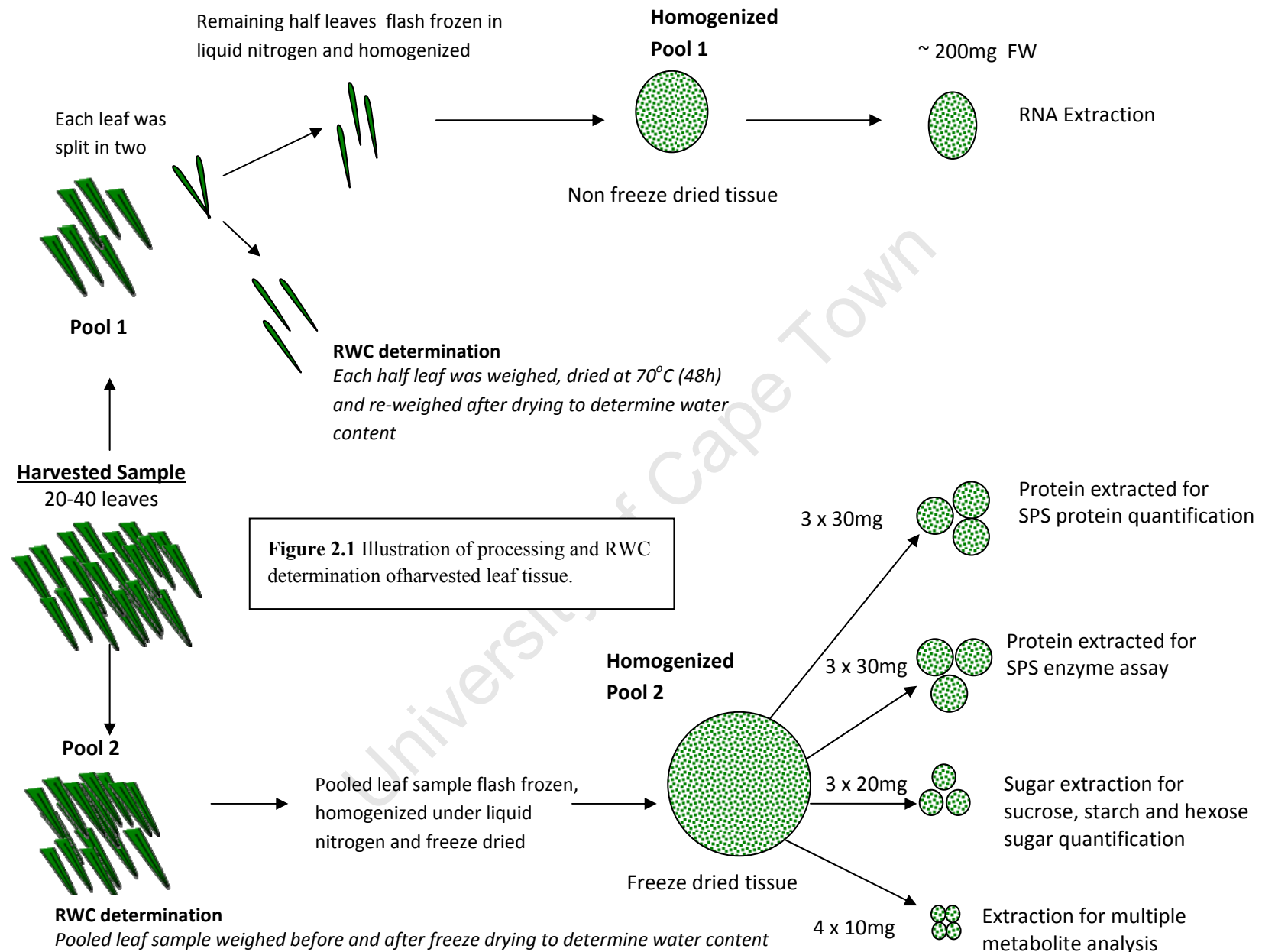
X. humilis is a slow growing ‘grass’ like species (see Appendix A for image). Due to its relatively small size it was not possible to obtain enough leaf tissue from an individual plant to allow for multilevel analysis (metabolite analysis, enzyme activity and gene expression) of leaf tissue at different stages of water loss. Consequently it was necessary to harvest leaves from different plants at each sampling point and to pool each group of harvested leaves. The pooled tissue provided enough material for the measurement of metabolite levels (current chapter), for the assessment of SPS activity (see Chapter 3) and for the analysis of gene expression (see Chapter 4) at multiple points during dehydration. As *X. humilis* leaves lose their chlorophyll during dehydration, when harvesting leaf tissue chlorophyll content was used as a rough indicator of tissue water loss (see Ingle et al., 2008). Hence at each harvest, leaves of similar chlorophyll content (estimated by eye) were selected. Thus, data obtained using this ‘leaf pooling’ strategy represents the average state of a pool of leaves which have similar water contents but which originate from different plants.

Processing and RWC estimation of harvested leaf tissue

Processing of the harvested leaf tissue and RWC estimation is illustrated in Figure 2.1. Briefly, at each sampling point during dehydration between 20 to 40 leaves were harvested from multiple plants. The Irradiance, temperature, time of day and days after withholding water were recorded at each harvest (see Results section of current chapter). From the total pool of harvested leaves, randomly selected leaves were removed and set aside for RNA extraction (described as Pool 1). After removal of these leaves, the remaining pool of leaves was used for protein and metabolite extraction (Pool 2).

Each leaf in Pool 1 was split vertically in two along the mid-vein and one leaf half used to determine RWC of the leaf tissue. Each half leaf was weighed before and after oven drying at 70 °C to determine water content. In this way the average ($\pm$ standard deviation (SD)) RWC of the pool of leaves in Pool 1 was obtained. The remaining half leaves in Pool 1 were flash frozen in liquid N₂ and homogenized. The frozen tissue was stored at -80 °C and used for RNA extraction and subsequent analysis of SPS transcript changes (see Chapter 4). All changes in transcript levels were related to the estimated RWC of Pool 1 leaf tissue (see Chapter 4).

The leaves in Pool 2 were weighed (collectively) prior to- and after freeze drying to determine the average RWC of the pool of leaf tissue. After weighing, the leaves were flash frozen in liquid N₂, homogenized and freeze dried under vacuum for 48 h. The tissue was re-weighed after freeze drying and the RWC of the pool of leaf tissue estimated. This method allowed for the determination of the average RWC of Pool 2 leaves but gave no estimate of variance in RWC between individual leaves within the pool. For the measurement of metabolite levels (current chapter), SPS activity (see Chapter 3) and SPS protein levels (see Chapter 4) the pool of homogenized freeze dried leaf tissue of Pool 2 was used. For all the above analyses changes were related to the RWC estimated for Pool 2 tissue.



Sucrose, glucose and fructose quantification

Twenty mg of homogenized freeze dried leaf tissue was ground in liquid nitrogen and extracted in cold 100 mM NaOH (in 50 % v/v ethanol/water). Chloroform (15 % v/v) was added and the samples incubated on ice for 10 min. Thereafter the pH was adjusted to 7.5 with 100 mM HEPES in 100 mM glacial acetic acid. After centrifugation for 20 min at 4 °C at 28,000 g, the supernatant was removed. A repeat extraction was performed on the tissue pellets. The supernatants were pooled and then centrifuged. Sucrose in the supernatant was calculated from the spectrophotometric measurement of NADPH production using a D-glucose/D-fructose sugar assay kit (Roche, Boehringer Mannheim Diagnostics, Germany) based on the methodology of Bergemeyer et al., 1974). The production of NADPH was determined spectrophotometrically at 340 nm (Beckman DU 6500) and used to calculate the quantity of sucrose, glucose and fructose in each sample. Each measurement was conducted in triplicate. For each pool of harvested leaf tissue a total of three 20 mg aliquots of freeze dried leaf tissue were extracted and the amounts of sucrose, glucose and fructose determined in each extract.

Starch quantification

Quantitative starch assays were conducted according to a modified method of Schulze et al., 1991). Freeze dried leaf tissue was ground in 70 % ethanol and extracted three times at 80 °C. Twenty mg of freeze dried leaf tissue was added to 250 µl 70 % ethanol (EtOH). The sample was shaken at 80 °C for 20 min and the pellet collected by centrifugation at 1400 rpm for 5 min. The pellet was washed once with 500 µl chloroform:methanol (1:1) and once with 500 µl acetone. The pellet was dried and re-suspended in 100 µl of 0.2 M sodium acetate (pH 5.5) and incubated for 1 h at 120 °C. After cooling the pellet was digested for 14 h at 37 °C in 0.2 M sodium acetate, pH 5.5, containing 1.4 units of amyloglucosidase (Sigma-Aldrich) and 0.11 units of alpha-amylase (Sigma-Aldrich). The digestion step was repeated and the supernatants pooled. Glucose in the supernatant was calculated from the spectrophotometric measurement of NADPH production using a D-glucose/D-fructose sugar assay kit (Roche, Boehringer Mannheim Diagnostics, Germany). The glucose content of the supernatant was then used to assess the

starch content of the sample. For each pool of harvested leaf tissue, extraction and measurement of starch was repeated a further two times. In total three 20 mg aliquots of freeze dried leaf tissue were extracted and the amounts of starch determined in each extract.

Metabolite analysis

All chemicals were purchased from Sigma (Taufkirchen, Germany), except [6-¹³C] sorbitol which was obtained from Isotech (Miamisburg, USA). Acetonitrile was obtained from J.T.Baker (Deventer, Netherlands).

Extraction procedure and sample preparation

Ten mg of powdered freeze dried leaf material was used for analysis. Metabolites were extracted as described in Weckwerth et al., (2004). For metabolite extraction, 1 ml of the extraction mixture containing methanol/chloroform/water (2.5:1:0.5 v:v:v) and 10 µl of an internal standard containing 2 mg/ml of [6-¹³C] sorbitol was added to the plant tissue. Soluble metabolites were extracted by mixing the solution at 4 °C for 10 min. After centrifugation for 6 min at 20,000 rpm, the supernatant was separated into chloroform and water/methanol phases. The aqueous phase was used for metabolite analysis.

For each pool of harvested tissue a total of four 10 mg aliquots of freeze dried leaf tissue were extracted for metabolites. The extracted metabolites (in the aqueous phase) were dried down under vacuum. After drying the samples were randomized and derivatized by dissolving the dried metabolite pellet in 20 µl of methoxyamine hydrochloride (40 mg/ml pyridine) and shaking the mixture for 90 min at 30 °C. After addition of 180 µl of N-methyl-N-trimethylsilyltrifluoroacetamid (MSTFA), the mixture was incubated at 37 °C for 30 min with vigorous shaking. A solution of even-numbered fatty acid methylesters, methylcaprylate (C8-ME), methylcaprate (C10-ME), methylaurate (C12-ME), methylmyristate (C14-ME), methylpalmitate (C16-ME), methylstearate (C18-ME), methyleicosanoate (C20-ME),

methyldocosanoate (C22-ME), lignoceric acid methylester (C24-ME), methylhexacosanoate (C26-ME), methyloctacosanoate (C28-ME), and triacontanoic acid methylester (C30-ME) (each 0.8 mg/ml CHCl₃) was spiked into the derivatized sample prior to injection into the GC.

GC-TOF-MS analysis

The GC-TOF-MS analysis was performed on an AGILENT[®] 6890 gas chromatograph. Samples were injected in random order. One µl of sample was injected at 230 °C injector temperature. GC was operated on a Factor Four[™] Capillary Column VF-5ms (30m x 0,25 mm ID; 0.25 µm film thickness + 10m EZ-Guard[™] Column (Varian, Inc., Lake Forest, USA) at constant flow of 0.6 ml/min helium. The temperature program started with 1 min isocratic at 70 °C, followed by temperature ramping at 9 °C/min to a final temperature of 350 °C which was held for 5 min. Data acquisition was performed on a Pegasus 4D TOF mass spectrometer (LECO, St. Joseph, USA) with an acquisition rate of 20 scans s⁻¹ after a solvent delay of 385 s in the mass range of m/z = 70–600.

Data pre-processing

Peak heights of the mass (m/z) fragments were normalized using the amount of the sample dry weight and the signal intensity of the stable isotope labeled internal standard [6-¹³C] sorbitol. This normalization was done prior to both the targeted metabolite analysis, which entailed the identification of each metabolite, and the un-targeted analysis, which involved classification of metabolites without individual identification of each metabolite.

Data pre-processing – un-targeted

For the analysis of unidentified compounds and their subsequent classification the publicly available Tagfinder (Luedemann et al., 2008) software was used. For each chromatogram 531 mass traces ranging from 70 to 600 m/z (with nominal mass solution) were extracted and plotted against the time axis. After definition of time groups correlating mass spectral tags (MSTs)

within each time group were determined. Correlating MSTs within each time group reflected one metabolite with a minimum of three correlating fragments considered as unique masses for quantification.

Data pre-processing - targeted

The raw chromatograms were analyzed at first by defining a reference chromatogram with the maximum number of detected peaks over a signal/noise threshold of 50. Afterwards all chromatograms were matched against the reference with a minimum match factor of 800. Compounds were initially annotated by retention index and mass spectra comparison to a user defined spectra library. Selected fragment ions specific for each individual metabolite were used for quantification. Each analytical replicate was subjected to a quality assessment. Essentially, after quantification the average value for each metabolite across all replicates was calculated. If the quantified value of a metabolite in a particular replicate fell outside of 1.5 standard deviations of the calculated average for all replicates it was designated as an outlier. After designating all outliers across all measured metabolites, the total number of outlier metabolites in each replicate was determined. If 50-100 % of the values in a replicate were designated as outliers, the replicate was deemed to be of poor quality and therefore excluded from the calculation of the mean relative amount of each metabolite. The process was formalized in R script and applied to data in a MS Excel (Microsoft) spreadsheet.

Statistical analysis and Independent Component Analysis (ICA)

The ‘un-targeted’ data set was statistically analysed by one way ANOVA using Matlab 6.5 (The Mathworks, Natick, MA). Differences were scored as significant at the $P < 0.05$ level. Only MSTs showing significant changes over all the relative water contents were used in further analysis. Data were transformed as follows: i) missing values were replaced by the median (over all desiccation states) of each specific compound; ii) all values were then divided by the median and iii) data were log transformed (the numerical value of the missing values is now zero). Independent component analysis (ICA) was applied in combination with principal components

analysis (PCA) according to (Scholz et al., 2004). The PCA analysis was applied to reduce the dimensionality of the data prior to ICA analysis (Hyvarinen et al., 2001)

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Results

The Irradiance, temperature, time of day and days after withholding water were recorded at each harvest and the information is displayed in Table 2.1.1. From the pool of harvested leaves, randomly selected leaves were removed for RNA extraction (Pool 1, refer to Figure 2.1, pg 24). After removal of these leaves, the remaining leaves were used for protein and metabolite extraction (Pool 2). While it was not possible to estimate variation in RWC in Pool 1 (as individual leaves were not weighed), variation was estimated in Pool 2 (see Table 2.1.2). The RWC values for each pool were found to be similar (within 2 % of each other) and are displayed in Table 2.1.2. In Pool 2 leaves the co-efficient of variation did not exceed 8 % indicating that most of the harvested leaves were of a similar RWC. When addressing changes in metabolite levels, the leaf tissue RWC estimation is based on Pool 1 leaves. To facilitate discussion, tissue dehydration is divided into three broad stages, namely an early stage from 100 to 60 % RWC, a middle stage from 60 to 40 % RWC and a late stage from 40 to 5 % RWC.

Changes in sucrose, hexose sugars (glucose and fructose) and starch were measured using the appropriate enzyme based quantification method. For sucrose and starch, equivalent measurements were also made on a control group of plants (maintained in fully hydrated state). For all other metabolite measurements, compounds were identified and quantified by GC-MS-TOF analysis. However in the case of the GC-MS-TOF analysis no equivalent control samples were analysed. Changes in photosynthesis were measured in both the experimental group of plants and the control group of plants.

Table 2.1.1 Time and environmental conditions recorded at each leaf harvest. The number of days between withholding water and leaf harvest are indicated

Sample	1	2	3	4	5	6	7
Hrs into light period (h)	3½	3½	3½	8	3½	8	3½
Time of day	10:30 am	10:30 am	10:30 am	3pm	10:30 am	3pm	10:30 am
Irradiance ($\mu\text{mol m}^{-2} \text{sec}^{-1}$)	400	425	750	571	554	500	450
Temperature (°C)	18	19	24	23	22	19	20
Days after withholding water	0	6	9	10	12	14	15

Table 2.1.2 Estimated RWC for leaf tissue of each sample. For each sample between 20-40 leaves were harvested. The average RWC was estimated independently for the pool of leaves used for protein and metabolite extraction, and for the pool of leaves used for RNA extraction.

Sample	^Leaves for RNA extraction (Pool 1)			*Leaves for protein & metabolite extraction (Pool 2)	
	No. of leaves	RWC (%)	SD	No. of leaves	RWC (%)
1	6	100	± 3.6	30	100
2	6	72	± 4.3	25	70
3	8	60	± 3.5	30	60
4	8	53	± 4.1	31	54
5	10	41	± 3.0	24	40
6	11	20	± 2.9	20	20
7	13	5	± 0.3	28	5

* RWC determined by weighing pool of leaves before and after freeze drying

^ RWC determined by weighing each half leaf before and after drying in 70 °C oven

Changes in sucrose content

During tissue dehydration in *X. humilis* leaf the major sucrose accumulating periods occur in the early phase of dehydration (between 100 and 60 % RWC) and in the late phase of dehydration (below 20 % RWC) (Figure 2.2 (A)). In the early phase sucrose content increases to almost twice that of the control tissue and in the late phase there is a further doubling in sucrose content from the 20 % RWC to the 5 % RWC mark (see Figure 2.2 (D)). In the middle to late stages of dehydration, between 60 and 20 % RWC, there is relatively little change in the sucrose content of drying leaf tissue. As it was uncertain if the change in sucrose content was linear during the periods of accumulation it was not possible to estimate the rate of sucrose accumulation (change in sucrose over change in time). However, to allow for comparison between the phases of sucrose accumulation, the change in sucrose content was expressed relative to the change in RWC (Figure 2.3). As can be seen in Figure 2.3 the most rapid period of sucrose accumulation occurs in the late stage of dehydration

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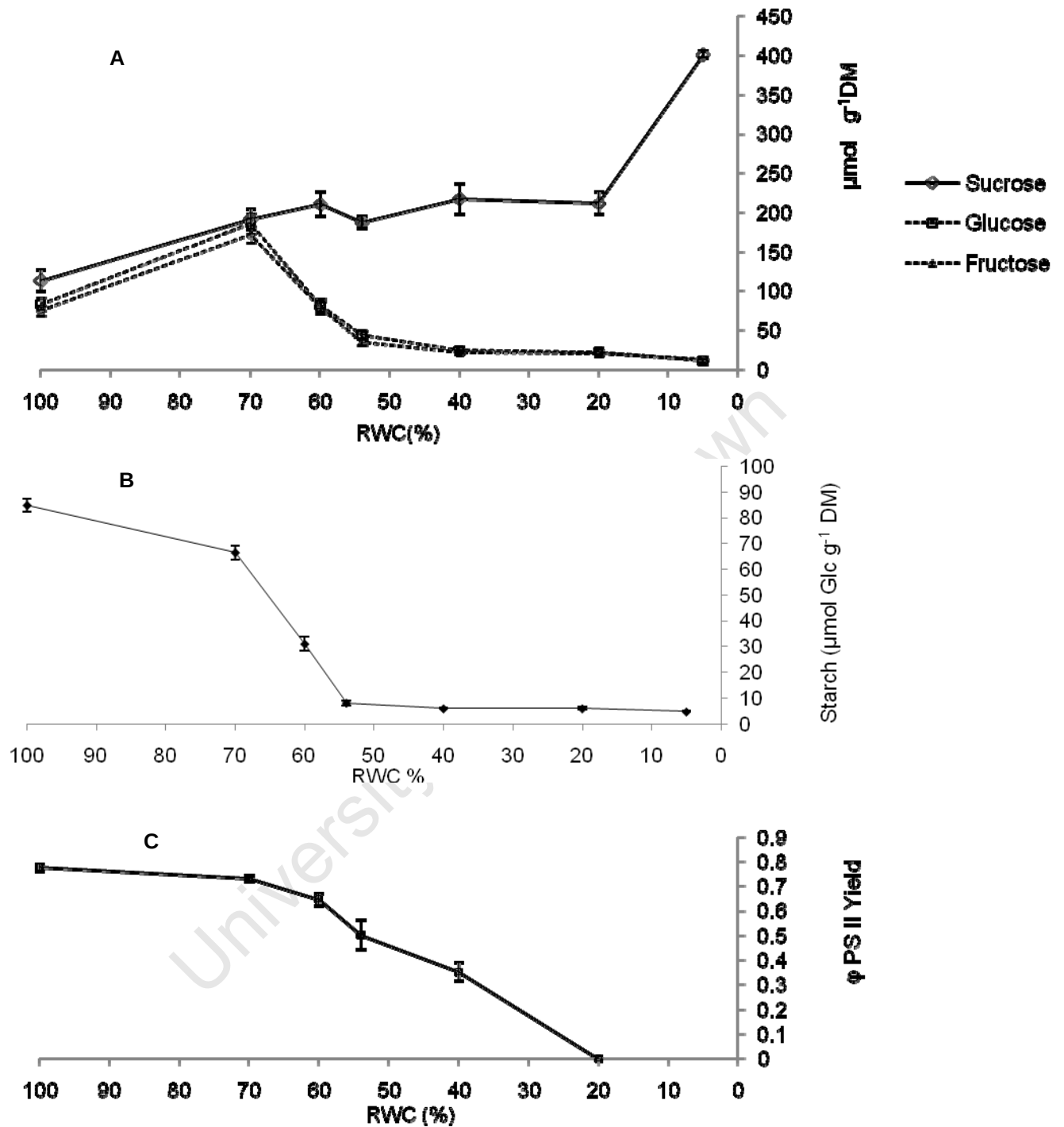


Figure 2.2(A) Changes in sucrose, glucose and fructose content in leaf tissue during dehydration. Each value is the mean $\pm$ SD of 3 measurements of a pool leaf sample (leaf $n > 6$). **(B)** Change in leaf starch content during dehydration. Each value is the mean $\pm$ SD of 3 measurements of a pool (leaf $n > 6$) of leaf tissue. **(C)** Change in quantum yield (ϕ PS II) during dehydration. Each value is the mean $\pm$ SD of 4 measurements of individual leaves.

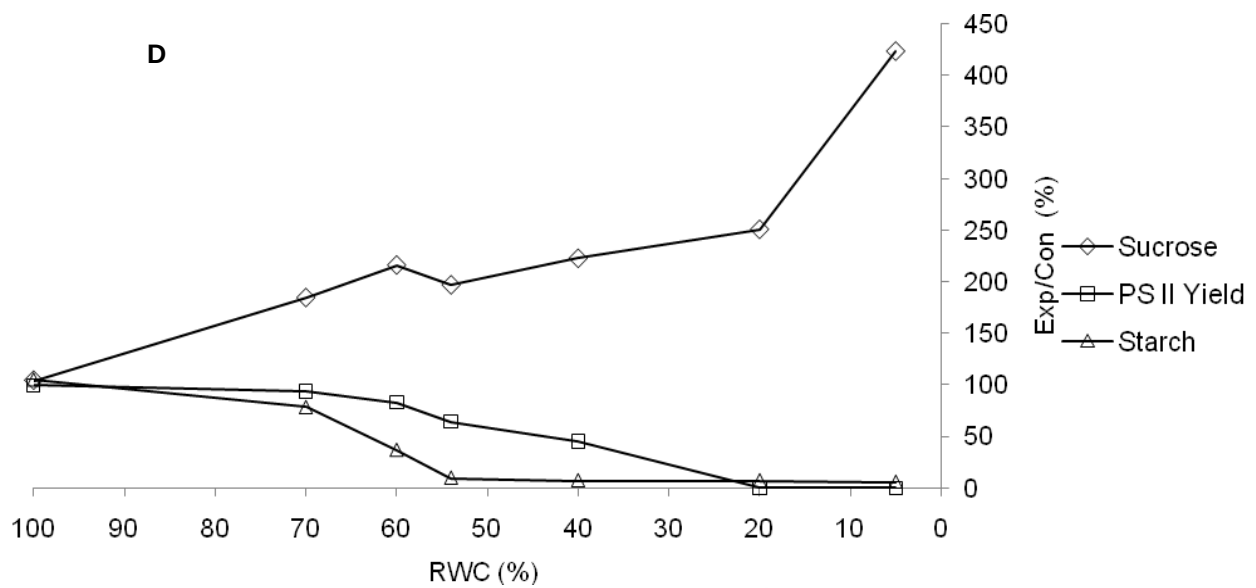


Figure 2.2 (D) Changes in sucrose content, starch content and ϕ PS II yield expressed as a percentage of values in control tissue.

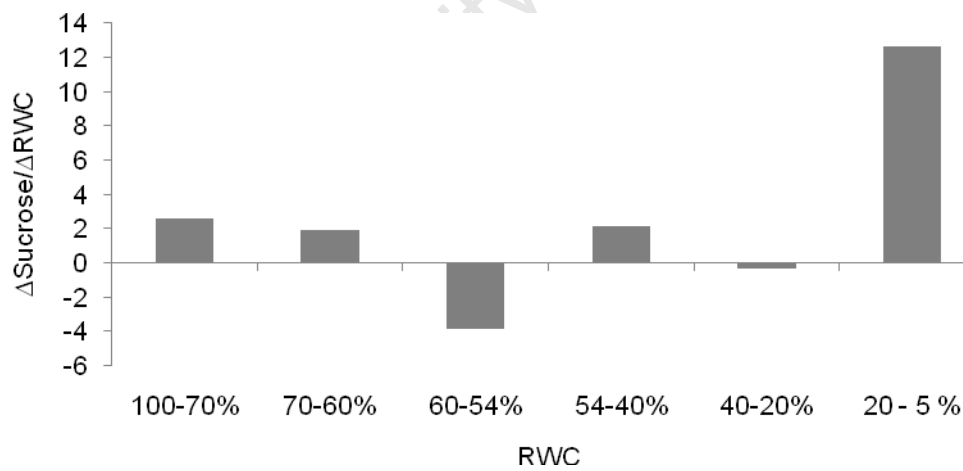


Figure 2.3 Change in sucrose content expressed relative to change in water content.

Carbon sources supporting sucrose accumulation

Photosynthesis

Changes in ϕ PSII yield (derived from fluorescence measurements) were used as a measure of PSII photochemical activity in dehydrating leaf tissue. In the glasshouse Irradiance ranged between 400 and 750 $\mu\text{mol } \mu\text{mol m}^{-2} \text{ sec}^{-1}$ over the period of sampling (Table 2.1.1). Quantum yield starts to change at the 70 % RWC mark where the first reduction in yield is noted (see above Figure 2.2 (C)). ϕ PSII yield continues to decrease reaching 50 % of its initial value at the 40% RWC mark and ceased when leaf tissue water content reached 20 % RWC (Figure 2.2 (C)). The reduced yield indicates the closure of the reaction centres in PS II and hence the reduced operation of the photosystem.

Carbohydrate carbon sources

In *X. humilis* leaf tissue dehydration results in a significant decline in starch content (see above Figure 2.2 (B)). Starch levels drop gradually initially, falling about 25 % in the early stage of tissue water loss (from 100 to 70 % RWC), but there is a major decline between 70 % RWC and 54 % RWC with starch levels falling from 66 $\mu\text{mol Glc g}^{-1} \text{ DM}$ to 8 $\mu\text{mol Glc g}^{-1} \text{ DM}$. The initial decline in starch content is correlated with the increase in sucrose in the early stages of dehydration indicating that starch metabolism may contribute to the observed increases in sucrose levels. However, despite continued starch decline between 60 % RWC and 54 % RWC there is a slight decrease in sucrose content, suggesting that the breakdown of starch is not solely linked to sucrose accumulation and may contribute to other carbon reserves as well. Furthermore, during the most rapid phase of sucrose accumulation, from 20 % RWC to 5 % RWC, there is very little starch to contribute carbon to the observed increases in sucrose content. Hence, starch metabolism may contribute directly to sucrose accumulation in the early phase of water loss, but beyond the 60 % RWC mark there appears no direct link between starch breakdown and sucrose accumulation. Regarding hexose sugars, both fructose and glucose levels initially increase in response to dehydration and follow a similar pattern to that of sucrose accumulation in the early phase of dehydration. However as water loss proceeds, the hexose

sugar levels decrease significantly, falling from approximately 180 $\mu\text{mol g}^{-1}$ DM at 70 % RWC to approximately 35 $\mu\text{mol g}^{-1}$ DM at 54 % RWC. Thus, as was observed for starch, hexose sugars may contribute directly to increased sucrose levels in the early phase of tissue dehydration but the most rapid period of sucrose accumulation does not coincide with the major decline in hexose sugar levels.

Changes in metabolite profile during dehydration

To establish if other metabolites, aside from starch and hexose sugars, could be linked to the observed sucrose accumulation changes in a broad range of metabolites were monitored by GC-MS-TOF analysis. For each pool of leaf tissue at a particular RWC (e.g. pool of leaf tissue with an average RWC of 70 % RWC), 4 independent metabolite extractions were conducted (see Figure 2.1, pg 24). These analytical replicates were then subjected to a quality assessment (see Methods for details) and the outlier replicates excluded from further analysis. Consequently between 2-4 measurements were obtained for each pool of leaf tissue. The co-efficient of variation for analytical replicates was below 20 %.

To obtain information from GC-MS-TOF raw data both an un-targeted and targeted approach was adopted. The un-targeted approach involved the classification of metabolites without the actual identification of each metabolite. The goal of the targeted approach was to identify and quantify as many individual metabolites as possible. While the former approach did not identify specific compounds it did permit changes in a large number of metabolites to be tracked. Thus the un-targeted approach allowed for a more robust statistical analysis of the data set while the targeted approach provided information on changes in specific metabolites.

Un-targeted metabolite analysis

From the un-targeted analysis of metabolite changes, a total of 522 MSTs (representing 522 unidentified metabolites) showed significant changes across all relative water contents

(ANOVA, $P < 0.05$). In each sample the relative metabolite levels will reflect both the biological and analytical variation of the sample. To visualize the effect of the tissue treatment (water loss) on the separation of the samples, independent component analysis (ICA) was applied to the 522 MSTs (Figure 2.4). If the biological variance due to the tissue treatment (water loss) is larger than the analytical variation then one would expect the samples to separate according to their relative water contents. Prior to the ICA analysis, principle component analysis (PCA) was applied to the data to reduce the dimensionality of the data set. The results of the ICA show a clear separation based on RWC for most of the samples. The largest shift was found between the 20 and 5 % RWC samples while the least separation is observed between the 100 and 70 % RWC samples, indicating that the metabolite profiles of these two samples (100 and 70 % RWC) are quite similar.

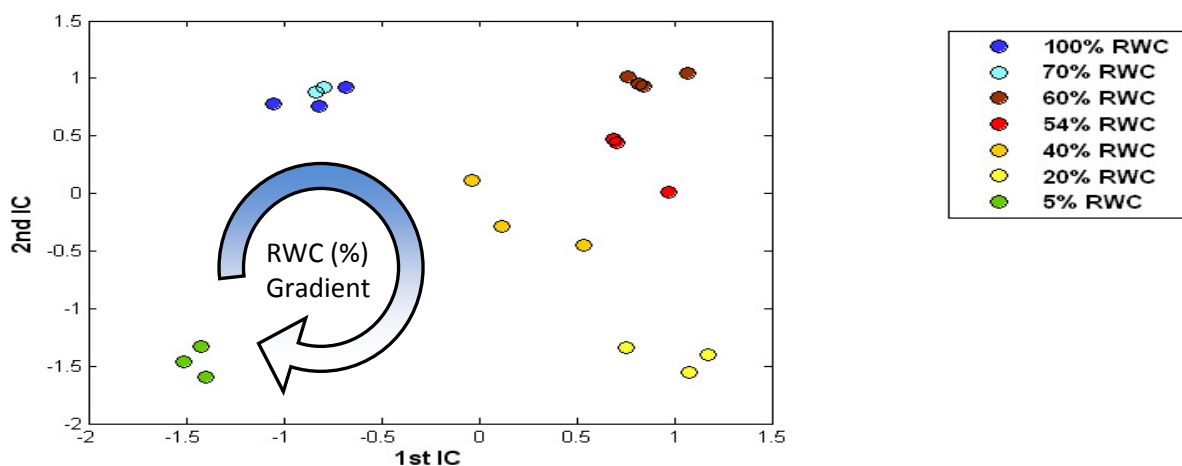


Figure 2.4 ICA of log-transformed un-targeted metabolite data from leaf samples harvested at 100 %, 70 %, 60 %, 54 %, 40 %, 20 % and 5 % RWC. The graphical representation of the ICA results allows for the separation of the samples to be visualized. Each dot represents one measurement of a pool of leaf tissue (leaf $n > 12$) at a particular RWC. In each measurement a total of 522 unidentified metabolites (represented by 522 MSTs) were quantified. These metabolites were found to differ significantly (ANOVA, $P < 0.05$) across all seven RWC samples. The circular arrow describes the RWC gradient as indicated by the separation of the samples.

While the untargeted analysis of metabolites did not provide quantification information on specific compounds, it was possible to use the data set to obtain information on changes in general carbohydrate pools during dehydration. Of the identified 522 MSTs, 146 displayed

spectra similar to carbohydrates. Figure 2.5 shows a heatmap of the changes in these metabolites. Notably, there is a general decrease in the hexose sugar pool and an increase in the disaccharide, e.g. sucrose, and trisaccharide sugar pools during dehydration. This suggests that sucrose accumulation may be supported by carbon transfer from the hexose sugar pool.



Figure 2.5 Heat map of changes in 146 MSTs showing similarity to carbohydrates. Groups of carbohydrates are indicated above the heat map. The log colour scale ranges between 0 and 1.74. The direction of the RWC gradient is indicated on the right of the heat map.

Targeted metabolite analysis: changes in identified metabolites

In total 52 metabolites could be confidently identified and quantified from the targeted analysis of the GC-TOF-MS based metabolite profiling experimental data. The changes in levels of each of these metabolites during dehydration were expressed relative to the amounts measured in the 100 % RWC leaf tissue sample (see Figure 2.6 and Table 2.2). The data presented in Figure 2.6 is based on relative changes in each of the metabolites and does not describe absolute amounts in leaf tissue. To obtain data on absolute changes in metabolite levels the analysis would have required the use of metabolite standards which were not available at the time of analysis. Hence only relative data is described. As is noted in the Figure 2.6 the data on relative changes in sucrose, glucose and fructose content was obtained from the enzymatic quantification on each of these metabolites. In principle, relative quantification of these metabolites could have also been obtained from the GC-TOF MS analysis. However, due to the high technical variation (coefficient of variation greater than 20 %) observed when these metabolites were analysed by the GC-TOF MS method, only the enzymatic data is presented. **cont/ pg 41**

Mannitol							
Ononitol							
Ribitol							
Arabitol							
Xylitol							
Erythritol							
Threitol							
Xylose							
Lyxose							



Figure 2.6 (preceding page) Primary metabolic pathway showing fold changes (relative to the value in the 100% RWC sample) in identified metabolites during dehydration. For all metabolites, except for sucrose, glucose, fructose and starch (*), data were derived from GC-TOF-MS analysis. Data for sucrose, glucose, fructose and starch was derived from enzyme based quantification. Each block (1-7) represents the mean fold change of 2-4 measurements of a pool of leaf tissue, (multiple plants, leaf n > 12) at a particular RWC. The RWC in each set of blocks decreases from left to right with block numbers 1-7 representing the RWC marks of 100- 5 % RWC respectively. Metabolites that have not been identified in the current study (e.g. Spermine, Cysteine) are displayed as reference points. Multiple arrows between compounds indicate multiple reactions. Arrows with dashed lines indicate a link between a particular compound and class of compounds. Identified metabolites which have been quantified but are not mapped are displayed in Tables A, B and C. **Table A** contains polyols and Xylose and Lyxose, **Table B** contains glycerol related compounds and **Table C** contains organic acids.

Abbreviations: Glu-glucose, P-phosphate, BP- bisphosphate, 3PGA- 3-phosphoglycerate, RuBP- ribulose-1,5-bisphosphate, PEP-phosphoenolpyruvate, Glyc-phospho-glycerol - glycerophosphoglycerol, DAP - dihydrodipicolinate, SAM - S-adenosyl-l-methionine.

Table 2.2 Fold changes in identified metabolites. Changes expressed relative to the measured value in the 100 % RWC sample. Each value represents the mean fold change of 2-4 measurements of a pool of leaf tissue at a particular RWC (multiple plants, leaf n > 12) at a particular RWC. The co-efficient of variation for replicate measurements of each sample was below 15%.

Metabolite	Fold change					
	70% RWC	60% RWC	54% RWC	40% RWC	20% RWC	5% RWC
Succinic acid	1.107	1.439	1.486	1.377	-2.096	-1.356
Malonic acid	-1.309	-1.18	-1.071	-1.118	-1.395	-1.567
Glutaric acid	1.834	5.783	2.509	1.488	1.075	1.977
Malic acid	1.762	1.033	1.2	1.59	1.927	-2.854
Threonic acid	-1.288	-1.215	-1.661	-3	-3.389	-12.85
Glyceric acid	-2.711	-2.543	-3.633	-6.351	-11.894	-7.295
Erythronic acid	1.63	-2.181	-1.164	-2.37	-2.657	-4.433
Citric acid	-1.374	-1.222	-1.604	-1.131	1.165	-1.986
Shikimic acid	-1.721	-2.164	-2.522	-8.395	-2.377	-2.284
Ribonic acid	-1.101	-1.266	-1.456	-3.786	-1.535	-1.125
Glycerol	-1.101	-1.274	1.312	8.176	11.57	6.167
Mannitol	-1.011	-1.387	-1.39	-1.135	-1.06	1.009
Inositol	-1.966	-3.097	-3.534	-6.223	-3.737	-2.409
Ononitol	-1.317	-1.625	-1.635	-2.291	-2.785	-1.692
Ribitol	-1.509	-1.113	-1.2	-1.715	-1.218	1.179
Arabitol	-2.125	-2.582	-1.216	-1.431	-1.135	1.511
Xylitol	-1.213	-1.101	-1.027	-1.175	1.013	10.921

Table 2.2 continued/

Metabolite	Fold change					
	70% RWC	60% RWC	54% RWC	40% RWC	20% RWC	5% RWC
Erythritol	-1.746	-1.946	-1.401	-1.18	-2.085	1.014
Threitol	1.907	1.516	1.012	-1.585	-2.74	-3.457
Ethanolamine	-1.353	-1.208	1.14	1.199	1.593	1.146
Putrescine	-1.126	-1.165	1.2	-1.481	-1.186	1.121
Threonine	-1.765	-1.063	2.245	2.159	4.172	-1.508
Glutamic acid	-1.553	-1.034	-1.104	1.094	1.842	-3.794
Tyrosine	-1.079	2.214	1.635	2.162	2.606	1.478
Aspartic acid	-1.431	1.338	1.667	2.962	3.297	-2.887
Alanine	-1.082	1.04	1.237	1.271	-1.48	1.14
β -Alanine	-1.253	2.066	5.004	5.254	4.983	18.554
Valine	1.281	3.19	3.271	3.51	2.19	-2.46
Lysine	-1.815	-1.229	-1.665	-1.151	1.087	-1.444
Glutamine	1.227	1.473	1.257	1.108	-1.368	-2.292
Glycine	-1.737	-1.779	-4.269	-3.494	1.004	-1.587
Tryptophan	1.979	5.087	6.325	6.242	11.084	6.764
Asparagine	-1.427	2.002	5.66	15.664	17.254	1.975
Alanine	-1.082	1.04	1.198	1.271	-1.504	-1.244
Phenylalanine	1.122	4.995	1.393	1.255	1.173	-4.677
Serine	-1.881	-1.551	-1.013	-2.221	2.3	-3.099
Ornithine	-1.264	-1.976	-2.215	-3.01	-1.989	-2.802
Isoleucine	-1.702	3.756	2.847	1.842	2.318	-1.189
Galactosylglycerol	-3.26	-3.503	-4.513	-6.85	-6.131	-16.828
Glyc-3-P	-1.133	1.684	1.932	1.915	1.829	1.59
Glyc-P-glycerol	2.529	9.853	7.778	8.668	8.952	9.999
Glc-6-P	1.194	-1.139	-1.487	-1.988	-4.897	-13.928
Fru-6-P	1.097	-1.546	-1.758	-3.063	-6.157	-18.154
Maltose	1.1	-1.36	-1.152	-1.253	-1.141	1.413
Mannose	1.387	-2.804	-8.307	-14.449	-20.005	-16.696
Xylose	-1.252	-3.949	-7.935	-5.212	-8.547	-6.711
Lyxose	-1.029	-1.515	-2.061	-2.513	-2.574	-1.984
Raffinose	-1.171	1.255	1.344	-1.143	1.941	2.068
Dodecanoic acid	-1.173	-1.404	-1.093	-1.067	-1.159	-1.291
Salicin	-1.16	-2.2	-1.656	-2.203	-2.389	-1.315
Coniferylalcohol	3.205	1.811	1.124	1.121	1.502	-1.674
Quinic acid	-1.2	-1.364	-1.377	-1.742	-1.676	-1.477

Abbreviations: Glyc-3-P Glycerol-3-Phosphate, Glyc-P-glycerol Glycerophosphoglycerol, Glc-6-P Glucose-6-Phosphate, Fru-6-P Fructose-6-Phosphate

In the introduction to this study it was suggested that dehydration may result in the increases in the levels of the metabolite fructose-2,6-bisphosphate. This signal metabolite is considered important in regulating the rate of sucrose synthesis during photosynthesis. Based on the GC-TOF MS analysis this metabolite could be identified, but due to high technical variation in the quantification of this metabolite (co-efficient of variation for technical replicates was above 20%) it was not possible to obtain data on changes in fructose-2,6-bisphosphate levels in response to dehydration.

Metabolites identified as possible carbon sources for sucrose accumulation

Regarding possible soluble sugar carbon sources, major declines are noted in the hexose phosphate (Fru-6-P and Glc-6-P) pool and mannose (Figure 2.6). The decrease in hexose phosphate sugars is gradual during most of dehydration but there is a notable drop off in the late stages of dehydration. Mannose levels increase initially when water content drops to 70 % RWC but this is followed by major decreases as tissue dehydration progresses. A general decline in sugar polyols is also observed during dehydration but notable increases in arabinitol and especially xylitol are noted in the late stages of dehydration. Thus, it is possible that during dehydration the accumulation of sucrose is supported by carbon from the hexose phosphate pool, from mannose and to some extent from the sugar polyol pool. However, as noted for starch and hexose sugars, reductions in these metabolites (polyols) are not always associated with increases in sucrose levels.

In addition to sugars, carbon skeletons for sucrose accumulation may also be obtained via gluconeogenesis from the amino acid pool. From about 60 % RWC mark a number of amino acids, including tyrosine, tryptophan, isoleucine, valine, threonine, aspartic acid, asparagine, increase their levels. These amino acids continue to increase through the middle stages of dehydration (60 – 40 % RWC) but in most cases significant declines are noted in the final stage of tissue dehydration - from 20 % RWC to 5 % RWC. Strikingly, after increasing almost 17 fold

from the 60 % RWC mark to the 20 % RWC mark, asparagine levels return close to their pre-dehydration levels at 5 % RWC. Based on this ‘bell’ shaped pattern of amino acid levels it appears that while carbon may flow into amino acid accumulation during the middle stages of dehydration, at least part of this carbon may be re-mobilized to support sucrose accumulation in the final stage of dehydration. Thus, if starch and hexoses serve as a carbon source for sucrose accumulation, amino acids may serve as a transient ‘store’ for metabolized carbon during the middle stages of tissue dehydration.

General changes in metabolite levels during dehydration

In addition to accumulation of sucrose, the targeted metabolite analysis also revealed that dehydration results in the accumulation of raffinose, glutaric acid, β -alanine, glycerol, glycerophosphoglycerol and glycerol-3-phosphate. To a lesser extent dehydration also induced an increase in two of the TCA intermediates, malate and succinate, but there is a general decline in the level of citrate. As is to be expected with the inhibition of photosynthesis, there is a general reduction in the photorespiratory intermediates, namely glycerate, serine and glycine during dehydration. Notable declines are also observed for shikimic acid, threonate, ornithine, erythronic acid and galactosylglycerol.

Discussion

Changes in sucrose content during dehydration

Sucrose accumulation is a widely reported response to dehydration in most desiccation tolerant plant systems. However, despite this common response, there is no clear link between the accumulation of a particular level of sucrose and desiccation tolerance. Based on *in vitro* work high sucrose concentrations have been shown to be able to protect dry membranes and proteins (e.g. Crowe et al., 1987; Caffrey et al., 1988) but exactly what the lower threshold concentration may be in plant tissue has not been estimated. In many desiccation tolerant seeds high concentrations of sucrose (above 5% dry mass) are generally noted but the actual range of sucrose content of mature orthodox seeds is quite broad, extending from 0.15 % to 11 % of dry mass (Vertucci and Farrant, 1995). Similarly in angiosperm resurrection species the reported sucrose content of most dried tissue ranges between 1.6 and 12 % dry mass (Bianchi et al., 1993; Albini et al., 1994; Müller et al., 1997; Ghasempour et al., 1998, Whittaker et al., 2001; Illing et al., 2005; Zikovic et al 2005). The notable exception being *Craterostigma plantagineum* where sucrose can constitute an exceptional 40% of the dry mass (Bianchi et al., 1991a). The broad range suggests that while in some cases sucrose accumulation may be in excess (*C. plantagineum*); at the lower end of the concentration range (1.6 %) the protection afforded by sucrose during dehydration may be complemented by other cellular components. In the current study *X. humilis* leaf tissue sucrose content was found to increase almost four fold from fully hydrated to dry (< 5 % RWC) tissue, attaining a final level of approximately 12% of dry mass. With the exclusion of *C. plantagineum*, *X. humilis* can therefore be placed at the high end of the sucrose accumulation range of angiosperm resurrection plants.

A four fold increase in sucrose content between fully hydrated and dry tissue was also reported previously in *X. humilis* leaf tissue (Farrant et al., 2003). However, due to a much lower initial pre-dehydration tissue sucrose level, the final tissue sucrose content attained was only 7% of dry mass in this previous study. The exact reasons for this discrepancy are unclear but it is likely that differing plant growth conditions resulted in the different initial sucrose contents. In the

current study plants were maintained in a glasshouse where light intensities ranged between 200 and 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ during the light period. In the previous study by Farrant et al., (2003) plants were maintained under a constant Irradiance of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ during the dehydration treatment. These different light regimes could have affected the rates of leaf carbon fixation and consequently the initial sucrose content of the leaf tissue. Discrepancies between reports of sucrose content in dried tissue have also been noted in two other resurrection plant species. In the closely related species, *Xerophyta viscosa*, Whittaker et al., (2001) report a sucrose content of 7.5 % of dry mass in desiccated tissue while Peters et al., (2007) observed sucrose accumulation of only 2.4 % of dry mass in fully dehydrated tissue. In *S. stapfianus* the amount of sucrose accumulated in dry tissue (~ 12 % dry mass) measured by Whittaker et al., (2001) was almost 4 fold higher than that previously recorded by Ghasempour et al., (1998). Notably, in the Whittaker et al., (2001) study plants were maintained under high light intensities and the initial pre-dehydration tissue sucrose content was higher than that observed in the Peters et al., (2007) and the Ghasempour et al., (1998) studies. Hence, there appears to be a range of possible sucrose contents in the dried state with the final sucrose content being partly dependent on the initial levels in fully hydrated tissue.

In *X. humilis* leaf tissue notable increases in sucrose content occur between 100 and 60 % RWC and between 20 % and 5 % RWC. The occurrence of large increases in sucrose content below the 30 % RWC mark is in line with previous work on *X. humilis* (Bajic, 2006) and on another resurrection species, *Craterostigma wilmsii* (Cooper and Farrant, 2002). In both of these previous studies the bulk of the sucrose accumulation occurred below the 25 % RWC mark. However, aside from the work on *X. humilis* and *C. wilmsii*, in most angiosperm resurrection species the main period of sucrose accumulation occurs between the 60 and 30 % RWC mark (e.g. Farrant, 2007). In *X. viscosa*, sucrose content increases between 2 and 3 fold from 90 to 40 % RWC but there is very little change below this water content (Whittaker et al., 2001; Peters et al., 2007). In *S. stapfianus*, there is a five fold increase in sucrose content from 70 to 30 % RWC but there are no further increases below this relative water content (Whittaker et al., 2001).

The occurrence of large increases in sucrose content below a RWC of 20 % is surprising as one may expect there to be an insufficient amount of water to support the necessary metabolism. However, based on the actual water content of the leaf tissue at 20 % RWC it does appear that enough water is present to allow for active accumulation to occur. It is generally accepted that loss of bulk water in plant tissue occurs below $0.3 \text{ g H}_2\text{O g}^{-1} \text{ DW}$ (reviewed in Hoekstra et al., 2001). In the current study the actual water content of leaf tissue at 20 % RWC is $0.47 \text{ g H}_2\text{O g}^{-1} \text{ DW}$. In seeds, a water content of between 0.45 and $0.3 \text{ g H}_2\text{O g}^{-1} \text{ DW}$ is described as an ‘intermediate’ water concentration (Berjak et al., 2007) or as Type III water (Vertucci and Farrant, 1995). At this stage water concentrations are still high enough to allow for respiration, protein synthesis and reserve accumulation to continue (Vertucci and Farrant, 1995; Berjak et al., 2007). Hence, while the cellular milieu is likely to be highly viscous at 20 % RWC in leaves, there should be sufficient free water to allow for limited metabolism to occur.

Carbon sources for sucrose accumulation

Results from the present study indicate that sucrose accumulation occurs despite reduced photosynthetic activity and continues even after photosynthesis is shut down (at 20 % RWC). Furthermore, in a study investigating the effect of light on desiccation tolerance, *X. humilis* leaf sucrose content increased to comparable levels in leaves dried in the light and in the dark (Farrant et al., 2003). Thus while photosynthesis may contribute a limited amount of carbon, it appears that dehydration induced sucrose accumulation is not completely dependent on carbon supplied directly by photosynthesis. In an attempt to identify possible carbon sources, changes in a broad range of metabolites, including starch, soluble sugars, amino acids and organic acids, were measured during dehydration.

In parallel with sucrose accumulation in the early phase of dehydration (100-60 % RWC) there is a dramatic decline in the leaf starch, suggesting that starch breakdown may provide the necessary hexose substrates for sucrose synthesis at this stage. However, even if all the glucose obtained from the breakdown of the starch reserve was used for sucrose synthesis this would only account

for half of the accumulated sucrose. During this period it is possible that the deficit may be made up by photosynthesis, which is still active, and by carbon translocation into the leaf tissue. As sucrose accumulates in *Ramonda* the starch content of the leaf also declines but, as in the current study, starch metabolism does not account for all of the accumulated sucrose (Müller et al., 1997).

As noted previously, in the later stages of tissue dehydration there is a final rapid increase in sucrose content from the 20 % to 5 % RWC. At this stage of dehydration carbon translocation is unlikely, photosynthesis is completely shut down and all of the leaf starch is depleted. Hence carbon must be obtained from non-starch derived internal cellular sources. In *X. viscosa* glucose and fructose accumulate initially in response to dehydration but as the plant approaches the desiccated state, the hexoses are metabolized and sucrose accumulates as the dominant carbohydrate. In the current study an initial increase in glucose and fructose is also observed, with hexose sugar contents more than doubling between 100 and 70 % RWC. This initial increase is followed by a steady decline in hexose sugar content from 70 to 20 % RWC. Similarly the hexose sugar phosphate pool increases in the early stages of dehydration, decreases through the middle stages and declines dramatically below the 20 % RWC mark. These observations suggest that the hexose sugar pool may be a source of carbon for sucrose accumulation. However, during the middle stages of dehydration (60 – 40 % RWC), tissue sucrose content remains relatively stable indicating that the hexose sugar pool is not being converted directly into sucrose. Interestingly, after a general increase in the many amino acids, including tyrosine, aspartic acid, valine, tryptophan and asparagine during this middle period, major declines in amino acid levels are observed between the 20 % RWC and the 5 % RWC marks. Thus, while carbon from hexose pool may support the increase in amino acids during the middle stages of dehydration, these amino acids may then serve as substrates for gluconeogenesis and subsequent sucrose synthesis in the final stage of water loss. Major decreases in the hexose phosphate sugar pool with parallel increases in a similar group of amino acids have also been noted prior to the onset of desiccation tolerance in *A. thaliana* seed tissue (Fait et al., 2006).

Aside from starch and hexoses, alternate carbon sources proposed in previous reports include 2-octulose in *C. plantagineum* leaf tissue (Bianchi et al., 1991b; Ingram et al., 1997) and stachyose in *C. plantagineum* root (Norwood et al., 2003) and in *Xerophyta villosa* leaf tissue (Ghasempour et al., 1998). Octulose is a very rare plant carbohydrate and to date has only been identified in *C. plantagineum*. Stachyose is more commonly reported but it could not be identified in the GC-TOF MS metabolite analysis conducted in the current study and it is therefore uncertain if it may also serve as a carbon source in *X. humilis*. Of all the metabolites profiled the most likely sugar that may serve a similar function to that of 2-octulose in *C. plantagineum* is mannose. In response to dehydration leaf mannose levels initially increase but this is followed by a considerable drop as dehydration progresses. Assuming that the necessary enzyme complement, including mannose phosphate isomerase, is present in *X. humilis* leaf tissue then mannose can serve as a substrate for gluconeogenesis. Alternatively the conversion of mannose to mannose-6-phosphate may sequester cellular phosphate and thereby reduce the inhibitory effect of this metabolite on SPS activity, the primary sucrose synthesizer (Weiner et al., 1992). Increased mannose-6-phosphate synthesis would thereby promote increased sucrose synthesis.

Mannan, a polysaccharide composed of mannose subunits, is known to serve as a carbon store in seeds of some plants (Carpita and Gibeaut, 1993) and in the vegetative structures in certain species, including Orchidaceae (e.g. Wang et al., 2006) - a close relative to the Pandanales, which include *X. humilis*. In the desiccation tolerant fern species *Mohria caffrorum* it is postulated that mannans may serve as a carbohydrate source supporting the metabolic changes that occur during the transition from a desiccation sensitive to a desiccation tolerant state (Farrant, 2009 *in press*). Hence it is possible that mannans may serve as a carbon source supporting dehydration induced sucrose accumulation in *X. humilis* leaf tissue. However, while it was possible to extract information regarding changes in mannose from the GC-TOF MS analysis it was not possible to obtain data regarding changes in mannan during dehydration.

Changes in metabolite profile during dehydration

The attempt to identify possible carbon sources for the observed sucrose accumulation also afforded the opportunity to look at changes in the metabolite profile of *X. humilis* leaf tissue during dehydration. Changes in the metabolite profile were considered using both an un-targeted (metabolites classified but each compound was not identified) and a targeted approach (each compound identified). The un-targeted approach allowed for changes in a large number of metabolites to be monitored and hence facilitated more powerful statistical analysis of the data set. Independent component analysis (ICA) of significantly changing metabolites in the un-targeted data set (represented by 522 MSTs) showed that there was a strong separation between most samples with a gradient in RWC being clearly visible. The exceptions were the 100 and 70 % RWC samples which clustered very closely together indicating that there is not much difference between the metabolite profiles of these two RWC marks. This is in line with transcript analysis of *X. humilis* leaf tissue which also indicates that there are no major global changes in transcript expression in the initial stages of tissue dehydration (Arthur Shen, University of Cape Town, South Africa, pers comm). After classification of 146 MSTs showing similarity to carbohydrates it was also evident that dehydration resulted in a general decrease in hexose sugars and an increase in di- and trisaccharides. While the un-targeted analysis provided limited information on global metabolite changes, to gain insight as to the effect of tissue water loss on metabolism a smaller set of identified metabolites (targeted) were tracked during dehydration with fold changes being expressed relative to the initial metabolite content at 100% RWC.

In addition to sucrose, oligosaccharide accumulation in response to dehydration is also a commonly reported response to dehydration in orthodox seeds and angiosperm resurrection plants (Koster and Leopold, 1988; Bachman et al., 1994; Brenac et al., 1997; Taji et al., 2002; Pennycooke et al., 2003; Peters et al., 2007). In the current study, raffinose was observed to increase as tissue water loss progressed, reaching a final tissue content double that measured at

the 100 % RWC mark. There is a general reduction in the level of most sugar alcohols measured during the initial and middle stages of dehydration, with the exception of threitol which increases between 100 and 60 % RWC and decreases as water loss continues. Notably, there is a striking increase in the level of xylitol in the later stages of dehydration between the 20 and 5 % RWC marks with a 10 fold increase in levels being observed. Increases in arabitol and ribitol are also noted but these changes are not to the same extent as xylitol. Rizhsky et al., (2004) reported an over three fold increase in xylitol levels when *A. thaliana* leaf was subjected to water deficit stress but this is the first report of a major increase in xylitol levels in an angiosperm resurrection species. In yeast the accumulation of xylitol has been linked to improved desiccation tolerance (Krallish et al., 1997) and hence its accumulation may be of functional importance in desiccation tolerance in *X. humilis* leaf tissue.

In response to dehydration there was also a significant increase in another polyol not commonly associated with stress tolerance in resurrection plants, namely glycerol. In *X. humilis* tissue glycerol levels increase dramatically at the 40 % RWC mark with a 7 fold increase in glycerol content being observed with glycerol levels remaining relatively high as water loss progresses. In studies on the effect of glycerol on plant metabolism, high glycerol accumulation appears to disrupt the flux of carbon through glycolysis and is therefore described as cytotoxic (Leegood et al., 1988; Aubert et al., 1994). Nevertheless, glycerol is a known osmoprotectant that may provide protection against the stresses associated with dehydration (Hasegawa et al., 2000). In yeast and algae, the accumulation of glycerol has also been reported to ameliorate the effects of salt, hyperosmotic and oxidative stresses (Brown and Simpson, 1972; Tamas et al., 1999; Hohmann and Mager, 2003; Pahlman et al., 2001) and in insects and amphibians (Lee et al., 1991; Storey, 1997) glycerol serves as a cryo-protectant. Further, in *A. thaliana* temperature stress is associated with increased glycerol levels (Rhizsky et al., 2004; Kaplan et al., 2004) and in glycerol insensitive mutants, the accumulation of glycerol has been shown to increase tolerance to a variety of abiotic stresses, including water deficit stress (Eastmond, 2004). In *X. humilis* leaf glycerol accumulation is initiated relatively late in dehydration, when normal cellular growth is already negatively affected by loss of tissue water. Hence, at this point in

dehydration the protective effects of glycerol accumulation may outweigh the negative effects on plant growth. Glycerol accumulation in response to dehydration has also recently been noted in a desiccation tolerant fern (Farrant et al., 2008). In this work, the authors propose that glycerol may provide mechanical stabilization by replacing lost water in the vacuoles of drying cells, as suggested for angiosperm resurrection plants (Farrant 2000; 2007). If glycerol is also partitioned to the vacuoles in *X. humilis* then its accumulation should have a limited effect on metabolism in the cytoplasm.

The metabolite analysis also revealed the accumulation of a previously unreported (in plants) derivative of glycerol, namely glycerophosphoglycerol (GPG). This polyol-phosphodiester has only been encountered in hyperthermophiles thriving in very hot environments but is absent in mesophiles (Santos and da Costa, 2001; Santos et al., 2007a). Glycerophosphoglycerol has been reported in the hyperthermophilic *Archaeoglobus fulgidus* (Martins et al., 1997) where it is proposed to be used for osmoadaptation over a range of temperatures (Borges et al., 2006; Lamosa et al., 2006). A putative thermo-protective function *in vivo* has also been ascribed to GPG, and its action as a protein stabilizer *in vitro* has been confirmed (Lamosa et al., 2003; Santos et al., 2007b). Both the osmo-adaptive and thermo-protective function of GPG will be of benefit to *X. humilis*, which in its natural environment may need to endure temperatures above 50 °C in the dried state.

Increased concentration of several amino acids including valine, isoleucine, asparagine, aspartate, tryptophan, tyrosine, phenylalanine, threonine and β -alanine was observed in *X. humilis* leaf tissue during the initial and middle to late stages of dehydration. Similar increases in amino acids in response to heat and drought stress have also been reported previously (Saini and Srivastava, 1981; Mayer et al., 1990; Rizhsky et al., 2004; Kaplan et al., 2004; Brosche et al., 2005). Further, accumulation of aromatic amino acids (tryptophan, phenylalanine and tyrosine) has also been observed in the desiccation period in *A. thaliana* seed (Fait et al., 2006). One possible function of these amino acids is to provide precursors for the synthesis of pathogen

defense metabolites. These metabolites are derived from secondary metabolism, such as the phenylpropanoid, isoprenoid, alkaloid, or fatty acid/polyketide pathways (Dixon, 2001) but precursors of these defense compounds originate in primary metabolism. For example, branched-chain amino acids (isoleucine, leucine, and valine) serve as precursors for cyanogenic glycosides (Vetter, 2000) while aromatic amino acids serve as precursors for indole glucosinolates, phytoalexins, alkaloids, lignins, flavonoids, isoflavonoids, and hydroxycinnamic acids (Dixon, 2001). Thus accumulation of these amino acids may drive increased synthesis of the metabolites necessary for pathogen defense in drying tissue.

Interestingly, despite initial accumulation many of the amino acids noted above decrease in concentration in the final stage of dehydration from the 20 % RWC to the 5 % RWC mark. A general 'bell' shaped pattern of initial increase followed by a decline in the levels of isoleucine, tyrosine and tryptophan has also been observed in *S. stapfianus* (Martinelli et al., 2007). In this previous study the decline in these amino acids was more pronounced in younger desiccation tolerant leaf tissue as opposed to older desiccation sensitive tissue, suggesting that their metabolism may be linked to the increased tolerance of the younger leaf tissue. At present the purpose of this amino acid turnover can only be the subject of speculation. In the present study it is postulated that there may be a movement of carbon from the amino acid pool and into the carbohydrate pool, specifically the sucrose pool. However, turnover of the amino acid pool may also support a number of other processes involved in protection of plant tissue during.

During dehydration desiccation tolerant systems are known to accumulate protective proteins such as late LEAs and dehydrins, hence the observed amino acid turnover may be necessary to support increased synthesis of these proteins. If this is the case then one would expect there to be a strong draw on amino acid pools which contribute most to LEA and dehydrin synthesis. LEAs are classified into four groups, Group 1-4, with Group 2 also described as dehydrins (Rorat, 2006). All four LEA groups are rich in glutamine and Group 1 and Group 2 are particularly rich in glycine, lysine and glutamate (Zhang et al., 2000; Wise, 2003). Based on the

metabolite analysis in the present study there is a gradual decrease in glycine and only a slight decrease in lysine during dehydration. No data was obtained for changes in glutamine, but there was a general decrease in the levels of glutamate. Notably only glutamate levels exhibits a 'bell' like pattern with an increase observed at the 20% RWC mark followed by a dramatic decrease from the 20% to the 5% RWC marks. Thus, in addition to the postulated role as a carbon store for carbohydrate synthesis, the turnover of these particular amino acids may also contribute to LEA and dehydrin synthesis. However, even if LEA synthesis was significant, carbon for carbohydrate synthesis may still be sourced from the amino acid pools that contribute less to LEA synthesis such as valine and asparagine.

Of the amino acids showing initial increases in concentration, β -alanine and asparagine remained relatively high in dry tissue (< 5 % RWC). β -Alanine is the precursor of β -alanine-betaine, a quaternary ammonium compound that has similar osmoprotective function as glycine-betaine and accumulates in species belonging to the highly salt tolerant family *Plumbaginaceae* (Rathinasabapathi et al., 2000) and in the salt tolerant tree species *Populus euphratica* (Brosché et al., 2005). β -alanine has also been demonstrated to protect against heat inactivation of proteins by acting as a molecular chaperone (Mehta and Seidler, 2005). Hence the accumulation of β -alanine in *X. humilis* leaf tissue during dehydration may assist in protecting tissue against damage caused by water loss. In addition to β -alanine, a high fold increase in the nitrogen rich asparagine is also noted during dehydration. The accumulation of asparagine in response to dehydration has also been reported in *S. stapfianus* (Martinelli et al., 2007) and during the desiccation period of *A. thaliana* seed development (Fait et al., 2006). Aside from serving as a possible source of carbon and nitrogen, the possible function of asparagine accumulation in stress tolerance is unclear.

Conclusion

In response to dehydration *X. humilis* leaf accumulated sucrose to a final level of 12 % DM in desiccated leaf tissue. The accumulation occurred in two distinct phases with a large percentage

of the sucrose being accumulated in the later stages of dehydration (beyond 40 % RWC). In the first phase of sucrose accumulation possible carbon sources included photosynthesis, starch breakdown and the hexose sugar. In the second phase of accumulation it is proposed that carbon may be mobilized out of the amino acid pool to support increases in sucrose content. The analysis of changes in metabolite levels during dehydration identified a number of previously reported metabolites as well as new metabolites that could play a role in protection of leaf tissue against extreme water loss. Thus it appears that sucrose works in conjunction with a number of other compounds to provide the necessary cellular protection during dehydration. Having confirmed an increase in sucrose content in response to water loss the next part of the project looked at changes in SPS activity in response to tissue dehydration.

Introduction

Sucrose accumulation in response to dehydration is commonly observed in both desiccation tolerant (Ghasempour et al., 1998; Peters et al., 2007; Whittaker et al., 2007) and desiccation sensitive angiosperms (Zrenner and Stitt, 1991; Pelleschi et al., 1997; Ferrario-Mery et al., 1998; Yang et al., 2002; Niedzwiedz-Siegien et al., 2004; Correia et al., 2005). Furthermore, in a number of orthodox seeds increased sucrose accumulation coincides with the onset of desiccation tolerance (e.g. Leprince et al., 1990 and reviewed by Vertucci and Farrant, 1995). In *Xerophyta humilis* significant sucrose accumulation in leaf tissue in response to drying has been confirmed in the present work (see Chapter 2) and is supported by similar observations in previous work (Bajic, 2006). While it is likely that a combination of factors affecting both the synthesis and breakdown of sucrose contribute to the observed accumulation, increased sucrose levels in photosynthetic (Jeong and Housley, 1990; Tognetti et al., 1990; Guy et al., 1992; Holaday et al., 1992) and non-photosynthetic tissues (Geigenberger et al., 1995) are frequently linked to increased activity of sucrose phosphate synthase (SPS) - a key regulator of sucrose synthesis (Barber, 1985; Stitt et al., 1988; Lunn and ap Rees, 1990). Notably, SPS activity is up regulated in response to dehydration in both desiccation sensitive, e.g. spinach (Zrenner and Stitt, 1991) and in desiccation tolerant species, e.g. *Craterostigma plantagineum* (Ingram et al., 1997) and *S. stapfianus* (Whittaker et al., 2007). The focus of the following section of work is on the response of SPS activity to dehydration in leaf tissue of *Xerophyta humilis*. To place the work in context a brief review of what is known on the response of SPS activity to water deficit stress in photosynthetic tissue will be presented. Included in this review will be general aspects of SPS regulation and issues regarding the accurate measurement of SPS activity.

SPS regulation in photosynthetic tissue

The mechanisms utilized to regulate SPS activity have been found to be complex and include: i) changes in protein level, described as coarse control and ii) changes in the kinetic properties of

the enzyme, described as fine control (reviewed in Huber and Huber, 1996; Huber and Winter, 2000). Alterations in transcription, translation and protein turnover contribute to coarse control while fine control can be attributed to both interaction with metabolic effectors (Siegl and Stitt, 1990) and to post-translational modification (PTM) of SPS protein (Walker and Huber, 1989).

Much of our initial understanding of the fine control of SPS activity in photosynthetic tissue stems from work carried out on spinach SPS. The activity of spinach SPS is strongly influenced by the metabolic effectors inorganic phosphate (Pi), which inhibits activity (Harbron et al., 1981; Amir and Preiss, 1982) and Glucose-6-Phosphate (Glc-6-P) which activates spinach SPS (Doehlert and Huber, 1983a; Doehlert and Huber, 1983b). Similarly in maize (Crafts-Brandner and Salvucci, 1989; Lunn and Hatch, 1997) and wheat (Trevanion et al., 2004) Pi and Glc-6-P have been found to inhibit and activate SPS respectively. However, especially in the case of Pi, the sensitivity of SPS to these metabolites has been found to vary between species (see Lawlor and Cornic, 2002). Both Pi and Glc-6-P are believed to bind at sites other than the active site and are therefore described as allosteric regulators of SPS activity. In spinach, SPS activity displays a distinct light dark modulation, being activated by exposure to light and deactivated when kept under dark conditions (Stitt et al., 1988; Huber et al., 1989; Huber and Huber, 1991; Huber and Huber, 1992). Light activation of SPS is also observed in other species but the extent and nature of this activation has been found to vary between species (see below for more detailed discussion). In general, SPS in its activated state is less inhibited by Pi and exhibits greater affinities for its substrates: UDP-Glucose and Fructose-6-P (Fru-6-P) and its activator Glc-6-P (Stitt et al., 1988). In addition to light activation, spinach SPS is also reported to be activated in response to water deficit stress (Zrenner and Stitt, 1991; Toroser and Huber, 1997). Water deficit stress activation of SPS has also been noted in a number of other species including rice (Yang et al., 2002) and wheat (Niedzwiedz-Siegen et al., 2004; Fresneau et al., 2007) but once again the nature and extent of activation appears to differ between species.

The mechanism of activation of spinach SPS has been demonstrated to involve a post-translational modification (PTM), and specifically the phosphorylation of particular serine sites

(Walker and Huber, 1988; Huber and Huber, 1989). To date three phosphorylation sites have been associated with the post translational regulation of SPS activity. On the spinach amino acid sequence these sites are found at Serine₁₅₈ (Ser₁₅₈), Serine₂₂₉ (Ser₂₂₉) and Serine₄₂₄ (Ser₄₂₄). Based on kinase recognition motifs, analogous sites to all three spinach sites have been identified in other plant species, e.g. Serine₁₆₂ in maize and rice SPS is considered equivalent to the Ser₁₅₈ site in spinach (see Chapter 4 of current work). In terms of regulatory significance of each site, Ser₁₅₈ has been demonstrated to be involved in light/dark regulation of SPS activity (McMichael et al., 1993; McMichael et al., 1995a; Toroser et al., 1999), Ser₂₂₉ is believed to be involved in the regulation of 14-3-3 binding to SPS (Toroser et al., 1998; Huber et al., 1998; Moorhead et al., 2001) and Ser₄₂₄ is considered important in osmotic activation (Toroser and Huber, 1997; Huang and Huber, 2001). Interestingly, the protein kinases and phosphatases thought to be involved in the phosphorylation/dephosphorylation of the above sites are also proposed to be under the regulation of the metabolites Glc-6-P and Pi, the known metabolite effectors of SPS.

In spinach, Ser₁₅₈ is phosphorylated *in vitro* by both a calcium dependent kinase, designated protein kinase I (PKI) (McMichael et al., 1995b) (see Figure 3.1) and a calcium independent kinase, designated protein kinase III (PKIII) (McMichael et al., 1995b; Sugden et al., 1999; Huang and Huber, 2001). PKI has been identified as a calmodulin-like domain protein kinase (CDPK) (Douglas et al., 1998) and PKIII kinase has been demonstrated to be a SNF1-related protein kinase (SnRK1) (Douglas et al., 1997; Sugden et al., 1999). While both PKI and PKIII are able to phosphorylate spinach SPS *in vitro*, the major kinase responsible for *in vivo* phosphorylation of spinach SPS at the Ser₁₅₈ is believed to be PKIII. In addition to the Ser₁₅₈ site, PKI has also been shown to phosphorylate the Ser₂₂₉ site *in vitro* (Toroser et al., 1998). The third phosphorylation site, Ser₄₂₄, is proposed to be phosphorylated by another CDPK, designated PKIV (Toroser and Huber, 1997). Of the three phospho-serine sites, the phosphatase involved in the dephosphorylation of Ser₁₅₈ is the most well studied. *In vitro* Ser₁₅₈ has been shown to be dephosphorylated by a type 2A protein phosphatase (PP2A) but not by type 1 phosphatases (Siegl et al., 1990). Notably, in spinach the activity of PKIII (SnRK1) (kinase) appears to be inhibited by Glc-6-P (McMichael et al., 1995b; Toroser et al., 2000) while PP2a

(phosphatase) is activated by Pi (Weiner et al., 1993) (see Figure 3.1). Thus the metabolites Glc-6-P and Pi may affect SPS activity by both direct interaction with SPS protein and via their effect on the kinases and phosphatases involved in regulating SPS activity.

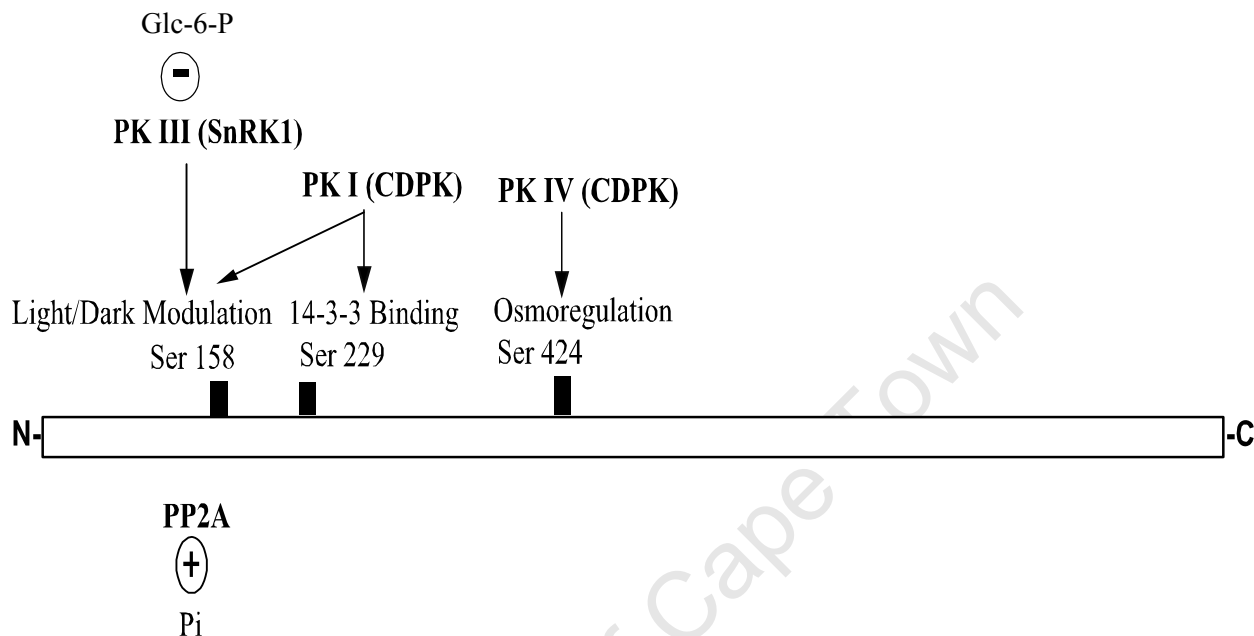


Figure 3.1 Important phosphoregulatory sites in spinach SPS protein. The associated kinases for Ser₁₅₈, Ser₂₂₉, and Ser₄₂₄ and the phosphatase associated with Ser₁₅₈ are displayed. For the Ser₁₅₈ site the effect of Glucose-6-Phosphate (Glc-6-P) on PKIII activity and of inorganic phosphate (Pi) on PP2A activity is indicated by (-) for inhibition and (+) for activation.

Accurate assay of SPS activity

In spinach light and water deficit activation of SPS cannot be detected when SPS activity is assayed with substrate saturating conditions. Rather activation is only observed when activity is assayed under substrate limiting conditions and in the presence of inorganic phosphate (Pi) (Huber et al., 1989; Zrenner and Stitt, 1991). Therefore, in order to detect changes in activation state of SPS it is necessary to assay SPS activity under two conditions: i) substrate saturated conditions (V_{max}) and ii) under substrate limiting conditions and in presence of the inhibitor Pi

(V_{lim}). In principle, the V_{max} assay will measure total extractable SPS activity while the V_{lim} assay will selectively measure the active form of SPS. The activated form of SPS is less sensitive to Pi inhibition and hence, due to the inclusion of Pi in the V_{lim} assay, the activated form of SPS should contribute most to the V_{lim} measured activity (Stitt et al., 1988; Siegl and Stitt, 1990). Thus, by expressing V_{lim}/V_{max} the percentage of SPS in the active form can be estimated and used to describe the 'activation state' of SPS. The V_{max} and V_{lim} assay conditions that are widely used to measure SPS activity were originally derived from the properties of spinach leaf SPS (Stitt et al., 1988). However, as our knowledge of the regulatory properties of non spinach SPS enzymes has increased it has become evident that the non-optimized use of these assays can be problematic.

Discrepancies regarding light activation of SPS in different species serve to illustrate the potential pitfalls of applying non-optimized assay conditions. Huber et al., (1989) proposed that plants could be divided into three groups based on nature of light activation of SPS activity. In group I species, e.g. maize and barley, light activation of SPS is detected as both changes in V_{lim} and V_{max} activities, while in group II species only the V_{lim} activity is altered in response to light while V_{max} activity remains unchanged. In group III species, e.g. soybean, pea, tobacco and *A. thaliana*, light has no effect on SPS activity. However, the inclusion of maize in group I has been questioned by the report of Lunn and Hatch, (1997) which showed no change in V_{max} activity in response to light in maize leaf. This is in contrast to earlier work which noted both changes in V_{max} and V_{lim} assays in response to light (Sicher and Kremer, 1985; Huber et al., 1987; Kalt-Torres and Huber, 1987). Rather, Lunn and Hatch (1997) propose that the V_{max} assay used previously measured SPS activity at sub-saturating levels of UDP-Glucose concentrations and was therefore inaccurate. The UDP-Glucose concentration used (10 mM UDP-Glucose), while being sufficient to saturate spinach SPS, was considerably below the saturation levels estimated for maize (at least 50 mM UDP-Glucose) by Lunn and Hatch (1997). There is also an indication that the other member of group I - barley, may respond in a similar manner to that of maize (Hatch and Lunn, 1997). Thus, the creation of group I may be based more on flaws in the assay conditions utilized then on real differences in the regulation of SPS activity. Furthermore,

within the group of species that show light/dark regulation of SPS, species specific variation in the kinetic properties affected by light activation is also noted. In spinach light activation has a pronounced affect on sensitivity to Pi inhibition but in many monocotyledonous species, e.g. maize, the enzyme's affinity for UDP glucose is increased significantly while its sensitivity to inhibition by Pi is only marginally altered (Lunn and Hatch, 1997). Based on these observations there is a clear need for tailoring of the spinach based $V_{\max}$ and $V_{\lim}$ assay conditions to each studied species.

Fluctuations in SPS activity

Another important aspect to consider when measuring SPS activity is the possible natural fluctuation in SPS activity. As discussed previously (Chapter 1) sucrose synthesis in the cytosol is closely coupled with the fixation of carbon in the chloroplast. It is therefore not surprising that fluctuations in SPS activity have been linked to changes in irradiance, temperature, stomatal closure and tissue sucrose contents (Stitt et al., 1988), factors which also have a direct influence on photosynthesis. Further, in addition to changes initiated by environmental cues there is also evidence of the involvement of endogenous rhythms in SPS activity. In a range of species, e.g. tomato (Jones and Ort, 1997), soybean (Kerr et al., 1985) and cotton (Hendrix and Huber, 1986) fluctuations in SPS activity are noted to persist even under constant environmental conditions. In many species, post-translational modifications such as phosphorylation/dephosphorylation (fine control) are thought to underpin the observed fluctuations of SPS activity. However, there are also cases where changes in protein level contribute to alterations in SPS activity e.g. in spinach prolonged increases in irradiance results in increased expression of SPS protein (Klein et al., 1993), and in soybean the endogenous fluctuation in SPS activity has been attributed more to changes in SPS protein levels than to alterations in SPS kinetic properties (Kerr et al., 1987). Thus, when considering the response of SPS activity to stresses such as water deficit it is necessary to account for possible non-treatment related fluctuation.

SPS activity and desiccation tolerance

To date, the relationship between desiccation tolerance and SPS activity has been considered in only two angiosperm resurrection species, namely *Craterostigma plantagineum* (Ingram et al., 1997) and *Sporobolus stapfianus* (Whittaker et al., 2007). In both species, tissue sucrose content and SPS activity increase in response to dehydration. In *C. plantagineum* SPS activity (measured using $V_{\max}$ assay only) continues to increase throughout tissue water loss while in *S. stapfianus* SPS activity ($V_{\max}$ and $V_{\lim}$ activity) increases to the mid-point of tissue water loss and then decreases as drying progresses. For both *C. plantagineum* and *S. stapfianus* the authors attribute the change in SPS activity to both increases in protein levels and to an increased activation state of SPS.

In desiccation tolerant seeds, the relationship between sugar accumulation and desiccation tolerance has been studied in many species but detailed studies on the link between actual SPS activity and desiccation tolerance are rare. In seeds of *Vicia faba*, increased levels of SPS mRNA transcript, SPS protein, and total SPS activity are observed at the onset of maturation drying (phase associated with seed desiccation tolerance) (Weber et al., 1997). However, the observed increase in SPS activity is not accompanied by a concomitant increase in sucrose levels and it is therefore difficult to explain the significance of the increased SPS activity in the *V. faba* study.

Aims

The overall goal of this section of work was to characterize the relationship between SPS activity and dehydration in *X. humilis* leaf tissue. Having confirmed increases in sucrose content in response to dehydration (in Chapter 2), the primary aim was to establish if the observed pattern of sucrose accumulation could be correlated with changes in SPS activity during dehydration.

Methods

Plant material

Plants were maintained as described in Chapter 2.

Two groups of plants, an experimental and a control were maintained in a greenhouse under ambient light and temperature conditions. Irradiance averaged $520 \mu\text{mol m}^{-2} \text{s}^{-1}$ and ranged from $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ to $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$, temperatures averaged 18°C and ranged from 16°C to 25°C . The experimental group was dried by withholding water while the control group was kept in a hydrated state. At various points during drying, leaves of similar chlorophyll content (estimated by intensity of green) were harvested from plants in the experimental group. As *X. humilis* leaves lose chlorophyll during desiccation, changes in the colour of *X. humilis* leaves were used as an indicator of leaf tissue water content (see Ingle et al, 2008). At each leaf harvest from the experimental group, leaves were also removed from plants in the fully hydrated control group.

Determination of relative water content (RWC)

RWC was determined as described in Chapter 2

SPS activity

Harvesting and processing of leaf tissue

Preliminary experiment: Comparison between freeze dried and non freeze dried tissue

For comparison of SPS activity in freeze dried and non-freeze dried tissue, leaves (between 10 and 16) were harvested from multiple plants in their fully hydrated state (100 % RWC) and in their fully dehydrated state (5 % RWC). All leaves were immediately flash frozen in liquid nitrogen (-196°C) and half of the leaves were freeze dried. Protein was extracted (described below) from freeze dried and non freeze dried tissue.

Optimization of SPS assay conditions

All plants used to optimize the SPS assay conditions were maintained in a fully hydrated state (100 % RWC). Leaves (between 10 and 16) were harvested after 4 h into the light period (Light treated sample) and after 4 h into the dark period (Dark treated sample). All harvested tissue was freeze dried and protein extracted as described below.

Main experiment: Changes in SPS activity during dehydration

The processing of leaf tissues and the determination of leaf tissue RWC prior to measurement of SPS activity is illustrated and described in Chapter 2 (Figure 2.1, pg 24).

Protein extraction from freeze dried tissue for SPS assay

Protein was extracted according to method of Huber et al., (1989) with slight modifications. Thirty mg of freeze dried leaf powder was re-ground under liquid nitrogen in the presence of PVPP (2 % w/v). Ice cold extraction buffer (50 mM MOPS NaOH (pH 7.5), 15 mM MgCl₂, 1 mM EDTA, 2.5 mM DTT and 0.1 % (w/v) Triton X-100, 1 mM PMSF, 1mM Benzamidine) was added in a 1:7 tissue to buffer ratio. The extract was transferred to a pre-cooled centrifuge tube and vortexed for 15 s. The extract was centrifuged at 14000g for 3 min at 4 °C. Two ml of the supernatant was removed and de-salted with G-25 Sephadex (Sigma-Aldrich) spin column, final bed volume of 5 ml. The column was pre-equilibrated in extraction buffer without Triton X-100. The de-salted extract was eluted from the column by centrifugation in a swing out centrifuge at 1800 rpm (2000g) for 2 min. The de-salted extract was used immediately for assay of SPS activity.

Protein extraction from non-freeze dried tissue for SPS assay (for comparison experiment)

Protein was extracted as for freeze dried tissue, except that 100 mg (fresh weight) of leaf tissue was extracted and extraction buffer was added in a 1:3 tissue to buffer ratio.

SPS activity assay procedure

SPS activity was assayed using a modified method of Huber et al., (1989). Forty five μ l of desalted extract was added to 25 μ l of pre-warmed (30 °C assay buffer (components described below) to start the reaction. The reaction was incubated at 30 °C and stopped with the addition of 1 ml ice cold 30 % KOH at time 0 and 15 min respectively. The reaction was linear over at least the first 45 min. To destroy any un-reacted hexose-phosphate the reactions were incubated at 100 °C for 10 min in 2 ml clip lid eppendorf tubes after the reaction was stopped (Huber et al., 1983). After cooling the reactions on ice, sucrose-6^F-P and sucrose formed during the assay period was detected with anthrone reagent (0.14 % Anthrone (Sigma-Aldrich) in 85 % sulphuric acid). One ml freshly prepared anthrone reagent was added to the reaction and colour development allowed for 20 min at 40 °C. Absorbance was read at 620 nm and the sucrose-6^F-P quantified based on a sucrose standard curve. Sucrose standards for the curve were made up in extraction buffer without Triton X-100. All assays were conducted in triplicate.

Replication of SPS activity measurements

Preliminary experiment: Comparison between freeze dried and non-freeze dried

After processing of harvested tissue, two homogenized pools of leaf tissue were obtained (each pool with multiple plants, leaf n >10). One pool contained freeze dried tissue while the other pool contained non-freeze dried tissue. From the freeze dried pool three 30 mg aliquots of leaf tissue were extracted for protein and the activity of SPS measured in each extract. For non-freeze dried tissue, three ~100 mg aliquots of pooled frozen tissue were extracted for protein and the activity of SPS measured in each extract. Thus, three independent activity measurements of the pool of freeze dried and the pool of non-freeze dried tissue were obtained.

SPS optimization

For the optimization of the SPS assay, protein was extracted from three independent biological replicates and SPS activity measured in each extract. Each replicate consisted of 2-3 leaves from one plant.

Main experiment: Changes in SPS activity during dehydration

To determine changes in SPS activity during dehydration three independent protein extractions were conducted on each pool of homogenized freeze dried leaf tissue (multiple plants, leaf $n > 6$). Three 30 mg aliquots of freeze dried leaf tissue from each pool of harvested leaf tissue, (see Figure 2.1 on pg 24) were extracted for protein and the activity of SPS measured in each extract. Thus, three independent activity measurements of each pool (all leaves at a particular RWC) of homogenized leaf tissue were obtained.

Concentrations of substrates and effectors used in assay buffers for optimization of SPS assay

All concentrations given are a final concentration in a reaction volume of 70 μ l (45 μ l de-salted extract + 25 μ l assay buffer). The concentrations of MOPS NaOH, $MgCl_2$ and DTT were maintained at the same concentration used in the extraction buffer. To estimate V_{max} and K_m for Fructose-6-Phosphate (Fru-6-P), UDP-Glucose was used at 10 mM, Fru-6-P concentration was increased from 0.25 to 10 mM (0.25, 0.5, 1, 2, 4, 8 and 10 mM) and Glucose-6-Phosphate (Glc-6-P) was used in a ratio of 5:1 (Glc-6-P to Fru-6-P). When crude extract is used the SPS assay is complicated by the presence of phosphoglucose isomerase (PGI) which inter-converts Glc-6-P and Fru-6-P (Doehlert and Huber, 1983). Therefore, Glc-6-P and Fru-6-P were included at a ratio of 5:1 (Glc-6-P to Fru-6-P) as this was shown to be close to the thermodynamic equilibrium of PGI (Dyson and Noltmann, 1968). To estimate V_{max} and K_m for UDP-Glucose, Fru-6-P was used at 8 mM, Glc-6-P was used at 40mM and UDP-Glucose was increased from 2 – 50mM UDP-Glucose (2,4,8,16,32 and 50 mM). The variation in substrate concentrations was used to determine both saturating and limiting substrate concentrations. To determine the effect of inorganic phosphate (Pi) concentration on SPS activity the assay contained substrates at limiting concentrations with 8mM UDP-Glucose, 0.5 mM Fru-6-P and 2.5 mM Glc-6-P. Pi (added in the

form of KH_2PO_4) was increased from 0 to 14 mM (1,2,4,6,8,10,12,14 mM). The total protein concentration of the de-salted extract was determined using the method of Bradford (1976) bovine serine albumin (BSA) was used to make up standards for construction of the standard curve.

Concentrations of substrates and effectors used in the final, optimized $V_{\max}$ and $V_{\lim}$ SPS assays

To measure the $V_{\max}$ activity of SPS the assay buffer mixture contained UDP-Glucose at 30 mM, Fru-6-P at 8 mM and Glc-6-P 40 mM. No Pi was included in the reaction. To determine $V_{\lim}$ activity, UDP-Glucose concentration was reduced to 6 mM, Fru-6-P concentration to 0.5 mM and the Glc-6-P to 2.5 mM and Pi was included in the form of KH_2PO_4 at a concentration of 5 mM. The total protein concentration of the de-salted extract was determined using the method of Bradford (1976) bovine serine albumin (BSA) was used to make up standards for construction of the standard curve.

Results

Effect of freeze drying on SPS activity

Leaf tissue was freeze dried to allow for easier handling of the homogenized pooled leaf samples and for more accurate mass measurement of tissue used for protein extraction. In order to confirm that freeze drying did not have a severe detrimental effect on SPS protein, SPS activity was measured in protein extracted from freeze dried and non-freeze dried leaf material. The results of the experiment (refer to Figure 3.2 A & B) confirmed that there was not a significant difference in SPS activity between the treatments. This was true for protein extracted from both fully hydrated (100 % RWC) leaf tissue and dehydrated leaf tissue (5 % RWC). Similarly the quality of protein extracted from cotton leaf tissue was found to be unaffected by lyophilisation (Saha et al., 1997)

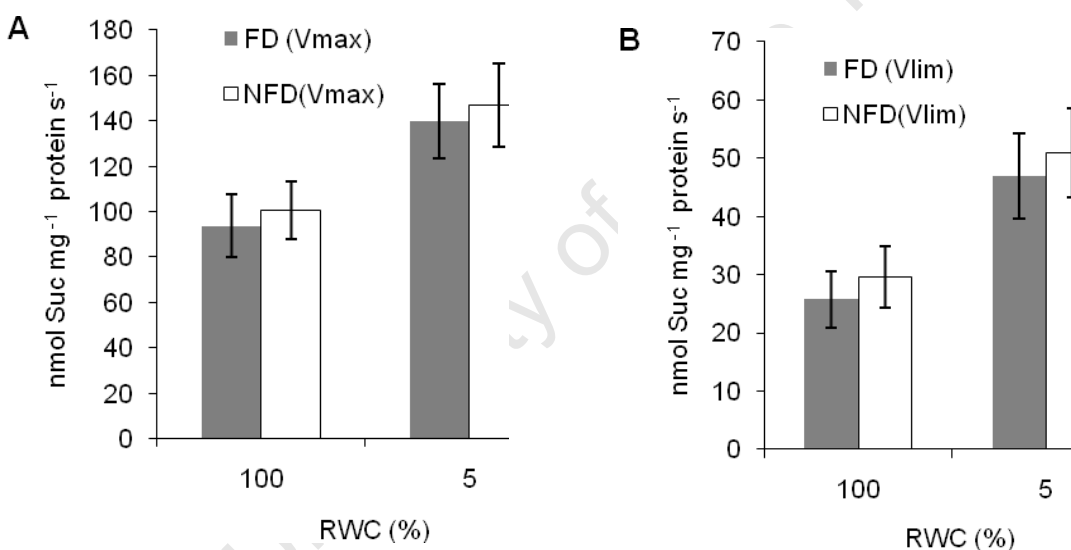


Figure 3.2 The effect of freeze drying on SPS activity. **(A)** V_{max} activity assay. **(B)** V_{lim} activity assay. Leaf tissue was harvested from fully hydrated plants (100 % RWC) and from plants at 5 % RWC. The harvested tissue was either freeze dried (FD) or only flash frozen (non-freeze dried (NFD)). Leaves were harvested from multiple plants (leaf, n>6). All fully hydrated leaves (100 % RWC) were pooled and all dehydrated leaves (5% RWC) were pooled. Three separate protein extractions were performed for each pool of leaves and SPS activity measured in each protein extract. The graph displays the mean $\pm$ SD of the three measurements.

SPS assay optimization

SPS activity was assayed at both substrate saturating levels without the inhibitor inorganic phosphate ($V_{\max}$ assay) and at substrate limiting levels in the presence of the inhibitor Pi ($V_{\lim}$ assay). The $V_{\max}$ assay, in principle, measures the total extractable activity of SPS while the $V_{\lim}$ assay is designed to estimate the contribution of the activated form of the enzyme to total SPS activity. To establish the most appropriate substrate saturating and limiting concentrations, SPS activity was measured at varying concentrations of either UDP-Glucose or Fru-6-P. For optimization of the $V_{\lim}$ assay, SPS activity was also measured with varying concentrations of Pi.

Based on the literature (see review of Huber and Winter, 2000), many plant systems display a diurnal variation in enzyme activity with SPS activity usually being highest during the day and lowest at night. Thus, to cover the range of activities that may be encountered, SPS was extracted from both light and dark treated fully hydrated leaf tissue for optimization of the SPS assay conditions. The substrate concentrations at which SPS became saturated ($V_{\max}$ values) were estimated from the data presented in Figures 3.3 and 3.4 and is shown in Table 3.1. The affinity of SPS (described by K_m values) for UDP-Glucose and Fructose-6-Phosphate (Fru-6-P) was also estimated and is included in Table 3.1. The affinity of SPS for both UDP-Glucose and Fru-6-P was observed to increase slightly in response to light, but the main difference between light and dark treated tissue is the significantly higher $V_{\max}$ activity measured for light treated SPS (refer to Table 3.1 and to Figures 3.3 and 3.4). Ideally, SPS should be purified to remove interfering molecules prior to study of its enzyme kinetics. However due to a limited amount of *X. humilis* leaf tissue being available, SPS could not be purified in sufficient amounts and hence the $V_{\max}$ and K_m values presented in Table 3.1 can only be considered as rough estimates. Nevertheless, these kinetic values did allow for a more informed choice of the SPS assay conditions. To determine the most appropriate concentration of inorganic phosphate (Pi) to use in the $V_{\lim}$ assay, SPS activity was assayed with increasing amounts of inorganic Pi (Figure 3.5). For light treated tissue, 14 mM Pi resulted in approximately 56 % reduction in uninhibited SPS activity while the reduction in dark treated SPS was approximately 68 %.

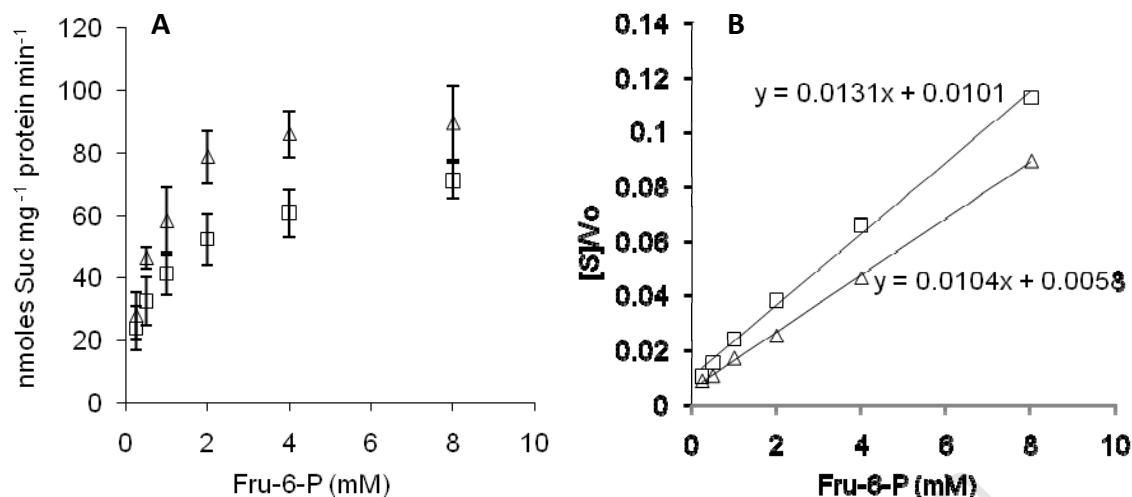


Figure 3.3 Effect of Fru-6-P concentration on the initial velocity (V_o) of SPS from leaves maintained under lighted conditions ($400\text{--}600 \mu\text{mol m}^{-2} \text{s}^{-1}$) (triangle) and from leaves kept in the dark for 4 h (square). The activity of SPS was measured with 20 mM UDP-Glucose and Glu-6-P was used at a 5:1 ratio with Fru-6-P concentration. The results are presented as changes in initial velocity (**A**) and as a Hanes plot (**B**)-with lines fitted by linear regression to calculate values for the K_m and V_{max} . In (**A**), values are the means $\pm$ SD of three biological replicates (leaf number, $n < 3$). Suc - Sucrose.

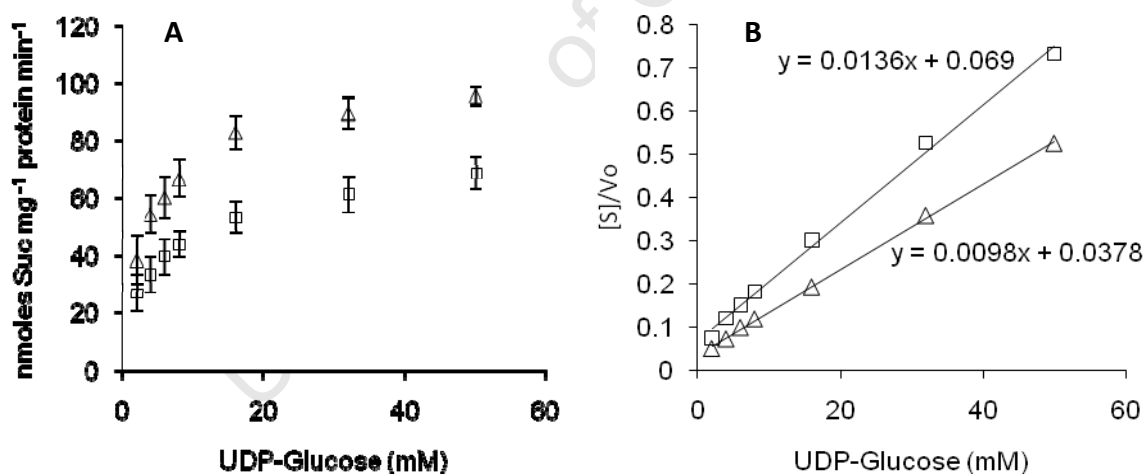


Figure 3.4 Effect of UDP-Glucose concentration of the initial velocity (V_o) of SPS from leaves maintained under lighted conditions ($400\text{--}600 \mu\text{mol m}^{-2} \text{s}^{-1}$) (triangle) and from leaves kept in the dark for 4 h (square). The activity of SPS was measured with 8 mM Fru-6-P and 40 mM Glc-6-P. The results are presented as changes in initial velocity (**A**) and as a Hanes plot (**B**)-with lines fitted by linear regression to calculate values for the K_m and V_{max} . In (**A**), values are the means $\pm$ SD of three biological replicates (leaf number, $n < 3$). Suc - Sucrose.

Table 3.1 The K_m (UDP-Glucose & Fru-6-P) and V_{max} of SPS in crude leaf extract. Leaves harvested from light treated (4 hrs into light period $400-600 \mu\text{mol m}^{-2} \text{sec}^{-1}$) and dark treated (4 h into dark period) tissue, calculations are based on data presented in Figures 3.3 and 3.4 Suc-Sucrose

Treatment	UDP-Glucose		Fru-6-P	
	K_m (mM)	V_{max} (nmol Suc mg^{-1} protein min^{-1})	K_m (mM)	V_{max} (nmol Suc mg^{-1} protein min^{-1})
Light	3.8	100.5	0.55	96
Dark (4 h)	5	73	0.77	76

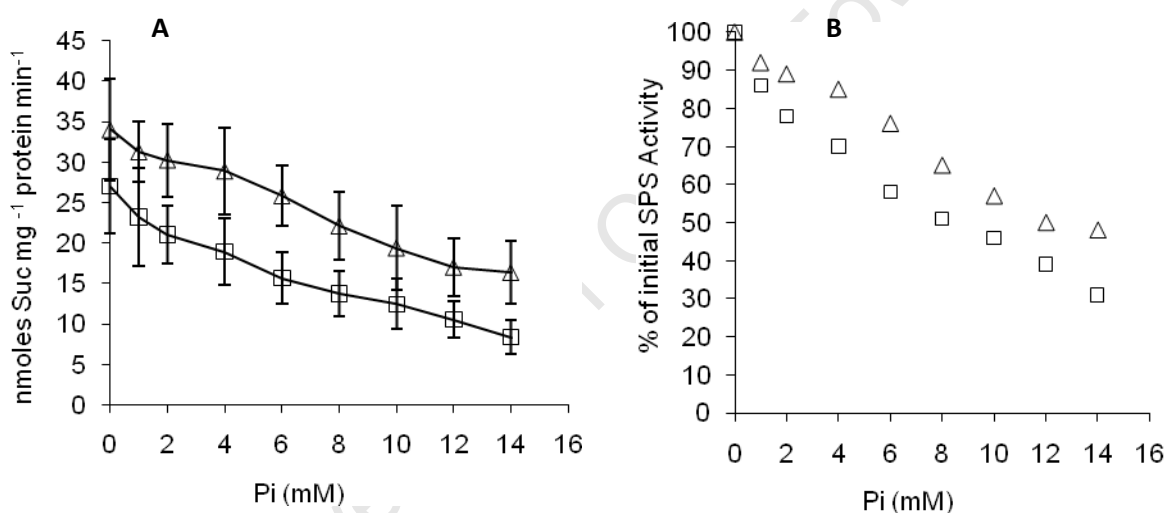


Figure 3.5 Inhibition of SPS activity by inorganic phosphate. SPS was extracted from leaves maintained under lighted conditions ($400-600 \mu\text{mol m}^{-2} \text{s}^{-1}$) (triangles) and from leaves kept in the dark for 4 h (square). The activity of SPS was measured with 6mM UDP-Glucose, 0.5 mM Fru-6-P and 2.5 mM Glc-6-P in the assay reaction. Leaves were harvested from multiple plants. In (A), values are the means $\pm$ SD of three biological replicates (leaf number, $n < 3$). In (B) data is presented as a percentage of the initial activity at 0 mM Pi. Suc - Sucrose

Based on the above kinetics studies on SPS enzyme from crude leaf extract, a substrate saturating assay (V_{max}) would require concentrations of UDP-Glucose in excess of 100.5 mM, but this high concentration was found not to be practical due to the interaction between UDP-Glucose and the detection reagent used to quantify sucrose (Anthrone reagent). It was

established empirically that the sensitivity of the assay was affected when UDP-Glucose concentrations were in excess of 35 mM; therefore only concentrations below this threshold were utilized. However, even if a reduced concentration of 30 mM is used it still provided a much improved estimate of the $V_{\max}$ activity (~ 88 % of total substrate saturating activity - $V_{\max}$) in crude leaf extract when compared to the estimate that would have been obtained using the standard 10 mM UDP-Glucose (~62 % of total substrate saturating activity - $V_{\max}$). As Glc-6-P was added in a 5:1 ratio the main constraint on the amount of Fru-6-P that could be used was the affect of the corresponding amount of Glc-6-P on the sensitivity of the assay. As was observed for UDP-Glucose, high concentrations of Glc-6-P (beyond 50 mM) reduced the sensitivity of the assay. Hence the Fru-6-P concentration was not increased beyond 8 mM.

For the $V_{\lim}$ assay, substrate concentrations were reduced to limiting levels and the inhibitor Pi (in the form of KH_2PO_4) was included at a concentration of 5mM. A concentration of 10 mM Pi was initially used, however at this concentration no difference in $V_{\lim}$ activity between light and dark SPS could be distinguished (data not shown). At 5 mM Pi the difference in $V_{\lim}$ activities between light and dark SPS, while still not being dramatic, is more apparent (data not shown). The substrate and effector concentrations adopted for the assay of SPS activity in *X. humilis* leaf tissue are summarized in Table 3.2

Table 3.2 Optimized substrate and effector concentrations used to measure changes in SPS activity leaf tissue during desiccation.

Assay	UDP-Glucose (mM)	Fructose-6-Phosphate (mM)	Glucose-6-Phosphate (mM)	Phosphate (mM)
$V_{\max}$	30	8	40	/
$V_{\lim}$	6	0.5	2.5	5

SPS activity and dehydration

In the two previous studies on the relationship between SPS activity and dehydration in *C. plantagineum* (Ingram et al., 1997) and *S. stapfianus* (Whittaker et al., 2007), SPS appears to play a role in dehydration induced sucrose accumulation. However, in a number of non-

desiccation tolerant species dehydration has no effect on- and in some cases may actually reduce SPS activity. To characterize the relationship between dehydration and SPS activity in *X. humilis* leaf, changes in SPS activity during dehydration were measured using the optimized $V_{\max}$ and $V_{\lim}$ assays. To account for possible non-dehydration related fluctuations, SPS activity was measured in both an experimental and control group of plants. The control plants were maintained in a fully hydrated state while the experimental group were allowed to dry. Changes in activity in the experimental group were related to changes in activity in the control group by dividing experimental activity by control activity (Exp/Con). This relative activity was used to describe trends observed during dehydration. To facilitate discussion tissue dehydration is divided into three stages, namely an early stage from 100 to 60 % RWC, a middle stage from 60 to 40 % RWC and a late stage from 40 to 5 % RWC.

At each leaf harvest the light, temperature and number of hours into the light period was recorded and is displayed in Table 2.1.1 (refer to Chapter 2 pg 30). Table 2.1.1 also displays data on the time of day of the harvest and on the time taken for the tissue to dehydrate. Changes in SPS activity during tissue water loss were calculated on a per g dry mass (DM) and on a per mg total protein basis. Figure 3.6 shows the protein content of the leaf tissue at each RWC. Protein content fluctuates slightly during the early and middle stages of water loss but the most notable change is a decrease in protein content from 20 mg g⁻¹ DM to 16 mg g⁻¹ DM between 20 % RWC and 5 % RWC.

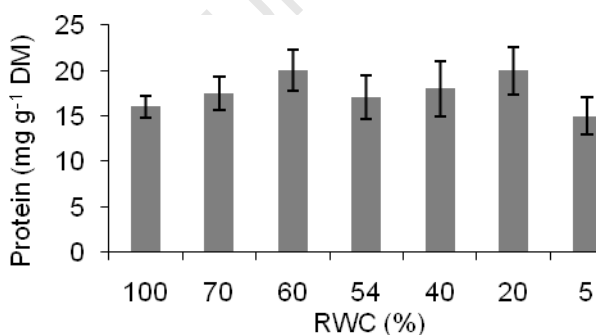


Figure 3.6 Protein content of leaf tissue during dehydration. Values are the mean $\pm$ SD of the four independent measurements of a pool of leaves (multiple plants, leaf n >6) at each RWC.

The $V_{\max}$ assay is designed to measure total extractable SPS activity and in principle should estimate changes in total SPS protein in dehydrating leaf tissue. In Figure 3.7 A(i) the actual changes in $V_{\max}$ activities (expressed on a per g DM basis) of both the experimental group and the control group are shown while in Figure 3.7 A (ii), the relative change in activity (Exp/Con) is displayed. In the control tissue a notable increase in activity occurs between the 70 % RWC and 60 % RWC marks (with reference to the RWC of the corresponding experimental sample). The notable difference between the control sample at the 70 % RWC mark and the control sample at the 60 % RWC mark is likely due to the higher irradiance and temperature recorded at the time of harvest (see Table 2.1.1, pg 30). Hence it appears that the increased Irradiance and temperature results in higher SPS $V_{\max}$ activity.

Based on the data displayed in Figure 3.7 A (ii), relative $V_{\max}$ activity (Exp/Con) is observed to increase notably from 100 to 70 % RWC. At the 70 % RWC mark activity was almost 2 fold higher than that observed in fully hydrated tissue (100 % RWC). Activity levels peaked at 70 % RWC and then began to decrease as dehydration progressed. At the 54% RWC mark $V_{\max}$ activity had returned to pre-dehydration levels (Figure 3.7 A (ii)). While the magnitude is much smaller than the initial increase, relative $V_{\max}$ activity increases once again in the late stage (below 40 % RWC) of tissue dehydration reaching a final level of ~130 % of control activity. When SPS activity is expressed on a per mg total protein basis (Figure 3.8) the increase in the late stage of tissue water is more pronounced suggesting that the change in the specific SPS quantity (percentage of SPS protein in total protein) is greater than the change in SPS protein per gram dry mass.

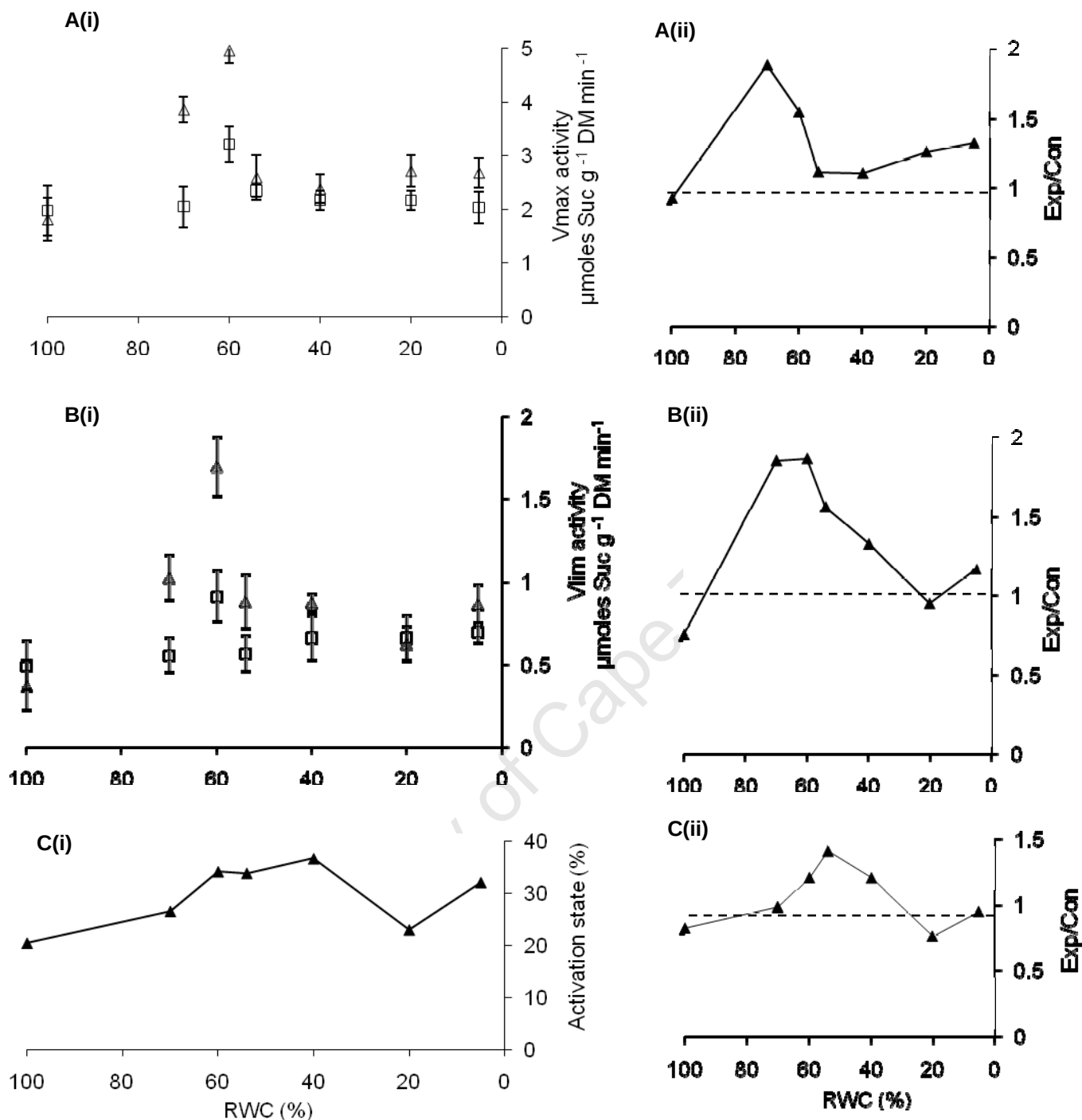


Figure 3.7 Changes in SPS activity during dehydration. **(A)** V_{max} SPS activity, in experimental (triangle) and control (open square) leaf tissue measured under saturating substrate conditions. **(B)** V_{lim} SPS activity, experimental (triangle) and control (open square) measured under limiting substrate conditions and in presence of inhibitor (Pi). **(C)** Changes in activation state of SPS. The activation state of SPS is calculated as percentage of V_{lim}/V_{max} activities. The results in **(A)**, **(B)** and **(C)** are presented as changes in the experimental group in **(i)** and as a ratio of experimental to control (Exp/Con) in **(ii)**. The dashed line in graphs (ii) indicates ratio of 1. At each RWC, a pool of leaves ($n > 6$) was used for measurement of SPS activity. Three separate protein extractions were performed from each pool of leaves. SPS activity was measured in each protein extraction. Graphs **A (i)** and **B (i)** display the mean $\pm$ SD of the three measurements.

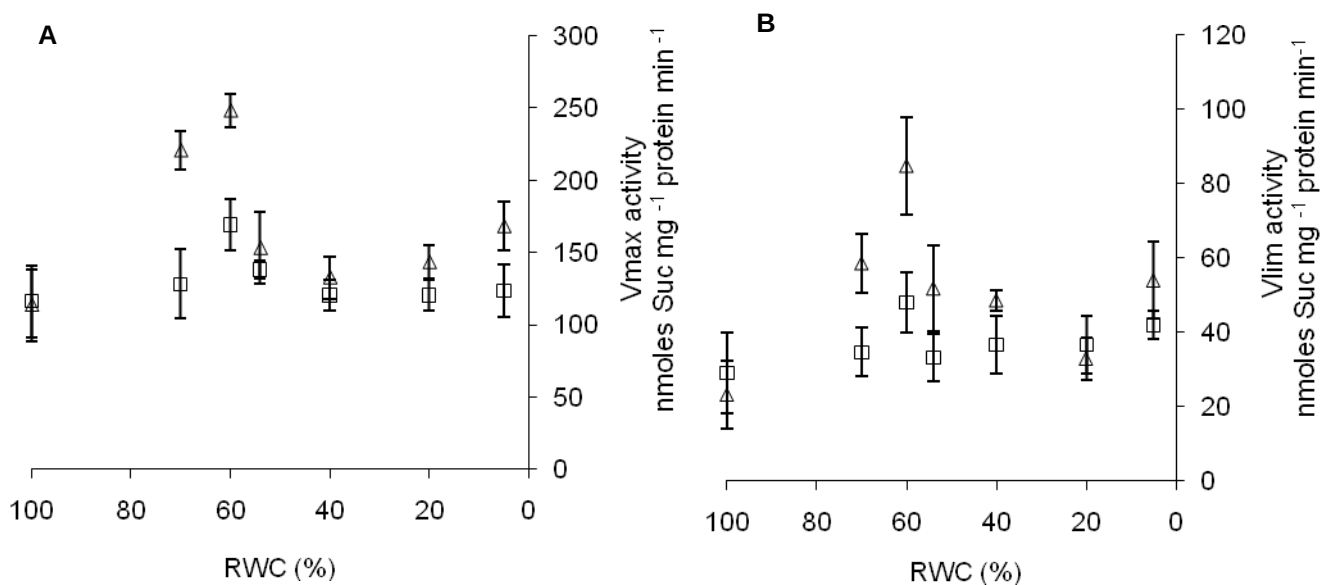


Figure 3.8 Changes in specific SPS activity. **(A)** $V_{\max}$ SPS activity, in experimental (triangle) and control (open square) leaf tissue measured under saturating substrate conditions. **(B)** $V_{\lim}$ SPS activity, experimental (triangle) and control (open square) measured under limiting substrate conditions and in presence of inhibitor (Pi). At each RWC, a pool of leaves from multiple plants (leaf $n > 6$) was used for measurement of SPS activity. Three separate protein extractions were performed from each pool of leaves. SPS activity was measured in each protein extraction. Each value is the mean $\pm$ SD of the three measurements.

$V_{\lim}$ activity

$V_{\lim}$ activity data is presented as the actual changes in $V_{\lim}$ activity in experimental and control groups in Figure 3.7 B (i) and as the relative change in activity in Figure 3.7 B (ii). As noted previously for changes in $V_{\max}$ activity in control tissue, there is also a notable increase in $V_{\lim}$ activity at the corresponding 60 % RWC experimental mark. This may also be attributed to the increased Irradiance and temperature recorded at the time of harvest (see Table 2.1.1, pg 30). The trend of relative $V_{\lim}$ activity during dehydration is similar to that observed for $V_{\max}$ activity (Figure 3.7 B (ii)). As observed for relative $V_{\max}$ activity, relative $V_{\lim}$ activity also rises quickly in response to dehydration with an almost two fold increase from 100 % to 70 % RWC. Relative $V_{\lim}$ activity continues to increase gradually after the 70% RWC mark peaks at the 60 % RWC mark, and begins to decline as dehydration progresses. The decline is relatively gradual initially but there is a sharp drop in activity from 40 % RWC to 20% RWC. This drop in activity is followed by a recovery between 20 and 5 % RWC with $V_{\lim}$ experimental activity reaching ~115

% of V_{lim} control activity at 5 % RWC. Thus, as observed for V_{max} activity, V_{lim} activity increases in the early stage of tissue dehydration, declines through the middle stage and increases again in the late stage of drying. A similar trend is observed when SPS activity is expressed on a per g DM basis and on per mg total protein (Figure 3.8) basis. The peak of V_{lim} activity is slightly offset when compared to the V_{max} activity peak, occurring at the 60 % RWC mark and not at the 70 % RWC mark.

Activation state of SPS

Due to the limiting substrate concentrations and presence of Pi the V_{lim} assay conditions will selectively measure the activated form of SPS. Thus by expressing the V_{lim} activity as a percentage of the V_{max} activity (which reflects total SPS activity) a measurement of the activation state of the SPS protein population is obtained. The activation state of SPS in the experimental group increases from ~20 % at full tissue hydration to ~35 % at the 40 % RWC mark (Figure 3.7 C (i)). There is a sharp fall in activation state from the 40 % to the 20 % RWC and a subsequent recovery between the 20 % and 5 % RWC marks. The trend differs when the fluctuation in the activation state of the control group is taken into account. To represent the relative activation state, the activation state of SPS in the experimental group was divided by the activation state of the control group and expressed as a ratio (Exp/Con) (Figure 3.7 C (ii)). The activation state of SPS in the control group fluctuates between ~25 % and ~35 % but it is difficult to link the pattern to changes in light and temperature (Figure 3.7 C and see Table 2.1.1 pg 30). Nevertheless, the change in relative activation state (Exp/Con) shows a similar trend to that described for V_{max} and V_{lim} activity - with an early stage increase, a middle stage decline and late stage recovery of activation state. The notable difference is the position of the peak in relative activation state which occurs at the 54 % RWC mark and does not coincide with either the V_{max} or V_{lim} activity peaks.

Correlation between changes in SPS activity and changes in sucrose content

To allow for direct comparison between changes in sucrose content and changes in SPS activity, experimental measurements were expressed as a percentage of control values (Figure 3.9). Increases in sucrose content were found to correlate positively with increases in SPS activity (see Figure 3.9). The correlation (Pearson correlation) between the pattern of sucrose accumulation and changes in $V_{\max}$ SPS activity was 0.73 while the correlation was slightly less for $V_{\lim}$ activity, at 0.66. The correlation between sucrose accumulation and SPS activity was strongest in the early stages of water loss (before 50 % RWC) but was weaker in the later stages of water loss.

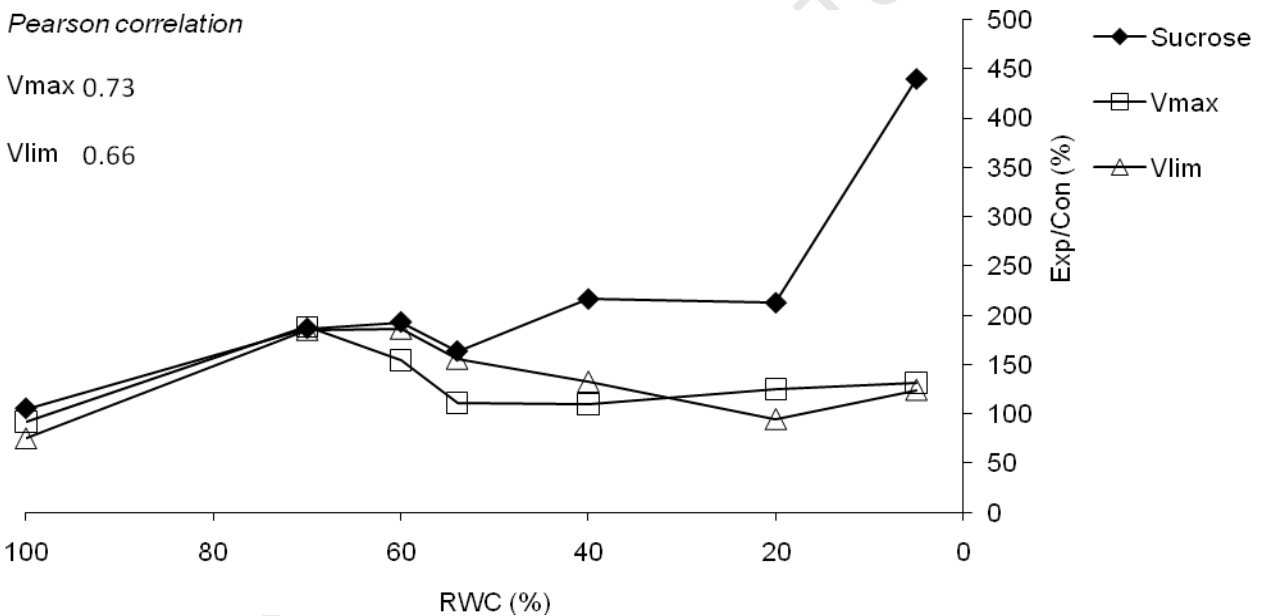


Figure 3.9 Sucrose accumulation and SPS activity during desiccation. Change in sucrose content, $V_{\max}$ activity and $V_{\lim}$ activity expressed as a percentage of control values. Original data for experimental and control tissues is found in Figure 2.2, pg 30 for sucrose and Figure 3.7 for SPS activity. Sucrose content and SPS activity is calculated on a per gram dry mass basis. Pearson correlation values are shown for the comparison between changes in sucrose content and SPS activity.

Discussion

SPS assay optimization

In many studies on SPS activity the $V_{\max}$ and $V_{\lim}$ assay conditions derived from kinetic studies of spinach SPS (e.g. Huber et al 1983; 1984; Stitt et al ., 1988) have been applied to other species without much modification. However, the non-optimized use of these assay conditions can be problematic (Lunn and Hatch, 1997 and discussed in Introduction to this chapter). Consequently, before assaying SPS activity in *X. humilis* it was necessary to obtain some understanding of SPS enzyme kinetics in *X. humilis* leaf tissue. Ideally, SPS should be purified to reduce possible interfering agents but due to a scarcity of sample tissue the enzyme kinetic studies were conducted on non-purified crude leaf extracts. SPS enzyme kinetics were characterized in terms of substrate saturation activity ($V_{\max}$) and affinity (K_m) for the substrates UDP-Glucose and Fructose-6-Phosphate (Fru-6-P). As all analysis was done on crude leaf extract only rough estimates of the kinetic properties (reflected in $V_{\max}$ and K_m values) of SPS in *X. humilis* leaf tissue were obtained. To establish the most appropriate concentration of the inhibitor Pi to use, the affect of the Pi concentration on SPS activity was also investigated. Based on the assumption that light/dark modulation of SPS occurs in *X. humilis*, SPS was extracted from light and dark treated leaf tissue to reflect the possible range of SPS activities. The information obtained was used to guide selection of the most appropriate $V_{\max}$ and $V_{\lim}$ assay conditions.

The selected substrate and effector concentrations have been described in the Results section but the differences between the assay conditions chosen in the current work and those used previously in spinach are worth highlighting. In the current work a final concentration of 30 mM UDP-Glucose was used in the $V_{\max}$ assay, which is 3 times higher then the concentration used to estimate spinach $V_{\max}$ activity (10 mM UDP-Glucose) (e.g. Huber et al., 1989). In theory for full $V_{\max}$ activity a concentration of ~100.5 mM UDP-Glucose would be necessary to saturate *X. humilis* SPS in crude leaf extract; however, due to practical limitations a reduced concentration had to be used in the final assay. Nevertheless, even this reduced concentration still provided a much improved estimate of the $V_{\max}$ activity (~ 88 % of total substrate saturating activity - $V_{\max}$) in crude leaf extract when compared to the estimate that would have been obtained using 10 mM

UDP-Glucose (~62 % of total substrate saturating activity - $V_{\max}$). In the case of Fru-6-P and Glc-6-P the concentrations used in the $V_{\max}$ assay of the current study and those used previously did not differ significantly. The most notable difference between the $V_{\lim}$ assay of the current work and that used in spinach is the reduced amount of Fru-6-P used. For spinach a concentration of 3 mM Fru-6-P is used in the limiting assay (e.g. Huber et al., 1989). In the current study a concentration of only 0.5 mM Fru-6-P is used, which based on the estimated K_m values in crude leaf extract (ranged between ~0.5 mM for light SPS and ~0.8 mM for dark SPS) is more substrate limiting than a concentration of 3 mM. Another difference between the current $V_{\lim}$ assay and that used in spinach is the concentration of inorganic Pi used. In spinach a concentration of 10 mM Pi was used (e.g. Huber et al., 1989) while in the current study a concentration of 5 mM Pi was used. According to the kinetics of inhibition by Pi a 10mM concentration appeared to be appropriate for *X. humilis* SPS, however at a concentration of 5 mM the difference in $V_{\lim}$ activity between activated (light SPS) and de-activated SPS (dark SPS) was found to be more pronounced. Hence the lower concentration was selected.

When comparing the kinetics of light and dark SPS, the most prominent difference is the higher estimated $V_{\max}$ (for both UDP-Glucose and Fru-6-P) of light SPS when compared to dark SPS (Table 3.1). The affinity for both substrates also decreases in dark SPS (increased K_m) but the reduction is relatively minor. This is in contrast to a number of other studies on species including spinach (e.g. Huber et al., 1989), maize (Lunn and Hatch, 1997), rice (Takahashi et al., 2000) and wheat (Trevanion et al., 2007) where $V_{\max}$ activity (measured in crude leaf extract) remains relatively unchanged in response to light. However, the affect on light activation on $V_{\max}$ activity is controversial. In many early studies on species including maize, $V_{\max}$ activity was found to increase in response to light (Sicher and Kremer, 1985; Huber et al., 1987; Kalt-Torres et al., 1987). In the case of maize, Lunn et al., (1997) have demonstrated that the UDP-Glucose concentrations previously used in the $V_{\max}$ assay (either 10 mM or 25 mM UDP Glucose) were likely to be sub-saturating for maize SPS. When increased concentrations of UDP-Glucose (50 mM) were used no change in $V_{\max}$ activity between light and dark treated SPS was detected in maize (Lunn and Hatch, 1997). In the current study the issue of sub-saturating

substrates was addressed and hence the estimates of $V_{\max}$ activities in light and dark treated tissues should be reflective of SPS activity in crude leaf extract.

SPS activity in response to dehydration

In *X. humilis* leaf tissue there is a strong initial response of SPS activity to dehydration with both $V_{\max}$ and $V_{\lim}$ activities increasing almost two fold between 100 to 60 % RWC. This initial increase is followed by a decline in activity as tissue water loss progressed. By the 40 % RWC mark both $V_{\max}$ and $V_{\lim}$ activities had returned to their pre-dehydration levels. In the final stage of water loss (below 20 % RWC) increases in $V_{\max}$ and $V_{\lim}$ activities are noted again, but they are not of the same magnitude as those observed in early dehydration. When expressed relative to control activity, $V_{\max}$ activity peaked at 70 % RWC, $V_{\lim}$ activity peaked at 60% RWC and the activation state of SPS ($V_{\lim}/V_{\max}$) was highest at the 54 % RWC mark. This response of SPS activity to dehydration follows a similar trend to that observed for sucrose accumulation (Chapter 2) where there is an early phase of sucrose accumulation (100 % to 60 % RWC) followed by a late phase of accumulation below 30 % RWC. In Figure 3.9 (pg 75) sucrose content, $V_{\max}$ activity, $V_{\lim}$ activity and SPS activation state are presented as a percentage of their corresponding control values. The Pearson correlation co-efficients are 0.73 for $V_{\max}$ activity and 0.66 for $V_{\lim}$ activity (see Figure 3.9) suggesting that there is a certain degree of correlation between changes in sucrose content and SPS activity during desiccation. However, the maximum SPS activity does not coincide with the maximum rate of sucrose accumulation with peak sucrose accumulation occurring between 20 % RWC to 5 % RWC while SPS activity is at a maximum between the 70 % -60 % RWC marks (see Figure 3.9). This incongruence suggests that, especially in the late phase of water loss (below 30 % RWC), changes in SPS activity alone may not account for the observed sucrose accumulation during desiccation.

Rather counter intuitively $V_{\max}$ activity increases first in response to dehydration and is followed by increases in $V_{\lim}$ activity. One might expect that the post-translational changes (e.g. phosphorylation of Ser₄₂₄) involved in altering $V_{\lim}$ activity would occur faster than the

translational changes required for altered $V_{\max}$ activity. However, it is also possible that some form of post-translational modification may reflect as changes in $V_{\max}$ activity. In *A. thaliana* it has been demonstrated that phosphorylation mediated binding of 14-3-3 proteins to SPS can control proteolytic degradation of SPS (Cotelle et al., 2000). Hence a post-translational modification such as phosphorylation may reflect as changes in $V_{\lim}$ and $V_{\max}$ activity by altering both the kinetics of SPS and the total amount of SPS protein. One future approach to assess the effect of a PTM on $V_{\max}$ activity may be to inhibit protein synthesis and to establish if changes in $V_{\max}$ activity still occur. Unfortunately, interpretation of these results will be complicated by secondary effects of suppression of protein synthesis and especially by possible side-effects on the kinases involved in the phosphorylation of SPS. Intriguingly, while in many desiccation sensitive plants the initial response to water deficit is to activate SPS (observed as changes in $V_{\lim}$ activity), in all the desiccation tolerant angiosperms studied, including *X. humilis*, the initial changes in SPS activity are detectable in both the $V_{\max}$ and $V_{\lim}$ assays.

Changes in SPS activity in response to dehydration have also been assessed in two other angiosperm resurrection species, namely the C3 dicot- *Craterostigma plantagineum* and the C4 grass *S. stapfianus*. In leaf tissue of *C. plantagineum* total SPS activity increases steadily throughout tissue dehydration, doubling activity from 100 % RWC to 60 % RWC and finally reaching a maximum of three times the fully hydrated activity at 10 % RWC (Ingram et al., 1997). This trend of steady increase in SPS activity is in contrast to the current work where there is a drop in activity around the mid-point of dehydration followed by a recovery at lower relative water contents. In the *C. plantagineum* study it would appear (based on lack of Pi in assay) that activity was only measured under $V_{\max}$ conditions and there is no data presented on actual changes in the activation state of SPS. A relationship between sucrose accumulation and SPS activity can also not be commented on as data on the change in sucrose content is not presented.

In *S. stapfianus* both $V_{\max}$ and $V_{\lim}$ activity were measured. Once again it is unclear as to how close the estimate of $V_{\max}$ activity is to actual total SPS activity as the substrate concentrations used (10mM UDP glucose and 10 mM Fru-6-P) are not demonstrated to be saturating for *S.*

stapfianus SPS. If *S. stapfianus* SPS shares similar kinetic properties to other C4 grasses such as maize, then much higher UDP-Glucose concentrations may be required. Nevertheless, $V_{\max}$ activity in *S. stapfianus* leaf was found to increase from the 90 % to 70 % RWC marks with activity peaking at 70 % RWC and then decreasing to pre-dehydration levels by the 10 % RWC (Whittaker et al., 2007). Notably, the peak of SPS activity was found to coincide with the highest rate of sucrose accumulation. The activation state of SPS was also observed to increase steadily from 90 to 45 % RWC, reaching a level almost five times that of SPS at full tissue hydration. Thus, similar to the current study, there is an increase in both $V_{\max}$ and $V_{\lim}$ activities in response to initial tissue dehydration. The main difference between the activity trends in *X. humilis* and *S. stapfianus* is the increase in total SPS activity ($V_{\max}$) in the final stages of dehydration which is observed in the current study but is not noted in the study on *S. stapfianus*.

Increases in SPS activity in response to dehydration have also been noted in desiccation sensitive species but the relationship between dehydration and SPS activity is not simple. In species such as soybean (Cheikh and Brenner, 1992), rice (Yang et al., 2002) and wheat (Niedzwiedz-Siegen et al., 2004; Fresneau et al., 2007) SPS activity ($V_{\max}$) increases in response to dehydration while in other species including *Phaseolus vulgaris* (Vassey and Sharkey, 1989), maize (Pelleschi et al., 1997; Foyer et al., 1998) and barley (Villadsen et al., 2005) total SPS activity ($V_{\max}$) is reduced in response to dehydration. Further, the relationship becomes even more complex when both $V_{\max}$ and $V_{\lim}$ activities are considered. In maize, while the $V_{\max}$ SPS activity has been shown to decline (Pelleschi et al., 1997; Foyer et al., 1998), there is no agreement on changes in $V_{\lim}$ activity in response to dehydration with $V_{\lim}$ SPS activity reported to increase (Foyer et al., 1998) and to decrease (Pelleschi et al., 1997). In *Phaseolus* leaves a decrease in RWC from 100 to 80% caused a corresponding drop of 60% in $V_{\max}$ SPS (Vassey & Sharkey 1989). Castrillo (1992) also measured decreased $V_{\max}$ SPS activity but increased $V_{\lim}$ activity in the same species under mild water deficit stress. In spinach, water deficit stress results in an increased activation state (increased $V_{\lim}$ activity relative to $V_{\max}$ activity) of SPS but the total activity ($V_{\max}$) remains unaffected (Quick et al., 1989; Zrenner and Stitt 1991). Thus, in some

species dehydration induces a change (either an increase or decrease) in total SPS activity while in other species total activity remains unchanged while the activation state of SPS is altered.

Alternative mechanisms (to SPS) contributing to sucrose accumulation in late dehydration

As highlighted in the introduction it is unlikely that sucrose accumulation would only be due to increased sucrose synthesis. More probably, sucrose accumulation in leaf tissue results from changes in a combination of factors. These factors may include increased synthesis, increased sucrose import and decreased sucrose utilization. Due to the low water content at 20 % RWC (actual water content of $\sim 0.47 \text{ g H}_2\text{O g}^{-1}$) one would expect sucrose translocation into the leaf tissue not to be a major contributor to the observed sucrose accumulation during the late stage of water loss. Thus internal sources of carbon must be utilized to support sucrose accumulation. Based on the results presented in Chapter 2 proposed internal carbon sources include hexose sugars, amino acids and polyols. Notably in the late stage of water loss (between 20 and 5 % RWC), there is a sharp decline in a number of amino acids suggesting that these amino acids may serve as a carbon source below 20 % RWC. If amino acids do provide the necessary carbon skeletons then the combination of increased substrate (by conversion of amino acids to hexose sugars) and the slight increases in SPS activity may lead to substantial increases in sucrose in the late stages of tissue water loss. However this proposal is weakened by the observation that there is a major decline in hexose sugar between 20 and 5 % RWC (See Chapter 2). Nevertheless, if the utilization of hexose sugars is rapid then the transient increase in their levels may have been missed.

Another possible factor which may contribute to increased sucrose accumulation in the late stages of water loss is the reduction in the degradation of sucrose. In many systems a cycle of sucrose synthesis and sucrose breakdown is noted (e.g. Dancer et al., 1990; Geigenberger and Stitt, 1991; Wendler et al., 1991). Hence sucrose will accumulate if there is a decrease in the activity of the enzymes involved in sucrose degradation. The two enzymes considered to be important in sucrose turnover in plant tissue are invertase and sucrose synthase. Changes in the

activity of invertase (neutral and acid invertase) have been studied in the resurrection plant species *S. stapfianus* and *X. viscosa* (Whittaker et al., 2001). In the study, invertase activity was found to remain relatively unchanged during the periods of net sucrose accumulation suggesting that decreased sucrose degradation by invertase was not a major determinant of sucrose accumulation in these species. While SuSy activity was not measured in the Whittaker et al., 2001 work, in another resurrection species, *C. plantagineum*, no major decrease in SuSy activity was noted in response to dehydration (Kleines et al., 1999). Thus, based on previous literature it appears that the downregulation of the sucrose degrading enzymes may not contribute significantly to sucrose accumulation in resurrection plant species. Nevertheless, further investigation will be required to establish if this holds true for invertase and SuSy activity in drying *X. humilis* leaf.

SPS activity and photosynthesis during dehydration

Under non-stressed conditions sucrose synthesis in the cytoplasm is linked to the production of photosynthate in the chloroplast. Thus, when considering the response of SPS activity to dehydration an important aspect to take into account is the affect of dehydration on photosynthesis. An early reaction of most species to dehydration is the closure of stomata to reduce water loss (Lawlor and Cornic, 2002). Stomata closure leads to a reduction in internal carbon dioxide (CO₂) concentrations and consequently a reduction in photosynthetic rate. In species such as *Phaseolus vulgaris* (Vassey and Sharkey, 1989; Castrillo, 1992) and maize (Pelleschi et al., 1997) the reduction in photosynthetic rate caused by stomatal closure is paralleled by a decrease in extractable SPS activity. In the case of *Phaseolus*, Vassey and Sharkey (1989) linked the reduction in SPS activity directly to the reduction in photosynthetic rate. In a later *Phaseolus vulgaris* study it was also observed that several hours of exposure to elevated CO₂ reversed the effects of water deficit on SPS activity (Vassey et al., 1991). Under saturating CO₂ conditions, photosynthesis is relatively resilient to water stress and in many species is only affected when water content drops below ~70 % RWC (Lawlor and Cornic, 2002). In spinach, experiments were conducted under CO₂ saturating conditions and thus, even after stomatal closure the internal CO₂ concentration was sufficient for photosynthesis to

continue and consequently for $V_{\max}$ SPS activity to remain high. It would appear that in some species photosynthesis and SPS activity remain tightly coupled (e.g. maize), while in other species (e.g. soybean and possibly spinach) the link between photosynthesis and SPS activity may be disrupted under water deficit stress.

Interestingly in the current study the major declines in $V_{\max}$ and $V_{\lim}$ activities both coincide with the down regulation of photosynthesis at the ~60 % RWC mark - as indicated by a drop in quantum yield. Similarly in *S. stapfianus*, the initial increase in SPS activity is followed by a decline in activity as photosynthesis is down regulated (Whittaker et al., 2007). Thus, in both species it appears that SPS activity and photosynthesis may still be partially linked during tissue dehydration. This link is described as partial as SPS also increases independently of photosynthesis in the early (for *X. humilis* and *S. stapfianus*) and late (only *X. humilis*) stages of tissue dehydration. In contrast to both the current study and the work on *S. stapfianus*, SPS activity continues to increase during and after the shutdown of photosynthetic activity in *C. plantagineum* (Ingram et al., 1997). In *C. plantagineum* photosynthesis is reported to be inhibited by stomatal closure between 70 and 50 % RWC and by direct inhibition of photosynthetic reactions beyond 50 % RWC (Schwab et al., 1989). Ingram et al., (1997) reported no dip in SPS activity at the 50 % RWC mark indicating that SPS activity was not linked to the falling photosynthetic rate.

Conclusion

In response to tissue water loss, SPS activity increases strongly in the early stage of dehydration, decreases during the middle stages and increases slightly in the late stage of water loss. This pattern of SPS activity is evident when measured with both the $V_{\max}$ and $V_{\lim}$ assay, suggesting that SPS activity is altered by both changes in total protein and by changes in the activation state of the enzyme. Particularly in the early to middle stages of water loss there is a good positive correlation between increases in SPS activity and increases in tissue sucrose content. However in the late stage of tissue water loss there appears to be a disproportionately large increase in

sucrose content relative to the increase in SPS activity. In the early stage of water loss (100-60 % RWC) both sucrose content and SPS activity increase two fold when compared to control values (see Figure 3.7, pg 72). In contrast, while sucrose content doubles again between 20 and 5 % RWC, the increases in $V_{\max}$ activity (relative value, see Figure 3.7 B (ii)) and activation state (relative value, see Figure 3.7 C (ii)) are considerably less when compared to the changes noted between the 100 and 60 % RWC marks. Thus, it appears that in addition to changes in the maximal activity and activation state of SPS, other regulatory mechanisms are important for driving sucrose accumulation. Further investigation will be required to characterize all the underlying mechanisms of sucrose accumulation in the late stages of water loss. Nevertheless, it is clear that dehydration modulates SPS activity in *X. humilis* leaf tissue. Having established that SPS activity responds to dehydration, the focus of the work moved to the relationship between water loss and SPS gene expression. In the next chapter particular attention is paid to the possible up-regulation of a dehydration specific isoform.

Introduction

Having confirmed an increase in SPS activity in response to dehydration, especially in the initial stages of water loss, the possible transcriptional and translational changes underpinning the activity changes were investigated. In particular, the question regarding the expression of a dehydration specific isoform was addressed. The approach adopted was to identify all expressed SPS genes in leaf tissue and to characterize their expression during dehydration. To place this work in context a review on what is known about the molecular phylogeny and expression of the SPS gene family in angiosperms is given. Information obtained from the review was also used to predict the number of SPS genes to expect in *X. humilis*.

SPS gene phylogeny in angiosperms

The gene encoding SPS was first identified in *Zea mays* (maize) (Worrell et al., 1991) with the isolation of the *Spinacia oleracea* (spinach) SPS gene following soon after (Klein et al., 1993; Sonnewald et al., 1993). Initially it appeared that the clustering of SPS genes would follow a simple monocot/eudicot split and early studies also concluded that each species contained only one SPS gene (Worrell et al., 1991; Valdez-Alarcon et al., 1996). However, as more SPS genes were cloned and with the whole genome sequencing of species such as *Arabidopsis thaliana* and *Oryza sativa*, the picture of SPS genes in angiosperms has become much more complex.

Multiple SPS genes in single species were first noted in *Citrus unshiu* (satsuma orange) with three distinct SPS genes being identified (Komatsu et al., 1996). Studies on the monocot *Saccharum officinarum* (sugarcane) (Sugiharto et al., 1997) and on the eudicot resurrection species *Craterostigma plantagineum* (Ingram et al., 1997) also revealed the presence of at least two SPS genes in each species. In addition, the sequencing of the *A. thaliana* genome revealed the presence of four putative SPS genes. Notably, when subjected to phylogenetic analysis multiple SPS genes identified in either monocot or eudicot species did not cluster according to a

simple monocot/eudicot split. Rather, the first large scale phylogenetic analysis of SPS genes revealed the presence of at least three main groups of SPS genes, designated Family A, B and C (Langenkamper et al., 2002). Of the four *A. thaliana* SPS genes, two were found to belong to the A Family, one to the B Family and one to Family C. Following the Langenkamper et al., (2002) analysis several phylogenetic analyses of SPS genes have been published, these include the analyses of Castleden et al., (2004), MacRae and Lunn, (2006) and Lutfiyya et al., (2007) (see Figure 4.1). The initial analysis by Langenkamper et al., (2002) was restricted to eudicot SPS genes but the later analyses also included SPS genes from monocots. Similar to *Arabidopsis thaliana* most of the monocot species, including *Zea mays* (maize), *Oryza sativa* (rice), *Hordeum vulgare* (barley), and *Saccharum officinarum* (sugarcane) were found to contain at least one representative in each family.

As the number of identified SPS genes increased more light has been shed on the evolutionary history of SPS genes in plants. In Families A, B and C there are clear eudicot and monocot subgroups indicating that the duplications giving rise to these families occurred prior to the eudicot/monocot split (Langenkamper et al., 2002). It is therefore likely that most eudicot and monocot plants should contain a member in each family (Family A, B and C). Based on the phylogenetic analyses of Castleden et al., (2004) and MacRae and Lunn, (2006) it is also proposed that monocot species formed a fourth divergent group, designated Family D. The D Family appears to be grass monocot specific and it is proposed to have split from the Family A approximately 77 million years ago around the time of divergence of the grasses from other monocots (Castleden et al., 2004) (Figure 4.1). In addition to the phylogenetic analysis of MacRae and Lunn, (2006) a recent phylogenetic analysis of known plant and bacterial SPS proteins has also been conducted by Lutfiyya et al., (2007). The tree topology obtained by the Lutfiyya et al., (2007) work appears to follow the general Family A, B and C family groupings but differs regarding the Family D grouping. The bacterial SPS genes formed Group 1 and, based on the set of SPS genes found in each group, Group 2 corresponds to plant Family A, Group 3 to Family B and Group 4 to Family C. **cont/ pg 89**

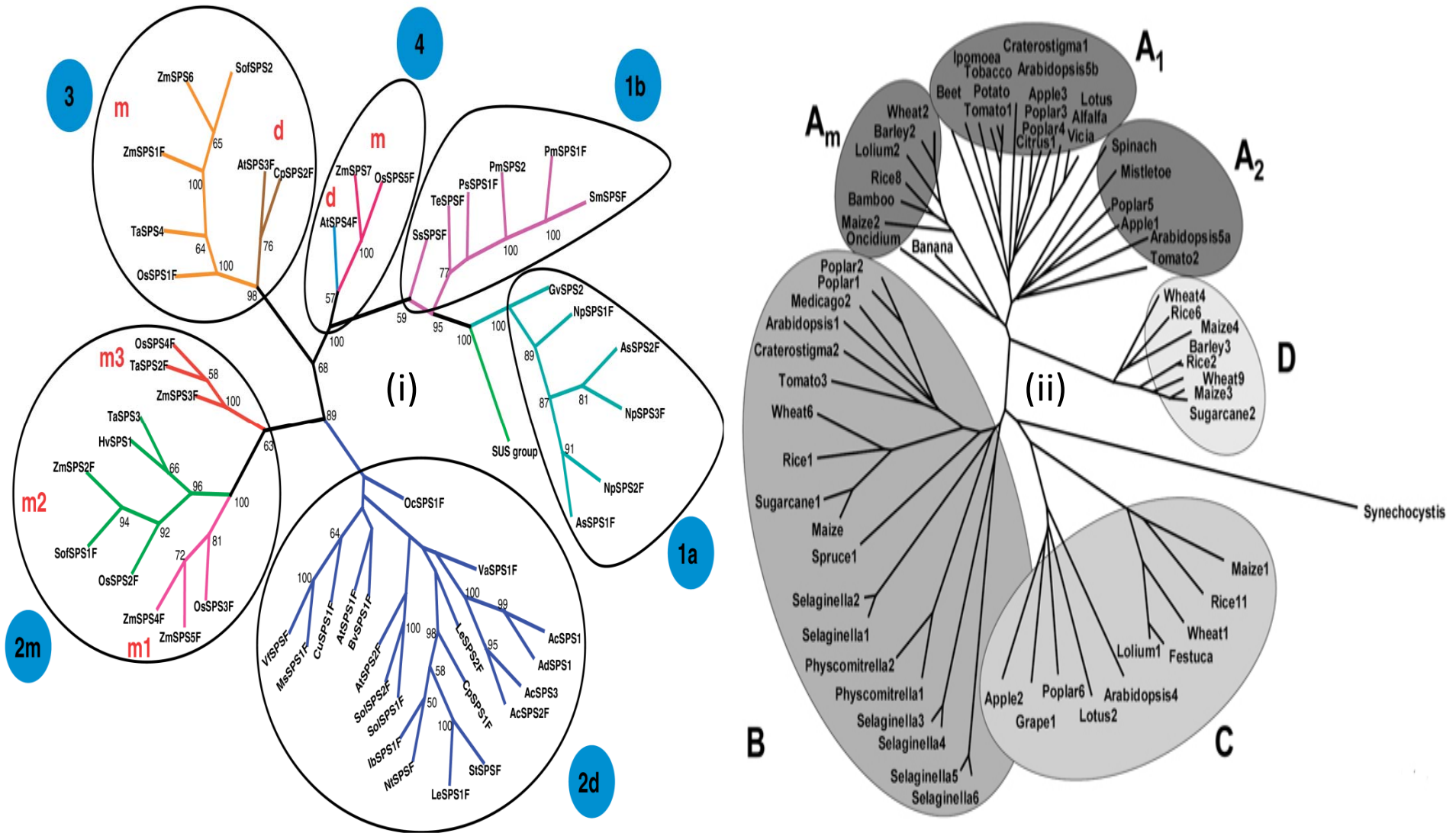


Figure 4.1 SPS gene families in plants predicted from phylogenetic analysis by (i) Lutfiyya et al., (2007) (for full species names refer to Table 3.1) and (ii) MacRae and Lunn, (2006) . Both trees are unrooted NJ phylogenetic trees. Tree (i) was generated from an alignment of the glucosyltransferase domain region of SPS. Bootstrap values greater than 50 (of 100 replicates) are indicated. Tree (ii) is based on an alignment of full length and partial length protein sequences. The tree in (ii) is supported by a previous analysis (Castleden et al., 2004) with good bootstrap support.

Table 4.1 A comparison of SPS phylogenetic groups proposed by Lutfiyya et al., (2007) and MacRae and Lunn, (2006). Names that could not be matched are indicated by (/) in the MacRae and Lunn, (2006) column.

*Lutfiyya et al., (2007)		MacRae & Lunn, (2006)		*Lutfiyya et al., (2007)		MacRae & Lunn, (2006)	
SPS gene	Grp	SPS gene	Grp	SPS gene	Grp	SPS gene	Grp
CpSPS1F	2d	Craterostigma 1	A	IbSPS1F	2d	Ipomoea	A
AtSPS1F	2d	Arabidopsis 5b	A	SolSPS1F	2d	Spinach	A
NtSPSF	2d	Tobacco	A	OcSPS1F	2d	Oncidium	A
StSPSF	2d	Potato	A	AtSPS2F	2d	Arabidopsis 5a	A
BvSPS1F	2d	Beet	A	LeSPS2F	2d	Tomato 2	A
LeSPS1F	2d	Tomato 1	A	OsSPS4F	2m3	/	A
CuSPS1F	2d	Citrus 1	A	TaSPS2F	2m3	Wheat 4	A
MsSPS1F	2d	Alfalfa	A	ZmSPS3F	2m3	/	A
ZmSPS4F	2m1	/	D	HvSPS1	2m2	Barley 3	D
ZmSPS6F	2m1	/	D	ZmSPS2F	2m2	Maize 3	D
OsSPS3F	2m1	Rice 6	D	SofSPS1F	2m2	Sugarcane 2	D
TaSPS3	2m2	/	D	OsSPS2F	2m2	Rice 2	D
CpSPS2F	3d	Craterostigma 2	B	ZmSPS1F	3m	Maize	B
AtSPS3F	3d	Arabidopsis 1	B	ZmSPS6	3m	/	B
SofSPS2	3m	Sugarcane 1	B	TaSPS4	3m	/	B
OsSPS1F	3m	Rice 1	B				
ZmSPS7	4m	/	C	ZmSPS7	4m	/	C
OSSPS6F	4m	Rice 11	C	AtSPS4F	4d	Arabidopsis 4	C

*The first two, or three letters in the name are an abbreviation of the genus/species name; Ac: Kiwifruit, *Actinidia chinensis*, Ad: Kiwifruit, *Actinidia deliciosa*, As: *Anabaena species*, At: *A. thaliana*, Br: Cabbage, *Brassica rapa*, Bv: Sugar beet, *Beta vulgaris*, Cp: *Craterostigma plantagineum*, Cu: Citrus, *Citrus unshiu*, Gv: *Gloeobacter violaceus*, Hv: Barley, *Hordeum vulgare*, Ib: Sweet potato, *Ipomoea batatas*, Le: Tomato, *Lycopersicon esculentum*, Ma: Banana, *Musa acuminata*, Mi: Mango, *Mangifera indica*, Ms: Alfalfa, *Medicago sativa*, Np: *Nostoc punctiforme*, Nt: Tobacco, *Nicotiana tabacum*, Oc: *Oncidium cv. 'Goldiana'*, Os: Rice, *Oryza sativa*, Pm: *Prochlorococcus marinus*, Pp: Pine, *Pinus pinaster*, Ps: *Pirellula sp. 1*, Sm: *Synechococcus marinus*, Sof: Sugarcane, *Saccharum officinarum*, Sol: Spinach, *Spinacia oleracea*, Ss: *Synechocystis sp.*, St: Potato, *Solanum tuberosum*, Ta: Wheat, *Triticum aestivum*, Te: *Thermosynechococcus elongates*, Va: Mistletoe, *Viscum album*, Vf: Fava bean, *Vicia faba*, Zm: Maize, *Zea mays*. The last letter 'F' in the name indicates that the sequence is full-length.

The cluster of genes which formed Family D in the MacRae and Lunn, (2006) analysis make up subgroups (2m1 and 2m2) within Group 2 (see Figure 4.1 and Table 4.1). Thus, in the Lutfiyya et al., (2007) analysis there are only three main plant SPS families and not four as proposed in the Castleden et al., (2004) and MacRae and Lunn, (2006) analyses.

The similar intron-exon structure of SPS genes points to a common evolutionary origin (Langenkamper et al., 2002; Castleden et al., 2004) and it is likely that SPS genes in plants evolved from a cyanobacterial-like enzyme inherited during the endosymbiosis of the ancestor of chloroplasts (Lunn, 2002). When SPS genes from extant cyanobacteria are included in phylogenetic analysis of plant SPS genes, topologies obtained indicate that Family C genes were the first to diverge from their bacterial ‘ancestors’ (Lutfiyya et al., 2007; MacRae and Lunn, 2006) (Figure 4.1). However, unexpectedly the most primitive types of plant SPS protein found in the moss *Physcomitrella patens* and the lycophyte *Selaginella moellendorffii* do not belong to Family C and rather cluster with Family B genes (MacRae and Lunn, 2006) (Figure 4.1). The occurrence of only Family B genes in these more primitive plants suggests that Family B and not Family C is the most ancient plant SPS family. Thus it is currently unclear as to which Family represents the most ancient SPS gene family.

As can be seen from a comparison of the naming schemes adopted by Lutfiyya et al., (2007) and MacRae and Lunn, (2006) (Table 4.1) there is no standard nomenclature for either the proposed SPS families or the specific SPS genes in each grouping. In Table 4.1 an attempt has been made to link corresponding Family and gene names, however, as not all the accession numbers for the SPS genes were reported it was not possible to match all reported SPS gene names. To avoid confusion, the nomenclature scheme adopted by MacRae and Lunn, (2006) will be used in this chapter to describe Family groups, if necessary reference will be made to Family and SPS gene names used in the Lutfiyya et al., (2007) work.

SPS protein structure

Figure 4.2 shows the generalized protein structures of 1) SPS proteins belonging to Families A, B and C, described as Family ABC type, 2) SPS protein from *Physcomitrella*, 3) SPS protein found in the proposed D Family and 4) SPS protein found in cyanobacteria. Two highly conserved regions in plant SPS enzymes are the glycosyltransferase and the sucrose phosphate phosphatase (SPP) like domains (see Figure 4.2). Both these domains are also present in SPS protein from cyanobacteria. The glucosyltransferase domain includes the UDP-Glucose binding site (Salvucci and Klein, 1993) and a putative Fructose-6-phosphate (Fru-6-P) binding motif (Salvucci et al., 1995). The main differences between most plant SPSs (belonging to Families A, B and C and described as ABC type) and bacterial SPSs are i) the occurrence of an 80-90 amino acid linker region found between the glycosyltransferase domain and the SPP domains and ii) the presence of regulatory phosphorylation sites. *Spinacia oleracea* (spinach) leaf SPS (SolSPS1F, Lutfiyya et al., (2007) nomenclature, see Table 4.1) contains three phosphorylation sites at Ser₁₅₈, Ser₂₂₉ and Ser₄₂₄, which are involved in light/dark regulation, 14-3-3 protein binding and osmotic stress activation, respectively (McMichael et al., 1993; Salvucci and Klein, 1993; Salvucci et al., 1995; Huber and Huber, 1996). Almost all known SPSs contain a consensus kinase recognition motif corresponding to the Ser₁₅₈ site in spinach. The serine (Ser₁₅₈) and the recognition elements critical for kinase function are highly conserved in SPS genes in families A, B and C. Similarly, phosphorylation motifs corresponding to spinach Ser₂₂₉ and Ser₄₂₄ are also present in most Family A, B and C angiosperm genes. The most notable exception is found in members of Family D (Castleden et al., 2004) which lack the corresponding Ser₂₂₉ and Ser₄₂₄ serines, and, while Ser₁₅₈ is present the basic and hydrophobic residues critical for kinase recognition are missing (Huber and Huber, 1996). D family enzymes are also smaller (~108 kDa) than ABC-type enzymes (~115 kDa-130 kDa) and lack the previously described linker region. Based on the absence of the phospho-regulatory sites and of the linker region, D family genes more closely resemble SPS from cyanobacteria than other plant SPSs. The loss of these features could suggest that either the D Family is the most ancient SPS group in plants, with the more complex regulatory features of ABC type SPS proteins having

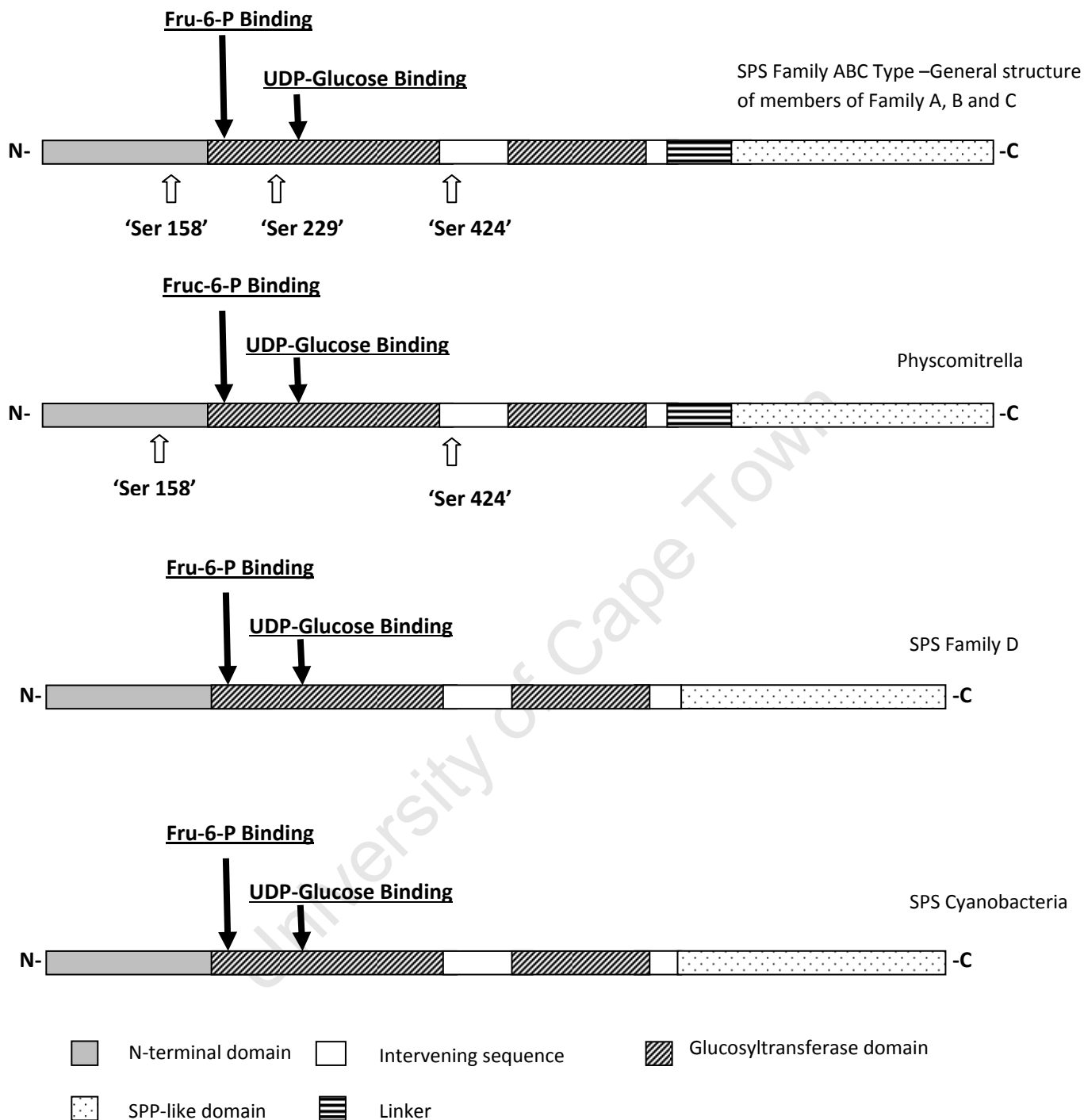


Figure 4.2 Basic protein structure of the Family ABC-type, *Physcomitrella*, Family D-type and bacterial SPS enzymes. The positions of the phospho-regulatory sites involved in light-dark regulation (Ser₁₅₈), 14-3-3 protein binding (Ser₂₂₉), and osmotic stress activation (Ser₄₂₄) of the plant ABC-type SPS are indicated by the arrows. The numbering is based on spinach amino acid sequence. The position of the Fructose-6-phosphate (Fru-6-P Binding) and UDP-Glucose binding sites are indicated.

evolved after divergence, or that Family D is the youngest SPS group, with the phospho-regulatory sites and linker regions having been lost from members of Family D.

SPS gene expression in angiosperms

Shared regulatory features such as the absence of all three phospho-regulatory sites in Family D and the presence of these sites in most of Family A is intriguing as it suggests that members of the same family may share common roles across different species e.g. constitutive, stress responsive and tissue specific. Family A transcripts are in general the most well represented in the EST database (MacRae and Lunn, 2006) and are usually constitutively expressed in a wide range of tissue types (MacRae and Lunn, 2006). In eudicots constitutive expression of Family A SPS genes has been reported for a number of species, including *Arabidopsis* SPS 5a (Gibon et al., 2004), Craterostigma 1 from *C. plantagineum* (Ingram et al., 1997) and Citrus 1 from *Citrus unshiu* (Komatsu et al., 1996) (see Figure 4.1 (ii) Lunn and MacRae (2006) nomenclature). In monocots constitutive expression of Family A members has also been reported for *Triticum aestivum* (wheat) (Castleden et al., 2004) *Zea mays* (maize) (Lutfiyya et al., 2007) *Saccharum officinarum* (sugarcane) (Sugiharto et al., 1997) and *Oncidium* cv. 'Goldiana' (Li et al., 2003) SPS genes. This expression data indicates that Family A type genes may function as the 'housekeeping' synthesizer of sucrose in plants. Notably, most Family A genes possess all three phospho-regulatory sites suggesting that the activity of this type of SPS isoform may be altered rapidly without the need for *de novo* protein synthesis. Thus, this type of isoform would be well suited to managing the dynamic sucrose needs of the cell. However, the inclusion of genes such as the stress induced *A. thaliana* gene, *Arabidopsis* 5b (see Figure 4.1 (ii)) (Gibon et al., 2004) also suggests that not all Family A genes are restricted to this 'housekeeping' role. It is possible that as more expression data becomes available more specific functional groupings within Family A may become apparent.

The *A. thaliana* B family gene, *Arabidopsis* 1 (see Figure 4.1 (ii)) appears to be expressed mainly in reproductive organs (stamens and developing seeds) (Gibon et al., 2004 supplemental

data). Similarly, the *Nicotiana tabacum* (tobacco) B family gene (see Figure 4.1 (ii)) is only lowly expressed in mature leaves and highly expressed in anthers and ovaries (Chen et al., 2005). The trend continues in *Hordeum vulgare* (barley) and *Triticum aestivum* (wheat) where B family members are only expressed in anthers and ovaries (Castleden et al., 2004). However, exceptions to the rule are found in *Z mays* (maize) (Lutfiyya et al., 2007) and *Oryza sativa* (rice) (Sakamoto et al., 1995) where B family genes are also the dominant transcripts in leaves. The picture is further complicated by B family genes which are induced by abiotic stress in *Actinidia deliciosa* (kiwi) (Fung et al., 2003) and in *C. plantagineum* (Ingram et al., 1997). Thus, while certain B Family genes are specific to reproductive structures, not all members of the family maintain this tissue specificity.

The C family *A. thaliana* gene, *Arabidopsis 4* (see Figure 4.1 (ii)), is highly expressed in leaves with transcript levels increasing during the night and decreasing during the day (Harmer et al., 2000; Gibon et al., 2004). This diurnal pattern of expression is also observed in the tobacco member of Family C genes (Chen et al., 2005) and it appears that these two Family C genes may be involved in night time sucrose synthesis from starch breakdown (Chen et al., 2005). In wheat and barley, Family C genes are highly expressed in leaf tissue (Castleden et al., 2004) but there is no data available on diurnal expression patterns. In contrast to wheat and barley, Family C genes are only poorly expressed in leaf tissues of rice, sugarcane, and *Sorghum bicolor* (sorghum) and not at all in maize (Castleden et al., 2004; Lutfiyya et al., 2007). In these species Family B genes are the dominant leaf tissue SPS genes.

The Family D cluster of genes lacks an *A. thaliana* representative and the group is populated only by grasses (Poaceae) at present. Temporal and spatial expression patterns of members of D-family vary greatly between species and there is no discernible shared pattern of expression. In many species, including sugarcane, wheat and maize, D family gene expression is detectable in leaves but these genes are not the dominant genes in leaf tissue (Castleden et al., 2004). One of the rice D family genes is upregulated in response to biotic stress (Castleden et al., 2004).

supplemental data) but based on all available expression data there appears to be no general relationship between D family genes and biotic stress.

The picture that emerges from the available expression data for SPS genes indicates that there may be a link between family groupings and shared functions, e.g. most of Family A genes being constitutively expressed and many of the Family B genes being specific to reproductive structures. However, as has been highlighted, not all members of a Family grouping necessarily share the same expression profile and it appears likely that there is a degree of functional redundancy amongst SPS genes. Thus, while the positioning of specific SPS genes in certain Family groups may hint at their functional roles, these roles will need to be confirmed by detailed expression studies.

SPS gene expression and desiccation tolerance

The expression of SPS genes during seed development has only been reported in a couple of species. In wheat seeds two SPS transcripts (TaSPSII and TaSPSIV) are upregulated in the late stage of seed development (Casteleden et al., 2004) but it is unclear whether this upregulation co-incides with the onset of desiccation tolerance. In *Vicia faba* seeds SPS transcript levels increase at the onset of desiccation tolerance but there is no information on the upregulation of particular genes (Weber et al., 1996). In angiosperm resurrection plants the expression of specific SPS genes has only been studied in one species, namely *Craterostigma plantagineum* (Ingram et al., 1997). In *C. plantagineum* two SPS encoding transcripts were identified, Cpsps1 and Cpsps2. In fully hydrated leaf tissue Cpsps2 levels are barely detectable (Northern Blot) but levels increase dramatically in response to dehydration. In this study changes in total SPS protein (Western Blot) were investigated but no data is provided on actual changes in the levels of the specific SPS isoforms. Ingram et al., (1997) propose that Cpsps1 performs a housekeeping function while Cpsps2 is involved in dehydration induced sucrose accumulation.

SPS gene expression: the need to measure both transcript and protein levels

In many studies it is often assumed that changes in gene expression at the transcript level translate into similar changes at the protein level. Hence, based on changes in transcripts alone particular genes are implicated in responses to external or internal stimuli. However, while significant changes in transcript levels are unlikely to have no biological significance, the relationship between changes in transcript levels and protein product may not always be simple. In yeast, comparisons of protein abundance and mRNA expression levels have revealed that protein levels can change independently of transcript levels (Horak and Snyder, 2002; Greenbaum et al., 2003). In *A. thaliana* changes in enzyme activities (indirect measure of protein abundance) were found to be dampened and strongly delayed when compared to changes in related transcript levels (Gibon et al., 2004; Gibon et al., 2006). In a more protein specific example, nitrate reductase (NR) protein increases twofold in leaves during the first part of the light period, whereas NR transcript decreases rapidly (Scheible et al., 1997). This discrepancy has been attributed to the effect of illumination on the synthesis and breakdown of NR protein (Kaiser et al., 1999; Weiner and Kaiser, 1999). Furthermore, in the study of resurrection species it has been observed that certain transcripts which are upregulated during dehydration are only translated during re-hydration (e.g. Illing et al., 2005). Thus, during dehydration the upregulation of particular transcripts may not necessarily have an immediate effect on the size of the associated protein pool. Taking the above into consideration it is clear that the measurement of transcript changes needs to be complemented with actual data on changes in protein level for a more complete picture of gene expression to be obtained.

Aims

The first aim of the following section of work was to identify all SPS genes in the monocot resurrection plant *X. humilis*. The transcriptome and the genome of *X. humilis* were searched using an RT-PCR/restriction fragment analysis based approach. Degenerate primers designed to highly conserved domains of known SPS proteins were used to amplify *X. humilis* SPS DNA. The resulting PCR products were subjected to RE digest to distinguish between different SPS

transcripts and all unique transcripts were sequenced to confirm their identity. Full length sequence information was obtained for all identified SPS genes and the phylogenetic relationships between *X. humilis* genes and other known SPS sequences established. Following on from the identification of all SPS genes, the hypothesis that dehydration results in the up regulation of a specific SPS isoform was investigated. Changes in both the transcript and protein levels of each of the identified SPS genes were monitored. Real-time quantitative PCR was used to determine the changes in the transcript levels of each identified SPS gene while a mass spectrometry based method, described as ‘Stable isotope absolute quantification’, was utilized to quantify changes in protein levels of each SPS isoform.

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Methods

Plant Material

Xerophyta humilis plants were maintained in a greenhouse with no supplementary lighting. Leaf and root tissue were harvested and immediately flash frozen in liquid nitrogen. Tissue samples were stored at -80 °C. Plants were dehydrated by withholding water. For leaf tissue relative water content (RWC) of tissues was determined as before (please refer to Chapter 2, pg 23). When root tissue was analysed, RWC was determined by the same method used to determine RWC in leaf tissue. Root and leaf tissue were not assumed to have the same RWC and hence the RWC of each tissue was determined independently.

RNA Extraction

Leaf tissue- TRI Reagent® extraction

Fifty to one hundred mg of leaf tissue was homogenized under liquid nitrogen with ~0.1 % w/w insoluble polyvinylpyrrolidone (PVPP) (Sigma). The frozen leaf powder was added piece meal to 1 ml TRI Reagent® (Molecular Research Centre, Inc.) in a 2 ml eppendorf tube. The extract was mildly shaken at 25 °C for 20 min and centrifuged at 12000g at 4 °C for 5 min to pellet insoluble material. The supernatant was transferred to a fresh new tube and kept at 25°C for 5 min. To separate the phases 1-bromo-3-chloropropane (BCP) was added to the supernatant at 0.1 ml BCP per 1 ml TRI Reagent®. The sample was shaken for 15 s and incubated at 25 °C for 10 min. After centrifugation (1200g at 4 °C for 15 min) 3 phases were visible, the upper RNA containing aqueous phase, the interphase and the lower phenol phase. The upper phase was transferred to a new tube and the RNA precipitated with 0.5 ml isopropanol per ml TRI Reagent® for 5-10 min at 25 °C. Precipitated RNA was collected by centrifugation at 12000g for 8 min at 25 °C. Following aspiration of the supernatant the RNA pellet was washed once with 1 ml 75 % ethanol and allowed to dry at room temperature. To re-suspend the RNA, the pellet was incubated in DEPC treated water at 55 °C for 10 min. The integrity of RNA was checked by agarose gel electrophoresis. RNA was quantified by UV spectrophotometry.

Root tissue -CTAB extraction

Modified method of Zeng and Yang, (2002).

Three ml of RNA Extraction Buffer (REB) (2 % CTAB, 2 % PVP (mol wt 25,000), 100 mM Tris-HCl (pH 8), 25 mM EDTA, 2 M NaCl, 0.05 % spermidine trihydrochloride, 2 % β -mercaptoethanol) was pre-warmed to 65 °C. One hundred to two hundred mg root tissue was ground under liquid nitrogen and the frozen root powder transferred directly to warm REB. The sample was mixed by tube inversion and incubated at 65 °C for 10 min with occasional vigorous shaking. An equal volume of chloroform-isoamylalcohol (24:1) was added to the sample and the sample was mixed by vigorous shaking. Insoluble material was pelleted by centrifugation at 10 000g for 10 min at 4 °C. The upper aqueous phase was transferred to new tube and re-extracted with an equal volume of chloroform-isoamylalcohol (24:1) followed by a repeat centrifugation at 10 000g for 10 min at 4 °C. The upper aqueous phase was transferred to a new tube and centrifuged at 30 000g for 20 min at 4 °C to pellet all insoluble material. The supernatant was transferred to new tube and 0.25 vol of 10 M LiCl added. The sample was mixed by inversion and stored at 4 °C overnight. Precipitated RNA was recovered by centrifugation at 30 000g for 30 min at 4 °C. After removal of the supernatant, the RNA pellet was washed in 75 % ethanol 3 times and allowed to dry. To re-suspend the RNA, the pellet was incubated in DEPC treated water at 55 °C for 10 min. The integrity of RNA was checked by agarose gel electrophoresis. RNA was quantified by UV spectrophotometry.

Genomic DNA Extraction

Modified method of Dellaporta et al., (1983)

One gram of leaf tissue was homogenized under liquid nitrogen and the frozen powdered leaf was transferred to a tube containing 15 ml DNA extraction buffer (DEB) (100mM Tris-Cl pH 8, 50mM EDTA pH 8, 0.5 M NaCl, and 0.01 M β -mercaptoethanol). One ml of 20 % SDS was added and the extract mixed vigorously. The extract was incubated at 65 °C for 10 min after which 5 ml of 5 M potassium acetate was added to the solution and the tube was shaken vigorously. The extract was incubated on ice for 20 min and centrifuged at 25 000g for 20 min.

The supernatant was transferred to a new tube and mixed with 10 ml isopropanol. After mixing by inversion the extract was incubated at -20 °C for 30 min to precipitate the DNA. The precipitated DNA was collected by centrifugation at 20 000g for 15 min. After aspiration of the supernatant the pellet was allowed to dry for 10 min. The pellet was re-suspended in 0.7 ml 50 mM Tris, 10 mM EDTA (pH 8.0). All insoluble material was removed by centrifugation at 12000g for 10 min. Fifty µl 3M sodium acetate and 100 µl 1 % CTAB was added to the supernatant to precipitate the nucleic acids and the DNA collected by centrifugation at 12000g for 5 min. After removal of the supernatant the pellet was washed once with 70 % ethanol and re-dissolved in 400 µl Tricine-EDTA (TE). To precipitate DNA, 50 µl 3 M sodium acetate and 1 ml ethanol was added and incubated at -20 °C for 30 min. The sample was centrifuged at 12000g to obtain final pellet. The pellet was allowed to dry and re-suspended in 10 mM Tris, 1 mM EDTA (pH 8.0).

Standard first strand cDNA synthesis

In a reaction volume of 20 µl, 2.5 µg total RNA was combined with oligo (dT) (125 ng) and random hexamer primers (125 ng) (Promega) and heated at 70 °C for 10 min. The sample was quick chilled on ice to maintain RNA in denatured state. Superscript® III 1st Strand Buffer (Invitrogen) was added to a final concentration of 1X. DTT was added to a final concentration of 10 mM and dNTPs were added to a final concentration of 5 µM. Twenty units RNasin® (Promega) inhibitor was added to prevent RNA breakdown. The sample was made up to a final volume of 20 µl with Rnase free water (Sigma). The reaction mixture was gently vortexed and equilibrated at 37 °C for 2 min. Two hundred units of Superscript® III reverse transcriptase (Invitrogen) was added and the reaction was allowed to proceed at 37 °C for 1 h. After 1 h an additional 200 units of Superscript® III reverse transcriptase (Invitrogen) was added and the reaction allowed to proceed at 37 °C for a further 1 h. The reaction was stopped by heat inactivation at 70 °C for 10 min.

Degenerate primer design

Thirty three known SPS amino acid sequences were aligned using ClustalW (Thompson et al., 1994). The universal primer set, SPS uf1 and SPS ur1a, was designed to anneal to highly conserved sites flanking a 200 amino acid variable region (see Figure 4.3). The forward universal primer, SPS uf1 was designed to anneal within the putative Fructose-6-P binding site while the reverse primer, SPS ur1a, was designed to anneal to a highly conserved region approximately ~700 bp downstream of this site (Figure 4.3). Based on the predicted intron/exon structure of all available *A. thaliana* and *Oryza sativa* (rice) full length genomic SPS sequences (see Appendix B), the forward universal primer, SPS uf1, was found to straddle an exon/intron boundary. Thus, to amplify SPS DNA from a genomic DNA template a new forward primer, SPS guf1, was designed to anneal ~20 bp upstream of SPS uf1 (Figure 4.3). To increase the specificity of the PCR reaction a new reverse universal primer, SPS gur1, was also designed with a lower degeneracy and a higher annealing temperature than the previously designed SPS ur1a. Table 4.2 displays the sequence of each degenerate primer and the expected product sizes of each primer set. The corresponding amino acid sequences are highlighted in Figure 4.3.

Table 4.2 Degenerate primers with minimum and maximum expected PCR product sizes. The level of degeneracy and the annealing used in the PCR for each primer is given.

Primers	^a Degen	^b Sequence (5'-3')	^c Expected size ^{cdNA}		^d Expected size ^{genomic}		^e Tm(°C)
			Min (bp)	Max	Min (bp)	Max (bp)	
SPS uf1	48	GAYACIGGTGGHCAGRTIAA					
SPS ur1a	128	CCAYTGYKCWTCWATYTCY	610	720	/	/	40
SPS guf1	6	GCGIGGIGARAAYATGGARC					
SPS gur1	32	GCCAYTGYTCITCWATYTCY	630	730	730	1130	56

^a Degeneracy, number of sequences in primer mix

^b Where I=Inosine, R=AG, Y=CT, M=AC, K=GT, W=AT, S=CG, B=CGT, D=AGT, H=ACT, V=ACG, N=ACGT

^c Based on alignment of 33 known SPS nucleotide sequences

^d Based on alignment of all available *A. thaliana* and *O. sativa* (rice) full length genomic SPS sequences (see **Appendix A**)

^e Annealing temperature used in PCR reaction

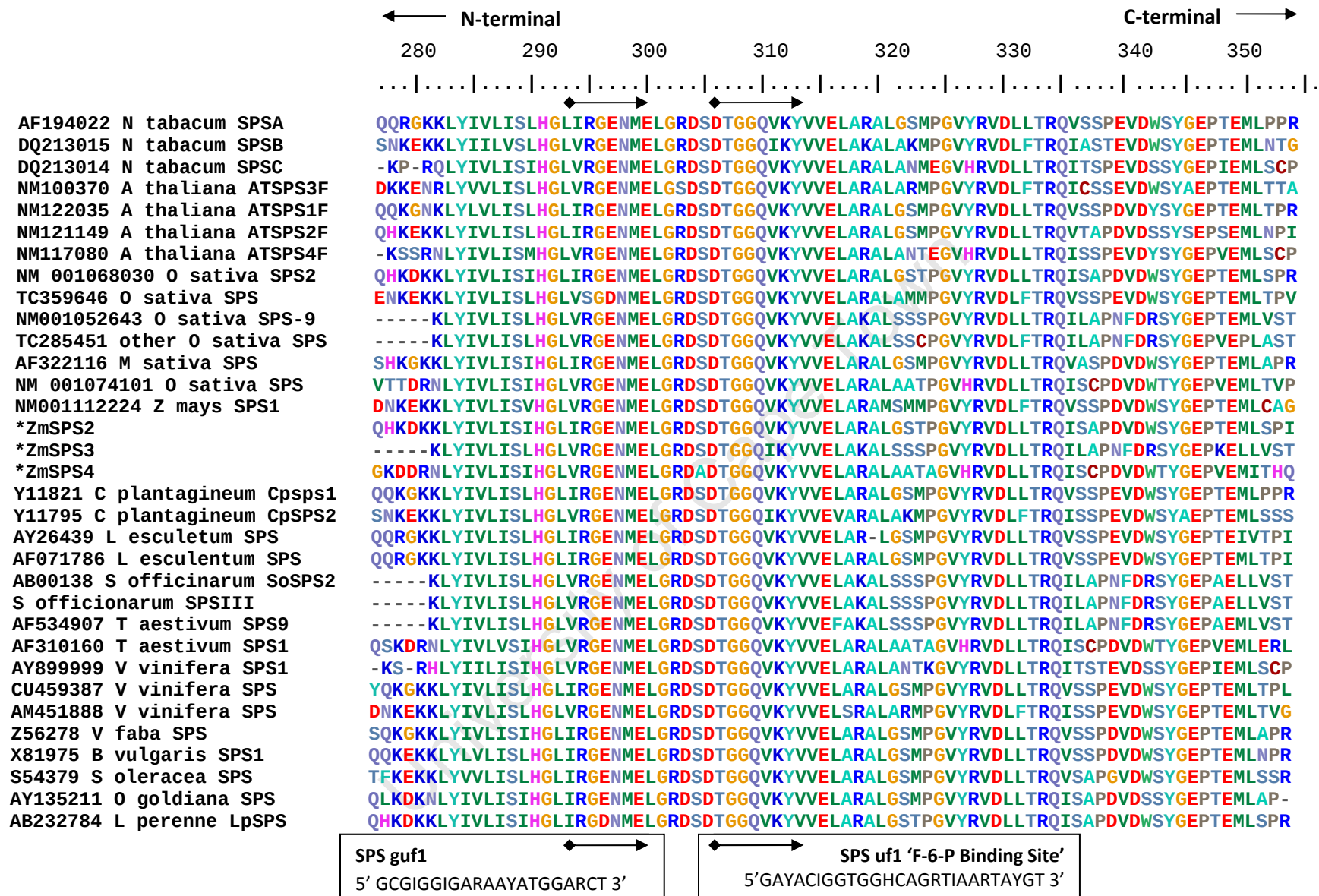


Figure 4.3 Primer positioning on aligned SPS amino acid sequences. Thirty three full length SPS amino acid sequences were aligned with ClustalW. The region between amino acid 278 and 356 (*N. tabacum* SPSA numbering) is shown. The degenerate nucleotide sequences for the primers SPS guf1 and SPS uf1 are displayed, where I=Inosine, R=AG, Y=C/T, M=A/C, K=G/T, W=A/T, S=C/G, B=C/G/T, D=A/G/T, H=A/C/T, V=A/C/G, N=A/C/G/T

* Designates *Z. mays* sequences obtained from supplemental data of Castleden et al., (2004)

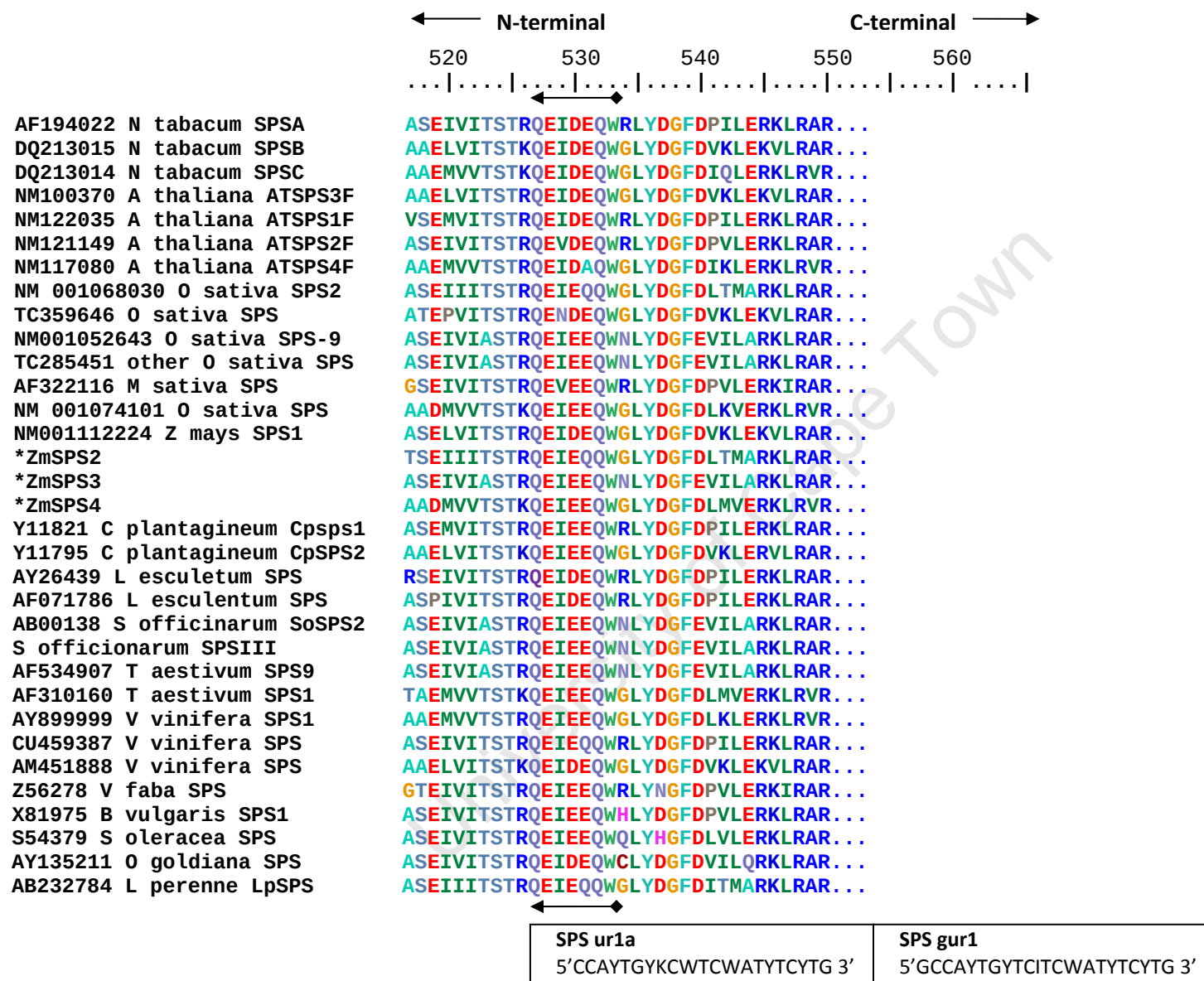


Figure 4.3 cont/ Primer positioning on amino acid sequence. The degenerate nucleotide sequences for the primers SPS ur1a and SPS gur1 are displayed.

* Designates *Z mays* sequences obtained from supplemental data of Castleden et al., (2004).

Amplification of SPS DNA by Polymerase Chain Reaction (PCR)

All PCR was conducted with SuperTherm Taq DNA Polymerase Mix (Southern Cross Biotechnology). All PCRs were performed in a GeneAmp PCR System 9700 (Perkin-Elmer). A total reaction volume of 25 μ l was used for each PCR reaction. The synthesized cDNA and extracted genomic DNA were diluted 1/100 and 2.5 μ l of each diluted template was used in their respective reactions. The PCR conditions and cycling parameters for each primer set are shown in Table 4.3. A final extension step of 7 min at 72 °C was added after the last cycle.

Table 4.3 PCR conditions and cycling parameters for universal primer sets.

Primers	Template	Primer conc (μ mol)	dNTPs (mM)	MgCl ₂ (mM)	Cycles	PCR Cycling parameters		
						Denature	^a T _m	Extension
SPS ufl	cDNA	0.6	0.2	1.5	35	94°C for 30s	40°C for 40s	72°C for 1min
SPS ur1a								
SPS gufl	gDNA	0.6	0.2	1.5	35	94°C for 30s	56°C for 40s	72°C for 1min 20s
SPS gur1								

^a Annealing temperature used in PCR

Cloning of PCR products

PCR products obtained with each set of degenerate primers listed in Table 4.3 (PCR described in previous section) were fractionated on 1.5 % agarose gels and visualized by UV illumination of ethidium bromide stained gels. After fractionation, individual bands of DNA were excised and the DNA extracted with the GENECLEAN II gel extraction kit (BIO 101 Inc.). The extracted DNA fragments were ligated into the pGEM-T vector (Promega) as per the pGEM-T Easy cloning system (Promega) instruction manual. Competent *E. coli* cells transformed with the PCR product/pGEM-T ligations were grown on 5-bromo-4-chloro-3-indolyl α -D-galactopyranoside (X-Gal) (Sigma-Aldrich) and isopropyl-beta-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich) containing Luria Broth (LB) agar plates which allowed for blue/white screening of bacterial colonies. Only white colonies (insert containing) were picked for further analysis.

Restriction enzyme digests of clones

Digest of non-amplified inserts

White colonies were picked and grown overnight at 37 °C in five ml LB media. Two ml of the LB cultures were purified using the High Pure Plasmid purification kit (Roche Applied Science) as per instruction manual. Four µl (~900 ng DNA) of the purified plasmid was double digested with the endonucleases *EcoRI* and *XbaI* (Fermentas) in a 20 µl reaction containing 1X final concentration of the appropriate buffer (Tango[®] Buffer, Fermentas). The digestion reaction was allowed to proceed at 37 °C for 4 h and was stopped by heat inactivation (75 °C for 10 min). The pGEM-T (Promega) vector contains *EcoRI* cutting sites in the region flanking the multiple cloning site, thus digestion with *EcoRI* resulted in the release of the insert from the vector. The digested inserts were fractionated on either a 2% (for cloned PCR product from cDNA template) or a 1.5% agarose gel (for cloned PCR product from genomic DNA template) and visualized under UV after ethidium bromide staining. All clones exhibiting distinct restriction fragment patterns were sequenced.

Colony PCR

The primer set SP6 and T7 was used to amplify cloned inserts in pGEM-T. SP6 and T7 are specific to sites flanking the multiple cloning site in the pGEM-T vector (Promega). The primers amplify a region of approximately 50 bp of the vector sequence on either side of the insert, as illustrated in Figure 4.4

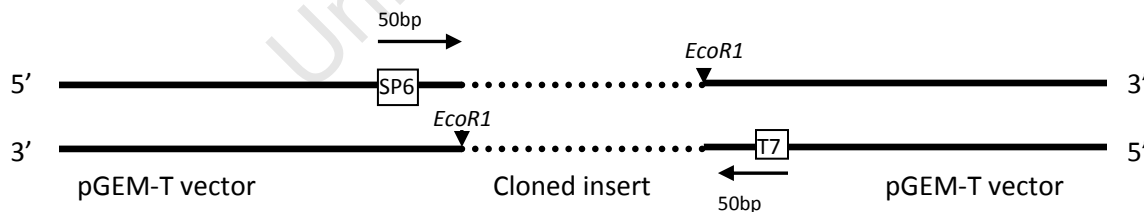


Figure 4.4 Positioning of SP6 and T7 primers on Pgemt vector. The *EcoRI* cutting sites area also indicated.

Colonies were picked and allowed to grow overnight in a 5 ml LB medium. One µl of the culture was used as the template in a 25 µl total volume PCR reaction. The SP6 and T7 primers were used at a final concentration of 0.5 µM, dNTPs at a final concentration of 0.2 mM and

MgCl₂ at a final concentration of 1.5 mM. A total of 25 cycles were carried out with an initial denaturation step of 94 °C for 30s, an annealing step of 52 °C for 30s and an elongation step of 72 °C for 1 min. A final extension step of 7 min at 72 °C was added after the last cycle.

Digest of PCR amplified inserts

Five µl of the PCR reaction (SP6 and T7 primers) was fractionated on a 1.5 % agarose gel to confirm amplification of the insert. Five µl of the PCR reaction (no purification) was double digested with the endonucleases *EcoRI* and *XbaI* (Fermentas) in a 20 µl reaction containing 1X final concentration of the appropriate buffer (Tango[®], Fermentas). The digestion reaction was allowed to proceed at 37 °C for 2 h and was stopped by heat inactivation (75 °C for 10 min). The digested products were fractionated on a 1.5 % or 2 % agarose gels and visualized with ethidium bromide stain. All clones exhibiting distinct restriction fragment patterns were sequenced.

Rapid Amplification of cDNA Ends (RACE)

RACE ready cDNA synthesis

A total reaction volume of 10 µl was used for preparation of 5'-RACE-Ready cDNA. cDNA was synthesized from RNA extracted from a pool of leaf tissue (at different stages of drying between 100 and 5 % RWC). Two and a half µg of total RNA was used and the 5'-CDS primer A and SMART II[™] A oligonucleotide (BD Biosciences Clontech) were added to a final concentration of 1 µM. The RNA and primers were denatured at 70 °C for 2 min and quick chilled on ice. Two hundred units of 'First strand buffer' was added to a final concentration of 1X (50 mM Tris-HCl (pH8.3), 75 mM HCl, 6 mM MgCl₂) (BD Biosciences Clontech), DTT was added to a final concentration of 2 mM and dNTPs were added to a final concentration of 1 mM. The reaction was started with the addition of 200U of SuperScript III M-MLV RNaseH⁻ (BD Biosciences Clontech) and allowed to proceed at 42 °C for 1.5 h. The reaction was diluted with 100 µl Tricine-EDTA Buffer (10mM Tricine-KOH (pH8.5), 1mM EDTA) and incubated at 70 °C for 7 min to stop the reaction. The 3'-RACE-Ready cDNA was synthesized using the same procedure

but with the replacement of the 5'-CDS and SMART II™ A oligonucleotide (BD Biosciences Clontech) with 1 µM of the 3'-CDS primer. Sequences of primers are shown in Table 4.4.

Table 4.4 RACE cDNA synthesis primers

SMART II™ A Oligonucleotide
5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'
3'-RACE CDS Primer A (3'-CDS)
*5'-AAGCAGTGGTATCAACGCAGAGTAC(T)30V N-3'
5'-RACE CDS Primer A (5'-CDS)
*5'-(T)25V N-3'

*(N = A, C, G, or T; V = A, G, or C)

RACE PCR

PCR was conducted in a total volume of 25 µl and 2.5 µl of 5'- or 3'- RACE-Ready cDNA was used as the template. The reaction included 1X universal primer mix (UPM), 0.2 µM gene specific primer (GSP), 1X Advantage 2 PCR buffer [40mM Tricine-KOH, pH 8.7; 15 mM KOAc; 3.5 mM Mg(OAc)₂; 3.75 µg/ml BSA; 0.005 % Tween-20; 0.005 % Nonidet-P40], 0.2 mM dNTPs and 1X Advantage 2 Polymerase mix (BD Biosciences Clontech). Cycling parameters as recommended by the BD SMART™ RACE cDNA Amplification Kit User Manual were used. The primers used are described in Table 4.5.

Table 4.5 5' and 3' RACE PCR primers

Primer	Primer
Universal Primer A Mix (UPM)	5' RACE Gene specific primers
Long 5' CTAATACGACTCACTATAGGGCAAGC -AGTGGTATCAACGCAGAGT 3'	XhSPS1GSP1 5'ATTACCATCTGCGTCATATTGGCCT 3'
Short 5' CTAATACGACTCACTATAGGGC 3'	XhSPS2GSP1 5'CACATCTGTACGAGGTGGAAGCATCTC 3'
Nested Universal Primer A (NUP)	
NUP 5' AAGCAGTGGTATCAACGCAGAGT 3'	
3' RACE Gene specific primers	
XhSPS1GSP2 5'ACTTGCCAGGGCATTGTCAATGATG 3'	
XhSPS2GSP2 5' TTGCAAGAGCTTTAGGTGCAATGC 3'	

Sequencing

DNA fragments were sequenced in two directions using Big Dye terminator v3.1 Cycle Sequencing kit as per manufacturer's protocols (Applied Biosystems). In each sequencing run a maximum of 700 bp could be confidently identified. All DNA fragments in excess of 700 bp were sequenced to completion by a process of primer walking. This process entailed extending sequence information in ~700 bp stretches. After each extension new primers were designed based on the new sequence. Once full sequence information was obtained and edited, regions of overlapping sequence were used to manually construct the full length DNA sequence.

Bioinformatics

Sequences of putative SPS clones were compared with known nucleotide databases at the NCBI (<http://www.ncbi.nlm.nih.gov>) using the BLAST (blastn) algorithm (Altschul et al., 1990). For re-construction of the phylogeny of all known full length SPS sequences, sequences were sourced by searching the NCBI amino acid database (<http://www.ncbi.nlm.nih.gov/>) with the keyword 'sucrose phosphate synthase'. A further 3 maize SPS full length protein sequences were sourced from EST compilations in the published supplemental data of Castleden et al., (2004). The TIGR EST database (Gene Index Project, <http://compbio.dfci.harvard.edu/tgi/>) was also searched using the BLASTn tool with a known monocot and eudicot SPS nucleotide sequence used as the query. Sourced sequences were aligned using ClustalW (Thompson et al. 1994), and alignment was manually edited in Bioedit (Hall, 1999) (see Appendix D). Only the coding regions, demarcated by start and stop codons, were used in the alignment. Phylogenetic analysis to determine the evolutionary relationship between SPS genes was conducted using *MEGA* version 4 (Tamura et al., 2007). For tree construction, evolutionary distances were calculated using Dayhoff matrix model (all gaps deleted) (Dayhoff et al., 1979). A neighbor-joining (NJ) tree was designed from these distances utilizing the neighbor joining algorithm (Saitou and Nei, 1987) as implemented by the *MEGA*. Bootstrap analysis (Felsenstein, 1985) was conducted on all trees with 1000 bootstrap replicates. After tree construction the line leading to the *Physcomitrella* SPS sequences was designated as the root branch.

SPS gene expression

Harvesting, processing and relative water content RWC determination of leaf tissue prior to RNA extraction is illustrated and described in Chapter 2 (see Figure 2.1, pg 24). For each pool of harvested leaf tissue approximately 200 mg (fresh weight) of non-freeze dried homogenized leaf tissue was extracted for total RNA (RNA extraction described previously in Chapter 3). Five µg of total RNA was used to synthesize cDNA (cDNA synthesis also described previously in Chapter 3). This synthesized cDNA was used as a template for Real-Time quantitative PCR. For each pool of leaves harvested at a particular point during desiccation a single RNA extraction and a single cDNA synthesis reaction was conducted. Real-Time quantitative PCR for each SPS gene was conducted for three technical repeats.

Real-Time quantitative PCR (RTqPCR)

RTqPCR was performed in 25 µl reaction mixtures containing Sensimix Taq DNA polymerase mixture (Quantace Ltd, UK), which included reaction buffer, heat-activated Taq DNA Polymerase, dNTPs, MgCl₂. The fluorescent dye SYBR® Green (Quantace Ltd) was added to a final 1X concentration and the MgCl₂ concentration was adjusted to a final concentration of 3mM. The concentration of each primer is shown in Table 4.6. cDNA template was diluted 1/10 and 2.5 µl of the diluted cDNA used in each reaction. Real-time reactions were conducted in 100 µl thin-walled tubes and monitored using a Rotor Gene 3000 (Corbett Research, Sydney, Australia). The primer sets (refer to Table 4.6) RTSPS1.2R/RTSPS1.2F and RTSPS2R/RTSPS2F were used to amplify a 100bp fragment of the XhSPS1 transcript and the XhSPS2 transcript respectively. The primer set Kdelr2F/Kdelr2R was used to amplify a reference gene (R1) which was used for normalization. For amplification of XhSPS1 thermal cycling consisted of an initial denaturation at 95 °C for 10 min followed by 50 cycles of denaturation at 94 °C for 14 s, annealing at 60 °C for 14 s, and extension at 72 °C for 15s. For amplification of R1 thermal cycling consisted of an initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 94 °C for 14 s, annealing at 60 °C for 15 s, and extension at 72 °C for 20 s. For amplification of XhSPS2 thermal cycling consisted of an initial

denaturation at 95 °C for 10 min followed by 45 cycles of denaturation at 94 °C for 14 s, annealing at 60 °C for 15 s, and extension at 72 °C for 16 s.

Table 4.6 Primers used for Real-Time quantitative PCR. The Primers were designed with the assistance of Primer 3 Software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). The final concentration of the primer in the PCR reaction is shown.

Primer Name	Sequence	Tm	Final primer conc.	Gene
SPS1.2R	5' GTGTGCTGCAGCCTCAATAA 3'	59.8	400nM	XhSPS1
SPS1.2F	5' TCTACCGACTTGCTGCAAAA 3'	60.1	400nM	XhSPS1
SPS2F	5' CATCTGAATGCCACCTGAAA 3'	60	500nM	XhSPS2
SPS2R	5' GCGTGATTCAAATCGAAACC 3'	59.9	500nM	XhSPS2
Kdelr2F	5' TCTACCGCGCTCTGTACCT 3'	58.6	500nM	Reference gene
Kdelr2R	5' AGAGAATGGTCTGGACGACAC 3'	59.2	500nM	Reference gene

Fluorescence data were acquired on the FAM channel (excitation at 470 nm, detection at 510 nm) at the end of each 72 °C extension step of the PCR. Following amplification, melting curves were acquired on the FAM channel using a hold of 45 s on the first step and 5 s on the next step from 72 to 95 °C. The differentiated data were analyzed using the Rotor Gene software (v6.0 Build 27) ©Corbett Research 2004 with the digital filter set as “none.” For accurate quantification of transcript levels, cDNA synthesized from RNA extracted from tissues at different RWCs was pooled and used for construction of the standard curve. The concentration of the pooled cDNA was determined spectrophotometrically. The pooled cDNA was used to make up standards of known cDNA concentrations. The standards were included in all Real-Time PCR runs and a standard curve was generated by Rotor Gene software (v6.0 Build 27) ©Corbett Research 2004. Transcript levels of XhSPS1 and XhSPS2 were normalized to transcript levels of the reference gene R1.

Analysis of SPS gene expression in A. thaliana

For vegetative tissue expression of the *A.thaliana* SPS genes data were collected from the **Genevestigator** (Zimmermann et al., 2004; 2005) website (www.genevestigator.ethz.ch).

SPS protein identification in crude leaf extract

Harvesting, processing and RWC determination of leaf tissue prior to protein extraction for SPS identification is illustrated and described in Chapter 2 (see pg 24). The overall procedure followed to identify SPS protein in crude leaf extract is illustrated in Figure 4.5

Protein extraction

Protein was extracted according to the method of Weckwerth et al., (2004) with slight modifications. Thirty mg freeze dried leaf tissue (homogenized material) was re-ground under liquid nitrogen in the presence of 2 % (w/w) PVPP. The re-ground tissue was added directly to a centrifuge tube containing ice cold methanol:chloroform:water in a ratio of 2.5:1:0.5. The tissue was washed 3X (10 min shaking per wash) to reduce soluble non-protein contaminants. All washes were done at 4 °C. After washing the remaining pellet was solubilised in protein extraction buffer (PEB) (50mM HEPES-KOH, 40 % Sucrose (w/v), 1.5 % β -mercaptoethanol, 4 μ M leupeptine, 1 mM benzamidine, 60 μ M NaF, 0.3 μ M microcystin). A commercially obtained protease inhibitor cocktail (Sigma-Aldrich) was also added to PEB at 5 μ l per ml PEB. The β -mercaptoethanol was added fresh. PEB was added to freeze dried leaf tissue in a 8:1 buffer to tissue ratio. Three volumes of TE-buffer equilibrated phenol was added to the extraction. The mixture was shaken at 4 °C for 30 min. The mixture was centrifuged in a swing out rotor at 2000 rpm (4000 g) for 10 min at 4 °C. The upper phenol phase was removed and collected in a new 15 ml tube. The remaining pellet was re-extracted with the addition of new phenol (1.5 X volume). The second phenol phase was combined with the first and was back-extracted with fresh PEB. The final phenol phase (after back extraction) was transferred to a pre-cooled centrifuge tube and proteins were precipitated by adding 5 volumes of ice cold 0.1 M Ammonium Acetate (in 100 % methanol). Proteins were allowed to precipitate at -20 °C for 2 – 12 h. Proteins were collected by centrifugation at 20 000g for 10 min and the pellet washed 1X with 0.1 M ammonium acetate (in 100% methanol) and 2X with 100 % methanol. A final wash was carried out with 80% acetone. Between washes, the mixture was incubated at -20 °C for 10min. After the final acetone wash, an aliquot of the pellet/acetone suspension was removed for total soluble protein quantification (described below). The remaining pellet was

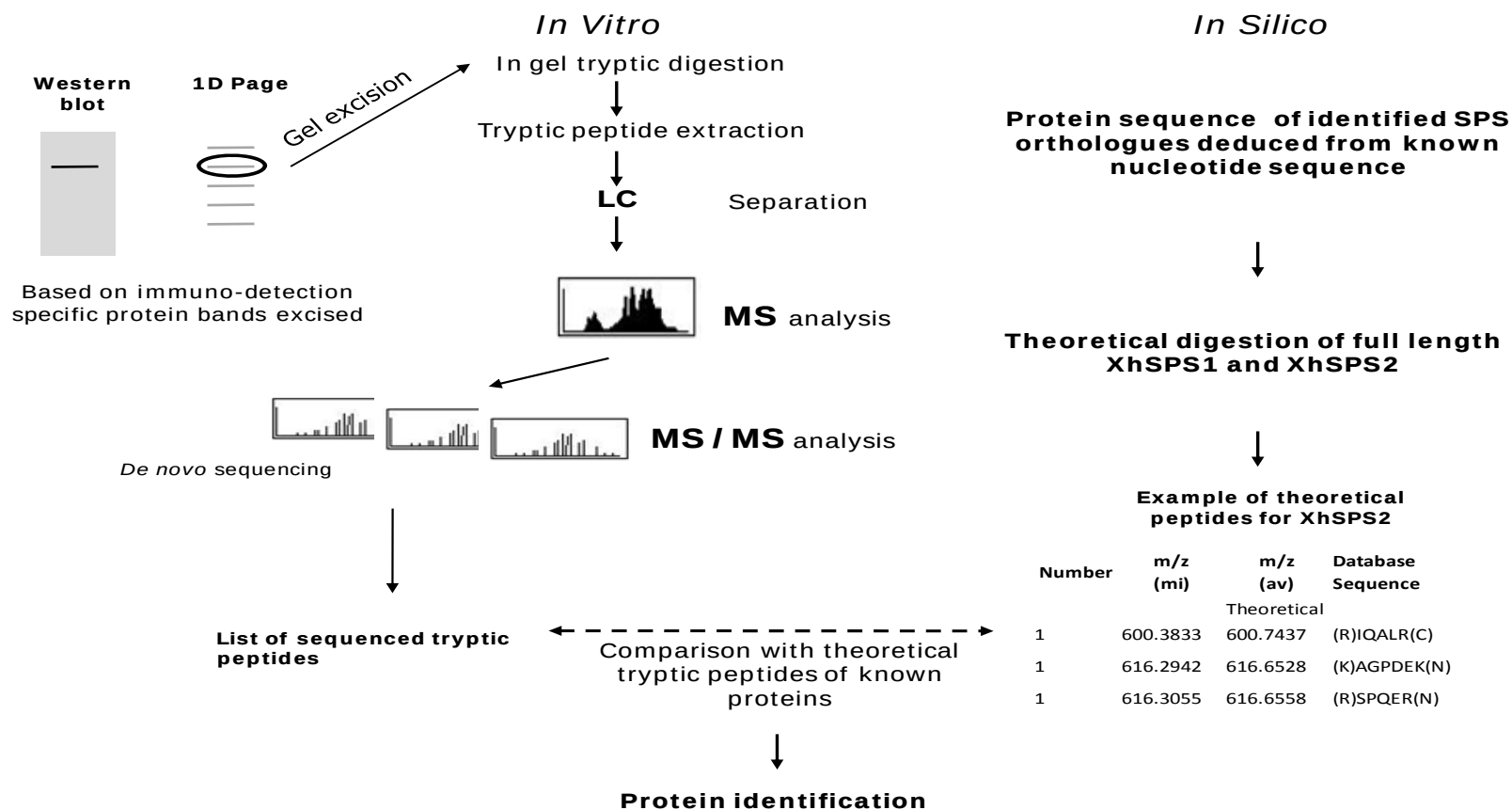


Figure 4.5 Flow diagram of experiments used to identify SPS protein in crude leaf extract. LC- Liquid chromatography, MS – Mass spectrometer, MS/MS Tandem Mass spectrometer

collected by centrifugation and the acetone removed. The pellet was allowed to dry but not to completion. The pellet was re-dissolved in 1X SDS sample loading buffer (2 % SDS, 15 % glycerol, 62.5 mM Tris-HCl (pH 6.8) , 0.01 % bromophenol blue and 5 % β -mercaptoethanol).

Total soluble protein quantification

To quantify the amount of total soluble protein extracted an aliquot of the final acetone protein wash was removed and the pellet collected by centrifugation. The pellet was allowed to dry but not to completion. The pellet was re-dissolved in 2 % SDS and total protein was quantified using the BCA TM Assay. Bovine serine albumin (BSA) was used to make up standards for construction of the standard curve.

Protein separation – 1-D polyacrylamide gel electrophoresis (1-D Page)

Proteins extracted from leaf tissue were separated on an 8% Polyacrylamide gel (8X10cm, 1 mm thickness) with an estimated 40 μ g of total protein loaded in each well (3 lanes per gel). A pre-stained broad range (6.5 – 175 kDa) marker (Cell Signalling Technology, Danvers, USA) was included as a size standard on each gel. Proteins dissolved in 1X SDS sample loading buffer (2% SDS, 15 % glycerol, 62.5mM Tris-HCl (pH 6.8) , 0.01 % bromophenol blue and 5% β -mercaptoethanol) were subjected to electrophoresis at a constant voltage of 100V for 1 h, followed by half an hour at 120V. Duplicate gels were run with one gel (referred to as ‘tpGel’) being stained for total protein using colloidal Coomassie (10 % Ammonium sulphate, 0.1 % Coomassie G-250, 3 % ortho-phosphoric acid, 20 % ethanol, stained for 8 h) while proteins from the accompanying gel (‘wbGel’) were transferred to a nitrocellulose membrane for western blot analysis.

Immunodetection of SPS protein (Western Blot)

After electrophoresis, the gel (‘wbGel’ see above) was washed in water and pre-equilibrated in methanol containing transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, 20 % methanol). The nitrocellulose membrane (GE, Minnetonka, USA) was pre-wetted in transfer buffer (5 min)

and placed in direct contact with the gel. Separated proteins were transferred to the nitrocellulose membrane using the semi-dry FastBlot (Biometra, Göttingen, Germany) system. The gel and membrane were sandwiched between sheets of blotting paper and placed in the Fastblot transfer apparatus. Transfer was conducted at 100V for 2 h at 4 °C. The membrane was blocked for 12 h in 5% fat free milk TBS (150 mM NaCl, 50 mM Tris) at 4 °C. After blocking the membrane was incubated with the primary antibody, global anti-sucrose phosphate synthase (Agrisera, Sweden), at a 1:2000 concentration for 1 h. Detection of primary antibody was conducted using the amplified alkaline phosphatase goat anti-rabbit immune-Blot assay kit (Biorad). Membrane washing, incubation with the secondary antibody and the final colourimetric assay were all conducted as per instruction manual of the kit. Both the primary and secondary antibody solutions were made up in 2 % fat free milk TTBS (150 mM NaCl, 50 mM Tris and 1 % Tween-20).

Excision of putative SPS protein bands and 'in gel' tryptic digestion

After staining with colloidal Coomassie the gel ('tpGel' refer to 1 D Page section above) was destained briefly with water to visualize protein bands. Based on the position and molecular weight of the putative SPS protein bands identified in the western blot analysis, protein bands of equivalent size were excised from the gel. The gel slices were cut into 1mm x 1mm squares and transferred to 1.5 ml eppendorf tubes. Gel pieces were washed with 200 µl water by vortexing for 15 min. The water was removed and replaced with 200 µl 100 mM ammonium bicarbonate (NH_4HCO_3). The mixture was vortexed for 10 min and 200 µl acetonitrile was added followed by a further vortex for 10 min. The liquid was removed and the gel pieces were washed a second time with 50 % acetonitrile/50 mM NH_4HCO_3 . All liquid was removed and the gel pieces were dried under vacuum for 5 min. Twenty five µg of trypsin (Trypsin Gold TM, Promega) was dissolved in 50 µL 1 mM HCl and kept on ice. Digestion buffer (25 mM NH_4HCO_3 , 10% acetonitrile, 5 mM calcium chloride (CaCl_2)) was added to the dissolved trypsin to give a final trypsin concentration of 12.5 ng per µl to make up trypsin buffer (TB). Approximately 30 µl TB was added to the dried spots. After twenty minutes an additional 5-10 µl of TB was added to ensure that all the gel pieces were covered by liquid. After ca. 45 min the gel pieces were

washed in buffer (without trypsin) by adding 100 µl buffer and immediately removing all liquid. A sufficient amount of fresh buffer to cover the gel pieces was then added and the gel pieces were incubated at 37 °C for 12-14 h.

Tryptic peptide extraction

Thirty µl of 5 % formic acid was added to the gel pieces and the mixture vortexed for 5 min. The liquid was transferred to a new 1.5 ml collection tube. This was followed by the addition of 30 µl of 1 % formic acid /5 % acetonitrile (ACN) and vortexing for 45 min. The liquid was transferred to the collection tube. The extraction was repeated in sequence with 30 µl 1% formic acid /50% ACN and 30 µl 1 % formic acid /90 % ACN. The collected extracts were dried down under vacuum and the extracted protein re-dissolved in 15 µl 5 % formic acid (FA).

Setup and running conditions of LC system and Mass spectrometer for protein identification

For the protein identification a reverse phase monolithic column (Merck, Darmstadt, Germany) of 15 cm length and 0.1 mm ID was coupled to the Orbitrap LTQ XL mass spectrometer (Thermo Electron). Peptides were eluted during a 50 minute gradient from 5 % to 100 % methanol / 0.1 % FA with a controlled flow rate of 0.5 µl per minute. Precursor mass was determined in the Orbitrap with a resolution of 3000 and an accuracy of 5 ppm. MS/MS was performed with the Orbitrap-LTQ in wideband mode. Spray voltage was set to 2.2 kV; temperature of the heated transfer capillary was set to 150 °C.

Protein identification by comparison to deduced SPS amino acid sequence

After mass spectrometric analyses, DTA files were created from raw files and were then searched against a user selected database for protein identification using Bioworks 3.2 software featuring the Sequest search algorithm (ThermoElectron, Dreieich, Germany). The database included all annotated *A. thaliana* proteins (<http://www.arabidopsis.org/>), the full length XhSPS1 and XhSPS2 SPS deduced amino acid sequences, a trypsin sequence and a keratin (human) sequence. Automatic analysis of SEQUEST results was performed using DTA Select (Tabb et

al., 2002) and a list of identified proteins was obtained using the following criteria: normalized difference in correlation score (DCn) of at least 0.08. The minimum accepted cross correlation (Xcorr) for peptides with a +1 charge state was 2.0. For peptides with +2 charge state the minimum Xcorr was 2.2 and for peptides with a +3 charged state an Xcorr of > 3.5 was required. At least three different peptides per protein were required for identification of the protein. For mass tolerances LTQ default settings were being used (precursor ion tolerance 1.4 amu; peptide tolerance 1.5 amu).

Absolute quantification of SPS protein during dehydration

Absolute quantification of the identified SPS isoforms was achieved using a modified method of Gerber et al., (2003). This mass spectrometer based quantification technique is described as ‘Stable Isotope Absolute Quantification’. Essentially, quantification of the targeted protein (more accurately a peptide of the protein) is achieved by diluting the biological sample with a known amount of a synthetic stable isotope (^{13}C , ^{15}N) labeled version of the peptide (synthetic peptide standard) and comparing abundances of the introduced peptide with that of the native peptide. These standards are identical to the analyte peptides of interest but are distinguished by the mass difference. Thus ^{13}C and ^{15}N isotopes ensure the co-migration of the labeled and unlabeled peptides during chromatography. After the chromatographic step(s) (Liquid Chromatography- LC) absolute quantification is achieved by comparison of the abundances (peak areas) of the internal standard peptide with the corresponding native counterpart utilizing multiple reaction monitoring (MRM) via tandem MS (Gerber et al., 2003).

Harvesting, processing and RWC determination of leaf tissue prior to protein extraction for SPS quantification is illustrated and described in Chapter 2 (see pg 24). The methods used for protein extraction and quantification of total soluble protein are the same as the methods used previously for the initial SPS protein identification. The overall procedure followed to quantify SPS protein in crude leaf extract is illustrated in Figure 4.6.

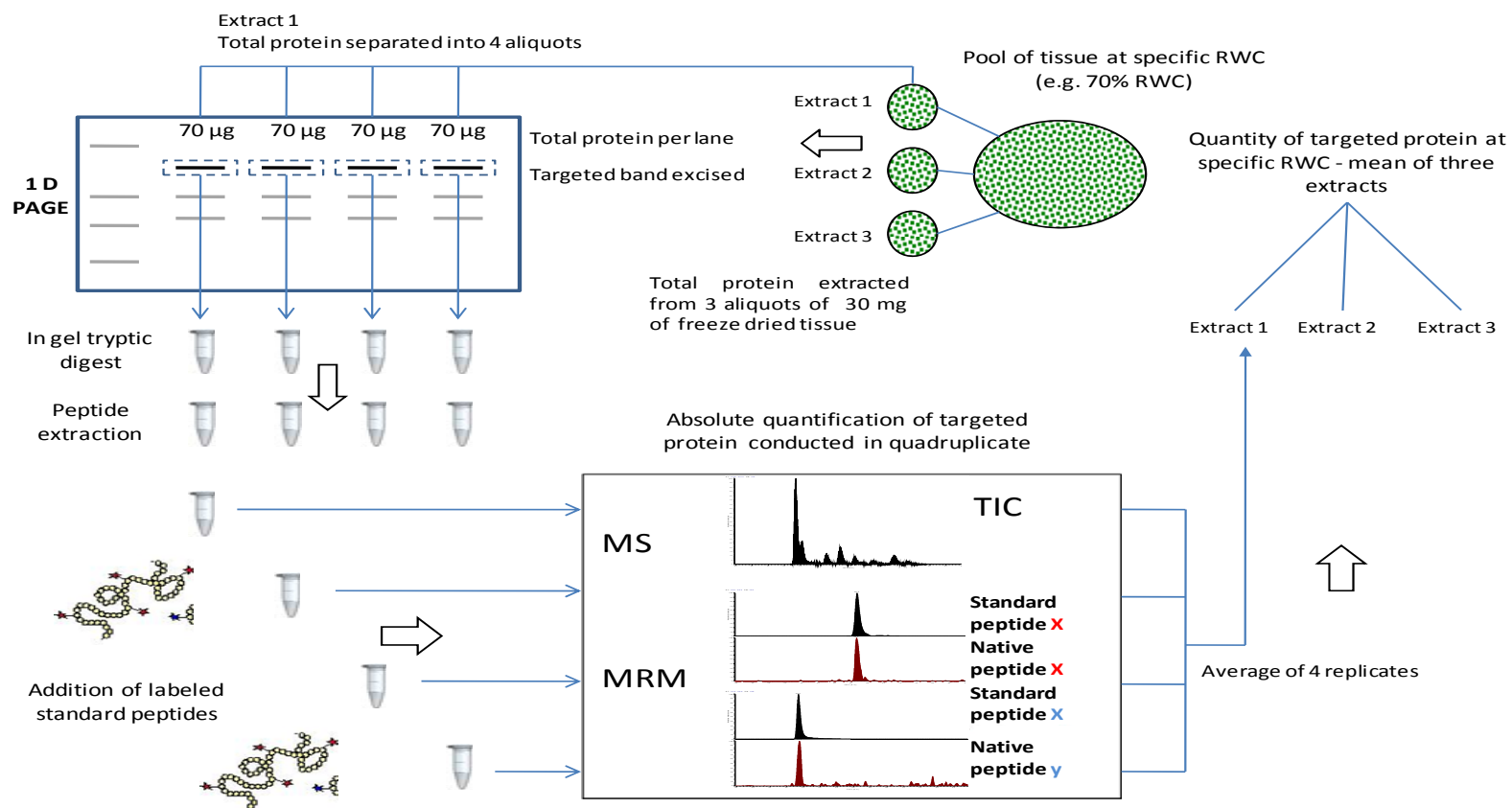


Figure 4.6 Illustration of quantification procedure for targeted proteins. MS-Mass spectrometer, TIC – Total ion current MRM- Multiple reaction monitoring. The illustration details the quantification of targeted protein (XhSPS1 and XhSPS2) in total protein obtained from a single protein extract (Extract1). Targeted protein was quantified in a total of three extracts (Extract 1, 2, and 3) for each pool of harvested leaf tissue.

For each pool of harvested leaf tissue, a total of three 30 mg aliquots of homogenized freeze dried leaf tissue were extracted for total soluble protein. Quantification of SPS protein in each of the three protein extracts was conducted in quadruplicate. Prior to quantification, proteins in crude leaf extract were separated by 1 D Page to reduce the complexity of the sample

Protein extraction and protein separation - 1 D Page

Thirty mg of homogenized freeze dried leaf tissue was extracted. Total protein was extracted and quantified as before (in section on SPS protein identification in crude leaf extract). Proteins were dissolved in 1X SDS sample loading buffer (2 % SDS, 15 % Glycerol, 62.5 mM Tris-HCl (pH 6.8), 0.01 % bromophenol blue and 5 % β -mercaptoethanol) and were subjected to electrophoresis on an 8 % Polyacrylamide gel (12X10cm, 1 mm thickness) at a constant voltage of 100V for 1 h, followed by half an hour at 120V. An estimated 70 μ g of total protein was loaded in each well (4 lanes per gel). A pre-stained broad range (6.5 – 175 kDa) marker (Cell Signalling Technology, Danvers, USA) was included as a size standard on each gel. After electrophoresis the gel was stained for total protein using colloidal Coomassie (10% Ammonium sulphate, 0.1 % Coomassie G-250, 3 % Ortho- Phosphoric acid, 20 % ethanol, stained for 8 h).

'In gel' tryptic digestion

After staining with colloidal Coomassie the gel was destained briefly with water to visualize protein bands. Based on the position and molecular weight of the identified SPS protein bands, protein bands of equivalent size were excised from the gel. In gel tryptic digestion was conducted as described previously (refer to pg 114 'Excision of putative SPS protein bands and 'in gel' tryptic digestion' in section on SPS protein identification in crude leaf extract).

Tryptic peptide extraction

Tryptic peptide extraction was conducted as described previously (refer to pg 115 'Tryptic peptide extraction' in section on SPS protein identification in crude leaf extract).

Generation of stable isotope-labelled internal standard peptides

Specific internal standard peptides for the LC/MS analysis of XhSPS1 and XhSPS2 were chosen for stable-isotope ($^{13}\text{C}/^{15}\text{N}$) labeling (Thermo Electron, Ulm, Germany). Based on the full nucleotide sequence obtained for each isoform in Chapter 4, the deduced amino acid sequences were tryptically digested in silico and specific peptides chosen for synthesis of the labeled and non labeled peptide standards. Detailed information about peptides and specific single reaction monitoring transition data are shown in Table 4.7. Figure 4.7 displays the positioning of the tryptic peptides on aligned regions of the XhSPS1 and XhSPS2 deduced amino acid sequences.

Table 4.7 List of target peptides and parameters for the quantification of four different SPS isoforms using LC/MS based high resolution multiple reaction monitoring. STD – Standard, CE-Collision energy

Name	Peptide Sequence	STD MW (Da)	Native MW (Da)	STD			Native		
				Precursor Ion (m/z)	Product Ions (m/z)	CE	Precursor Ion (m/z)	Product Ions (m/z)	CE
XhSPS1-Q1	SGAAVVEHFNPTR	1394.697	1384.697	697.8	1009.5	30	692.8	999.5	30
XhSPS1-Q2	LQVIPLLASR	1115.704	1109.704	558.3	662.4	20	555.3	656.4	20
XhSPS2-Q1	ALGAMPGVYR	1041.545	1034.545	521.2	591.3	20	517.7	591.3	20
XhSPS2-Q2	IGSAETFDWANQ QK	1675.787	1665.787	838.3	588.3	27	833.397	588.3	27

XhSPS2	82	---LLLRRERGRFSPT-AYFVEEVISGFDETDLYKSWVRAAATRSPQERNTRLENMCWRIW
XhSPS1	94	QKR <u>SGAAVVEHFNPTR</u> VYLRWRRLRGWTRPIFTGHGSRSSPHEAPANEARASRNCWRIW
<i>XhSPS1-Q1</i>		
<i>XhSPS2-Q2</i>		
XhSPS2	109	ARREAAADMSEDLSEGEKGDHAGDASAHGDSHRGRMPRI <u>IGSAETFDAWANQQ</u> KEKKLYIV
XhSPS1	121	GRRDATEDMQEDLSEGEKGDT---VSELSQSETPKKKLQRNVSDIQVWSDDNKSKKLYIV
<i>XhSPS2-Q1</i>		
XhSPS2	253	VVELAR <u>ALGAMPGVYR</u> VDLLTRQIQAPDVDWSYGEPTEMLPPRTDVLTPGE--SEEGLQV
XhSPS1	254	VVELARALSMMPGVYRVDLFTRQISSPDVDWSYGEPTEMLTSG-----QYDADGN
XhSPS2	978	CHAFKVKNSSLVPPITELRKLMRIQALRCHVIYSHDGTKLHAIPVLASRSQALRYLYVRW
XhSPS1	991	CVSFFIKDPSKAKAIDDLRQKLMRGLRCHLMYCRNSTR <u>LQVIPLLASRS</u> QALRYMFVRW
<i>XhSPS1-Q2</i>		

Figure 4.7 ClustalW alignment of regions of XhSPS1 and XhSPS2 used for synthesis of tryptic peptide standards. Tryptic peptides are under lined in the sequence. Trypsin cuts after an arginine (R) or a lysine (K). Names of tryptic peptides are in italics. Gaps in sequence indicated by (---).

Setup and running conditions of the LC system and MS for quantification of SPS isoforms

For the quantification of different isoforms a one-dimensional (1D) nano flow liquid chromatography (LC) system with pre-column (Agilent, Germany) was used. A monolithic column (Merck, Darmstadt, Germany) of 15 cm length and 0.1 mm internal diameter was coupled to the triple quadrupole mass spectrometer. Peptides were eluted during a 30 min gradient from 0 to 100 % MeOH / 0.1 % FA with a controlled flow rate of 0.7 µl per minute. One pmol per standard peptide was added prior to each sample digest analysis.

Mass spectrometry (MS) was performed on a TSQ Quantum Discovery MAX mass spectrometer (Thermo Electron) operated in the positive mode. The scan width for all MRMs was 0.7 mass units. The resolution for Q1 was 0.25 mass units; the resolution for Q3 was set to 0.7 mass units. The mass spectrometer was tuned to its optimum sensitivity for each standard peptide. Optimization procedure is detailed in Glinski and Weckwerth, (2005). Collision energies (CE) used for the recorded transitions are shown in Table 4.7. The dwell time per transition was 50 ms. Spray voltage was set to 1.8 kV; temperature of the heated transfer capillary was set to 150 °C.

The quantification function of the ABI Analyst software (version 1.4.2) was used to calculate the area under the peak for light and heavy peptides as well as their ratio. Peaks were manually checked and peak integrations were adjusted accordingly. The light to heavy peak area ratios were calculated for each transition and exported to Excel where the average of peak ratios for three transitions were calculated to yield the heavy to light peptide ratios. These calculations were done for quadruplicate MRM experiments to calculate the error for each ratio measurement and evaluate the reproducibility.

University of Cape Town

Results

Detection of *X. humilis* SPS genes

Screen of expressed transcripts

To find all SPS transcripts in *X. humilis* a degenerate PCR based approach was used to search the transcriptome of *X. humilis* leaf and root. To broaden the search RNA was extracted from leaf and root tissue at different relative water contents (RWC) during tissue dehydration. For leaves, tissue was harvested at 100, 80, 60, 40 and 5 % RWC and for roots tissue was harvested at 100, 50 and 5 % RWC. cDNA was synthesized from each pool of extracted RNA with the exception of the root 5 % RWC sample from which cDNA could not be synthesized. Based on known plant SPS sequences a set of degenerate primers was designed to amplify all SPS encoding transcripts (described as the universal SPS primer set). Figure 4.8 shows the PCR product obtained from the 100% and 5% RWC leaf samples while Figure 4.9 shows the product obtained from the root 100% and 50% RWC samples. A DNA fragment of 710 bp was amplified from leaf and root tissue cDNA templates. This fragment size is close to the upper expected size of 720 bp based on the alignment used for the primer design. A single product at the same size was also obtained from all other leaf samples.

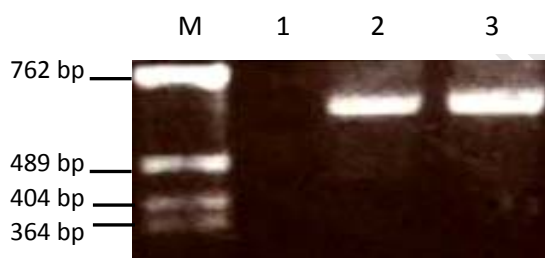


Figure 4.8 PCR amplified SPS DNA from leaf tissue. Primer set: SPS uf1 and SPS ur1a. DNA fractionated on a 1.5 % agarose gel. Lane M-*HapII* pGEM-T marker, Lane 1 - negative control (no template), Lane 2-fully hydrated leaf cDNA template (100 % RWC) and Lane 3 - desiccated leaf cDNA template (5 % RWC).

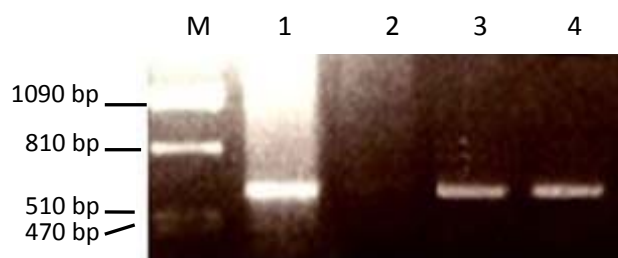


Figure 4.9 PCR amplified SPS DNA from root tissue. Primer set: SPS uf1 and SPS ur1a. DNA fractionated on a 1.5% agarose gel. Lane M- *PstI* lambda marker , Lane 1 - positive control (leaf 100 % RWC cDNA template), Lane 2 - negative control (no template), Lane 3 - fully hydrated root cDNA template (100% RWC) and Lane 4- drying root cDNA template (50 % RWC).

The amplified DNA from the 100 % RWC sample was ligated into the pGEM-T vector and the plasmid transformed into *E. coli* bacteria. To establish the identity of the amplified product, the cloned insert was sequenced and the sequence compared to known plant SPS sequences using BLAST (blastn) (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). The clone was found to share a 77 % identity with *Nicotiana tabacum* SPS B. This transcript was named Xh SPS1p (p designates partial sequence) as it was the first *X. humilis* SPS gene identified. Having confirmed that the primers were targeting SPS, the remaining clones were screened for any new SPS transcripts. As the universal primer set only amplified a product of one size (710bp), the clones were first screened by restriction fragment pattern analysis to identify good candidates for sequencing. The restriction enzymes used in the screening, *EcoR1* and *Xba1*, were chosen as both their cutting sites were present in the sequenced transcript Xh SPS1p. In total 6 distinct DNA fragment patterns could be distinguished from leaf and root cDNA. Examples of the actual restriction fragment patterns observed are shown in Figure 4.10 and each pattern is described in Table 4.8. Table 4.8 also includes data on the relative proportions of each restriction fragment pattern for each RWC tissue sample. Representatives of all clones exhibiting distinct restriction enzyme fragment patterns were sequenced. Each sequence was compared to known SPS sequences with the BLASTn tool (NCBI) to determine the identity of the sequence. Sequences sharing a greater than 50 % identity with known SPS sequence were then compared to previously isolated *X. humilis* SPS encoding transcripts to establish if the particular sequence represented a new *X. humilis* SPS gene. The cloning and screening process was repeated for cDNA amplified from the 100, 80, 60, 40 and 5 % RWC leaf samples and from the 100 and 50 % RWC root samples. In total 80 clones were screened and of these clones 24 were sequenced. From the screening of *X. humilis* cDNA two unique 710 bp SPS encoding transcripts were identified, the previously identified Xh SPS1p and a second transcript which was named Xh SPS2p (see Table 4.8). Based on the relative proportions (Table 4.8), Xh SPS2p appears to be dominant in fully hydrated (100% RWC) leaf and root tissue. In leaf tissue there is an increase in the proportion of Xh SPS1p in the late stages of dehydration, beyond 40 % RWC, and by 5 % RWC Xh SPS1p appears to be slightly more prevalent than Xh SPS2p. In root tissue the proportion of Xh SPS1p doubles from 10 to 20 % from fully hydrated tissue to tissue with a 50 % RWC but Xh SPS2p is still the dominant gene.

Table 4.8 Distinct restriction enzyme fragment patterns of clones obtained from cDNA template. The relative proportion of each pattern in each sample pool of screened clones is also given. For each sample of leaf tissue at different relative water contents (RWC), between 10-14 clones were screened.

Pattern	Fragments	Sizes (bp)	Clone	BLAST n Match	Identity	E-value	Relative proportions (%)						
							Leaf					Root	
							100%RWC	80%RWC	60%RWC	40%RWC	5%RWC	100%RWC	50%RWC
C1	3	336,296,212	/	No match with SPS	/	/	/	/	10	/	/	/	/
C2	2	342,274	/	No match with SPS	/	/	/	10	/	/	/	10	/
<u>C3</u>	2	336, 295	Xh SPS2p	<i>Musa acuminata</i> SPS Acc: AJ505606.1	76%	4e-123	70	60	70	40	40	80	70
C4	2	290,187	/	No match with SPS	/	/	/	/	/	10	/	/	10
<u>C5</u>	3	301,197,103	Xh SPS1p	<i>Nicotiana tabacum</i> SPSB Acc: DQ213015.1	75%	4e-116	30	30	20	40	50	10	20
C6	1	324	/	No match with SPS	/	/	/	/	/	10	10	/	/

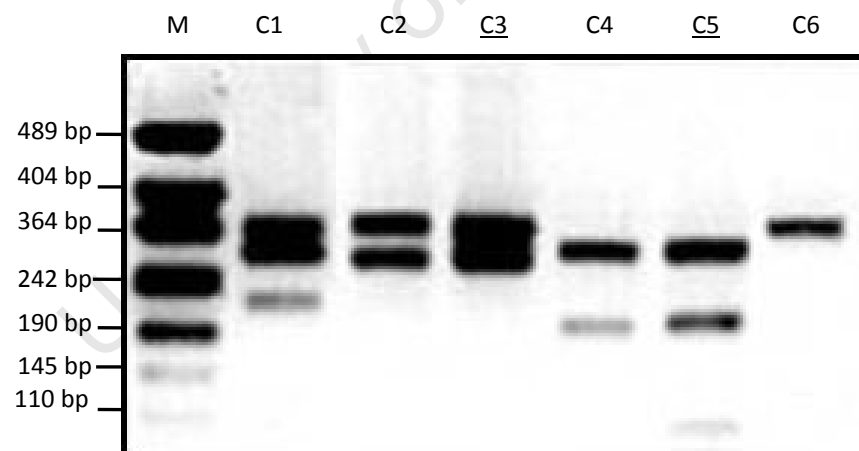


Figure 4.10 Examples of restriction enzyme fragment patterns obtained from amplified PCR product (cDNA template) digested with *EcoRI* and *XbaI*. DNA fractionated on 2 % agarose gel. Lane M-*HpaII* pGEM-T. SPS encoding clones are underlined.

Screen of *X. humilis* genome

In addition to the transcriptome of leaf and root *X. humilis* tissue, the search for new SPS genes was also extended to the genome of *X. humilis*. Initially the same universal primer set used in the cDNA screen (SPS uf1 and SPS ur1a) was used with *X. humilis* genomic DNA as the template. However, sequencing of the products amplified with these primers revealed a high degree of non-specificity with a large proportion of the sequenced clones sharing no homology with known SPS sequences. Further, based on an alignment of *A. thaliana* genomic SPS genes it appeared that the forward primer straddled an exon/intron boundary. Therefore, a new forward primer (SPS guf1) was designed for use with genomic DNA template. The new primer annealed ~18 bp upstream of the previous forward primer. In Figure 4.11 the two amplification fragments obtained with the new primer set, of 838 bp and 765 bp, are displayed. These fragments were cloned and the cloned inserts amplified with vector specific primers (SP6 and T7). Examples of the two product sizes obtained after insert amplification are shown in Figure 4.12.

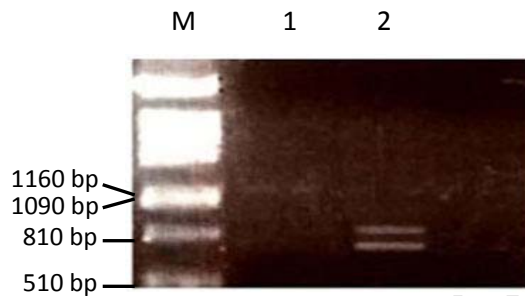


Figure 4.11 Amplified product from *X. humilis* genomic DNA. Primer set: SPS guf1 and SPS gur1. DNA fractionated on a 1% agarose gel. Lane M - *PstI* Lambda marker. Lane 1 – negative control (no template). Lane 2 – genomic DNA template.

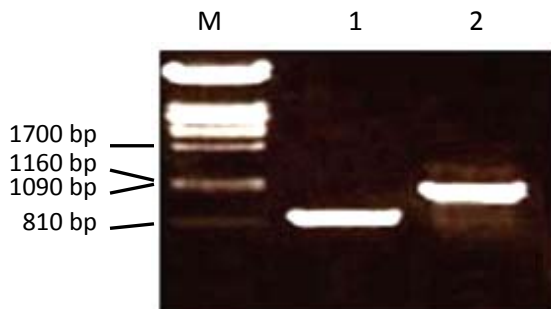


Figure 4.12 Example of sizes of cloned PCR products obtained from genomic DNA. DNA fractionated on a 1.5% agarose gel. Lane M - *PstI* Lambda marker. Lane 1 & 2 – amplified insert.

The sizes of the fragments in Figure 4.12 are larger by approximately 100 bp as the SP6 and T7 vector specific primers also amplified a 50 bp vector region flanking the insert. The flanking vector region contains *EcoR1* cutting sites on either side of the insert, hence after digestion with *EcoR1* the vector sequence is removed from the insert. Both the '838' and the '765' bp amplified fragments were digested with *EcoR1* and *Xba1*. Digestion of the longer fragment produced three distinct fragment patterns, namely G11, G12 and G13 while digestion of the shorter fragment produced two distinct fragment patterns, namely Gs1 and Gs2. Examples of these patterns are displayed in Figure 4.13. Representative clones of all distinct fragment patterns in Table 4.9 were sequenced. Of the five distinct fragment patterns obtained only two, namely G12 and G13, were found to represent clones which shared over 40 % sequence identity with known SPS sequences. Each sequence was also compared to the previously described SPS clones obtained from leaf and root cDNA (Xh SPS1p and Xh SPS2p). The clones represented by the patterns G12 and G13 shared a high homology (over 80 % identity) with the previously described Xh SPS2p and Xh SPS1p respectively, and were therefore named gXh SPS2p and gXhSPS1p. When the sequences of gXH SPS1p and gXhSPS2p were compared to those of Xh SPS1p and Xh SPS2p an intron sequence of approximately 120 bp in length was identified in both gXh SPS1p and gXh SPS2p. When the intron sequence was removed, gXh SPS1p and gXh SPS2p were found to share a 100 % identity with the previously described Xh SPS1 and Xh SPS2 respectively. The presence of the intron explains the larger sizes of both gXh SPS1p and gXh SPS2p. Thus, the genomic screen identified the two previously isolated SPS genes, Xh SPS1p and Xh SPS2p, but no new genes were found.

Table 4.9 Distinct restriction enzyme fragment patterns of clones obtained from genomic DNA template

*Pattern name	No. of fragments	Sizes (bp)	Clone name	BLAST n	Identity	E-value
Gs1	2	418,247	/	No ID with SPS	/	/
Gs2	1	350	/	No ID with SPS	/	/
Gl1	2	503, 308	/	No ID with SPS	/	/
<u>Gl2</u>	2	500,329	gXhSPS2p	<i>M acuminata</i> SPS Acc: AJ505606.1	75%	1e-103
<u>Gl3</u>	2	305, 284	gXhSPS1p	<i>N tabacum</i> SPSB Acc: DQ213015.1	75%	1e-112

* s – short cloned insert (765bp) l –long cloned insert (838bp)

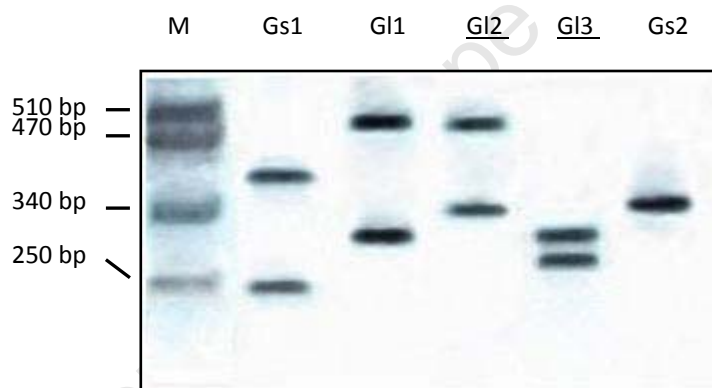


Figure 4.13 Examples of restriction enzyme fragment patterns obtained from genomic DNA template. PCR product (SP6 and T7 primers) digested with *EcoRI* and *XbaI*. DNA fractionated on 1.5% agarose gel. Lane M - *PstI* Lambda marker. SPS encoding clones are underlined.

Sequence of full length SPS genes

To obtain the full length of Xh SPS1p and Xh SPS2p, the initial partial length sequences were extended by 3' and 5' RACE PCR. The 3' RACE ends amplified for Xh SPS1p and Xh SPS2p were of a similar size (~2.8 kb) and are shown in Figure 4.14. The 5' RACE products, displayed in Figures 4.15 and 4.16, differed slightly with more of the 5' end being obtained for Xh SPS2p (810 bp product) when compared to Xh SPS1p (780 bp product).

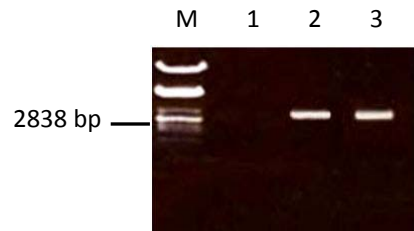


Figure 4.14 3' RACE product of Xh SPS1p & Xh SPS2p. Lane M - *PstI* Lambda marker. Lane 1 - negative control (no template). Lane 2 - amplified product from 3' RACE ready leaf cDNA using Xh SPS1p gene specific primer. Lane 3 - amplified product from 3' RACE ready leaf cDNA using Xh SPS2p gene specific primer.

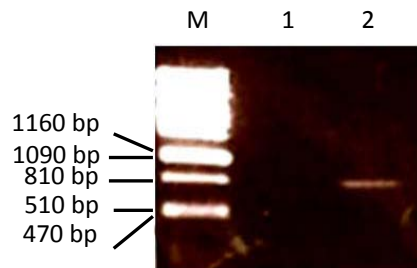


Figure 4.15 5' RACE product of Xh SPS1p. Lane M - *PstI* Lambda marker. Lane 1 - negative control (no template). Lane 2 - amplified product from 5' RACE ready leaf cDNA using Xh SPS1p gene specific primer.

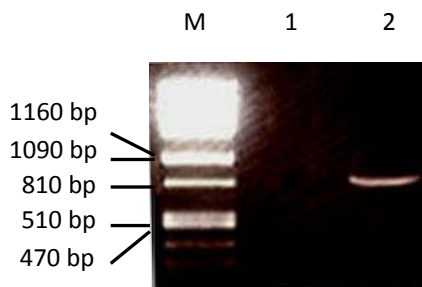


Figure 4.16 5' RACE product of Xh SPS2p. Lane M- *PstI* Lambda marker. Lane 1 - negative control (no template). Lane 2 - amplified product from 5' RACE ready leaf cDNA using Xh SPS2p gene specific primer.

After sequencing of the three PCR amplified fragments for each SPS gene - the initial 710 bp fragment, the 3' fragment and the 5' fragment, the full length sequence of each gene was constructed from a composite of the three fragments. The full length SPS genes were named XhSPS1 and XhSPS2. Over the full sequence length XhSPS1 shares the highest similarity with *N. tabacum* SPSB (71% identity) and the closest match in the database for XhSPS2 was *Oncidium cv 'Goldiana'* SPS (73 % identity). Notably, when compared to each other, the *X. humilis* genes only shared a 58% identity at the nucleotide level. The predicted protein sizes for both genes were found to be similar with XhSPS1 having a size of 121 kDa and XhSPS2 a size of 119 kDa. Similarly, the predicted pI values for each gene did not differ greatly with XhSPS1 having a pI value of 6.3 and XhSPS2 being slightly more basic at a pI of 6.6.

Comparison between X. humilis SPS and spinach SPS

To determine if the *X. humilis* genes shared conserved features with the well characterized SPS from spinach (*S. oleracea*), an alignment (ClustalW) of the two *X. humilis* sequences and the *S. oleracea* SPS sequence was conducted. The aligned amino acid sequences are displayed in Figure 4.17 and some important conserved regions are highlighted.

Important regions include the two substrate binding motifs, namely the putative Fructose-6-phosphate binding site (Asp₁₉₇ to Glu₂₀₆, *S. oleracea* amino acid sequence) (Salvucci et al., 1995) and the UDP-Glucose binding site (Gln₂₂₇ to Glu₂₃₉, *S. oleracea* amino acid sequence) (Salvucci and Klein, 1993), and the three phospho-regulatory sites, namely the light/dark regulatory Ser₁₅₈ site (McMichael et al., 1993; McMichael et al., 1995a; Toroser et al., 1999), the 14-3-3 binding Ser₂₂₉ site (Toroser et al., 1998; Moorhead et al., 1999) and the osmoregulatory Ser₄₂₄ site (Toroser and Huber, 1997; Huang and Huber, 2001). The substrate binding sites in *S. oleracea* were found to be highly conserved in both XhSPS1 and XhSPS2 (see Figure 4.17). However, of the three *S. oleracea* phospho-regulatory sites only one site, the Ser₁₅₈ site, was found to be conserved in both XhSPS1 and XhSPS2. The Ser₂₂₉ phospho-serine is present in XhSPS1 but is absent in XhSPS2 and both *X. humilis* genes lack the Ser₄₂₄ phospho-serine (see Figure 4.17 and Table 4.10).

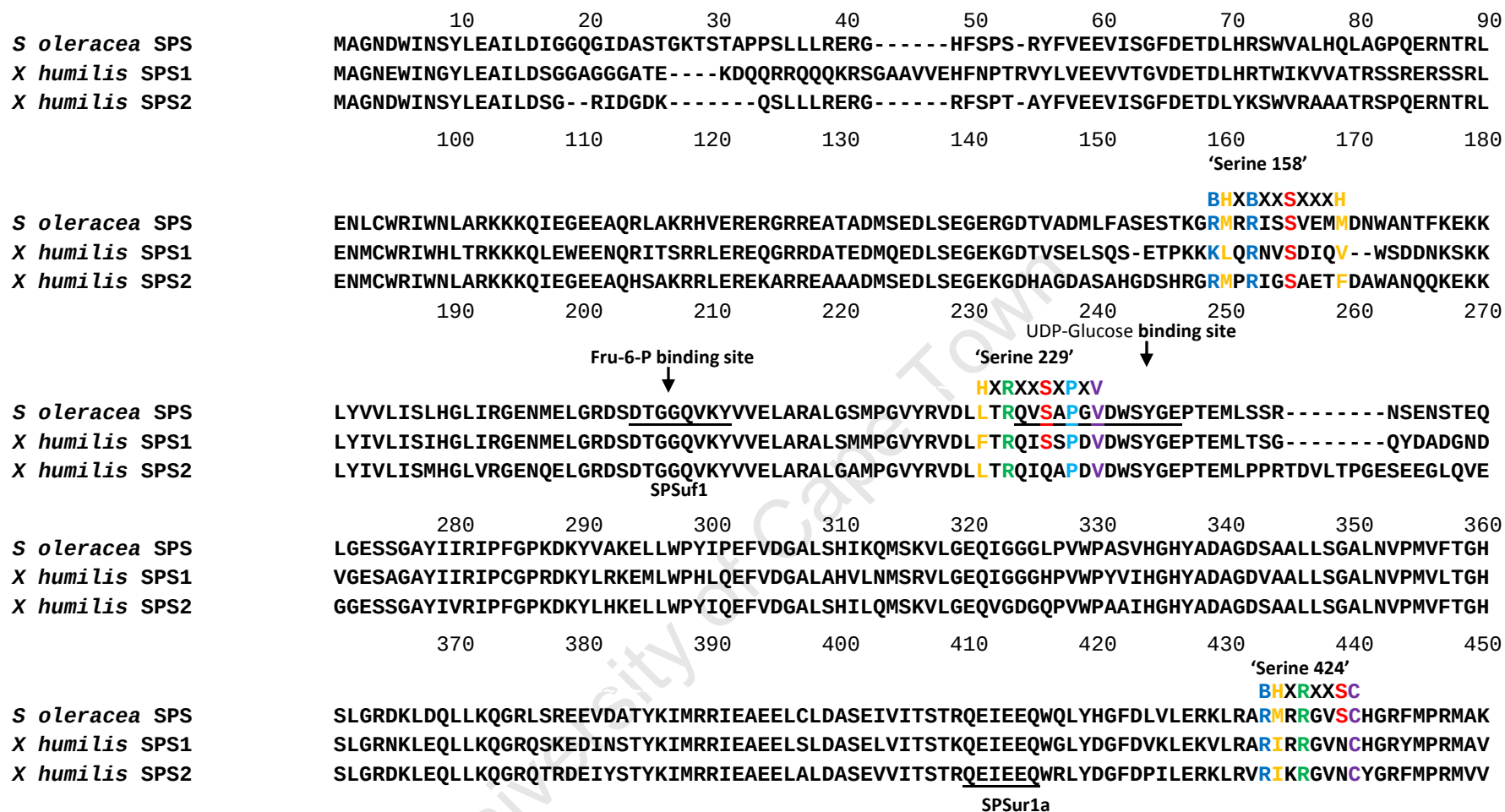


Figure 4.17 Alignment of full length amino acid sequence of *X. humilis* SPS1 (XhSPS1), *X. humilis* SPS2 (XhSPS2) and the published *S. oleracea* SPS (Acc: S54379, *S. oleracea* SPS) amino acid sequence (ClustalW). The two substrate binding regions are highlighted in blocks. The three phospho-regulatory sites corresponding to Ser₁₅₈, Ser₂₂₉ and Ser₄₂₄ of the *S. oleracea* sequence are labeled and the kinase recognition motif for each site is displayed. The phosphorylated serine is shaded in red. The positioning of the universal primer set (SPS uf1 and SPS ur1a) used in the initial PCR reaction is also shown.

* Kinase recognition motif, where X is any amino acid, B is a basic residue, H a hydrophobic residue and S the phosphorylated Serine.

	460	470	480	490	500	510	520	530	540
S oleracea SPS	IPPGMEFN-HIAPEDADMDTDIDGHKESN-ANPD--PVIWSEIMRFFSNGRKPMILALARPDPKKNLTTLVKAFGECRPLRELANLTLII								
X humilis SPS1	IPPGMDFSNVVAQEDAEADGELTAITGADGASPKSVPIIWQEVLRFFTNPHKPMILALSRPDPKKNITTTLLKAFGESRPLRELANLTLIM								
X humilis SPS2	IAPGMEFN-NIVVHDTDMEGEVD-LEDNP-ASPD--PPIWKKIMRFFTNPRKPMILALARPDPKKNLTTLVKAFGECRPLRELANLTLIM								
	550	560	570	580	590	600	610	620	630
S oleracea SPS	GNRDDIDEMSTTSSSVLISILKLIDKYDLYGQVAYPKHHKQSDVPDIYRLAAKTGKVFINPAFIEPFGLTLEAAAYGLPIVRTKNGGPV								
X humilis SPS1	GNRDDIDGMSTGNASVLTTLVLKLIDKYDLYGLVAYPKHHIQSDVPEIYRLAAKTGKVFINPALVEPFGLTLEAAAHGLPMVATKNGGPV								
X humilis SPS2	GNREEIDEMSSTNASVLTSLVLKLIDKYDLYGQVAYPKHHKQSEVPDIYRLAAKTGKVFVNPAFIEPFGLTLEAAAHGLPIVATKNGGPV								
	640	650	660	670	680	690	700	710	720
S oleracea SPS	DIIGVLDNGLLIDPHDQKSIADALLKLVDKQVWTKCRQNGLNHLFSWPEHCKNYLSRIASCKPRQPNWQRIDEGSENSDTSAGDSL								
X humilis SPS1	DIHRALNGLLVDPHDQNAISDALLKLVSSEKNLWHECRKNGWRNIHLFSWPEHCRTYLTRVAACRMHPQWQLDTPQDDMPLEESLGDSL								
X humilis SPS2	DIHRALDNGLLIDPHNQEAIDALLR-LADRQLWARCRQNGLNHLFSGPEHCKTYLSRITSCRPRQPQWRNEDGSEKSEPDSPSDSL								
	730	740	750	760	770	780	790	800	810
S oleracea SPS	RDIQDISLNLKLSLDAERTEGGNSFDDSLDSE-EANAKRKIENAVAKLSKSMKAQ-VDVG-----NLKFPAIRRRKCIFVIALD								
X humilis SPS1	MDVHESSL--RLSIDGDKSSSLERNPDGLESVANGDGKPDLDQVVKRILNRIKKQPPKDMNNKQSDALGSAIGRYPLRRRRRLFVIALD								
X humilis SPS2	RDIQDISLNLKFSLDGDKTEDASTLD-SVDT--ATDGKNKLDREVSKLSKGLDRGR-HKAGPDEKNEQTGNSSKLPAIRKRKHIVVIAVD								
	820	830	840	850	860	870	880	890	900
S oleracea SPS	C-----DVTSDLLQVIKTVISIVGEQRPTGSIG-FILSTSMTLSEVDSLLDSGGLRPADFDAFICNSGSELYYPS----TDYSESPFVLD								
X humilis SPS1	SYGEKGEPNKEMAHVIEVLRAIRLDSQMSRISGFALSTAMPVSETLDLLKSGKIPVTFDFDALICSSGSEVYYPG-TSQCMDSDGKFCAD								
X humilis SPS2	S-----DSNEDLMATVKKIFDATEKDRASGSIG-FVLSTALTIMEVHSALCSVDMPGTEFDAFICNSGSDLYYPSQNNEDNSSELPYVLD								
	910	920	930	940	950	960	970	980	990
S oleracea SPS	QDYYSIDYRWGGEGWLKTLVKWAASVNEKKGENAPNIVIADETSSTTHCYAFKVNDFTLAPPAKELRKMRIQALRCHAIYCQNGTWLN								
X humilis SPS1	PDYATHIEYRWGYDGVKRTIILMNSQDSQDVSRSENLEVEDAKSCNAYCVSFFIKDPSKAKAIDDLRQKLRMRLRCHLMYCRNSTRLQ								
X humilis SPS2	TDYHSQIEYRWGGEWLKTLIRWAASVVNINDEGEAQVVTEDADRSSAYCHAFKVNSSLVPPITELRKLRIQALRCHVIYSHDGTKLH								
	1000	1010	1020	1030	1040	1050	1060	1070	1080
S oleracea SPS	VIPVLASRSQALRYLFMRWGVLSNFVVFVGESGDTDYEGLLGGVHKTIVILKGIGSNTSN-FHATR-AYPMEHVMPVDSP-NMFQTGGCN								
X humilis SPS1	VIPLLASRSQALRYMFVRWGLNVANMYVILGERGDTDHEELISGSHKTIVIMKGIVERGESLLRTAGSYQKEDIVPGDSPLIVYTTEGIK								
X humilis SPS2	AIPVLASRSQALRYLYVRWGTELSNMVVFVGETGDTDYEGLLSGVHKSIVILKGVCCKSTSDRRFSSR-NYSLSDVVAFDNP-NILQIEP-E								
	1090	1100							
S oleracea SPS	IEHISDALSKIGCLKAQKSL								
X humilis SPS1	AEEIMKALKEASKAASAM--								
X humilis SPS2	CKDIQSALNKLGMMLKN---								

Figure 4.17 cont/.

Table 4.10 Conservation of SPS phospho-regulatory sites in *X. humilis* SPS genes. The sites were identified from an alignment of XhSPS1, XhSPS2 and *S. oleracea* SPS (see Figure 4.17). The kinase recognition motifs for the *S. oleracea*, Ser₁₅₈ (McMichael et al., 1993; McMichael et al., 1995b; Toroser et al., 1999), Ser₂₂₉ (Moorhead et al., 1999) and Ser₄₂₄ (Toroser and Huber, 1997; Huang and Huber, 2001) sites and the actual motif sequence for each *X. humilis* gene are displayed. The phosphorylation site is described as conserved when all critical elements of the kinase recognition motif are present.

Name	Phospho-regulatory sites					
	Ser ₁₅₈ Light/Dark		Ser ₂₂₉ 14-3-3 binding		Ser ₄₂₄ Osmoregulation	
	*Motif	Conserved	*Motif	Conserved	*Motif	Conserved
	BHXBXXSXXXH		HXRXSXPPXV		BHXRXSXC	
XhSPS1	KLQRNVSDIQV	Yes	FTRQISSPDV	Yes	RIRRGVNC	No
XhSPS2	RMPRIGSAETF	Yes	LTRQIQAPDV	No	RIKRGVNC	No

* Kinase recognition motif, where X is any amino acid, B is a basic residue, H a hydrophobic residue and S the phosphorylated Ser.

Phylogenetic analysis of SPS Proteins

A neighbor-joining phylogenetic tree (see Figure 4.18) was constructed from all known full length SPS sequences and the two *X. humilis* SPS sequences to i) determine the relationship of *X. humilis* SPS genes to SPS gene families and ii) to analyse the evolutionary history of the SPS gene family in plants. A total of 41 full length angiosperm and two bryophyte (*Physcomitrella patens*) SPS protein sequences were extracted from the available databases and used in the phylogenetic analysis. The tree displayed in Figure 4.18 was based on a ClustalW alignment of the amino acid SPS sequences (see Appendix D). Evolutionary distances between proteins were calculated with the Dayhoff Matrix substitution model. Trees were also generated with the JTT and Poisson Correction protein distance calculation models. All tree topologies were found to be similar to the one obtained in Figure 4.18. A phylogenetic tree generated using maximum parsimony (data not shown) also confirmed the major family groupings (designated Family A, B and C) observed in Figure 4.18. In addition, when the phylogenetic analysis was conducted with full length nucleotide sequences instead of amino acid sequences there was no significant change in the grouping of the SPS genes (data not shown).

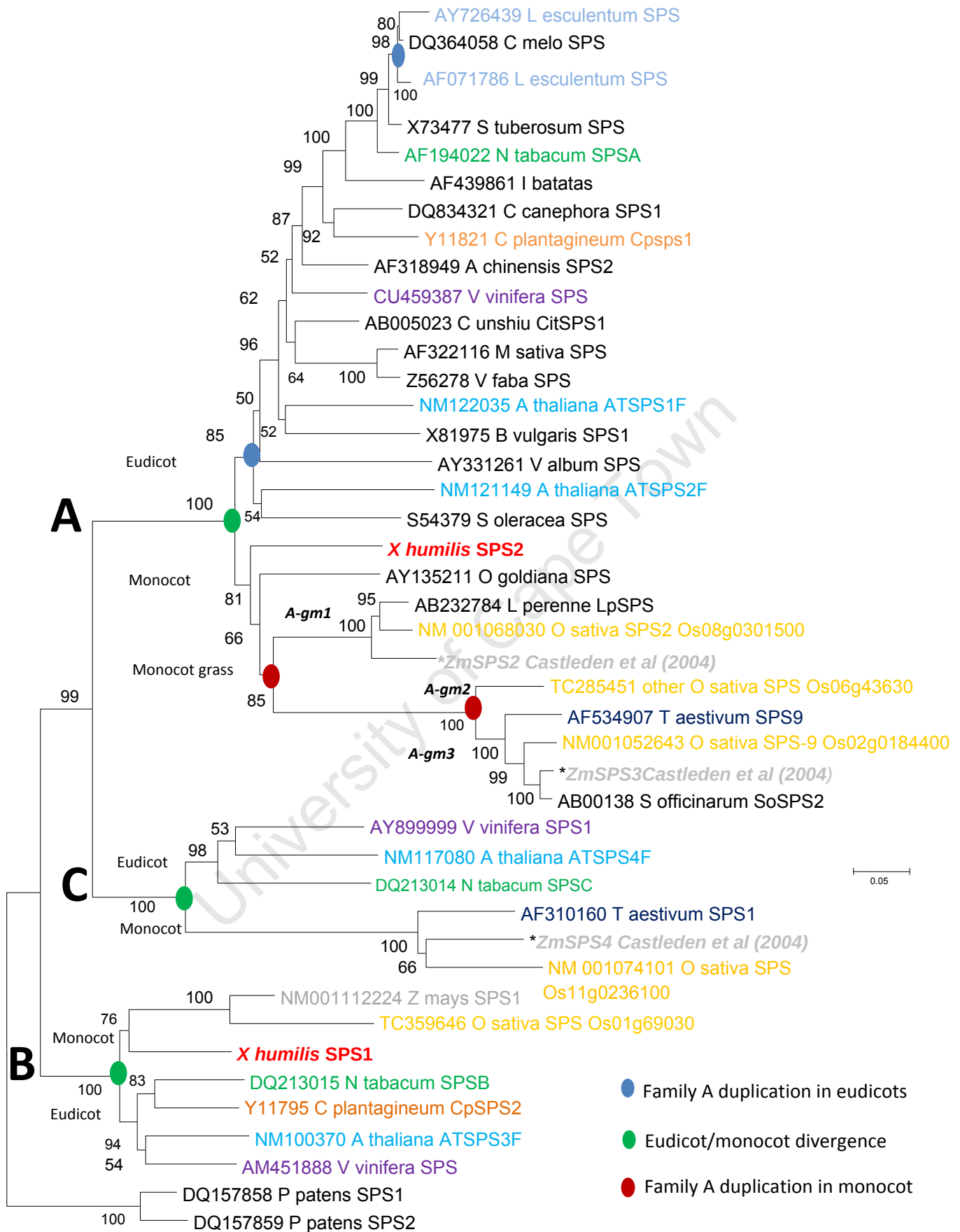


Figure 4.18 (preceding page) Phylogenetic analysis of 43 full-length SPS protein sequences. Sequences were aligned with ClustalW. The tree was constructed using the neighbor-joining method where all gaps were eliminated. Results of bootstrap test (1000 replicates) are shown. The moss *Physcomitrella* (PhySc) SPS sequences are designated as root. The three main family groupings are designated Family A, Family B and Family C. Eudicot and monocot subgroups within each family are indicated. Grass monocot groups are labeled 'gm'. Multiple SPS orthologues in the same species share the same colour font. Genbank accession numbers are given.

* Designates *Z. mays* sequences obtained from supplemental data of Castleden et al., (2004)

The clustering of the full length SPS proteins suggests the occurrence of three families of SPS genes. To stay in line with the nomenclature used in previous phylogenetic analyses (Langenkamper et al., 2002, Lunn and MacRae, 2003., Castleden et al., 2004., MacRae and Lunn., 2006) these groups have been designated Family A, Family B and Family C. There is high bootstrap support for these family divisions, greater than 70% in the tree rooted on the cyanobacterial *Synechostis* SPS sequence (data not shown) and 100% in the tree rooted on the moss *Physcomitrella* sequence. The lower bootstrap support in the *Synechostis* (used in previous analyses, e.g. Langenkamper et al., 2002) rooted tree results from the loss of sequence information due to the rooting on the smaller bacterial SPS. It is therefore proposed that the *Physcomitrella* sequence serves as a better outgroup for phylogenetic analyses of SPS genes. In each family there is a well supported split between the eudicot and monocot members of each group. In Family A further SPS duplication events are apparent in both the eudicots and the monocots. The presence of two *A. thaliana* and two *L. esculentum* SPS genes in Family A eudicots suggests the occurrence of two recent duplication events within this lineage. In the monocot grass line of the Family A the presence of multiple *Oryza sativa* and *Zea mays* SPS genes also points to the occurrence of at least two duplication events within this line. These duplications within Family A monocots appear to have given rise to the three monocot subgroups observed in Figure 4.18, labeled A-gm1, A-gm2 and A-gm3.

The identified *X. humilis* genes were found to separate into two main families with *X. humilis* SPS1 (XhSPS1) clustering closely with Family B (B monocot) genes while *X. humilis* SPS2 (XhSPS2) clustering with Family A (monocot) genes. In both Family A and Family B the *X. humilis* genes are positioned at the base of the monocot lines. The occurrence of eudicot and monocot sub-groups within each family suggests that the duplication events giving rise to the

three SPS families occurred prior to the eudicot/monocot split. Furthermore the occurrence of representatives from each family have also been noted in the fully sequenced poplar (*Populus trichocarpa*). Thus, most angiosperm plant species, including *X. humilis*, should contain at least one SPS representative in each of the major families. In the fully sequenced genomes of *O. sativa*, *A. thaliana* and *V. vinifera* this prediction holds true as each species contains representatives in each of the three major family groups (A, B and C). However, despite extensive clone screening, only Family A and Family B *X. humilis* SPS genes were isolated in *X. humilis*. The search for all SPS genes included the use of PCR primer sets biased to Family C genes but the expected third Family C SPS gene were still not isolated.

Comparison of conserved phospho-regulatory motifs in plant SPS proteins

Based on previous literature three phospho-regulatory sites, namely Ser₁₅₈, Ser₂₂₉ and Ser₄₂₄ (*S. oleracea* amino acid numbering) are believed to be important in the post-translational regulation of SPS activity (McMichael et al., 1993; Salvucci and Klein, 1993; Salvucci et al., 1995; Huber and Huber, 1996). To determine if the family grouping of SPS proteins (refer to Figure 4.18) could be related to the conservation of these regulatory sites the presence of these sites in each family was established. Figure 4.19 displays a non-contiguous ClustalW alignment of 41 known SPS protein sequences. The three phospho-regulatory sites are displayed as well as the conserved regions which are believed to be important in substrate binding, namely the putative Fructose-6-phosphate binding domain (Salvucci et al., 1995) and the UDP glucose binding domain (Salvucci and Klein, 1993). The conservation of these regions is addressed in detail in the Discussion section of this chapter.

		Ser 158	Fru-6-P Binding	Ser 224	UDPG Binding	Ser 424	
Eudicot	AF194022 N tabacum SPSA	KGRLPRISSVE	DTGGQVKY	LTRQVSSPEVDWSYGEPT	RARIKRNVSC	Family A	
	NM122035 A thaliana ATSPS1F	KPRLPRINSAE	DTGGQVKY	LTRQVSSPDVDYSYGEPT	RARIKRNVSC		
	NM121149 A thaliana ATSPS2F	KGRMSRISSVD	DTGGQVKY	LTRQVTAPDVDSSYSEPE	RARMKRGVSC		
	AF439861 I batatas	KGRLPRISSVE	DTGGQVKY	LTRQVSSPEVDWSYGEPT	RARIKRNVSC		
	DQ364058 C melo SPS	RGRLPRISSVE	DTGGQVKY	LTRQVSSPEVDWSYGEPT	RARIKRNVSC		
	AF071786 L esculentum SPS	RGRLPRISSVE	DTGGQVKY	LTRQVSSPEVDWSYGEPT	RARIKRNVSC		
	AY26439 L esculentum SPS	RGRLPRISGVE	DTGGQVKY	LTRQVSSPEVDWSYGEPT	RARIKRNVSC		
	X73477 S tuberosum SPS	RGRLPRISSVE	DTGGQVKY	LTRQVSSPEVDWSYGEPT	RARIKRNVSC		
	DQ834321 C canephora SPS1	RGRLPRISSVE	DTGGQVKY	LTRQVSSLEVDWSYGEPT	RARIKRNVSC		
	Y11821 C plantagineum Cpsps1	RGRLPRINSVD	DTGGQVKY	LTRQVSSPEVDWSYGEPT	RARIKRNVSC		
	AF318949 A chinensis SPS2	RGRLPRISSVE	DTGGQVKY	LTTQVSSPEVDWSYGEPT	RARIKRNVSC		
	CU459387 V vinifera SPS	RGRLPRISSVD	DTGGQVKY	LTRQVSSPEVDWSYGEPT	RARIKRNVSC		
	AF322116 M sativa SPS	KARLPRISSAD	DTGGQVKY	LTRQVSPDWDWSYGEPT	RARIKRNVSC		
	AB005023 C unshiu CitSPS1	RSRLPRISSVD	DTGGQVKY	LTRQVSAPDWDWSYGEPT	RARIKRNVSC		
	X81975 B vulgaris SPS1	RPRLPRINSID	DTGGQVKY	LTRQVSSPDVDWSYGEPT	RARIKRNVSC		
	AY331261 V album SPS	--RFPRISSVD	DTGGQVKY	LTRQVSAPDIHWSYGEPT	RARIKRNVSC		
	Monocot	S54379 S oleracea SPS	KGRMRRISSVE	DTGGQVKY	LTRQVSAPGVDWSYGEPT		RARIKRNVSC
Z56278 V faba SPS		KSRLPRISSAD	DTGGQVKY	LTRQVSSPDVDWSYGEPT	RARIKRNVSC		
<i>X humilis</i> SPS2		RGRLPRISSVE	DTGGQVKY	LTRQVSSPDVDWSYGEPT	RARIKRNVSC		
AY135211 O goldiana SPS		RGRLPRISSVD	DTGGQVKY	LTRQISAPDWDWSYGEPT	RARIKRNVSC		
AB232784 L perenne LpSPS		RRRMPRISSVD	DTGGQVKY	LTRQISAPDWDWSYGEPT	RARIKRNVSC		
NM 001068030 O sativa SPS2		RRRMPRISSVD	DTGGQVKY	LTRQISAPDWDWSYGEPT	RARIKRNVSC		
*ZmSPS2 Z mays		RTRMPRISSVD	DTGGQVKY	LTRQISAPDWDWSYGEPT	RARIKRNVSC		
TC285451 other O sativa SPS		TGNTPRISSVD	DTGGQVKY	FTRQILAPNFDWSYGEPT	RARIKRNVSC		
NM001052643 O sativa SPS-9		TGSSPKTSSVD	DTGGQVKY	LTRQILAPNFDWSYGEPT	RARIKRNVSC		
AF534907 T aestivum SPS9		TGSSPKTSSVD	DTGGQVKY	LTRQILAPNFDWSYGEPT	RARIKRNVSC		
*ZmSPS3 Z mays		TGSSPKTSSVD	DTGGQIKY	LTRQILAPNFDWSYGEPT	RARIKRNVSC		
Eudicot	AB00138 S officinarum SoSPS2	TGSSPKTSSVD	DTGGQVKY	LTRQILAPNFDWSYGEPT	RARIKRNVSC		
	DQ213015 N tabacum SPSB	-KRFQNFNSL	DTGGQIKY	FTRQIASPEVDWSYGEPT	RARIKRNVSC		
	NM100370 A thaliana ATSPS3F	-RQLQNLNSL	DTGGQVKY	FTRQICSSPEVDWSYGEPT	RARIKRNVSC		
	AM451888 V vinifera SPS	-KKFQNFNSL	DTGGQVKY	FTRQISSPEVDWSYGEPT	RARIKRNVSC		
	Y11795 C plantagineum CpSPS2	-KKYHRNFNSL	DTGGQIKY	FTRQISSPEVDWSYGEPT	RARIKRNVSC		
Monocot	<i>X humilis</i> SPS1	-KKLQNFNSL	DTGGQVKY	FTRQISSPDVDWSYGEPT	RARIKRNVSC		
	NM001112224 Z mays SPS1	-KKFQNFNSL	DTGGQVKY	FTRQVSSPDVDWSYGEPT	RARIKRNVSC		
	TC359646 O sativa SPS	-KKFQNFNSL	DTGGQVKY	FTRQVSSPEVDWSYGEPT	RARIKRNVSC		
Eudicot	DQ213014 N tabacum SPSC	HHVTRINSVT	DTGGQVKY	LTRQITSPDWDWSYGEPT	RARIKRNVSC		
	AY899999 V vinifera SPS1	KEQMTIRNSDM	DTGGQVKY	LTRQITSPDWDWSYGEPT	RARIKRNVSC		
	NM117080 A thaliana ATSPS4F	RDHMPRISEM	DTGGQVKY	LTRQISSPEVDWSYGEPT	RARIKRNVSC		
	*ZmSPS4 Z mays	DGRIRISGEA	DTGGQVKY	LTRQISCPDWDWTYGEPT	RARIKRNVSC		
Monocot	AF310160 T aestivum SPS1	RTRLARINSEV	DTGGQVKY	LTRQISCPDWDWTYGEPT	RARIKRNVSC		
	NM 001074101 O sativa SPS	LSRLFARINSDP	DTGGQVKY	LTRQISCPDWDWTYGEPT	RARIKRNVSC		
*Kinase recognition motif		BHXBXSSX		HXRXXSXP	BHXRXXS		
		Ser 158		Ser 224	Ser 424		

Figure 4.19 (preceding page) Non-contiguous alignment (ClustalW) of phospho-regulatory and substrate binding sites of 41 plant SPS proteins. Family groupings corresponding to the phylogenetic tree (Figure 4.18) are labeled. Internal monocot and eudicot sub-groups in each family are indicated and the grass only monocot sub-groups in Family A are highlighted. Sequences lacking elements shown to be critical for phosphorylation of the spinach SPS are indicated with strikethrough font (~~XXX~~).

* Kinase recognition motif where X is any amino acid, B is a basic residue, H a hydrophobic residue and S the phosphorylated Serine.

SPS gene expression in response to dehydration

SPS gene expression was investigated to determine if changes in SPS transcript and protein levels underpinned the observed fluctuations in SPS activity. Changes in both the identified SPS isoforms (XhSPS1 and XhSPS2) were measured at the transcript and protein levels. The information obtained was also used to establish which of the two isoforms was most strongly induced by dehydration.

Identification of SPS protein in crude leaf extract

Prior to quantification of each of the identified SPS isoforms (protein level), each SPS isoform had to be identified in crude leaf extract. To identify at which molecular weight SPS protein occurs in crude leaf extract, a commercially available global anti-SPS antibody was used to detect total SPS protein (Western Blot) (Figure 4.20). Total protein was extracted from fully hydrated leaf tissue and from a pool of drying leaf tissue (combined 70% RWC, 60 % RWC, 54% RWC, 40% RWC, 20 % RWC and 5 % RWC). The global antibody is designed to bind to a highly conserved region of the plant SPS protein, and in principle should detect all SPS protein present in *X. humilis* leaf tissue. Four protein bands were immuno-detected in crude *X. humilis* drying leaf extract, while two bands were detected in extract from fully hydrated leaf tissue (Figure 4.20). Standard curves were generated by plotting log molecular weight (MW) versus migration distance (Rf) of each band in the protein marker. Based on this curve the sizes of the 4 bands in the drying leaf extract were estimated at 124 kilodalton (kDa), 121 kDa, 75 kDa and 35 kDa. The fully hydrated leaf extract contained the two lower size bands but the larger 124 kDa and 121 kDa bands were not immunodetected. To determine which of these protein bands

contained SPS protein, protein bands corresponding to each of these sizes (124 kDa, 121 kDa, 75 kDa and 35 kDa) were excised from a duplicate polyacrylamide gel. As it was difficult to separate the 124 kDa and 121 kDa bands, a slice of gel in the 120-125 kDa range was removed. Even though no protein was immunodetected at the ~124 kDa size in fully hydrated leaf extract (Figure 4.20), a slice of gel in the region of the 120-124 kDa mark was also removed from the relevant gel lane. After protein extraction from the excised gel pieces, proteins were analysed (Mass spectrometer) to determine which of the excised protein bands contained SPS protein. Of the four molecular weight bands, only the 121 and 124 kDa protein bands were found to contain SPS protein with tryptic peptides of both XhSPS1 and XhSPS2 confirmed to be present in these protein bands. As it was not possible to effectively separate the 124 kDa and 121 kDa bands when cutting out the gel slices it was unclear as to which of the upper two immunodetected bands corresponded to XhSPS1 and XhSPS2. (see Table 4.5). SPS tryptic peptides were identified in both the full hydrated leaf extract and the drying leaf extract in the 120-124 kDa size region. Thus the lack of immunodetection of SPS in the full hydrated leaf extract (see Figure 4.20) was probably due to SPS protein levels being below the detection limit of the primary antibody. For all further protein quantification of XhSPS1 and XhSPS2 the 121 and 124 Kda protein bands were excised (as one slice) and extracted for protein.

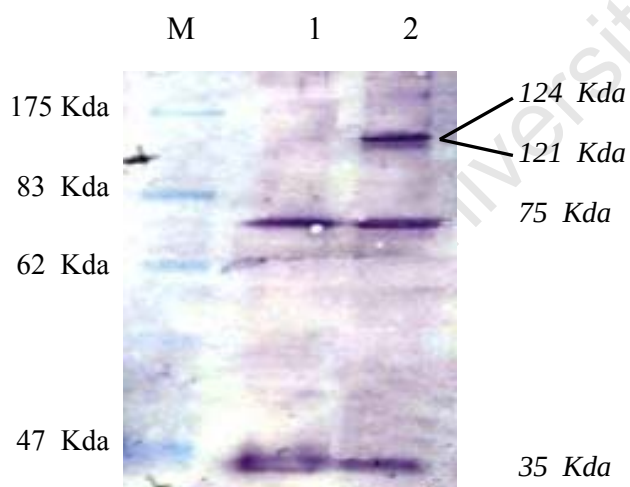


Figure 4.20 Western blot for SPS protein identification. Global anti-SPS used to detect SPS in extracts from leaf tissue. Lane 1 - 100% RWC leaf sample. Lane 2 - Combined samples of drying tissue (70%, 60%, 54%, 40%, 20% and 5% RWC). Protein separated on an 8% polyacrylamide gel.

Table 4.5 SPS tryptic peptides identified in crude leaf extract. 'Fully hydrated leaf extract' corresponds to Lane 1 and 'Drying leaf extract' corresponds to Lane 2 in Figure 4.20 (see above). **Z** – ion charge, **Xcorr** – cross correlation value.

Fully hydrated leaf extract					
Scan(s)	Isoform	Protein bands	Peptide sequences	Z	Xcorr
8530	XhSPS1	121&124 kDa	R.SGAAVVEHFNPTR.V	2	5.38
12370	XhSPS1	121&124 kDa	R.LQVIPLLASR.S	2	4.36
12504	XhSPS1	121&124 kDa	K.LIDKYDLYGLVAYPK.H	2	4.58
8885	XhSPS2	121&124 kDa	R.IDGDKQSLLLR.E	2	4.06
9430	XhSPS2	121&124 kDa	R.ALGAMPGVYR.V	2	6.32
10742	XhSPS2	121&124 kDa	K.LIDKYDLYGQVAYPK.H	1	3.5
11405	XhSPS2	121&124 kDa	K.NSSLVPPITELR.K	2	4.9
11430	XhSPS2	121&124 kDa	K.NSSLVPPITELR.K	3	6.45
11841	XhSPS2	121&124 kDa	R.LYDGFDPIER.K	2	4.7
Drying leaf extract					
Scan(s)	Isoform	Protein bands	Peptide sequences	Z	Xcorr
8951	XhSPS1	121&124 kDa	R.SGAAVVEHFNPTR.V	2	4.85
9147	XhSPS1	121&124 kDa	K.YVVELAR.A	1	3.7
11706	XhSPS1	121&124 kDa	K.QSDALGSAIGRYPLL.R	2	4.52
14978	XhSPS1	121&124 kDa	R.ISGFALSTAM*PVSETLDLLK.S	2	5.62
8885	XhSPS2	121&124 kDa	R.IDGDKQSLLLR.E	2	4.52
9430	XhSPS2	121&124 kDa	R.ALGAMPGVYR.V	2	4.83
9444	XhSPS2	121&124 kDa	R.ALGAMPGVYR.V	3	5.78
10142	XhSPS2	121&124 kDa	R.DKLEQLLK.Q	2	4.51
10148	XhSPS2	121&124 kDa	R.DKLEQLLK.Q	3	5.92
10938	XhSPS2	121&124 kDa	K.LIDKYDLYGQVAYPK.H	2	4.84
11142	XhSPS2	121&124 kDa	K.LIDKYDLYGQVAYPK.H	3	5.28
11655	XhSPS2	121&124 kDa	R.DIQDISLNLK.F	2	6.67
12221	XhSPS2	121&124 kDa	R.LYDGFDPIER.K	2	5.9
14577	XhSPS2	121&124 kDa	R.NYSLSDVVAFDNPNIQIEPEC#K.D	2	4.4

Changes in SPS transcript levels

Changes in transcript levels of the two SPS isoforms, XhSPS1 and XhSPS2 were measured during tissue water loss using quantitative Real Time PCR (Figure 4.21). A reference gene, R1, was used to normalize the data obtained for XhSPS1 and XhSPS2. The expression of this gene (R1) was shown to remain at a relatively constant level during dehydration in microarray studies (Sally-AnnWalford, University of Cape Town, South Africa, unpublished) and therefore it was chosen as a reference gene. The fluorescence emission, melting curves and standard curves for each gene are shown in Appendix C. Figure 4.21 B displays changes in XhSPS1 and XhSPS2 transcript normalized to the amount of R1 transcript. XhSPS1 and XhSPS2 initially respond similarly to dehydration with a two fold increase in transcript levels being noted between 100 and 72 % RWC. After the peak at the 72 % RWC mark there is a general reduction in transcript levels. At the 53 % RWC mark both transcript levels are significantly down regulated with XhSPS1 and XhSPS2 transcript levels dropping to less than half that observed in fully hydrated tissue. However, while XhSPS2 levels remain low there is a recovery in XhSPS1 levels from 53 % RWC to 19 % RWC, at which point XhSPS1 levels are similar to that measured in fully hydrated tissue. XhSPS1 levels then decrease again as the 5 % RWC mark is reached but still remain higher than XhSPS2 levels. Thus while XHPS2 is more dominant during the first half of tissue water loss (before 50 % RWC), in the second half XhSPS2 is down-regulated and XhSPS1 becomes the more highly expressed transcript.

Changes in SPS protein levels

The principle and method of the mass spectrometer based quantification process is described in detail in the Materials and Methods section of the current chapter. To reduce the complexity of the sample, proteins were first separated on a 8 % polyacrylamide gel prior to quantification by the mass spectrometer. Based on the previous identification and size determination of SPS protein in crude leaf extract, the targeted SPS protein bands were then excised and the protein quantified. After quantification of both XhSPS1 and XhSPS2 the general trend of total SPS protein expression was found to be similar to the trend in total extractable SPS activity ($V_{\max}$ activity) during dehydration (Figure 4.22).

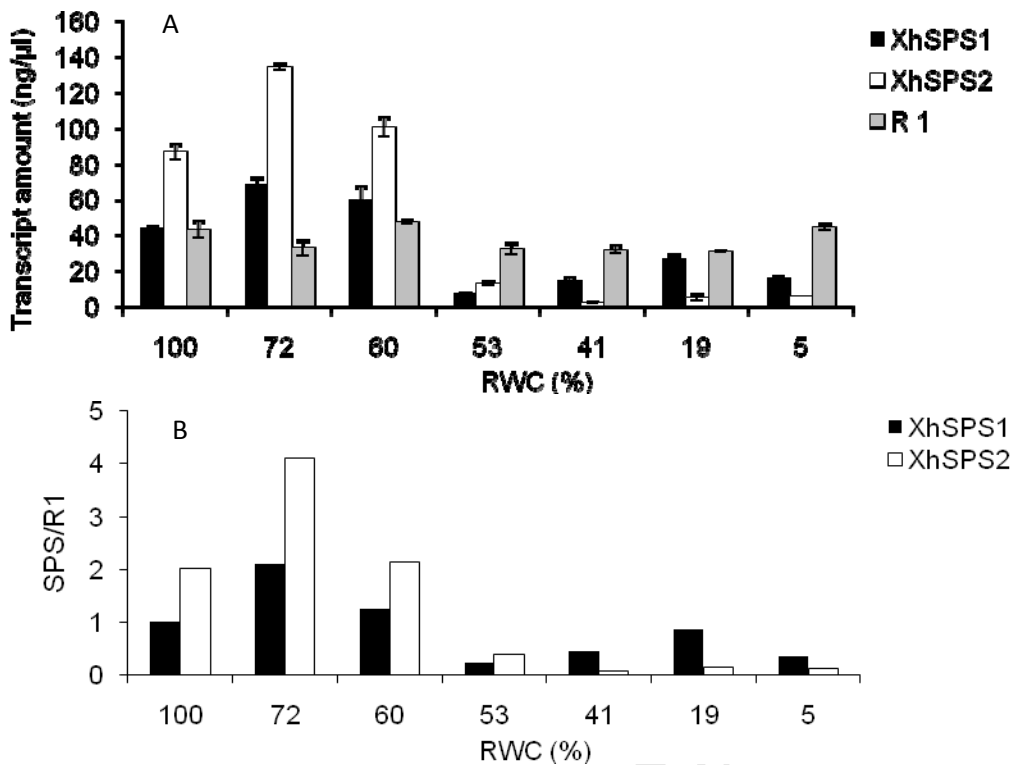


Figure 4.21 Change in transcript levels of XhSPS1 and XhSPS2 during dehydration. **(A)** Changes in transcript abundance of XhSPS1, XhSPS2 and R1. At each RWC point RNA was extracted from a pool of leaves (leaf $n > 6$) and 5 μ g of total RNA used for a single cDNA synthesis. Each value is mean $\pm$ SD of three technical replicates. **(B)** XhSPS1 and XhSPS2 transcript levels normalized to the reference gene R1 (SPS/R1). The value on the y-axis is the ratio of SPS transcript (XhSPS1 or XhSPS2) to the reference transcript (R1).

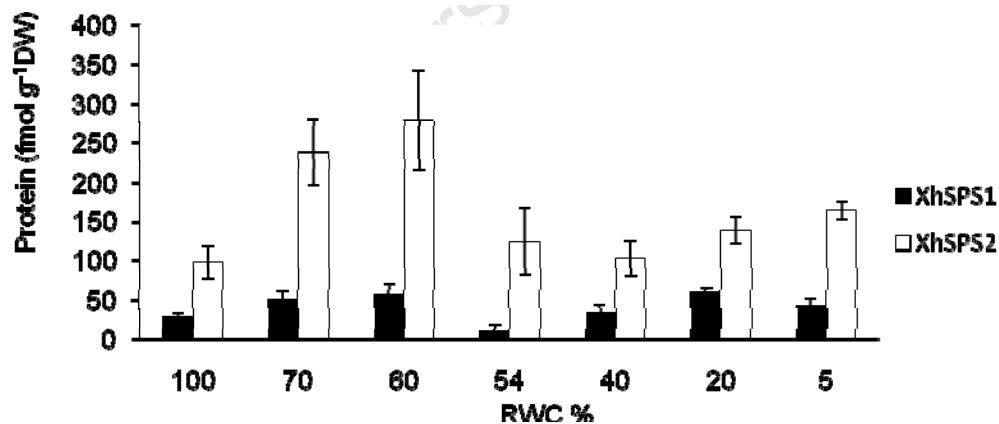


Figure 4.22 Changes in levels of XhSPS1 and XhSPS2 protein during dehydration. At each RWC point protein was extracted from a pool of homogenized freeze dried leaf tissue. Three independent protein extractions (30mg of leaf tissue per extraction) were performed on the pool of leaves. For each of the three protein extracts, the amount of XhSPS1 and XhSPS2 was quantified (see Figure 2.1, pg 24). Values are means $\pm$ standard deviation of three independent measurements of a pool of harvested leaf tissue at each RWC point.

In response to dehydration both XhSPS2 and XhSPS1 increase significantly with an almost three fold increase in XhSPS2 and a two fold increase in XhSPS1 being noted between 100 and 60 % RWC (Figure 4.22). At 54% RWC both isoforms are downregulated with a dramatic drop in XhSPS1 levels being observed. XhSPS2 levels continue to drop from 54% RWC to 40% RWC but increase again from 40 to 5 % RWC. However the magnitude of this latter increase is much smaller than that observed in the initial stages of dehydration. After the striking drop at the 54% RWC mark, XhSPS1 levels increase again in the later stages of water loss and by 20% RWC XhSPS1 levels exceed their pre-dehydration values. In both fully hydrated and drying leaf tissue the amount of XhSPS2 protein was found to be consistently higher than XhSPS1 (Figure 4.22), and in contrast to transcript levels there is no switch in dominance beyond 50 % RWC.

Changes in transcript levels of SPS genes in model system A. thaliana

With a well annotated genome and a large amount of expression data available online it was possible to conduct an in silico analysis of SPS gene expression in *A. thaliana*. The results in Figure 4.23 display a heatmap showing fold changes in each of the four SPS genes in response to a number of stresses. The map was produced in Genevestigator (<https://www.genevestigator.ethz.ch/>) and is based on normalized micro-array expression data. In the map it can be seen that the AtSPS1F (labeled as 245904_At in the heat map) isoform in particular is upregulated in response to a number of stresses - including salt, cold and osmotic stress (300 mM Mannitol treatment). However, in response to 'drought' stress (loss of 10% of fresh weight) there appears to be no major change in any of the SPS isoforms.

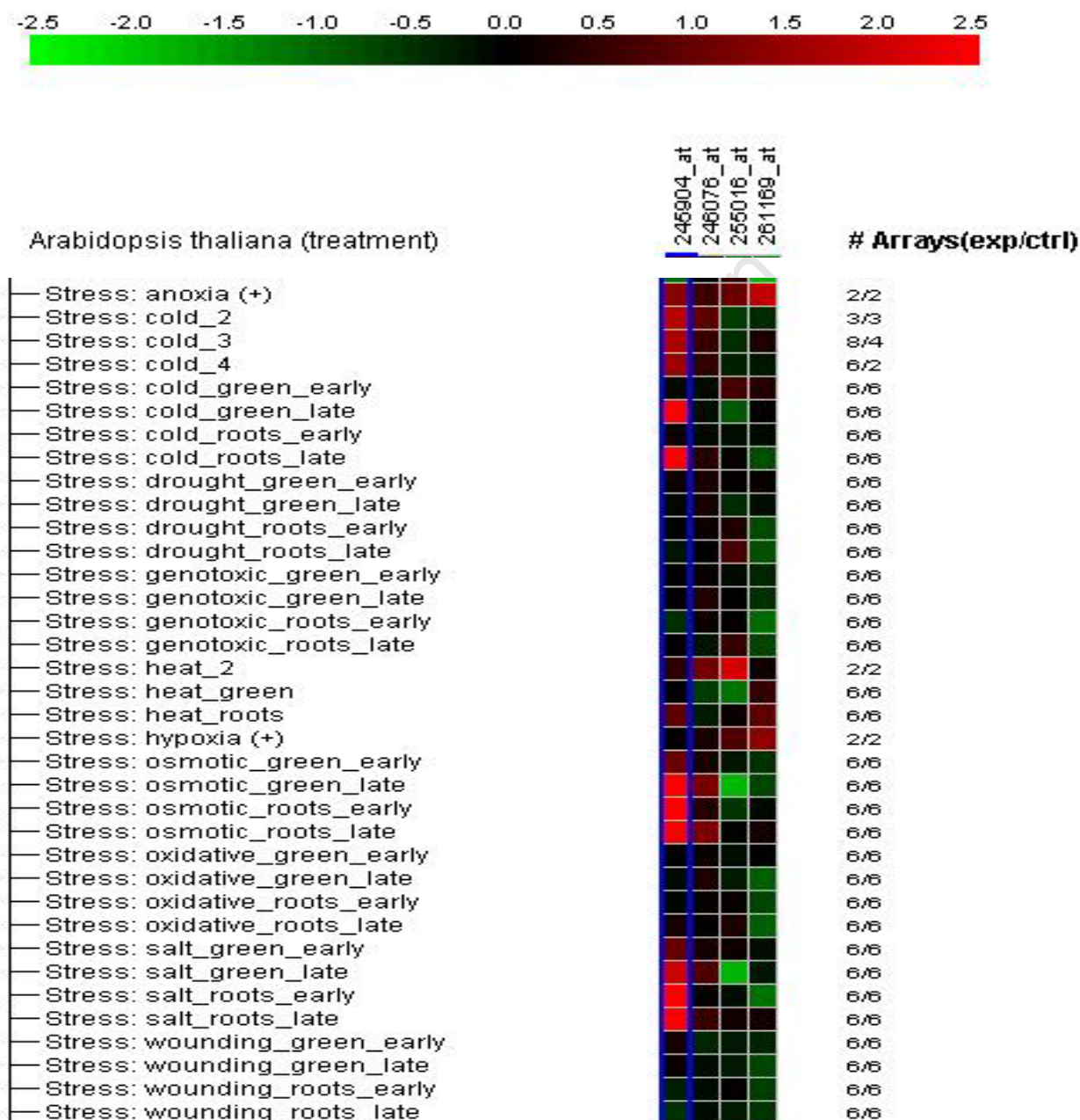


Figure 4.23 Expression of *A. thaliana* SPS isoforms in response to stress. Heat map produced by Genevestigator (<https://www.genevestigator.ethz.ch>) and based on normalized micro-array expression data.

Discussion

SPS genes identified in *Xerophyta humilis*

After an extensive screening of potential SPS encoding transcripts, two SPS genes were identified in *X. humilis*, namely XhSPS1 and XhSPS2. Both SPS genes were found to share a higher identity with SPS genes from other species than with each other (only 58 % identity at nucleotide level), suggesting that each gene may be functionally specialized. In the well studied *Spinacia oleracea* (spinach) SPS protein four important conserved regions have been characterized, namely the UDP-Glucose binding site, the putative Fructose-6-phosphate binding site, the phospho-regulated 14-3-3 binding site and two regulatory phosphorylation sites involved in light/dark and osmotic regulation of SPS activity (McMichael et al., 1993; Salvucci and Klein, 1993; Salvucci et al., 1995; Huber and Huber, 1996). With regards to the conserved substrate binding motifs there is little sequence variation between the *X. humilis* genes in both the putative fructose-6-phosphate and the UDP-Glucose binding sites. Of the three phospho-regulatory sites only the Ser₁₅₈ site is well conserved in both genes. Notably, both genes lack the corresponding osmoregulatory Ser₄₂₄ site. This suggests that either i) XhSPS1 and XhSPS2 are not under osmotic regulation or ii) osmotic activation of these *X. humilis* SPS genes does not occur via the phosphorylation/de-phosphorylation of the analogous Ser₄₂₄ site. When comparing conserved motifs between XhSPS1 and XhSPS2 the main difference is found in the proposed 14-3-3 binding site of the enzyme where the corresponding Ser₂₂₉ is substituted by glutamine in XhSPS2. While 14-3-3 proteins have been demonstrated to bind SPS, the regulation of 14-3-3 binding and the effect this binding may have on SPS activity is still a matter of debate (see below). Nevertheless, an intriguing observation regarding the relationship between 14-3-3 binding and SPS protein degradation has been made by Cotelle et al., 2000. The authors found that binding of 14-3-3 proteins in phosphorylation dependent manner inhibited *in vitro* cleavage of SPS. Thus, the observed difference between XhSPS1 and XhSPS2 in the 14-3-3 binding site could translate into differences in the turnover rates of the two genes.

Phylogeny of SPS genes in angiosperms

To establish the relationship of the identified *X. humilis* SPS genes to other known SPS genes a phylogenetic analysis of all available full length SPS protein sequences was conducted. The resulting phylogenetic tree suggests that SPS genes in higher plants can be grouped into three main families, designated Family A, Family B and Family C. In all the major families there are clear eudicot and monocot divisions indicating that the duplications giving rise to the current gene families probably occurred before the eudicot/monocot divergence. Based on the topology of the tree rooted on the cyanobacteria *Synechocystis* SPS (data not shown) it would appear that Family C was the first to diverge from the assumed ancestral bacterial SPS. However, in the MacRae and Lunn, (2006) analysis, which included sequence information from plants such as *Selaginella* and *Physcomitrella*, the more ‘primitive’ SPS sequences were all found in Family B. As can be seen in Figure 4.18 when the root is placed on the line leading to a *Physcomitrella* SPS genes the first family to diverge is Family B. It is therefore currently debatable as to which SPS gene family is the most ancient in higher plants.

When compared to previous analyses, the topology of the phylogenetic tree obtained in the present study is broadly consistent with previous tree structures. The most significant difference between the present work and the Castleden et al., 2004 and MacRae and Lunn, 2006 body of work is the absence of the proposed Family D group of SPS genes. Rather, the analysis in the current study is more consistent with the ‘Family D’ group of SPS genes forming part of the broader Family A and not constituting a new family grouping. Amongst the monocots of Family A, the presence of multiple *Oryza sativa* and *Zea mays* genes indicate recent duplication events within the monocot grass lineage. Within the monocot grass group these duplications have given rise to three subgroups, labeled A-gm1, A-gm2 and A-gm3 (Figure 4.18). Notably, the combined A-gm2 and A-gm3 groups contain all the SPS genes found in the proposed Family D grouping of Castleden et al., (2004) and MacRae and Lunn, (2006).

In the phylogenetic analysis conducted by Lutfiyya et al., (2007) three main groups of plant SPS genes are also identified, termed Group 2, Group 3 and Group 4 (Group 1 in the analysis contains only bacterial SPS). Based on the set of SPS genes in each group, Group 2 is equivalent to Family A, Group 3 to Family B and Group 4 to Family C. Similar to the current analysis (see Figure 4.1), there are clear monocot and eudicot subgroups in each major Family and the monocot subgroup of Group 2 (equivalent to Family A) is further divided into three subgroups: 2m1, 2m2 and 2m3. Based on the genes present in groups 2m1 and 2m2 it would appear that these groups are equivalent to sub-groups A-gm2 and A-gm3 of the current study and to Family D of the Castleden et al., (2004)/MacRae and Lunn, (2006) analysis. Group 2m1 contains additional *Z. mays* sequences which are not publicly available and hence are not present in the A-gm2 sub group of the current phylogenetic analysis. In line with the present study, groups 2m1 and 2m2 fall within the greater Group 2 (equivalent to Family A) family and are not designated as a new fifth Family (as is the case of Family D in Castleden et al., 2004). Thus, in both the current work and in the analysis of Lutfiyya et al., (2007) there is support for the 'Family D' cluster of genes being positioned within the broader Family A grouping.

In Family A eudicots recent duplication events in both *A. thaliana* and *L. esculentum* have resulted in two genes from each species being found in Family A. MacRae and Lunn, (2006) describe two subgroups (A1 and A2) (Figure 4.1) within the eudicots of Family A with a number of species including, *A. thaliana*, *Beta vulgaris* (sugar beet) and *L. esculentum* (tomato) containing A-type representatives in both sub group A1 and A2. However, in the current analysis these sub-groupings within Family A eudicots are unclear. The two *A. thaliana* genes are well separated in the present analysis but the two *L. esculentum* genes cluster quite closely together and there is only one full length *B. vulgaris* (sugar beet) sequence available (the sequence used in the MacRae and Lunn (2006) analysis was partial). Possibly with the addition of new full length sequences the subgroups will become more apparent. Within Family A one would expect, based on established phylogenetic relationships between angiosperm species (Figure 4.25) (Angiosperm Phylogeny Group II, 2003), that three groups would be evident. One group made is up of *Actinidia*, *Craterostigma*, *Lycopersicon*, *Nicotiana* and *Solanum*, a second including *Beta* and *Vicia* and a third consisting of *A. thaliana* and *Citrus*. The lack of these

typical groupings suggests that the SPS genes in Family A eudicots are under active duplication and divergence.

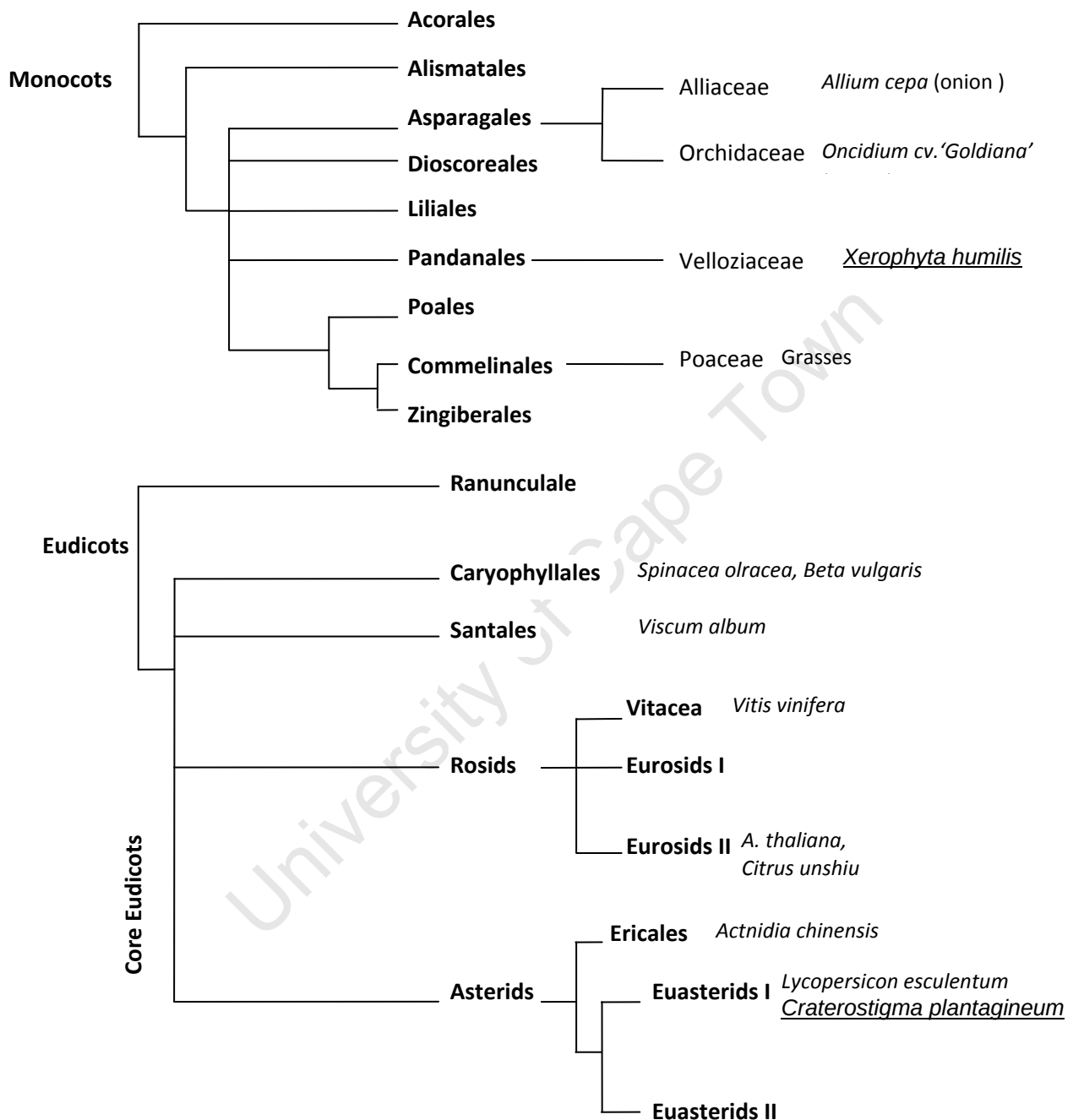


Figure 4.24 Phylogram displaying relationships of the major monocot and eudicot orders (adapted from Angiosperm Phylogeny Group 2, 2003). The position of select species used in the phylogenetic study are displayed. Resurrection species are underlined.

Compared to Family A, Families B and C contain much fewer SPS genes and hence these families are much less complex than Family A. As in Family A there is a clear clustering of eudicots and monocots in Family B and Family C. This separation is also supported by both the Castleden et al., (2004)/MacRae and Lunn, (2006) analyses and Lutfiyya et al., (2007) analysis. There is no indication of recent duplication events occurring in either Family B or Family C but it is likely that as more members of these families are isolated, the evolutionary history of each family will become more apparent.

Prior to the current investigation there was only one full length non-grass monocot sequence in the available databases, the orchid sequence, *Oncidium* cv 'Goldiana'. As can be seen in phylogenetic tree, one of the identified *X. humilis* SPS genes, XhSPS2, clusters closely with this sequence and they are both positioned at the base of the monocot line leading to the grasses in Family A. Family B contains the other identified *X. humilis* SPS gene, XhSPS1, and as is the case in Family A, this *X. humilis* gene is positioned at the base of the monocot line. Interestingly, when the almost full length (missing first ~80 amino acids) *Allium cepa* (onion) sequence is included in the phylogenetic analysis, it forms a distinct cluster with XhSPS1 within Family B and there is strong bootstrap support (over 80%) for the separation of non-grass (XhSPS1 and *A. cepa* SPS gene) and grass monocots in Family B (data not shown). Thus, in Family A and Family B there appears to be a separation between non-grass and grass monocots but more non-grass monocot sequences will be needed for a clearer definition of these sub-groups. As can be seen in Figure 4.25 *Allium* and *Oncidium* are both members of the order Asparagales which is closely positioned to Pandanales, the order which contains *X. humilis*. Thus, the clustering of these sequences at the base of the line leading to the monocot grasses fits well with the proposed relationships within the monocot orders.

Possible additional SPS genes in *X. humilis*

The phylogenetic relationships between known SPS proteins would suggest that *X. humilis* should have three SPS genes belonging to three different families. Hence, the identification of

only two SPS genes suggested that the screening process may have missed a gene. If the initial screening had missed a gene, then it is in Family C that a possible third *X. humilis* SPS gene would be expected, probably positioned at the base of the Family C monocot line. An attempt was made to isolate a *X. humilis* Family C member by designing primers biased towards Family C SPS proteins but no new SPS genes could be found. This suggests that either the screening process failed to isolate a third SPS gene or that *X. humilis* lacks a third SPS gene. It is possible that the expected Family C is only expressed in a very specific tissue, such as pollen, and hence the screening process would not have detected it as only leaf and root tissue were screened. Interestingly, in the only other resurrection plant studied by Ingram et al., (1997), the eudicot *C. plantagineum*, only two SPS encoding transcripts were identified in leaf tissues. Similar to *X. humilis*, *C. plantagineum* has a member in Family A, Cpsps1 and in Family B, Cpsps2.

Conserved motifs in SPS protein sequences

The presence of multiple SPS gene families could suggest that members of particular families may share common functions or are subject to common regulatory pathways. To gain some insight into the functional or regulatory significance of the family groupings, important conserved regions in plant SPS proteins were compared between different families. Four conserved regions are believed to be important in substrate binding and regulation of SPS in plants, the putative Fructose-6-phosphate binding domain (Salvucci et al., 1995), the UDP-Glucose binding domain (Salvucci and Klein, 1993), and the three conserved motifs thought to be involved in the phospho-regulation of SPS enzyme activity, the 'Ser₁₅₈', 'Ser₂₂₉' and 'Ser₄₂₄' sites (amino acid numbering based on *S. oleracea* (spinach) sequence) (McMichael et al., 1993; Salvucci and Klein, 1993; Salvucci et al., 1995; Huber and Huber, 1996). An alignment of all SPS protein sequence will reveal a number of other highly conserved regions but in most cases the functional significance of these regions is yet to be characterized.

Conservation of substrate binding domains

The putative fructose-6-phosphate binding region is highly conserved in most SPS sequences with the only variation being found in *C. plantagineum* CpSPS2 (Y11795), *N. tabacum* SPSB (DQ213015) and ZmSPS3 *Z. mays* (see Figure 4.18) sequences where the chemically similar isoleucine is substituted for valine. In the UDP-Glucose binding region (which includes the 'Ser' 229 site), there is more variation but in general the observed amino acid differences are not likely to have a major effect on the properties of the region. Amino acid substitutions which may affect the substrate binding properties of the region are found in members of the A-gm2 and A-gm3 sub-groups where there is i) the substitution of the neutral amino acid tryptophan with the basic arginine and ii) the substitution of the acidic aspartic acid (D) with the neutral asparagine. What effect the changes may have on the affinity of the region requires experimental demonstration but it has been observed that the saturation levels for UDP-Glucose are generally much higher in monocots, particularly grass monocots, when compared to eudicots such as spinach (Lunn and Furbank, 1997; Trevanion et al., 2004).

As will be described below, the members of A-gm2 and A-gm3 groups are also distinct from most other SPS genes in their lack of the three phospho-regulatory sites common to higher plant SPS proteins. In the introduction to this chapter it was noted that members of Family D (described by Castleden et al., 2004) more closely resemble bacterial SPS and it was postulated that Family D may represent the most ancient SPS gene family in plants. However, the phylogenetic analysis in the current study indicates that the A-gm2 and A-gm3 groups, which are equivalent to Family D, are most likely due to recent duplication events in Family A. Based on this observation it can be concluded that the phospho-regulatory features common to most higher plant SPS proteins have been lost in the members of A-gm2 and A-gm3 (Family D in Castleden et al., 2004 analysis). It is likely that the loss of these features will represent a functional adaptation, but this will require more in depth biochemical studies on A-gm2 and A-gm3 type SPS genes to characterize any possible functional specialization.

Conservation of phospho-regulatory sites

Ser₁₅₈-Light/Dark regulatory site

The Ser₁₅₈ site, shown to be involved in light dark regulation in spinach (McMichael et al., 1993), is present in all known SPS sequences with the exception of AY26439 *L. esculentum* SPS where the serine is substituted with a cysteine. While the actual phospho-serine is highly conserved in most angiosperm SPS proteins, there is significant variation in the kinase recognition motif surrounding this serine. It has been demonstrated that a basic residue at position -6 relative to the phosphorylated serine is a strong positive recognition element necessary for phosphorylation (Huang and Huber, 2001). In members of the A-gm2 and A-gm3 groups, both this basic residue and the accompanying hydrophobic residue at position -5 are absent. The basic residue at position -6 is also absent from two eudicot members of the Family C, *N. tabacum* (tobacco) and *V. vinifera* (grape). The absence of these recognition elements suggests that the degree of phosphorylation of these genes at the Ser₁₅₈ site may be altered relative to other genes. Interestingly, the *N. tabacum* (tobacco) member of Group C has been proposed to be the major SPS gene responsible for night time sucrose synthesis in tobacco leaf (Chen et al., 2005). As phosphorylation of the Ser₁₅₈ site is known to down regulate SPS activity in spinach at night (McMichael et al., 1993), the lack of a critical kinase recognition residue in the tobacco Family C member may allow this gene to maintain the activity levels necessary for night time sucrose synthesis in tobacco.

Much of the work related to Ser₁₅₈ phosphorylation has been conducted on spinach leaf extracts. From this work it was established that spinach SPS Ser₁₅₈ could be phosphorylated by both Ca²⁺ dependent and Ca²⁺ independent kinases (McMichael et al., 1995b). At present two kinases are thought to be involved in the phosphorylation of SPS, namely a calmodulin-like domain protein kinase (CDPK) and a sucrose non-fermenting 1-related protein kinase (SnRK1) (Huang and Huber 2001). The occurrence of a proline residue at position -4 has been demonstrated to significantly inhibit phosphorylation by CDPK (Huang and Huber, 2001) and thus genes containing this proline will be mainly phosphorylated by SnRK1. In Family A eudicots and Family A non-grass monocots, most genes contain the proline at position -4 suggesting that

SnRK1 is the main kinase responsible for light/dark regulation in these SPS enzymes. The notable exceptions are i) *S. oleracea* and *A. thaliana* in Family A eudicots and ii) *Oncidium cv. 'Goldiana'* in Family A monocots which do not have a proline residue at position -4 and may therefore be phosphorylated by CDPK. In contrast to most Family A eudicots and Family A non-grass monocots, the proline residue is absent in most members of Families B and C. Thus, it appears that certain groups of SPS genes may be targeted by specific classes of kinases and hence may be under the control of different regulatory networks.

Ser₂₂₉ - 14-3-3 binding site

The next conserved motif which may be of regulatory significance is the region corresponding to the Ser₂₂₉ site in the spinach SPS protein. The peptide motif RSXpSXP (where pS is the phosphorylated Serine and X is any residue) has been identified as a consensus binding site for 14-3-3 proteins (Muslin et al., 1996). While the sequence surrounding spinach Ser₂₂₉ (LTRQVSAP) is not an exact match, a phosphorylated form of this peptide has been shown to bind 14-3-3s *in vitro* (Toroser et al., 1998). Sequence analysis of spinach leaf SPS also reveals several other candidate binding sites for 14-3-3 proteins but only the putative site involving Ser-229 is conserved across species boundaries (consensus sequence HXRXXSXP, where H is a hydrophobic residue). It has been demonstrated that SPS interacts directly with 14-3-3 proteins in a phosphorylation and Mg²⁺-dependent manner (Toroser et al., 1998; Moorhead et al., 1999). 14-3-3 proteins are a highly conserved family of regulatory proteins which have been shown to interact with a number of primary metabolic enzymes including NR and SPS (Aitken, 1996; Roberts, 2000). The sequence surrounding Ser₂₂₉ (consensus sequence HXRXXSXP) is common to most plant SPS proteins with little variation being observed. The notable exceptions are members of Family A-gm2 and A-gm3, where Ser₂₂₉ is replaced by alanine. For a number of other 14-3-3 target enzymes it has been shown that serine to alanine substitutions within the binding motifs completely abolished 14-3-3 interaction in the two-hybrid system (Kanamaru et al., 1999; Igarashi et al., 2001). However, the necessity of Ser₂₂₉ phosphorylation for 14-3-3 binding has been questioned by results demonstrating that certain 14-3-3 genes may bind SPS independently of the phosphorylation state of the target peptide sequence (Bornke, 2005). The effect of binding of a 14-3-3 on SPS activity is also debated. Both partial inhibition of the

enzyme (Toroser et al., 1998) as well as activation (Moorhead et al., 1999) have been reported. Alternatively, 14-3-3 binding may modulate the rate of proteolytic degradation of SPS in response to changes in cellular carbohydrate status (Cotelle et al., 2000). Thus, while the *in vitro* interaction experiments and the high conservation of this putative 14-3-3 binding region suggest that the site may be of regulatory significance, the mechanisms and result of that regulation still require clarification. Nevertheless it is still intriguing that once again members of Family A-gm2 and A-gm3 differ from other SPS genes in this region.

Ser₄₂₄ – Osmoregulatory site

In addition to the site around Ser₁₅₈, the kinase recognition motif BHXBXS (where B is a basic residue) (McMichael et al., 1995b) is also noted around Ser₄₂₄. In all plant SPSs the recognition critical -3 and -6 basic residues are highly conserved but in certain instances the serine is replaced by asparagine. The substitution is observed in all members of Family A-gm2 and A-gm3 and in all eudicots of group B. In spinach phosphorylation of the Ser₄₂₄ site has been shown to be associated with osmotic stress activation of SPS activity (Toroser and Huber, 1997). The lack of a target serine in the above mentioned groups may indicate that these genes are either not activated by osmotic stress or are activated by an alternative mechanism.

SPS gene expression in response to dehydration

The methodology used, namely quantitative Real Time PCR for transcript analysis and Stable Isotope Absolute Quantification for protein analysis, allowed for specific changes in both XhSPS1 and XhSPS2 transcript and protein levels to be measured. Hence the response of each isoform to dehydration could be characterized at both the transcript and protein levels. Changes in transcript levels of the identified SPS genes, XhSPS1 and XhSPS2, were measured at 6 points during dehydration. In fully hydrated leaf tissue, XhSPS2 is the more dominant transcript and makes up 60 % of the total SPS transcript. In response to initial dehydration there is a two fold increase in both XhSPS1 and XhSPS2 but there is little change in the transcript demographic. As water loss progresses XhSPS2 remains as the dominant transcript down to a RWC of 53 % but a

switch occurs at 41 % RWC when XhSPS2 transcript levels drop below XhSPS1 levels. As tissue water loss progresses XhSPS1 remains as the dominant transcript, constituting more than 80% of the total SPS transcript. Hence, based on the transcript analysis alone, XhSPS1 appears to be a 'late' dehydration specific isoform. However, rather surprisingly, the switch in dominance from XhSPS2 to XhSPS1 noted at the transcript level does not translate into a similar change at the protein level. In both fully hydrated (100% RWC) and drying tissue (all stages of dehydration) the dominant SPS isoform was found to be XhSPS2. Further, even when XhSPS2 transcript levels dropped considerably below XhSPS1 levels, XhSPS2 protein remained at least two fold higher than XhSPS1 protein.

In the current study a dehydration specific isoform is defined as an isoform which is either absent or lowly expressed in fully hydrated tissue and becomes more prevalent in response to dehydration. In the previous study on *C. plantagineum*, the two identified SPS transcripts, Cpsps1 and Cpsps2, were found to exhibit distinct responses to dehydration. For Cpsps1, transcript levels are high in fully hydrated tissue, increase slightly in the early stage of dehydration (100 to 79 % RWC) but decline dramatically in the late stage of dehydration (25 % - 2 % RWC). In the case of Cpsps2, transcript levels are low in fully hydrated tissue (barely visible in Northern Blot) increase strongly at 79 % RWC and are barely detectable again in the late stage of dehydration (25 -5 %RWC). Thus, in *C. plantagineum* there appears to be a constitutively expressed SPS isoform and a dehydration specific isoform.

Despite the changes observed at the transcript level, it is clear from the protein analysis that XhSPS2 is the dominant isoform in both fully hydrated and drying tissue. Therefore it was concluded that in *X. humilis*, dehydration induced sucrose accumulation is not associated with an up-regulation of a specific SPS isoform. The reason for the disparities between the transcript analysis and the protein analysis in the current study is unclear. At the transcript level there is a definite switch in dominance from XhSPS2 to XhSPS1 in the late stage of dehydration but at the protein level XhSPS2 remains as the dominant isoform. One possible explanation is that XhSPS2 protein levels remain high due to decreased protein turnover in the late stages of dehydration. In *A. thaliana* phosphorylation of a specific serine has been demonstrated to affect

the rate of SPS breakdown (Cotelle et al., 2000) and possibly a similar post translational modification may affect the stability of XhSPS2. Notably the serine site equivalent to that found in *A. thaliana* (Ser₄₂₄-Spinach amino acid numbering) is only present in the XhSPS2 isoform. Further investigation will be required to establish if phosphorylation of this site may affect stability of XhSPS2.

Adopting a rather simple model of the relationship between gene transcript and gene product (protein) one may expect changes in protein levels to closely follow changes in transcript levels. While in many cases there is a correspondence between transcript and protein levels, the relationship between changes in transcript levels and changes in protein levels can be complex. In a detailed study on the correspondence between transcript levels and protein levels in *Arabidopsis*, Gibon et al., (2004) observed that in certain instances changes in transcript levels did not result in altered protein levels. Further, even when protein levels were altered there could be a significant delay between the initial change of transcript level and the alteration in protein level (Gibon et al., 2004). The incongruence between changes in transcript levels and protein levels observed in the current study provides further support for the need to consider both transcriptional and translational elements when describing a biological response to a stress or other stimulus.

In *C. plantagineum* the proposal that dehydration results in the up regulation of a specific SPS isoform was based solely on transcript analysis (Ingram et al., 1997). At the protein level only changes in total protein are reported and there is no indication of specific changes in each of the *C. plantagineum* isoforms. Furthermore, there is little correlation between changes in total transcript (sum of Cpsps1 and Cpsps2) and changes in total SPS protein during dehydration. Considering the strong up regulation of Cpsps2 in response to dehydration it is unlikely that the change is not of biological significance, nevertheless, the findings in the current work suggest that conclusions based on transcript analysis alone may need to be confirmed at the protein level. In the other well studied desiccation tolerant tissue, namely orthodox seeds, multiple SPS gene expression has been reported during seed development. Unfortunately, linking SPS gene

expression to onset of desiccation tolerance is not always possible. For example, in wheat seeds two SPS transcripts (TaSPSII and TaSPSIV) are upregulated in the late stage of seed development (Casteleden et al., 2004) but it is unclear whether this upregulation co-incides with the onset of desiccation tolerance. Further, due to lack of data on actual sucrose accumulation and SPS activity the significance of this upregulation is unclear. In *Vicia faba* seeds SPS activity (measured in V_{lim} assay), SPS protein levels and SPS transcript levels all increase at 55 days after flowering (DAF) around the onset of desiccation tolerance (Weber et al., 1996). However, these changes do not result in concomitant increases in sucrose content of the drying seed tissue. In *Vicia faba*, the period of active sucrose accumulation actually occurs during reserve storage phase (Heim et al., 1993) when SPS activity and SPS transcript levels are low (Weber et al., 1996). Hence the role of this up regulation of SPS activity is once again unclear.

In desiccation sensitive plants no studies aimed specifically at characterizing the expression of particular SPS isoforms in response to water deficit stress in photosynthetic tissue could be found in the literature. The only information that can be obtained is based on publicly available micro-array expression data. Due to the large amount of micro-array expression data, the most detailed information on SPS isoform expression is available for *A. thaliana*. The map was produced in Genevestigator (<https://www.genevestigator.ethz.ch/>) and is based on normalized micro-array expression data. In the map it can be seen that the AtSPS1F (labeled as 245904_At in the heat map) isoform in particular is upregulated in response to a number of stresses - including salt, cold and osmotic stress (300 mM Mannitol treatment). However, in response to 'drought' stress there appears to be no major change in any of the SPS isoforms. In addition to *A. thaliana*, a large amount of expression data is also available for maize. In maize, one of the seven identified isoforms (ZmSPS3F) is specifically induced by 'drought' treatment in leaf tissue (Lutfiyya et al., 2007). In the *A. thaliana* experiment 'drought' treatment resulted in a loss of 10% of fresh weight but no information on actual water loss in the maize experiment could be found.

From the above discussion it is clear that while multiple SPS gene expression is observed in a number of species, dehydration does not necessarily induce the up regulation of a specific SPS

isoform. In the case of *X. humilis* the XhSPS2 isoform is the major constitutive isoform in fully hydrated tissue and there appears to be little need for the induction of a specialist isoform in response to drying. The major down regulation of XhSPS1 at the 54 % RWC mark, a point which co-incides with the downregulation of photosynthesis, suggests that XhSPS1 expression may be linked specifically to photosynthetic activity. However, the recovery of XhSPS1 in the late stages of dehydration despite almost complete inhibition of photosynthetic activity complicates this interpretation. Further investigation will be required to determine the functional significance, if any, of the expression of two SPS isoforms in *X. humilis* leaf tissue.

Post-translational modification (PTM) of SPS

To investigate the possible regulation of SPS by post translational modification an attempt was made to measure changes in the phosphorylation state of XhSPS1 and XhSPS2. Based on sequence information both isoforms contain the Ser₁₅₈ kinase recognition motif which is involved in light dark modulation in spinach (Huber and Huber, 1996). In addition, XhSPS2 also contains the Ser₂₂₉ site thought to be involved in 14-3-3 binding (Toroser and Huber, 1997). However both isoforms lack the Ser₄₂₄ site which is proposed to be involved in osmoregulation in spinach (Toroser and Huber, 1997). In the current study an attempt was made to investigate the possible involvement of the Ser₁₅₈ and Ser₂₂₉ sites in dehydration induced SPS activation. To quantify changes in phosphorylation of these sites, tryptic peptides were designed which included the analogous phosphorylated serines in XhSPS1 and XhSPS2. A set of corresponding peptides were also designed which did not contain the post-translational modification. An attempt was made to measure the specific levels of these phosphorylated peptides by the previously described stable isotope quantification procedure. While the levels of the non-phosphorylated peptide could be measured, the phosphorylated forms were below the detection limit of the mass spectrometer used. A primary challenge when conducting studies on phosphorylated proteins is the low stoichiometry of the phosphorylated form of the protein when compared to the non-phosphorylated form (Glinski and Weckwerth, 2005). Thus, except when studying highly phosphorylated proteins it is usually necessary to employ a phospho-enrichment strategy prior to analysis. In the current study an attempt was made to enrich for phospho-

proteins using a modification Metal Oxide Affinity Chromatography (MOAC) (Wolschin and Weckwerth, 2005). Unfortunately due to high protein loss this strategy could not be applied to the current analysis. Limited availability of total *X. humilis* leaf tissue also prevented the use of alternative phospho-protein enrichment strategies. Consequently, it is uncertain if the lack of the targeted phospho-peptides is due to the inability to detect these peptides in a non-enriched (for phospho-protein) sample or due to their actual absence. One possible future strategy would be to use monoclonal antibodies to enrich for the phosphorylated forms of XHSPS1 and XhSPS2 prior to tryptic digestion and mass spectrometer analysis. Alternatively a novel phospho-protein enrichment strategy which reduces protein loss may need to be developed and utilized. An understanding of how each SPS isoform is regulated at the phosphorylation level will also assist in assigning possible functional roles to each isoform in general or stress metabolism.

Conclusion

In conclusion, two SPS genes, named XhSPS2 and XhSPS1, were identified in *X. humilis* leaf tissue. Changes in mRNA transcript abundance and protein levels revealed that dehydration resulted in the up-regulation of both SPS isoforms. The modulation of total SPS protein (XhSPS2 and XhSPS1) by dehydration was similar to that observed in total SPS activity ($V_{\max}$) in Chapter 3. XhSPS2 was the dominant protein in both fully hydrated and drying leaf tissue. At the transcript level a switch in dominance between XhSPS2 and XhSPS1 was observed in the later stages of water loss. However, this switch was not noted at the protein level and hence it was concluded that severe water loss does not induce a dehydration specific form of SPS.

The current work describes a multilevel study of the relationship between SPS and dehydration induced sucrose accumulation in the resurrection plant species *X. humilis*. In response to dehydration changes in SPS enzyme activity measurement and gene expression (at both transcript and protein level) were monitored in leaf tissues. In addition, changes in a broad range of metabolites were tracked in order to identify possible sources of carbon supporting the observed sucrose accumulation. To obtain enough leaf tissue for the multilevel analysis a group of *X. humilis* were dried down and leaves from several individuals were harvested and pooled at various points during leaf dehydration. Ideally a second dehydration series should have been conducted to confirm the findings, however due to the limited availability of plant material (*X. humilis* is not under cultivation) it was not practical to repeat the entire multilevel analysis, which required both an experimental and a control group of plants. Nevertheless, despite only acquiring data from a single dehydration series it should be noted that the results obtained represent the average response of at least three individual plants (leaf tissue was pooled at each harvest) to water loss. Furthermore, to ensure that the experimental procedures used were accurate all analyses were conducted with at least three technical replicates. Thus the conclusions drawn should be reflective of real molecular and biochemical changes during dehydration.

Sucrose accumulation in response to dehydration

In *X. humilis* leaf tissue sucrose levels increased almost four fold from fully hydrated tissue (100% RWC) to fully desiccated tissue (RWC <5%). Sucrose accumulation during tissue water loss was biphasic with a doubling in sucrose content observed in the early stages of dehydration (100 to 60 % RWC) and a further two fold increase occurring in the latter stages of dehydration (40 to 5 % RWC). These two phases of sucrose accumulation were separated by a period, during the middle stage of water loss (60 to 40 % RWC), when sucrose content remained relatively unchanged. The most rapid period of sucrose accumulation occurred between the 20 and 5 %

RWC marks where sucrose content doubled in less than two days. Desiccated leaf tissue contained a final stable sucrose content of almost 12 % (on a dry mass basis). A similar four fold increase in sucrose content has been observed previously in drying *X. humilis* leaf tissue (Farrant, 2003; Bajic, 2006) with most of the sucrose accumulation occurring below the 30 % RWC mark (Bajic, 2006). However this is the first report of two distinct phases of sucrose accumulation in *X. humilis*. Thus the results in the current study confirmed previous reports that dehydration induces sucrose accumulation in *X. humilis* leaf tissue.

Possible carbon sources supporting sucrose accumulation

To gain some insight as to possible sources of carbon for the observed sucrose accumulation, changes in a broad range of metabolites were monitored during tissue water loss. In the early stages of dehydration it appears that photosynthesis, starch breakdown, carbon translocation and the conversion of hexose sugars may all contribute to the observed increases in sucrose content. However, during the period of late sucrose accumulation photosynthesis ceases, translocation is minimal and the starch and hexose sugar pools are depleted. Hence access to new sources of carbon would be necessary to support continued sucrose accumulation. The metabolite analysis identified a transient increase in a number of gluconeogenic amino acids, including asparagine and valine, during dehydration. Accumulation of these particular amino acids follows a ‘bell’ shaped pattern with increases occurring in the middle stages of dehydration followed by decreased content in the late stage of dehydration (below 20% RWC). Based on this pattern it is postulated that amino acids may serve as a temporary carbon store with mobilization of carbon out of this pool supporting sucrose accumulation in the later stages of dehydration.

Changes in metabolite profile during dehydration

In addition to increases in the sucrose content of leaf tissue the metabolites raffinose, glycerol, glycerophosphoglycerol, xylitol and β -alanine also displayed notable increases. In the current study dehydration was found to induce a two fold increase in raffinose content. In a closely related species, *X. viscosa*, a comparable three fold increase in raffinose content has been

reported (Peters et al., 2007) in response to dehydration. Although dehydration-induced glycerol, β -alanine and xylitol accumulation has been previously reported previously in plants (Farrant et al., 2008; Rizhsky et al., 2004) this is the first report of glycerophosphoglycerol in a plant system and of the accumulation of this metabolite in response to stress. Glycerophosphoglycerol has been reported in the hyperthermophilic archaeon, *Archaeoglobus fulgidus* (Martins et al., 1997) where it is proposed to be used for osmoadaptation over a range of temperatures (Borges et al., 2006; Lamosa et al., 2006). A putative thermo-protective function *in vivo* has also been ascribed to glycerophosphoglycerol, and its action as a protein stabilizer *in vitro* has been confirmed (Lamosa et al., 2003; Santos et al., 2007b). Thus, it appears that *X. humilis* is not solely dependent on sucrose accumulation to provide macromolecular stabilization but utilizes a combination of different compounds to provide the necessary protection.

SPS activity and dehydration

In both photosynthetic (e.g. Guy et al., 1992; Holaday et al., 1992) and non-photosynthetic tissues (Geigenberger et al., 1995) increases in sucrose content are frequently linked to increased activity of sucrose phosphate synthase (SPS) - a key regulator of sucrose synthesis (Barber, 1985; Stitt et al., 1988; Lunn and ap Rees, 1990). Increases in SPS activity in response to dehydration have also been noted in both desiccation sensitive, e.g. spinach (Zrenner and Stitt, 1991) and in desiccation tolerant species, e.g. *Craterostigma plantagineum* (Ingram et al., 1997) and *S. stapfianus* (Whittaker et al., 2007). Hence it was hypothesized that the observed sucrose accumulation in drying *X. humilis* tissue is due to increased SPS activity. To investigate this hypothesis, the pattern of sucrose accumulation was related to the response of SPS activity to tissue water loss.

During dehydration changes in SPS activity follow a similar pattern to that observed for sucrose content (overall Pearson correlation of 0.73). There is a strong increase in activity during the early stage of dehydration, a drop off around the 50 % RWC mark and an increase in activity in the late stage of dehydration. This general pattern is observed when activity is assayed using both

the $V_{\max}$ assay conditions and the $V_{\lim}$ assay conditions. The data indicate that dehydration modulates both the total amount of SPS protein (reflected by the $V_{\max}$ assay) and the activation state of the enzyme (reflected by $V_{\lim}/V_{\max}$). In the early to middle stages of water loss (100 – 40 % RWC) a strong positive correlation between increases in SPS activity and increases in sucrose content was observed. However, in late stages of dehydration (below 20 % RWC) the link between increases in SPS activity and sucrose content appears much weaker. During this period the highest rate of sucrose accumulation was observed but the accompanying increases in SPS activity were comparatively low. This observation indicates that other mechanisms, aside from changes in SPS activity, may drive sucrose accumulation in the late stage of dehydration. Thus while increases in sucrose content are associated with increases in SPS activity during dehydration, changes in SPS activity do not appear to account for all the observed sucrose accumulation.

SPS activity and photosynthesis during dehydration

During photosynthesis it is well established that the synthesis of sucrose in the cytoplasm is coupled to carbon fixation in the chloroplast (e.g. Stitt et al., 1988). In *X. humilis* photosynthesis is known to be downregulated relatively early during dehydration (Farrant et al., 1999). However despite this downregulation of photosynthesis sucrose accumulation continues in the drying tissue. Hence the possible links between changes in photosynthetic activity and changes in SPS activity were considered. The relationship between SPS activity and photosynthesis during dehydration in *X. humilis* leaf was found to be complex. In *X. humilis* leaf a decrease in photosynthesis is noted around the 70% RWC mark with a major down regulation being observed between 60 and 40% RWC marks. This decline in photosynthetic activity (between 60 and 40 % RWC) is associated with both a decreased total SPS activity and an increase in activation state of SPS. Based on the classic understanding of the co-ordination of cytoplasmic sucrose synthesis with chloroplastic carbon fixation (see Introduction chapter) one would expect the downregulation of photosynthesis to be associated with an inactivation of SPS activity but not a decrease in total SPS activity. Thus, it appears that during dehydration SPS activity is not

subject to the classic regulatory mechanisms which are believed to link carbon fixation to sucrose synthesis during active photosynthesis.

In the introduction to this study it was suggested that dehydration may result in the increase in the levels of the metabolite fructose-2,6-bisphosphate. This signal metabolite is considered important in regulating the rate of sucrose synthesis during photosynthesis. Unfortunately, fructose-2,6-bisphosphate could not be identified in the metabolite analysis and hence no comment can be made on the effect of dehydration on the levels of this signal metabolite.

SPS gene expression

Based on the proposed evolutionary history of SPS gene families in higher plants, *X. humilis* was expected to contain 3 SPS genes, one in each of the three proposed SPS gene families (Families A, B and C). The search of both the transcriptome and genome of *X. humilis* only revealed the presence two isoforms, named XhSPS1 and XhSPS2. Although the presence of a third SPS gene in the *X. humilis* genome cannot be excluded, XhSPS2 and XhSPS1 were the most highly expressed isoforms in leaf tissue. Similarly, in the study of SPS gene expression in the resurrection species *C. plantagineum* only two SPS encoding transcripts were identified in leaf tissue.

A phylogenetic analysis of all known full length angiosperm SPS genes revealed that XhSPS1 clustered with Family B genes while XhSPS2 fell into the Family A group. Notably, XhSPS1 clustered closely with the stress responsive SPS isoform of *C. plantagineum*, Cpsps2, while XhSPS2 was located in the same Family as the constitutively expressed *C. plantagineum* SPS gene, Cpsps1. Thus, initially there was an indication that XhSPS2 may be the constitutively expressed isoform while XhSPS1 may be stress responsive. However, after the expression analysis of mRNA transcript abundance and protein levels of each SPS gene it was concluded that neither of the two identified SPS isoforms could be classified as dehydration specific. XhSPS2 was the dominant isoform in fully hydrated tissue and both isoforms responded in a

similar manner (transcript levels of both isoforms increased to similar levels) during initial tissue dehydration (from 100 - 54% RWC). In the later stages of water loss XhSPS2 transcript levels were down regulated and XhSPS1 became the dominant transcript. Despite the down regulation at the transcript level, XhSPS2 protein levels still remained higher than XhSPS1 levels throughout dehydration. The reason for the discrepancy between transcript and protein levels is unclear but it is postulated that reduced turnover of the XhSPS2 protein may have resulted in this isoform remaining dominant despite reduced transcript levels.

Conclusions

In summary, two phases of sucrose accumulation were identified during dehydration. In the first phase the proposed carbon sources include photosynthesis, starch breakdown and the hexose sugar pool. In the second phase changes in amino acid levels indicate that this metabolite pool may serve as a carbon source. During dehydration increases in sucrose content were accompanied by the accumulation of a number of known osmoprotectants, including raffinose, glycerol and xylitol. The accumulation of these metabolites suggests that cellular protection during tissue water loss is afforded by a combination of compounds. In the early stage of water loss there is a strong correlation between increases in SPS activity and increases in sucrose content. However, in the later stages of water loss changes in sucrose content were disproportionately large in comparison to changes in SPS activity. Based on this observation it was concluded that other mechanisms, in addition to increased SPS activity, are responsible for dehydration-induced sucrose accumulation. In *X. humilis* leaf tissue two SPS genes, named XhSPS1 and XhSPS2 were identified. In fully hydrated tissue XhSPS2 was found to be the dominant isoform at both the transcript and protein levels. In response to early dehydration similar increases in both isoforms were observed. During the later stages of dehydration XhSPS2 transcript levels decreased resulting in XhSPS1 becoming the dominant SPS transcript. Nevertheless, despite the changes in transcript abundance, XhSPS2 remained as the dominant SPS protein through all stages of water loss. Thus it was concluded that dehydration does not result in the upregulation of a specific form of SPS in *X. humilis* leaf tissue.

Future work

Functional characterization of XhSPS1 and XhSPS2 protein

Having obtained the full length DNA sequence of each isoform in the present study it will be possible to functionally characterize each isoform in future work. This could be achieved by knocking out all endogenous SPS genes in a plant system such as *Arabidopsis* and expressing either of the isoforms. Alterations in normal and/or stress phenotypes can then be used to characterize the *in vivo* function of each isoform. Alternatively, each isoform could be expressed in bacterial or yeasts systems and the purified proteins characterized *in vitro* at different water potentials. This future work may shed some light on the possible functional significance of each isoform in regulating dehydration induced sucrose accumulation in *X. humilis* leaf tissue.

Possible carbon sources for sucrose accumulation

The metabolite analysis provided some insight to the possible pattern of carbon allocation during dehydration. Based on changes in certain metabolite pools possible carbon sources supporting sucrose accumulation were proposed. To extend this investigation, and confirm these proposed carbon sources, it will be necessary to monitor the actual flow of carbon between these cellular carbon sources. One method would be to use $^{14}\text{CO}_2$ to label the primary carbon pools, via photosynthesis, and to track the distribution of the ^{14}C label during dehydration. The method could also be used to track the movement of carbon between different parts of the plant, e.g. roots and shoots, and thereby determine the contribution of translocated carbon to sucrose accumulation. This labeling work could be complemented by analysis of the enzymes involved in the conversion of possible carbon sources into the primary substrates (hexose sugars) for sucrose synthesis. For example, if carbon is re-mobilized out of the amino acid pool in late dehydration then one would expect an up-regulation of the particular enzymes involved in the amino acid based gluconeogenic pathways.

In addition to confirming proposed sources of carbon, future work could also be aimed at revealing new, alternative carbon sources. In *C. plantagineum*, 2-octulose is proposed to serve

as a carbon reserve to support the high levels of sucrose accumulation observed in this species (Bianchi et al., 1991a). In the current study it was hypothesized that stachyose may serve a similar function during dehydration. The conversion of stachyose, or a similar carbohydrate reserve, into sucrose may be of particular importance in the late stage of dehydration when the starch and hexose sugar pools are depleted. However stachyose could not be confidently identified in the current study and hence no conclusions were drawn concerning this metabolite. In future work the use of a targeted sugar analysis method, e.g. Dionex HPLC system, may enable more confident identification and quantification of stachyose and thereby provide more insight on its possible role as a carbon store.

Location of accumulated glycerol

When considering the changes in all the identified metabolites during dehydration, one of the most interesting observations is the large increase in glycerol and glycerophosphoglycerol (GPG). Glycerol is a known osmoprotectant in plant systems (e.g. Hinch and Hagemann, 2004; Eastmond, 2004) while GPG has been recently reported to be important in adaptation to stress in thermophilic bacteria (Santos et al., 2007b). The accumulation of glycerol is intriguing as high levels of glycerol accumulation in plant tissues are proposed to be toxic ((Leegood et al., 1988; Aubert et al., 1994). Farrant et al., (2008) suggest that glycerol may be accumulated in vacuoles and hence may not affect cytoplasmic metabolism. Thus determining the cellular location of glycerol accumulation may shed some light on how this compound may be accumulated without negatively affecting cellular metabolism.

Alternative mechanisms (to SPS) regulating sucrose accumulation

In the current work there appears to be a strong correlation between increases in sucrose content and increases in SPS activity in the early stages of dehydration. However, the rapid period of sucrose accumulation in the late stage of dehydration does not coincide with the highest rate of SPS activity. Thus it appears that other factors may contribute to the observed sucrose accumulation in the late stage of water loss. These other factors may include a reduction in

sucrose turnover and/or the production of sucrose via non-SPS mediated pathway. The enzymes invertase (neutral and acid) and sucrose synthase are considered important in regulating sucrose degradation. Hence, measuring their activity during dehydration will establish if reduced sucrose breakdown contributes to sucrose accumulation. Sucrose could also be produced as a result of the breakdown of more complex carbohydrates such as stachyose; a process which does not involve sucrose phosphate synthase. If this carbohydrate serves as a carbon reserve, then an up-regulation of the enzymes involved in its breakdown will be expected.

Based on the observed pattern of amino acid accumulation during dehydration, it is proposed that increases in sucrose content may be supported by carbon obtained from this amino acid pool. Thus, in the late stages of water loss one would expect an upregulation of: i) the enzymes involved in converting amino acids to substrates for gluconeogenesis and ii) the enzymes catalyzing gluconeogenesis itself, e.g. phosphoenolpyruvate carboxykinase (Leegood and Walker, 2003). The analysis of changes in the activity of these enzymes will shed more light on the possible role of amino acids as carbon reserves and on the regulation of the pathways converting amino acids to sucrose.

Post-translational modifications of SPS isoforms

In the well studied spinach SPS, three phosphoregulatory sites are proposed to be important in the regulation of SPS activity. These sites include the Ser₁₅₈ site, associated with light/dark regulation, the Ser₂₂₉ site, associated with 14-3-3 binding and the Ser₄₂₄ site, associated with osmoregulation. Analogous sites have been found in other species and based on the functional studies in spinach these sites have been assigned similar regulatory functions. Intriguingly, both the identified *X. humilis* SPS isoforms lack the proposed osmosregulatory site, Ser₄₂₄. Nevertheless, despite the lack of this site, observed changes in SPS activity still indicate that dehydration results in the activation of SPS. This suggests that either dehydration results in the phosphorylation of another known regulatory site (Ser₁₅₈ or Ser₂₂₉) or that a completely new site is phosphorylated. An attempt was made to determine if either of the classic phosphoregulatory

sites, Ser₁₅₈ or Ser₂₂₉, were phosphorylated in response to dehydration but due to technical difficulties no conclusions could be drawn. If future work can improve on the methods used, and especially on the phospho-enrichment strategy, then it will be possible to identify which sites are phosphorylated in response to dehydration. This work may lead to a review of the functional assignments of each of the proposed phosphoregulatory sites.

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Appendix A

Photographs of *Xerophyta humilis* in fully hydrated (A) and desiccated state (B)

A



B



Appendix B

Positioning of SPS guf1 (Forward primer) and SPS gur1 (Reverse primer) on genomic DNA of *Arabidopsis thaliana* and *Oryza sativa*. Sequence of the four *A. thaliana* SPS genes and the five *O sativa* SPS genes are shown . Alignment of mRNA and genomic DNA done with NCBI, SPIDEY software.

NM122035 A thaliana ATSPS1F

Exon 4: 1458-1521 (genomic); 522-585 (mRNA)

```

1458      ATGTATGCAGTCTTCATGGTCTCATACGTGGTGAAAACATGGAGCTCGGC
          |||||||||||||||||||||||||||||||||||||||||||||||
522      TCTTCATGGTCTCATACGTGGTGAAAACATGGAGCTCGGC
          L H G L I R G E N M E L G
                  Forward primer
1498      CGCGACTCTGACACCGGTGGACAGGTAATGATAC
          |||||||||||||||||||||
562      CGCGACTCTGACACCGGTGGACAG
          R D S D T G G Q
  
```

Exon 5: 1617-2315 (genomic); 586-1284 (mRNA)

```

1617 - 2157 .....
2157      TCTCTTGATGTTTCTGAAATGGTAATAACTAGCACTCGACAGGAGATTGA
          |||||||||||||||||||||||||||||||||||||||||||||||
1126      TCTCTTGATGTTTCTGAAATGGTAATAACTAGCACTCGACAGGAGATTGA
          S L D V S E M V I T S T R Q E I D
                  Reverse primer
2207      TGAGCAATGGAGACTGTATGATGGATTTGACCCCATATTGGAGCGCAAAT
          |||||||||||||||||||||||||||||||||||||||||||||||
1176      TGAGCAATGGAGACTGTATGATGGATTTGACCCCATATTGGAGCGCAAAT
          E Q W R L Y D G F D P I L E R K
  
```

NM121149 A thaliana ATSPS2F

Exon 4: 1047-1110 (genomic); 543-606 (mRNA)

```

1047      TGTCTTGCAGTCTTCATGGTTTGATACGTGGTGAAAACATGGAGCTTGGT
          |||||||||||||||||||||||||||||||||||||||||||||||
543      TCTTCATGGTTTGATACGTGGTGAAAACATGGAGCTTGGT
          L H G L I R G E N M E L G
                  Forward primer
1087      CGCGATTCTGATACTGGTGGCCAGGTATACTTCT
          |||||||||||||||||||||
583      CGCGATTCTGATACTGGTGGCCAG
          R D S D T G G Q
  
```

Exon 5: 1198-1890 (genomic); 607-1299 (mRNA)

```

1198-1738 .....
1738      GATGCTTCTGAGATTGTCATAACTAGTACAAGGCAAGAGGTAGACGAGCA
          |||||||||||||||||||||||||||||||||||||||||||||||
1147      GATGCTTCTGAGATTGTCATAACTAGTACAAGGCAAGAGGTAGACGAGCA
          D A S E I V I T S T R Q E V D E Q
                  Reverse primer
1788      ATGGCGCCTTTATGATGGTTTTGATCCGGTTTTGGAGCGAAAACCTCAGAG
          |||||||||||||||||||||||||||||||||||||||||||||||
1197      ATGGCGCCTTTATGATGGTTTTGATCCGGTTTTGGAGCGAAAACCTCAGAG
          W R L Y D G F D P V L E R K L R
  
```

NM100370 A thaliana ATSPS3F

Exon 3: 794-857 (genomic); 528-591 (mRNA)

794 TTTTTCAGTTTGCATGGGTTGGTTCGTGGAGAAAACATGGAGCTTGGT
|||||
528 TTTGCATGGGTTGGTTCGTGGAGAAAACATGGAGCTTGGT
L H G L V R G E N M E L G
Forward primer

834 AGCGATTCCGATACTGGTGGACAGGTACATTGTG
|||||
568 AGCGATTCCGATACTGGTGGACAG
S D S D T G G Q

NM117080 A thaliana ATSPS4F

Exon 4: 1202-1265 (genomic); 603-666 (mRNA)

1202 CCAACTGCAGCATGCATGGACTCGTGCCTGGAGAAAACATGGAGCTTGGA
|||||
603 CATGCATGGACTCGTGCCTGGAGAAAACATGGAGCTTGGA
M H G L V R G E N M E L G
Forward primer

1242 AGAGACTCTGATACTGGTGGGCAGGTATTAGCC
|||||
643 AGAGACTCTGATACTGGTGGGCAG
R D S D T G G Q

Exon 4: 939-1634 (genomic); 592-1287 (mRNA)

939-1479
1479 CTGGATGCTGCAGAGCTTGTATAACAAGCACGAGGCAGGAGATTGATGA
|||||
1132 CTGGATGCTGCAGAGCTTGTATAACAAGCACGAGGCAGGAGATTGATGA
L D A A E L V I T S T R Q E I D E
Reverse primer

1529 ACAGTGGGGACTTTATGATGGGTTTCGATGTAAACTCGAGAAAGTCTTGA
|||||
1182 ACAGTGGGGACTTTATGATGGGTTTCGATGTAAACTCGAGAAAGTCTTGA
Q W G L Y D G F D V K L E K V L

Exon 5: 1388-1587 (genomic); 667-866 (mRNA)

Exon 6: 1684-2164 (genomic); 867-1347 (mRNA)

1684-2024
2024 ATGGTGGTGACAAGCACACGACAAGAGATTGACGCGCAGTGGGGGCTGTA
|||||
1207 ATGGTGGTGACAAGCACACGACAAGAGATTGACGCGCAGTGGGGGCTGTA
M V V T S T R Q E I D A Q W G L Y
Reverse primer

NM 001074101 O sativa SPS Os11g0236100

Exon 4: 3238-3301 (genomic); 642-705 (mRNA)

```
3238      TTGTGTTTAGCATTTCATGGGCTCGTGCGGGGCGAGAACATGGAGCTCGGC
          ||||||||||||||||||||||||||||||||||||||||||||
642      CATTTCATGGGCTCGTGCGGGGCGAGAACATGGAGCTCGGC
          I H G L V R G E N M E L G
                  Forward primer

3278      CGAGACTCTGACACCGGGGGCCAGGTCAATCACA
          ||||||||||||||||||||||||
682      CGAGACTCTGACACCGGGGGCCAG
          R D S D T G G Q
```

Exon 5: 3413-4426 (genomic); 706-1719 (mRNA)

```
3413-4003

4003      CGACATGGTGGTGACGAGCACCAAGCAGGAGATCGAGGAGCAGTGGGGGC
          ||||||||||||||||||||||||||||||||||||||||||||
1296      CGACATGGTGGTGACGAGCACCAAGCAGGAGATCGAGGAGCAGTGGGGGC
          D M V V T S T K Q E I E E Q W G
                  Reverse primer
```

NM 001068030 O sativa SPS2 Os08g0301500

Exon 4: 4343-4406 (genomic); 576-639 (mRNA)

```
4343      TTTTTCGAGCATTTCATGGTCTTATACGTGGTGAAAATATGGAGCTTGGG
          ||||||||||||||||||||||||||||||||||||||||||||
576      CATTTCATGGTCTTATACGTGGTGAAAATATGGAGCTTGGG
          I H G L I R G E N M E L G
                  Forward primer

4383      CGTGATTCAGACACTGGTGGTCAGGTGCTTAAAT
          ||||||||||||||||||||||||
616      CGTGATTCAGACACTGGTGGTCAG
          R D S D T G G Q
```

Exon 5: 4581-5279 (genomic); 640-1338 (mRNA)

```
5121      TGTCTTGATGCATCTGAAATCATAATTACAAGCACTAGACAAGAGATAGA
          ||||||||||||||||||||||||||||||||||||||||||||
1180      TGTCTTGATGCATCTGAAATCATAATTACAAGCACTAGACAAGAGATAGA
          C L D A S E I I I T S T R Q E I E
                  Reverse primer

5171      ACAACAATGGGGATTATATGATGGTTTTGATTTAACCATGGCAAGGAAAC
          ||||||||||||||||||||||||||||||||||||||||||||
1230      ACAACAATGGGGATTATATGATGGTTTTGATTTAACCATGGCAAGGAAAC
          Q Q W G L Y D G F D L T M A R K
```

TC285451 other O sativa SPS Os06g43630

NM001052643 O sativa SPS-9 Os02g0184400

Exon 4: 3445-3508 (genomic); 528-591 (mRNA)		Exon 4: 2762-2825 (genomic); 633-696 (mRNA)	
3445	AATACTGCAGCCTTCATGGCCTGGTCCGTGGTGAGAACATGGAGCTTGGC	2762	CGTACTGCAGCCTACATGGTCTGGTCCGTGGTGAGAACATGGAGCTAGGA
528	CCTTCATGGCCTGGTCCGTGGTGAGAACATGGAGCTTGGC	633	CCTACATGGTCTGGTCCGTGGTGAGAACATGGAGCTAGGA
	L H G <u>L V R G E N M</u> E L G		L H G <u>L V R G E N M</u> E L G
	Forward primer		Forward primer
3485	CGTGATTCACTACTGGCGGACAGGTATTGTTTG	2802	CGAGATTCAGATACTGGTGGCCAGGTAATGCTGC
568	CGTGATTCACTACTGGCGGACAG	673	CGAGATTCAGATACTGGTGGCCAG
	R D S D T G G Q		R D S D T G G Q
Exon 5: 3700-4398 (genomic); 592-1290 (mRNA)		Exon 5: 3304-4002 (genomic); 697-1395 (mRNA)	
4240	GCTCTTGATGCATCTGAAATAGTTATAGCAAGCACTAGGCAAGAGATAGA	3844	TCTCTTGATGCATCTGAAATTGTTATTGCTAGCACTAGACAGGAGATAGA
1132	GCTCTTGATGCATCTGAAATAGTTATAGCAAGCACTAGGCAAGAGATAGA	1237	TCTCTTGATGCATCTGAAATTGTTATTGCTAGCACTAGACAGGAGATAGA
	A L D A S E I V I A S T R <u>Q E I E</u>		S L D A S E I V I A S T R <u>Q E I E</u>
	Reverse primer		Reverse primer
4290	AGAGCAATGGAATTTGTATGACGGTTTTGAGGTCATACTTGCAAGGAAAC	3894	AGAGCAGTGGAACCTTGTATGATGGTTTTGAGGTTATACTTGCAAGGAAGC
1182	AGAGCAATGGAATTTGTATGACGGTTTTGAGGTCATACTTGCAAGGAAAC	1287	AGAGCAGTGGAACCTTGTATGATGGTTTTGAGGTTATACTTGCAAGGAAGC
	<u>E Q W</u> N L Y D G F E V I L A R K		<u>E Q W</u> N L Y D G F E V I L A R K

TC359646 O sativa SPS Os01g69030

Exon 3: 1623-1686 (genomic); 600-663 (mRNA)

1623 TCTACTTTAGTCTTCATGGTCTAGTTCGTGGCGATAACATGGAAC TTGGT
 |||||
600 TCTTCATGGTCTAGTTCGTGGCGATAACATGGAAC TTGGT
 L H G L V R G D N M E L G
 Forward primer

1663 CGGGATTCAGATACTGGAGGACAGGTAAAACATG
 |||||
640 CGGGATTCAGATACTGGAGGACAG
 R D S D T G G Q

Exon 4: 1819-2511 (genomic); 664-1356 (mRNA)

2359 GATGCAGCGGAGCTTGT CATAACGAGTACAAGGCAGGAGATTGATGAGCA
 |||||
1204 GATGCAGCGGAGCTTGT CATAACGAGTACAAGGCAGGAGATTGATGAGCA
 D A A E L V I T S T R Q E I D E Q
 Reverse primer

2409 ATGGGGACTGTATGATGGCTTTGATGTCAAGCTAGAGAAAGTTT TGAGGG
 |||||
1254 ATGGGGACTGTATGATGGCTTTGATGTCAAGCTAGAGAAAGTTT TGAGGG
 W G L Y D G F D V K L E K V L R

Appendix C

Amplification cycles, melt curves and standard curves for Real-time PCR experiments

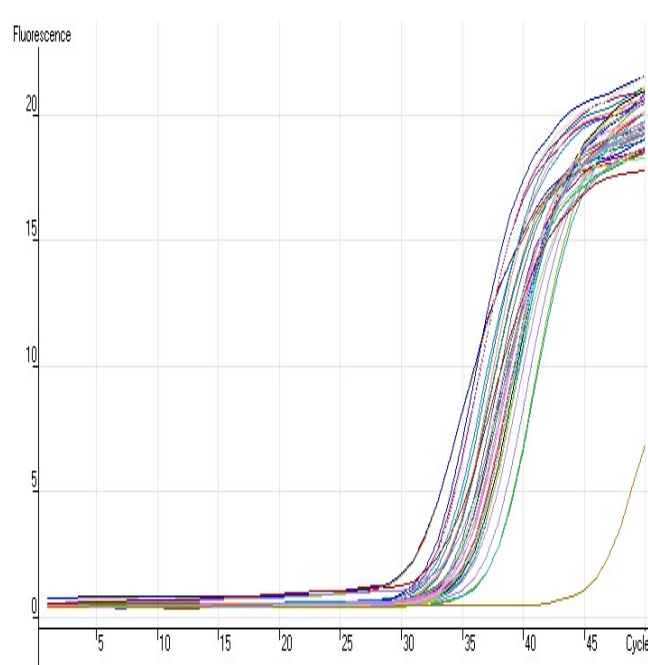


Figure B1 Amplification of XhSPS1. Plot of fluorescence vs cycle number

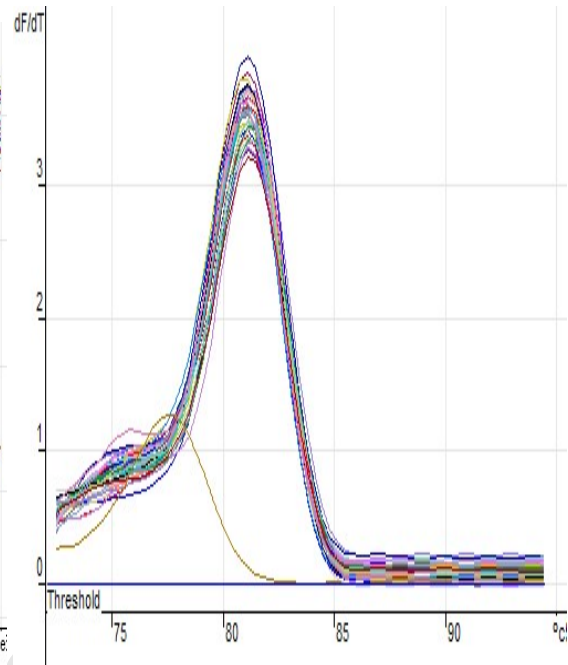


Figure B2 Melt curve for primers used to amplify XhSPS 1 transcript.

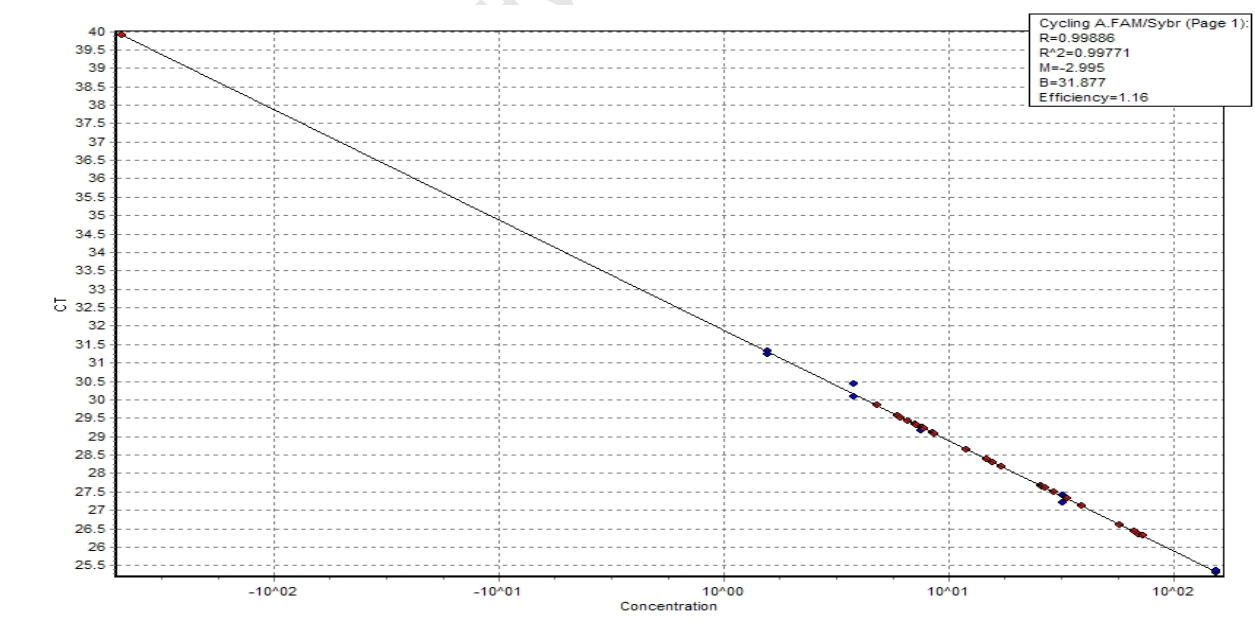


Figure B3 Standard curve used for XhSPS1 quantification. Efficiency and R^2 values are displayed. Blue dots are standards, red dots are samples

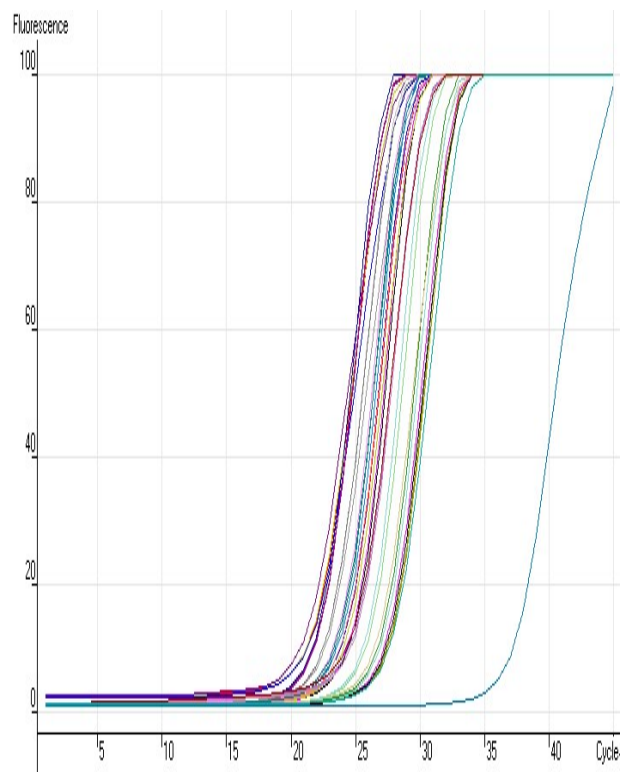


Figure B4 Amplification of XhSPS2. Plot of fluorescence vs cycle number.

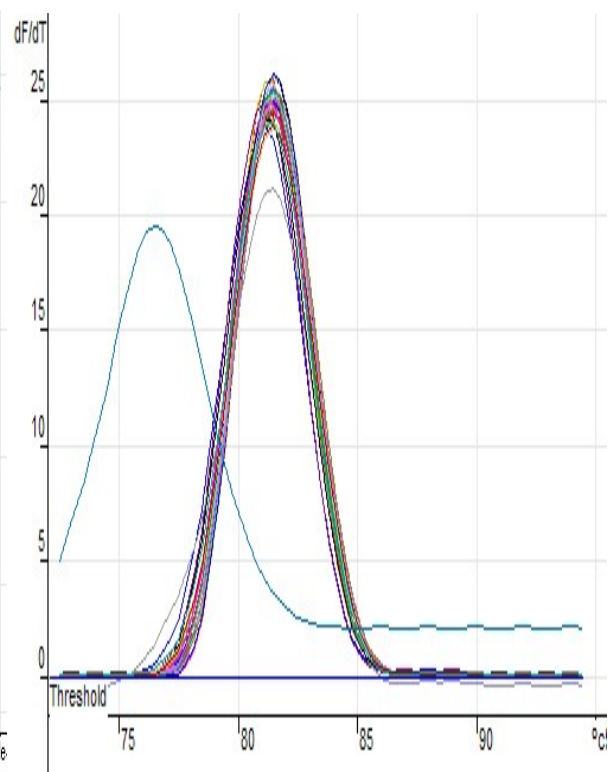


Figure B5 Melt curve for primers used to amplify XhSPS2.

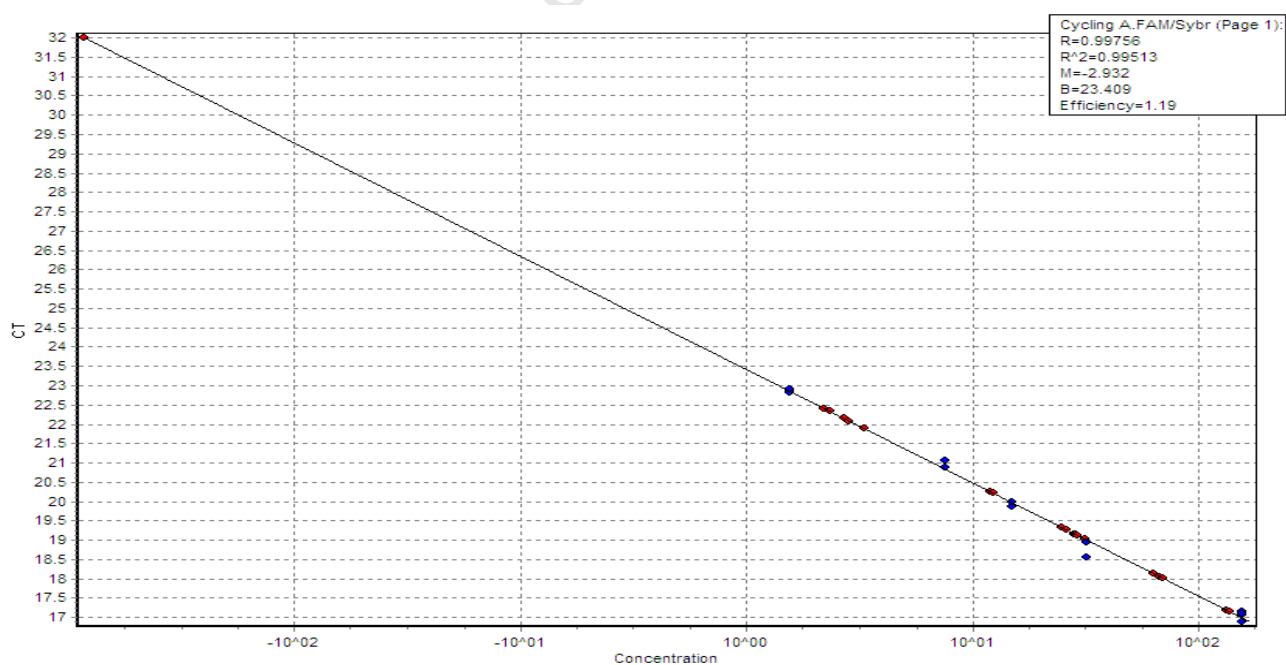


Figure B6 Standard curve used for XhSPS2 quantification. Efficiency and R^2 values are displayed. Blue dots are standards, red dots are samples

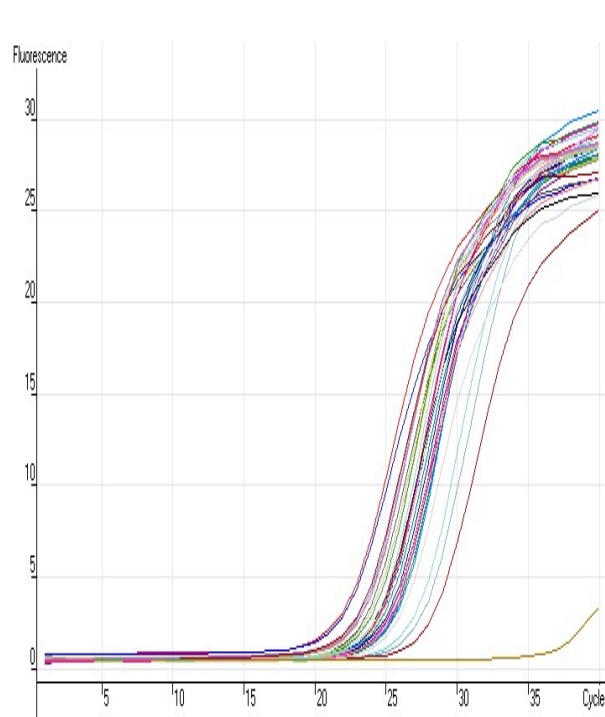


Figure B7 Amplification of R1. Plot of fluorescence vs cycle number.

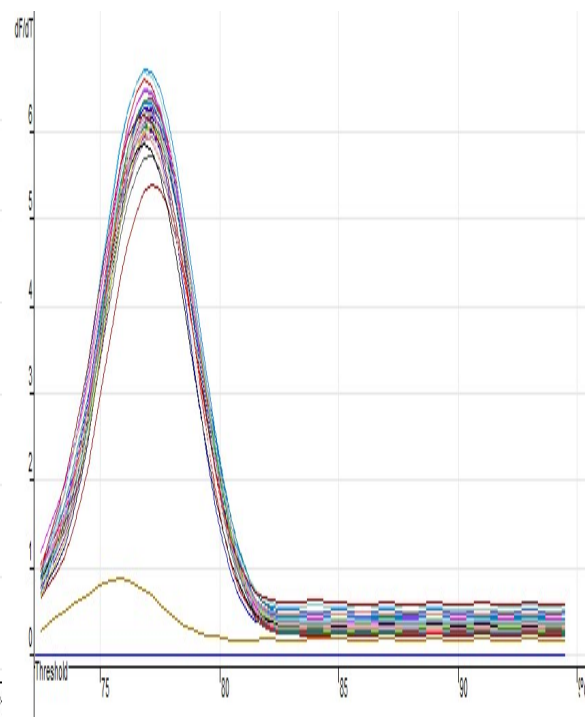


Figure B8 Melt curve for primers used to amplify R1.

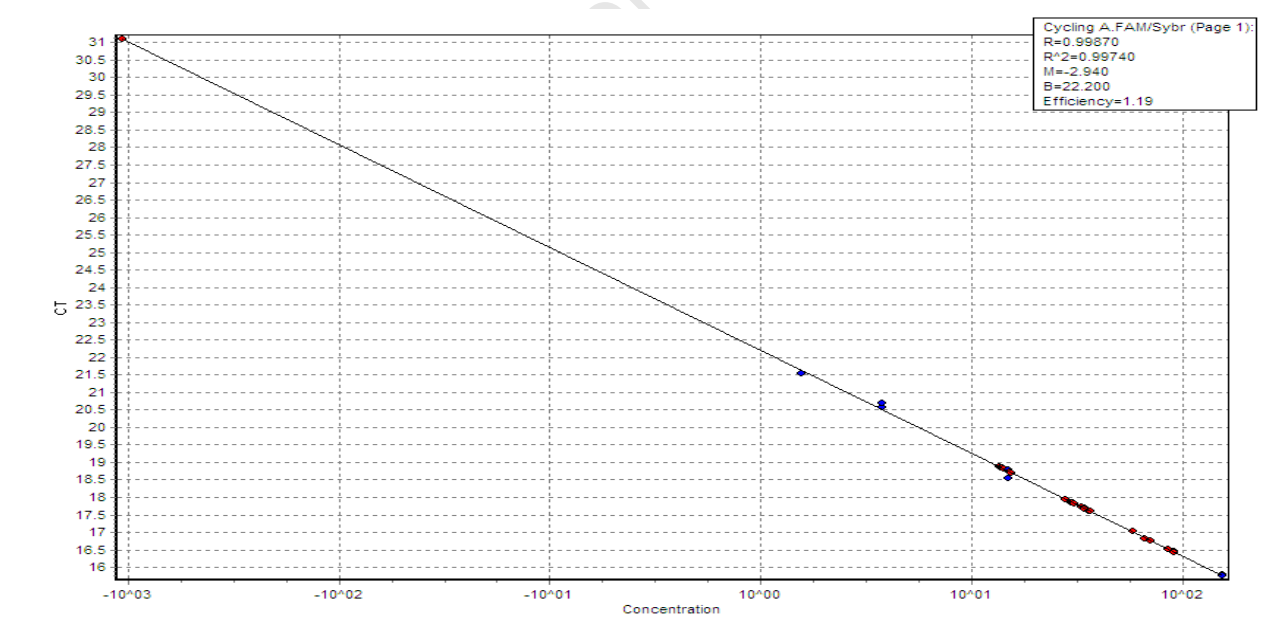


Figure B9 Standard curve used for R1 quantification. Efficiency and R^2 values are displayed. Blue dots are standards, red dots are samples

Appendix D Amino acid ClustalW Alignment of 42 Angiosperm SPS orthologues and 2 Bryophyte SPS orthologues .

AF194022 N tabacum SPSA	-MAGND-WINSYLEAILDVGP-----IEDKKSS-----LLLRRG---RFSPT-RYFV
NM122035 A thaliana ATSPS1F	-MAGND-WVNSYLEAILDVGG-----LDDARSSP-----SLLLRRG---RFTPS-RYFV
NM121149 A thaliana ATSPS2F	-MVGND-WVNSYLEAILAAEPGI-----ANSKPPGTGDSKSS-----LLLRRG---HFSPT-RYFV
AF439861 I batatas	-MAGND-WINSYLEAILDVGP-----IDDAKSS-----LLLRRG---RFSPT-RYFV
DQ364058 C melo SPS	-MAGND-WINSYLEAILDVGP-----LDDKKSS-----LLLRRG---RFSPT-RYFV
AF071786 L esculentum SPS	-MAGND-WINSYLEAILDVGP-----LDDKKSS-----LLLRRG---RFSPT-RYFV
AY26439 L esculentum SPS	-MAGND-WINSYLEAILDVGP-----LDDKKSS-----LLLRRG---RFSPT-RYFV
X73477 S tuberosum SPS	-MAGND-WINSYLEAILDVGP-----LDDKKSS-----LLLRRG---RFSPT-RYFV
DQ834321 C canephora SPS1	-MAGND-WINSYLEAILDVGP-----IDDAKSS-----LLLRRG---RFSPT-RYFV
Y11821 C plantagineum Cpsps1	-MAGND-WINSYLEAILDVGP-----IDEAKGS-----LLLRRG---RFSPT-RYFV
AF318949 A chinensis SPS2	-MAGND-WINSYLEAILDVGP-----IDDAKSS-----LLLRRG---RFSPT-RYFV
CU459387 V vinifera SPS	-MAGND-WINSYLEAILDVGP-----LDDAKTS-----LLLRRG---RFSPT-RYFV
AF322116 M sativa SPS	-MAGND-WLNSYLEAILDVGP-----LDDAKSS-----LLLRRG---RFSPT-RYFV
AB005023 C unshiu CitSPS1	-MAGND-WINSYLEAILDVGP-----LDDAKSS-----LLLRRG---RFSPT-RYFV
X81975 B vulgaris SPS1	-MAGND-WINSYLEAILDVGP-----LDDAKSS-----LLLRRG---RFSPT-RYFV
AY331261 V album SPS	-MAGND-WVNCYLEAILDADPG-----IGDAKSS-----LLLRRG---HFSPT-RYFV
S54379 S oleracea SPS	-MAGND-WINSYLEAILDIGGQ-----IDASTGKTSTAPPS-----LLLRRG---HFSPT-RYFV
Z56278 V faba SPS	-MAGND-WLNSYLEAILDVGP-----LDDAKSS-----LLLRRG---RFSPT-RYFV
X humilis SPS2	-MAGND-WINSYLEAILDSG-R-----IDGDKQS-----LLLRRG---RFSPT-AYFV
AY135211 O goldiana SPS	-MAGND-WINSYLEAILDAGPS-----IDASKSS-----LLLRRG---RFSPT-KYFV
AB232784 L perenne LpSPS	-MAGND-WINSYLEAILDAGGAAGDLSAAAGAGGGDGAGPGAGEKRDKSS-----LMLRRG---RFSPA-RYFV
NM 001068030 O sativa SPS2	-MAGND-WINSYLEAILDAGGAAGEISAAAG-GGGDGA-AATGEKRDKSS-----LMLRRG---RFSPA-RYFV
Castleden et al (2004) ZmSPS2	-MAGND-WINSYLEAILDAGGAAGDLSAAAG--SGDGRDGTAVEKRDKSS-----LMLRRG---RFSPA-RYFV
NM001052643 O sativa SPS-9	-MAGNDNWINSYLDAILDAGK-----AAIGG-----DRPSLLLRRG---HFSPA-RYFV
TC285451 other O sativa SPS	-MYGNDNWINSYLDAILDAGKGA-----AASASASAVGGGGGAGDRPSLLLRRG---HFSPA-RYFV
AF534907 T aestivum SPS9	-MVGNDNWINSYLDAILDAGK-----SAVGG-----DRPSLLLRRG---HFSPA-RYFV
Castleden et al (2004) ZmSPS3	-MAGNDNWINSYLDAILDAGK-----AAIGG-----DRPSLLLRRG---HFSPA-RYFV
AB00138 S officinarum SoSPS2	-MAGNDNWINSYLDGILDAGK-----AAIGG-----NRPSLLLRRG---HFSPA-RYFV
NM100370 A thaliana ATSPS3F	-MAGNE-WINGYLEAILDSQAQ-----GIEETQ-QKPQASVNLREGDQ-----YFNPT-KYFV
DQ213015 N tabacum SPSB	-MAGNE-WINGYLEAILSSGAS-----AIEDKTPSSSTTSSHLNLAERA---NFNPT-KYFV
X humilis SPS1	-MAGNE-WINGYLEAILDSGGAG-----GGATEKDQRRQQQKRSGAAVVE---HFNPTRVYLV
AM451888 V vinifera SPS	-MAGNE-WINGYLEAILVSGAS-----AIED-----SKATPIALREGG---HFNPT-KYFV
Y11795 C plantagineum CpSPS2	-MAGNE-WINGYLEAILDTGAS-----AIDENSGGGKTAQAQKGRHHDH---HFNPT-KYFV
NM001112224 Z mays SPS1	-MAGNE-WINGYLEAILDSHTSS-----RGAGGGGGGGDPRSPK-AASPRGAHMNFNPS-HYFV
TC359646 O sativa SPS	-MAGNE-WINGYLEAILDSGG-A-----AGGGGGGGVDPRSPAAGAASPRGPHMNFNPT-HYFV
DQ213014 N tabacum SPSC	-MAENE-WLNGYLEAILDAGTDRNGTQKERKASSIEDRNNLKNTSVRDNNKIEETLRFKFEIQKEAEKLFSP-TYFV
AY899999 V vinifera SPS1	-MAGNE-WINGYLEAILDAGSSRNGLRVVEDG--DEKSNKNNGSRRRRFVEGKVRIGRLEEKEKEEVEFNPT-KYFV
NM117080 A thaliana ATSPS4F	-MARND-WINSYLEAILDVGTSKKKR-----FESNSKIVQKLGDISKDHQEKVFGDMN-GKDHQEKVFSPI-KYFV
Castleden et al (2004) ZmSPS4	MAAGNE-WINGYLEAILDAGT-----RLRGPWQQQGAASLTAALPRLLAEGGQQGAAAYSPT-RYFV
AF310160 T aestivum SPS1	MAVGNE-WINGYLEAILDAGS-----KLRVQGVSLPP-----LEPAPALASEESSAAYNPT-RYFV
NM 001074101 O sativa SPS	MVHHYS-MSCPDLEAIEQVEW-----EFSRQLSRR-----RLEQELGSREAAADLS-----
DQ157858 P patens SPS1	MAVGNE-WINGYLEAILDTGEK-----ITDHKHIG-EGVN---DFKAA-KYFV
DQ157859 P patens SPS2	MAAGNE-WINGYLEAILDTGEK-----ITDHKHIGDEGVN---DFKAA-KYFV

AF194022 N tabacum SPSA
 NM122035 A thaliana ATSPS1F
 NM121149 A thaliana ATSPS2F
 AF439861 I batatas
 DQ364058 C melo SPS
 AF071786 L esculentum SPS
 AY26439 L esculentum SPS
 X73477 S tuberosum SPS
 DQ834321 C canephora SPS1
 Y11821 C plantagineum CpSPS1
 AF318949 A chinensis SPS2
 CU459387 V vinifera SPS
 AF322116 M sativa SPS
 AB005023 C unshiu CitSPS1
 X81975 B vulgaris SPS1
 AY331261 V album SPS
 S54379 S oleracea SPS
 Z56278 V faba SPS
 X humilis SPS2
 AY135211 O goldiana SPS
 AB232784 L perenne LpSPS
 NM 001068030 O sativa SPS2
 Castleden et al (2004) ZmSPS2
 NM001052643 O sativa SPS-9
 TC285451 other O sativa SPS
 AF534907 T aestivum SPS9
 Castleden et al (2004) ZmSPS3
 AB00138 S officinarum SoSPS2
 NM100370 A thaliana ATSPS3F
 DQ213015 N tabacum SPSB
 X humilis SPS1
 AM451888 V vinifera SPS
 Y11795 C plantagineum CpSPS2
 NM001112224 Z mays SPS1
 TC359646 O sativa SPS
 DQ213014 N tabacum SPSC
 AY899999 V vinifera SPS1
 NM117080 A thaliana ATSPS4F
 Castleden et al (2004) ZmSPS4
 AF310160 T aestivum SPS1
 NM 001074101 O sativa SPS
 DQ157858 P patens SPS1
 DQ157859 P patens SPS2

LSE-----GEKGDVVS DIPSHGE----STKGR LPRISSVETMEAWVN---QQRGKKLYIVLISLHGLIRGENME
 FSE-----GEKGDII SDISTHGE----STKPR LPRINSAESMELWAS---QQKGNKLYLVLSLHGLIRGENME
 FSE-----GEKADLPGEIPTPSDN--N--TKGRMSRISSVDVFENWFA---QHKEKKLYIVLISLHGLIRGENME
 LSE-----GEKGDAISDISAHGE----SIKGR LPRISSVETMESWAN---QQKGKKLYIVLISLHGLIRGENME
 LSE-----GEKGDIVTDMSSHGE----STRGR LPRISSVETMEAWVS---QQRGKKLYIVLISLHGLIRGENME
 LSE-----GEKGDIVADMSSHGE----STRGR LPRISSVETMEAWVS---QQRGKKLYIVLISLHGLIRGENME
 LSE-----GEKGDIVTDMSSHGE----STRGR LPRISCVETMEAWVS---QQRGKKLYIVLISLHGLIRGENME
 LSE-----GEKGDIVADMSSHGE----STRGR LPRISSVETMEAWVS---QQRGKKLYIVLISLHGLIRGENME
 LSE-----GEEGDTVGDFLAHGE----SNRGR LPRISSVETMEAWAS---QQKEKKWYIVLISLHGLIRGENME
 LSE-----GEKGDIVVDHSHHGE----SNRGR LPRINSVDTMEAWMN---QQKGKKLYIVLISLHGLIRGENME
 LSE-----GEKGD TVSDLSAHGE----SNRGR LPRISSVETMEAWVS---QQKGKRLYIVLISLHGLIRGENME
 LSE-----GEKGD TVSDISAHGD----SIRGRMPRISSVDMETWVS---YQKGKKLYIVLISLHGLIRGENME
 LSE-----GERGDPVSDVSAHGG---ESTKAR LPRISSADAMETWAH---SHKGKKLYIVLISIHGLIRGENME
 LSE-----GEKGDIVSDVSAHGD----STRSR LPRISSVDMETWIS---QQKGKKLYIVLISIHGLIRGENME
 LSE-----GEK-----DISAHGD----STRPR LPRINSLDAMETWIS---QQKEKKLYLVLSLHGLIRGENME
 LSE-----GEKGDFTGDL SAHSDR-----RFPRISSVDVMENWIN---QHKEKKLYIVLISLHGLIRGENME
 LSE-----GERGDTVADMLFASES----TKGRMRRISSVEMMDNWAN---TFKEKKLYVVLISLHGLIRGENME
 LSE-----GERGDPVSDVSTHGGG---DSVKSRLPRISSADAMETWVN---SQKGKKLYIVLISIHGLIRGENME
 LSE-----GEKGDHAGDASAHGD----SHRGRMPRIGSAETFDWAN---QQKEKKLYIVLISMHGLVRGENQE
 LSE-----GEKGDVVGELSSHGD----SSRGRMHRISSIDALDAWAS---QLKDKNLYIVLISIHGLIRGENME
 LSE-----GEKVENINESS-IHDE--S--TRRRMPRIGSTDAIEAWAS---QHKDKKLYIVLISIHGLIRGDNME
 LSE-----GEKGENINESSSTHDE--S--TRGRMPRIGSTDAIEAWAS---QHKDKKLYIVLISIHGLIRGENME
 LSE-----GEKGETNNEPS-IHDE--S--MRTMRPRIGSTDAIDTAN---QHKDKKLYIVLISIHGLIRGENME
 LFE-----GEKGEDAGDPSVAYG---DSTTGSSPKTSSID-----KLYIVLISLHGLVRGENME
 LFE-----GEKGEDAGDPSVAYG---DSTTGNTPRISSVD-----KLYIVLISLHGLVRGENME
 LFE-----GEKGEDAGDPSVAYG---DSTTGVS PKTSSVD-----KLYIVLISLHGLVRGENME
 LFE-----GEKGEDAGDPSVAYG---DSTTGSSPKTSSID-----KLYIVLISLHGLVRGENME
 LFE-----GEKGEDAGDPSVAYG---DSTTGSSPKTSSID-----KLYIVLISLHGLVRGENME
 LSE-----GEKGDGLGEIV-QPET--PR--RQLQRNLSNLEI---WSD---DKKENRLYVVLISLHGLVRGENME
 LSE-----GEKGDVLGETP-TLDS--PR--KRFQRNFSNLEV---WSD---SNKEKKLYIILVSLHGLVRGENME
 LSE-----GEKGD TVSELS-QSET--PK--KKLQRNVSDIQV---WSD---DNKS KKLYIVLISIHGLIRGENME
 LSE-----GEKGETVGELL-PGET--PK--KKFQRNFSNLEV---WSD---DNKEKKLYIVLISLHGLVRGENME
 LSE-----GEKGDVMGETPVALDS--PRGNKKYHRNFSNLEV---WSD---SNKEKKLYIVLISLHGLVRGENME
 LSE-----GEKGD TIGELA--PVE--T-TKKKFQRNFSDLTV---WSD---DNKEKKLYIVLISVHGLVRGENME
 LFE-----GEKADTVGELA--QQD--TPMKKKFQRNFSSELTVS--WSD---ENKEKKLYIVLISLHGLVSGDNME
 LSE-----GEKEKTDVN-----TSDSHHVISRINSVTQMWPDED-----KP-RQLYIVLISIHGLVRGENME
 LSE-----GEKEKGDPNQ-----IEPVKEQMTRINS DMHIWSDDD---KS-RHLYIILISIHGLVRGENME
 LSE-----GEKDKNDGEKEKSEVVTTLEPPRDHMPRISEM QIWSEDD---KSSRNLYIVLISMHGLVRGENME
 EKD-----GAPDAAQQPV-----SVAAPDGRIARIGSEARIVSDDEGGDGGKDDRNLIVLISIHGLVRGENME
 SEGEKETVPKPDGAAAHLSADE-----QQPQQRTRLARINSEVRLVSDDE--DEQSKDRNLIVLVSIHGLVRGENME
 DGG-----GGDHQQQQQQ-----PPPHQLSRFARINS DPRIVSDEE--EEVTTDRNLIVLISIHGLVRGENME
 LSE-----GEKETTPADTMPRVES--SL--ALASSNVGEITT---PEK---EKPDKRLYIVLVSLHGLVRGDNME
 LSE-----GEKESIPQDCIPRVES--AL--TLASSNFGESIS---PEK---EKPEKRLYIVLISLHGLVRGDNME

AF194022 N tabacum SPSA	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQVSSPEVDWSYGEPTMLPPRS-----TEGLMT---EMGE
NM122035 A thaliana ATSPS1F	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQVSSPDVDYSYGEPTMLTPRD-----SEDFSD---EMGE
NM121149 A thaliana ATSPS2F	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQVTPADVDSSYSEPSEMLNPID-----TDIEQE---N-GE
AF439861 I batatas	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQVSSPEVDWSYGEPTMLTPIN-----SEGLMT---EMGE
DQ364058 C melo SPS	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQVSSPEVDWSYGEPTMLTPIS-----TDGLMS---EMGE
AF071786 L esculentum SPS	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQVSSPEVDWSYGEPTMLTPIS-----TDGLMS---EMGE
AY26439 L esculentum SPS	LGRDSDTGGQVKYVVELAR-LGSMGPVYRVDLLTRQVSSPEVDWSYGEPTMIVTPIS-----TDGLMS---EMGE
X73477 S tuberosum SPS	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQVSSPEVDWSYGEPTMELA-PIS-----TDGLMT---EMGE
DQ834321 C canephora SPS1	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQVSSLEVDWSYGEPTMLPPRN-----SE-GLN---EMGE
Y11821 C plantagineum Cpsps1	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQVSSPEVDWSYGEPTMLPPRN-----SENMD---EMGE
AF318949 A chinensis SPS2	LGRDSDTGGQVKYVVELAAALGSMGPVYRVDLLTTQVSSPEVDWSYGEPTMLPPRN-----SDVLM---EMGE
CU459387 V vinifera SPS	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQVSSPEVDWSYGEPTMLTPLN-----SESFME---DMGE
AF322116 M sativa SPS	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQVASPDVDWSYGEPTMLAPRN-----TDEFGD---DMGE
AB005023 C unshiu CitSPS1	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQVSAPDVDWSYGEPTMLTPRN-----SDDFMD---DMGE
X81975 B vulgaris SPS1	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQVSSPDVDWSYGEPTMLNPRD-----SNGFDDDDDEMG
AY331261 V album SPS	LGRDSDTGGQVKYVVELARALGTMPIYRVDLLTRQVSAPDIHWSYGEPTMLNHGN-----PENLIE---ERGE
S54379 S oleracea SPS	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQVSAPGVDWSYGEPTMLSSRN-----SENSTE---QLGE
Z56278 V faba SPS	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQVSSPDVDWSYGEPTMLAPRN-----TDEFGD---DMGE
X humilis SPS2	LGRDSDTGGQVKYVVELARALGAMPVYRVDLLTRQIQAPDVDWSYGEPTMLPPRTDVL-----PGESEGLQVEGGE
AY135211 O goldiana SPS	LGRDSDTGGQVKYVVELARALGSMGPVYRVDLLTRQISAPDVDSSYGEPTMLAP-----SHSEN---FHMG
AB232784 L perenne LpSPS	LGRDSDTGGQVKYVVELARALGSTPGVYRVDLLTRQISAPDVDWSYGEPTMLSPRN-----SENFH---EMGE
NM 001068030 O sativa SPS2	LGRDSDTGGQVKYVVELARALGSTPGVYRVDLLTRQISAPDVDWSYGEPTMLSPRN-----SENFH---DMGE
Castleden et al (2004) ZmSPS2	LGRDSDTGGQVKYVVELARALGSTPGVYRVDLLTRQISAPDVDWSYGEPTMLSPIS-----SENFGL---ELGE
NM001052643 O sativa SPS-9	LGRDSDTGGQVKYVVELAKALSSSPGVYRVDLLTRQILAPNFDERSYGEPTMLVSTS-----FKNSKQ---EKGE
TC285451 other O sativa SPS	LGRDSDTGGQVKYVVELAKALSSCPGVYRVDLFTQILAPNFDERSYGEPEPLASTS-----FKNFKQ---ERGE
AF534907 T aestivum SPS9	LGRDSDTGGQVKYVVEFAKALSSSPGVYRVDLLTRQILAPNFDERSYGEPAEMLVSTT-----FKNSKQ---EKGE
Castleden et al (2004) ZmSPS3	LGRDSDTGGQIKYVVELAKALSSSPGVYRVDLLTRQILAPNFDERSYGEPEKELLVSTS-----GKNYKQ---EKGE
AB00138 S officinarum SoSPS2	LGRDSDTGGQVKYVVELAKALSSSPGVYRVDLLTRQILAPNFDERSYGEPAELLVSTS-----GKNKSKQ---EKGE
NM100370 A thaliana ATSPS3F	LGSDSDTGGQVKYVVELARALARMGPVYRVDLFTQICSSPEVDWSYAEPTMLTTA-----EDCDGD---ETGE
DQ213015 N tabacum SPSB	LGRDSDTGGQIKYVVELAKALAKMPGVYRVDLFTQIASTEVDWSYGEPTMLNTG-----PEDGDDT---DLGE
X humilis SPS1	LGRDSDTGGQVKYVVELARALSMMPGVYRVDLFTQIISPVDVDWSYGEPTMLTSG-----QYDADGN---DVGE
AM451888 V vinifera SPS	LGRDSDTGGQVKYVVELSRALARMGPVYRVDLFTQIISPPEVDWSYGEPTMLTVG-----AEDADGT---DVGE
Y11795 C plantagineum CpSPS2	LGRDSDTGGQIKYVVEVARALAKMPGVYRVDLFTQIISPPEVDWSYAEPTMLSSSSTTAGEAHEPEEEEEEE---DLGE
NM001112224 Z mays SPS1	LGRDSDTGGQVKYVVELARAMSMMPGVYRVDLFTQVSSPDVDWSYGEPTMLCAG-----SNDGE---GMGE
TC359646 O sativa SPS	LGRDSDTGGQVKYVVELARALAMMPGVYRVDLFTQVSSPEVDWSYGEPTMLTPVP-----LTERE---AVRV
DQ213014 N tabacum SPSC	LGRDSDTGGQVKYVVELARALANMEGVHRVDLLTRQITSPEVDSSYGEPIEMLSCPS-----HAFGS-----
AY899999 V vinifera SPS1	LGRDSDTGGQVKYVVELARALANTKGVYRVDLLTRQITSTEVDSSYGEPIEMLSCPS-----DGGGS-----
NM117080 A thaliana ATSPS4F	LGRDSDTGGQVKYVVELARALANTEGVHRVDLLTRQIISPPEVDYSYGEPEVEMLSCPP-----EGSDS-----
Castleden et al (2004) ZmSPS4	LGRDADTGGQVKYVVELARALAATAGVHRVDLLTRQISCPDWDWYGEPEVEMITHQA-----DDGD-----GS
AF310160 T aestivum SPS1	LGRDSDTGGQVKYVVELARALAATAGVHRVDLLTRQISCPDWDWYGEPEVEMLERLS-----SGDD---GDESG
NM 001074101 O sativa SPS	LGRDSDTGGQVKYVVELARALAATPGVHRVDLLTRQISCPDWDWYGEPEVEMTVPA-----ADADDEDGGGGS
DQ157858 P patens SPS1	LGRDSDTGGQIKYVVELARALALMPEVYRVDLLTRQICSPDVDWSYGEPTMLSMG-----SYDDVE---DVGE
DQ157859 P patens SPS2	LGRDSDTGGQIKYVVELARALALMPEVYRVDLLTRQICSPDVDWSYGEPTMLSLG-----SYDDFE---DVGE

AF194022 N tabacum SPSA
 NM122035 A thaliana ATSPS1F
 NM121149 A thaliana ATSPS2F
 AF439861 I batatas
 DQ364058 C melo SPS
 AF071786 L esculentum SPS
 AY26439 L esculentum SPS
 X73477 S tuberosum SPS
 DQ834321 C canephora SPS1
 Y11821 C plantagineum Cpsps1
 AF318949 A chinensis SPS2
 CU459387 V vinifera SPS
 AF322116 M sativa SPS
 AB005023 C unshiu CitSPS1
 X81975 B vulgaris SPS1
 AY331261 V album SPS
 S54379 S oleracea SPS
 Z56278 V faba SPS
 X humilis SPS2
 AY135211 O goldiana SPS
 AB232784 L perenne LpSPS
 NM 001068030 O sativa SPS2
 * ZmSPS2
 NM001052643 O sativa SPS-9
 TC285451 other O sativa SPS
 AF534907 T aestivum SPS9
 Castleden et al (2004) ZmSPS3
 AB00138 S officinarum SoSPS2
 NM100370 A thaliana ATSPS3F
 DQ213015 N tabacum SPSB
 X humilis SPS1
 AM451888 V vinifera SPS
 Y11795 C plantagineum CpSPS2
 NM001112224 Z mays SPS1
 TC359646 O sativa SPS
 DQ213014 N tabacum SPSC
 AY899999 V vinifera SPS1
 NM117080 A thaliana ATSPS4F
 Castleden et al (2004) ZmSPS4
 AF310160 T aestivum SPS1
 NM 001074101 O sativa SPS
 DQ157858 P patens SPS1
 DQ157859 P patens SPS2

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AF194022 N tabacum SPSA
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 NM121149 A thaliana ATSPS2F
 AF439861 I batatas
 DQ364058 C melo SPS
 AF071786 L esculentum SPS
 AY26439 L esculentum SPS
 X73477 S tuberosum SPS
 DQ834321 C canephora SPS1
 Y11821 C plantagineum Cpsps1
 AF318949 A chinensis SPS2
 CU459387 V vinifera SPS
 AF322116 M sativa SPS
 AB005023 C unshiu CitSPS1
 X81975 B vulgaris SPS1
 AY331261 V album SPS
 S54379 S oleracea SPS
 Z56278 V faba SPS
 X humilis SPS2
 AY135211 O goldiana SPS
 AB232784 L perenne LpSPS
 NM 001068030 O sativa SPS2
 Castleden et al (2004) ZmSPS2
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 TC285451 other O sativa SPS
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 NM100370 A thaliana ATSPS3F
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 X humilis SPS1
 AM451888 V vinifera SPS
 Y11795 C plantagineum CpSPS2
 NM001112224 Z mays SPS1
 TC359646 O sativa SPS
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 Castleden et al (2004) ZmSPS4
 AF310160 T aestivum SPS1
 NM 001074101 O sativa SPS
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AF194022 N tabacum SPSA
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 AF439861 I batatas
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 SAGKFPALRRRKQIFVIAVDCDS-----STGLFENVRKIFAAVEAE-GMEGSIGFHIGHFIQYIRSAFFSDFRGHEST
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 FDAYICNSGSDLYYPSLN-----SEDRLFVGDLYFHSHIEYRWGGEGRLRKTLLRWAASIT---DKKGESNEQIVSPVEQ
 FDAFICNSGSDLYYSTLN-----SEDGPFVVDFFYYHSHIEYRWGGEGRLRKTLLRWAASQV---DKKAESGEKVLTPAEQ
 FDAYICNSGSELYYSSLN-----SEESNIIADSDYHSHIEYRWGGEGRLRKTLLRWAASIT---EKNGENEEQVITEDEE
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 FDAFICNSGSELYYPST---D---YSESPFVLDQDYSHIDYRWGGEGRLWKTLLVKAASVN---EKKGENAPNIVIADET
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 FDALICSSGSEVYYPGTSS-----EEHGKLYPDPDYSSHIEYRWGGEGRLRKTLLRWAASIT---GKEEKSVTCAIEEDVK
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 FDALICSSGSEVYYPGTYT-----EEDGKLLPDPDYASHIDYRWGGEGRLRKTLLRWAASIT---GGKSKNPSKPIEEDGK
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 FDALICSSGAEICYPWK-----ELTADEEYSGHVAFRWPGDHVKTVPRLGKAED-----AQASDLAVDVS
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AF194022 N tabacum SPSA
 NM122035 A thaliana ATSPS1F
 NM121149 A thaliana ATSPS2F
 AF439861 I batatas
 DQ364058 C melo SPS
 AF071786 L esculentum SPS
 AY26439 L esculentum SPS
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 TC285451 other O sativa SPS
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 Castleden et al (2004) ZmSPS3
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 TC359646 O sativa SPS
 DQ213014 N tabacum SPSC
 AY899999 V vinifera SPS1
 NM117080 A thaliana ATSPS4F
 Castleden et al (2004) ZmSPS4
 AF310160 T aestivum SPS1
 NM 001074101 O sativa SPS
 DQ157858 P patens SPS1
 DQ157859 P patens SPS2

NSADYCYT - -FKVRKLGTVPPAKELRKLMRIQALRCHAVYCQNGSRINVIPVLASRSQALRYLYLRWGMDSLKL VVFVGE
 LSTDYCYT - -FTVKKPAAVPPVRELKLLRIQALRCHVVYSQNGTRINVIPVLASRIQALRYLFVRWGIDMAKMAVVFVGE
 SSTNYCLS - -FKVKDPALMPPMKELRKLMRNQLRCHNAVYCQNGARLNVIPVLASRSQALRYLLVRWGIDLSNMVVFVGD
 NSADYCYT - -FKVQKSGGDPVKELRKS MRIQALRCHVVYCQNGSRINVIPVLSSRSQALRYLYLRWGMDSLKL VVFVGE
 NSADYCYT - -FKVCKPGKVPPAKELRKVMRIQALRCHAVYCQNGSRINMIPVLASRSQALRYLYLRWGMDSLKL VVFVGE
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 NSADYCYT - -FKVCRPGVPPVRELRKVMRIQALRCHVIYCQNGSKINVIPVLAARCQALRYLYLRWGMDSLKV VVFVGE
 TSADYCYT - -FKVQKPNVPPVKEARKVMRIQALRCHVVYCQNGKINVIPVLASRAQALRYLYLRWGMELSKT VVFVGE
 ISTNYCYA - -FKVQKAGKVPVKEIRKLMRIQALRCHVIYCQNGKINVIPVLASRSQALRYLYLRWGMDSLKM VVFVGE
 VLTEYCYA - -FKVQKPGMPPVKELRKLMRIHALRCHVIYCQNGTKLNVIPIMASRSQALRYLYVRWGMDSLNI VVFVGE
 LSTDYCYA - -FKVRKPGMAPPLKELRKLMRIQALRCHPIYCQNGTRNLNVIPVLASRSQALRYLYVRWGFELSKM VVFVGE
 LSTNYCYA - -FSVQKPGMTPPVKELRKVLRIQALRCHVIYCQNGSRVNVIPVLASRSQALRYLYLRWGMELSKM VVFVGE
 VSTGYCFA - -FKIKNQNKVPPTKELRKS MRIQALRCHVIYCQNGSKMNVIPVLASRSQALRYLYVRWGMELSKM VVFVGE
 GSTSHCHA - -FNVRNPDLVGPVRELRKSMRIQALRCHVVYCQNGYKMNVIPVLASRSQALRYLSIRWGMDSLNAV VFTGE
 SSTTHCYA - -FKVNDFTLAPPAKELRKMMRIQALRCHAIYCQNGTWNVIPVLASRSQALRYLFMRWGMELSNF VVFVGE
 LSTDYCYA - -FNVKAGMAPPLKELRKLMRIQALRCHPIYCQNGTRNLNVIPVLASRSQALRYLYVRWGFELSKM VVFVGE
 RSSAYCHA - -FKVKNSSLVPPITELRKLMRIQALRCHVIYSHDGTKLHAIPVLASRSQALRYLYVRWGTEL SNM VVFVGE
 RSSTYCHA - -FKLKNPALVPPIKELRKLMRIQALRCHVLYSYDCTKLHVIPILASRSQALRYLHVRWDTDL SNL VVFVGE
 CSSTYICIS - -FSVQKPGMTPPVKELRKVLRIQALRCHVIYCQNGSRVNVIPVLASRSQALRYLYLRWGMELSKM VVFVGE
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 CSSTYICIS - -FKVSNNTAAAPPVKEIRRTMRIQALRCHVLYSHDGSKLNVIPVLASRSQALRYLYIRWGMELSNIT VIVGE
 HSSTYCLA - -FRVVPNHLPLKELRKLMRIQSLRCNALYNHSATRLSVVPIHASRSQALRYLCIRWGIELPNVAVLVGE
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 SSNSHCVA - -YMIKDRSKVMRVDLQKLRRLRGLRCHPMYCRNSTRMQIVPPLASRSQALRYLFVRWRLNVANMYVVVGD
 SSNSHCIS - -YLIKDRSKAKKVDMDRQKLRMRGLRCHLMYCRNSTRMQVVPPLASRSQALRYLFVRWRLNVANMCVILGE
 SCNAYCVS - -FFIKDPKAKAIDDLRQKLRMRGLRCHLMYCRNSTRLQVIPLASRSQALRYMFVRWGLNVANMYVILGE
 SSNAHCVS - -YLIKDLKSKVKKVDDLQKLRMRGLRCHPMYCRNSTRLQVIPLASRAQALRYLFVRWRLNVTNMYVILGE
 SSNSHCLS - -YAIKDPKAKKVDMDRQKLRMRGLRCHLMYCRNSTSMQVVPPLASRSQALRYLFVRWRLSVANMYVILGE
 SSNAHCVA - -FLIKDPQKVKTVDEMRELRMRGLRCHIMYCRNSTRLQVVPPLASRSQALRYLSVRWGVSVGNMYLITGE
 SCNPHCVS - -FFIKDPNKVRTADEMRELRMRGLRCHLMYCRNSTRLQVVPPLASRSQALRYLFVRWGLSVGNMYLIVGE
 ACSSRCYS - -YSITPGAKVPKVNLDLQKLRMRGLRCHVYTHAASRLNVTPLFASRSQALRYLSVRWGVGLSSM VVFVGE
 VCSTRCYS - -YGVKPGAKTRRIDLHQRMRMRGFRCNLVYTHATSRLNVVPLFASRAQALRYLSVRWGIDLSKM VVFVGE
 SCSTRCYA - -ISVKQGVKTRRVDLQKLRMRGLRCHVYTHAATRLNVIPLCASRIQALRYLSIRWGIDMSKT VFFLGE
 ACSGPCHA - -YAAAGASKVKKVDSIRQSLMRGFRCNLVYTRACTRLNVIPLSASRPALRYLSIQWIDLDK VAVLVGD
 AGSVHCHA - -YAAATDASKVKKVDSIRQALMRGFRCNLVYTRACTRLNVIPLSASRPALRYLSIQWIDLDK VAVLVGD
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 NCNSHCLA - -YVTNSDIAPTVDQLRQRLMRGLRCHVMFCRNASRLHVLPLASRSQALRYFFARWNVVDVANMF VVVGE
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 NM117080 A thaliana ATSPS4F
 Castleden et al (2004) ZmSPS4
 AF310160 T aestivum SPS1
 NM 001074101 O sativa SPS
 DQ157858 P patens SPS1
 DQ157859 P patens SPS2

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NM122035 A thaliana ATSPS1F	-----
NM121149 A thaliana ATSPS2F	-----
AF439861 I batatas	-----
DQ364058 C melo SPS	-----
AF071786 L esculentum SPS	-----
AY26439 L esculentum SPS	-----
X73477 S tuberosum SPS	-----
DQ834321 C canephora SPS1	-----
Y11821 C plantagineum Cpsps1	-----
AF318949 A chinensis SPS2	IALRVSLSMVVIWSFLNASRSLTFFGKYIHHHSWKCEACVWFLHENLVTIHSFVFLPVSLDQKSILRWFCWHIESNLSC
CU459387 V vinifera SPS	-----
AF322116 M sativa SPS	-----
AB005023 C unshiu CitSPS1	-----
X81975 B vulgaris SPS1	-----
AY331261 V album SPS	-----
S54379 S oleracea SPS	-----
Z56278 V faba SPS	-----
X humilis SPS2	-----
AY135211 O goldiana SPS	-----
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X humilis SPS1	-----
AM451888 V vinifera SPS	-----
Y11795 C plantagineum CpSPS2	-----
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X73477 S tuberosum SPS	-----
DQ834321 C canephora SPS1	-----
Y11821 C plantagineum Cpsps1	-----
AF318949 A chinensis SPS2	TSIVIFVLERIMEPYRVKT
CU459387 V vinifera SPS	-----
AF322116 M sativa SPS	-----
AB005023 C unshiu CitSPS1	-----
X81975 B vulgaris SPS1	-----
AY331261 V album SPS	-----
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TC359646 O sativa SPS	-----
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