The analysis of starch degradation in *Solanaceae* species

by

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Declaration

The experimental work in this thesis was supervised by Dr. JR Lloyd and was conducted at the Institute for Plant Biotechnology, at Stellenbosch University, South Africa. The results presented are original, and have not been submitted in any form to another university.

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in parts been submitted at any other university for a degree.

ME Samodien

February 2014

Abstract

This project involved the analysis of genes in *Solanaceae* species that have previously been shown to be involved in the phosphorylation of starch or its subsequent dephosphorylation. Both these processes are essential for normal starch mobilization. A tomato conditional mutant lacking the starch phosphorylating enzyme glucan water dikinase was analyzed. It is known that starch accumulates transiently in tomato fruit and is degraded throughout the ripening process. The study aimed to determine the effect of inhibited starch degradation on fruit development. Unfortunately no effect on starch mobilisation was found in the fruit of the mutant. Immunoblot analysis revealed expression of Glucan Water Dikinase (GWD) within the fruit of the tomato mutant indicating that the conditionality of the mutation was compromised.

The second set of experiments analyzed the roles of Starch Excess4 (SEX4), Like Sex Four-1 and Like Sex Four-2 (LSF1 and LSF2) in starch degradation in potato and *Nicotiana benthamiana*. These enzymes have, thus far, only been studied in *Arabidopsis*, with the proposed role for SEX4 and LSF2 being that they are involved in dephosphorylation of the C-6 and C-3 positions of starch breakdown products. The role of LSF1 is unclear, although it is not thought to be a phosphatase.

SEX4, *LSF1* and *LSF2* were repressed individually while the expression of *SEX4* and *LSF2* were also inhibited simultaneously. Using a transient repression system in *N. benthamiana* it was shown that all of the genes play a role in leaf starch degradation. The SEX4 and LSF2 enzymes were shown to influence the proportion of phosphate located on the starch which contained an altered ratio of C-3/C-6 phosphate.

Stably transformed potato plants were produced where *SEX4* and *LSF2* were successfully repressed in potato leaves and tubers. Although *AtLSF2* had been shown not to be essential for normal starch degradation on its own, in potato plants when *LSF2* was repressed, the plants developed a starch-excess phenotype. Taken together with the *N. benthamiana* data this indicates that *LSF2* plays a bigger role in leaf starch degradation in Solanaceae than in *Arabidopsis*.

The ratio of C-3/C-6 phosphate was also altered in tuber starch from some of the silenced plants. Starch from *SEX4* repressed potato plants contained increased amounts of glucose-6-phosphate and increased glucose-3-phosphate in the tuber when compared to the WT. An increase in the proportion of C-6 or C-3 phosphate is not surprising with SEX4 being characterized as a phosphatase specific for C-6 position and LSF2 for the C-3 position in Arabidopsis, however the combined increase in C-3 and C-6 amounts in StSEX4 silenced plants is interesting. The differences seen in the phosphate alteration in both *N. benthamiana* leaves and potato tubers indicates that in *Solanaceae* species these proteins may have a slightly altered specificity when compared with Arabidopsis, although they are undoubtedly involved in starch degradation.

The effect of silencing SEX4 or LSF2 on cold-induced sweetening was also investigated, with no effect being found. This may be because of functional redundancy between the proteins and a better approach in terms of blocking cold sweetening would be to simultaneously repress *SEX4* and *LSF2*.

Overall, these enzymes seem to play similar roles in leaves of Solanum species as has been described in Arabidopsis. The starch from the engineered plants did have an altered phosphate ratio and further analysis is needed to determine if this leads to improved or additional functionality.

Opsomming

Die projek omhels die ontleding van gene van die Solanaceae spesie wat voorheengetoon het dat hulle deel neem in fosforilering of defosforilering van stysel. Altwee van hierdie reaksies is belangrik vir normale stysel metabolisme. ‘n Tamatie konditionele mutant was geanaliseer waarin die stysel fosforilering ensiem glucan water dikinase nie teenwoordig was nie. Die doel van die studie was om te ondersoek watter effek het n gebrek in stysel afbraak op die rypwording en ontwokkeling vrugte. Ongelukkig was geen effek op stysel metabolism in die munant se vrugte gesien. Immunoklad analise het getoon dat GWD protein wel uitdruk word in die vrugte en dus die mutant nie heeltemal effektief was nie.

Die tweede stel van experimente het in aartappels en tabak die rol van SEX4, LSF1 en LSF2 in stysel afbraak ondersoek. Hierdie ensieme was huidiglik nog net deeglik in Arabidopsis bestudeer, waar daar gewys was dat SEX4 and LSF2 in die defosforilering van stysel by die C-6 en C-3 posisie deel neem. Die rol van LSF1 is nog onbekend, maar daar word huiglik gelgo dat dit is nie ‘n fosfatase nie.

SEX4, *LSF1*, en *LSF2* was onderdruk op sy eie, waar *SEX4* en *LSF2* gelyktydig onderdruk was. Met behulp van n verbygaande onderdrukking in tabak, was dit getoon dat al die bogenoemde gene n gedeeltelike rol speel in die afbraak van stysel. Dit was getoon dat SEX4 and LSF2 ensiemedie verhouding van waar fosfaat op stysel gelee is beinvloed en het n verandering in die C-3/C-6 phosphaat verhouding ook gehad.

Aardappels was stabiel getransformeer en daar was suksesfol plante waar *SEX4* en *LSF2* onderdruk was in blare en knolle geproduseer. Alhoewel daar getoon was dat AtLSF2 op sy eie nie n groot rol speel in stysel katabolisme nie was daar wel gesien dat in aardappel wanner hierdie geen afgeskakel was dat daar n stysel oorskot fenotiepe ontwikkel. As die tabak resultate saamgevat word met die aardappel wil dit voorkom asof LSF2 n groter rol binne die stysel katabolisme in Solanaceae speel as in Arabidopsis. Daar was gevind dat die verhouding van C-3/C-6 fosfaat was in die knolle verander in perty van die lyne waar geen afskakeling wel plaasgevind het.

Die verhouding van C-3/C-6 fosfaat was verander in knolle stysel van sommige stilgemaak plante. Sysel van SEX4 stilgemaak plante het hoër vlakke glukose-6-fosfaat en glukose-3-fosfaat in die knolle gehad wanner dit met die WT vergelyk was. n Toename in die persentasie van C-6 fosfaat is nie verbasend nie, SEX4 word gekenmerk as die spesifieke fosfatase verantwoordelik vir die fosfaat by die C-6 posisie en LSF2 spesifiek vir die C-3 posisie in Arabidopsis. Die gekombineerde toename in beide C-6 en C-3 bedrae in StSEX4 stilgemaak plante is wel heel interesant. Verandering in beide tabak blare and aartapple knolle dui daarop dat in solanacea spesie hierdie proteiene, n effens verandering in spesifisiteit kan hê as dit met Arabidopsis vergelyk word. Daar kan wel nie getwyfel word dat hulle wel n rol speel in stysel afbraak nie. Die effect wat*SEX4* of *LSF2* op koue-geinduseerde soetheid het is ook ondersoek maar daar was geen effek gevind nie. Dit mag wees asgevolg van die funksionele onslag tussen die twee proteien en better benadering on die koue-soetheids effek te verhoed sou wees om beide protein op die selfde stadium aft e skakel. As daar in gegeheel gekyk word lyk dit asof hierdie protein die selfde rolle het in die Solanum spesies as in Arabidopsis.Die stysel van hierdie die ontwerpte plante het ‘n veranderde fosfaat verhouding getoon en veder analise is nodig om te bepaal of dit lei tot verbeterde einskappe of bykommende funksies.

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Chapter 1 General Introduction

1.1 The importance of starch

Starch functions as the primary carbohydrate storage reserve in the leaves of most plants, being synthesized during the day as a product of photosynthesis and subsequently degraded at night and converted to sucrose. This is transported to storage organs, such as tubers and seeds, where it is converted back to starch in order to function as a long-term carbon reserve. This starch-to-sucrose conversion in leaves is regarded as one of the largest carbon fluxes which occurs daily on the planet (Niittylä *et al.*, 2006).

Starch is a renewable resource that is used in many industries (Kossmann and Lloyd, 2000), for example as a thickener in food products as well as being useful in the textile, paper-manufacturing and pharmaceuticals industries (Ramesh and Tharanathan, 2003; Delcour *et al.*, 2010, Santelia and Zeeman, 2011). In all these instances, the starch has to be modified through physical or chemical treatments to make it amenable for that particular industrial application. Examples of modifications include oxidation and esterification which are often required to stabilize the glucan polymers during processing. The introduction of functional groups, such as phosphate, to achieve this purpose has also been described (BeMiller, 1997a). Starches which contain elevated phosphate levels are extremely valuable due to their usefulness in various industries. These types of starches have increased swelling power and form stable, clear, gelatinous pastes (Santelia and Zeeman, 2011).

Modification of the structure of starch within the plant can be achieved using biotechnological tools by targeting one, or a series of enzymes involved in the pathway, in order to create a specific type of starch, tailor made for industry. Understanding the mechanisms involved in starch metabolism on a molecular level gives insight into how to

produce novel starches with altered properties, hopefully resulting in improved functionality (Santelia *et al.*, 2011).

1.2 Starch structure

Starch exists in a granular form located within the plastids and consists of two polymers of glucose, amylose and amylopectin. These are structurally diverse with amylose being the unbranched constituent comprised mainly of α-1,4-linkages. Amylopectin contains both α-1,4-bonds and α-1,6 branch points which are arranged in an ordered structure almost certainly similar to the 'cluster' model described more than 40 years ago by Hizukuri *et al.*, (1970), where short chains cluster together in ordered arrays of densely packed double helices and are linked together through longer chains. Amylopectin constitutes about 70-90% of starch and its ordered structure confers the semi-crystallinity to the starch molecule. Small angle X-ray scattering experiments have shown that all starches analysed contain a 9 nm repeat structure, comprising one crystalline and one amorphous layer (Waigh *et al.*, 1998). These repeats are thought to contain a single layer of double helical clusters forming the crystalline layer interspersed with amorphous amylose.

Starch granules exhibit various degrees of crystallinity, due to the ordering of the double helices known as allomorph of which A and B types exist. The former is found in cereal seed starches and is described as being more compact than the B type which is found, for example, in potato tuber starch (Gallant *et al.*, 1997; Gèrard *et al.*, 2001; Hejazi *et al.*, 2008). The C type has also been described, which is essentially a mixture of the A and B types (Bogracheva *et al.*, 2001; Imberty *et al.*, 1991) and this type of double helical arrangement is found in legume starches. Figure 1.1 illustrates starch structure and how the various components of the starch molecule are arranged.

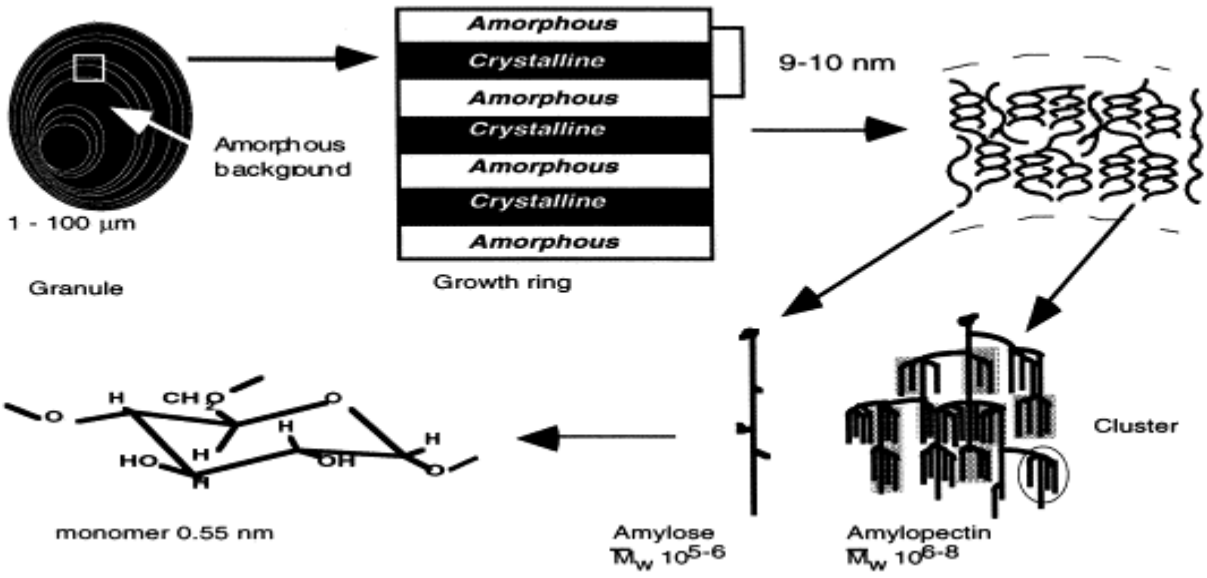

Figure 1.1: Starch structure and organisation. Illustration for the organisation of the starch granule, how the amorphous and crystalline layers are arranged, also showing the structure of starch components amylose and amylopectin are how these arranged. Reproduced with permission from Buléon *et al.*, (1998).

The phosphorylation that starch undergoes is the sole covalent modification described within plants (Santelia *et al.*, 2011). The extent varies quite considerably, being about 0.1% in leaf starch but much greater in some tuber starches, such as potato (*Solanum tuberosum L.*) and Curcuma (*Curcuma zedoaria* Rosc), which contain about 0.2% to 0.5% phosphorylated glucose residues. In cereal starches covalently bound phosphate levels are close to being undetectable (Tabata and Hizukuri, 1971; Blennow *et al.*, 2000; Yu *et al.*, 2001; Ritte *et al.*, 2004).

Phosphate is located on the glucose monomers at either the C-6 or C-3 positions of the amylopectin fraction (Hizukuri *et al.*, 1970; Tabata *et al.*, 1975; Baldwin *et al.*, 1997; Jane *et al.*, 1999; Blennow *et al.*, 2000, 2002). The physical characteristics that starches exhibit is significantly influenced by the amount of phosphate they contain (Muhrbeck and Eliasson, 1991). The genes involved in phosphorylating starch have been identified (described below) which has led to the ability to manipulate the starch phosphate content in transgenic and mutant plants. Elimination of phosphate in potato tuber starch resulted in a molecule with low

paste viscosity (Lorberth *et al.*, 1998), while increasing phosphate in cereal endosperm starch led to a higher paste viscosity (Zeeman *et al.*, 2010; and references therein). Potentially, more precise manipulation of enzymes involved in phosphorylating and dephosphorylating starch may provide a means whereby the exact amount of phosphate can be controlled as well as influencing the ratio in which these phosphate groups exists on the C-3 or C-6 positions. A model of a crystalline starch molecule which has been phosphorylated at the C-3 or C-6 position is shown in Figure 1.2.

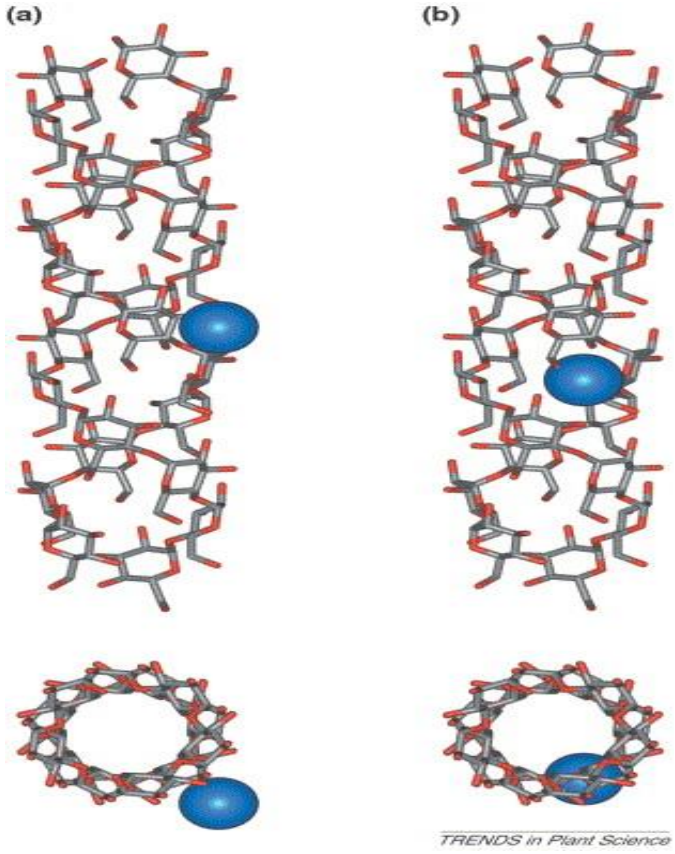

Figure 1.2: Crystalline domain of a starch molecule phosphorylated at the C-3 (a) C-6 (b) position. Reproduced with permission from Blennow *et al.*, (2002)

Although much has been learned about the way starch is synthesized, there are still some aspects which remain unclear especially with respect to how the different isoforms involved contribute to this process. Although this project deals exclusively with enzymes

involved in starch degradation, the important aspects involved in the synthesis of starch will be briefly discussed below.

1.3 Starch Synthesis

As previously mentioned, starch is comprised of two distinct α-glucan polymers, amylose and amylopectin (Bulèon *et al.*, 1998). Activities of three enzyme classes, starch synthases (SS), branching enzymes (BE) and debranching enzymes (DBE) are required for its proper synthesis (Ball *et al.*, 1998; Myers *et al.*, 2000; Deschamps *et al.*, 2008). The mechanisms involved are complex, with the actions of these enzymes being interlinked; for example SS produces linear chains which are the substrates for BE that lead to the production of short chains which SS can then act upon (Zeeman *et al.*, 2007; Liu *et al.*, 2009, and references therein). To further complicate matters, multiple isoforms of starch synthases exist with, for example, *Arabidopsis* containing four soluble starch synthases (SS1-SS4) as well as one granule bound isoform (GBSS). Mutants in the genes encoding all of the soluble SS proteins have been studied in *Arabidopsis*, with fragmentary studies of the isoforms in other species such as maize, rice and potato. The use of *Arabidopsis* as a model organism only allows the study of the role of these proteins in transitory starch synthesis. This has, however, allowed the production of a model for the roles of these proteins to be proposed. Analysis of amylopectin structure in lines of *Arabidopsis* mutants revealed that SS1 primarily functions in the synthesis of short chains (degree of polymerization, DP, up to 10; Delvalle *et al.*, 2005), with SS2 involved in generating longer chains with a DP of about 20 (Zhang *et al.*, 2008). These two isoforms function in combination to synthesize chains which form the crystalline lamellae. SS3 is involved in longer chain synthesis and shares some overlapping functions with SS2 (Zhang *et al.*, 2005 and 2008). The activity of SS4 normally does not contribute to amylopectin synthesis, however, it is important for granule initiation as *ss4*

mutants accumulate one large starch granule per chloroplast compared with several smaller ones in control plants (Roldán *et al.*, 2007). SS3 is also involved in granule initiation as *ss3/ss4* double mutants are essentially starchless (Szydlowski *et al.*, 2009) but, when all other SS isoforms are removed through mutation, SS4 activity can contribute to amylopectin synthesis (Szydlowski *et al.*, 2009).

Branching enzymes (BE) catalyse α-1,6-bond synthesis within the polymer (Sivak and Preiss, 1998) and act by increasing the number of non-reducing ends, aiding SS chain elongation. Branching Enzymes are found in plants with two or three isoforms normally present (Mizuno *et al.*, 1992; Nakamura *et al.*, 1992; Fisher *et al.*, 1996; Larsson *et al.*, 1996 and 1998; Morell *et al.*, 1997). Two classes of BE have been characterized with respect to their protein sequences; these are BEI (the B family) and BEII (the A family; Burton *et al.*, 1995). BE1 activity has been demonstrated to be more active on longer linear chains, such as amylose, while BEII activity preferentially branches the shorter chains found within amylopectin (Dumez *et al.*, 2006 and references therein). The activities of these enzymes are influenced by the presence of phosphate, which has been described in previous potato and wheat studies (Blennow, 1992; Morell *et a.*, 1997). Phosphate interacts with the substrate, changing its structure and enhancing the enzymes activity, however the importance of this in vivo is still to be investigated (Rydberg *et al.*, 2001). Starch granule bound phosphate is crucial to the activity of enzymes involved in its degradation (discussed more in detail below). It would therefore not be surprising if phosphate interacting with a particular substrate in plants could be important in stimulating enzymes involved in starch synthesis.

Three isoforms of starch branching enzyme (BE1-3) have been identified with *Arabidopsis* genome. *BE1* is part of the class I family while *BE2* and *BE3* were characterized as class II type of starch branching enzymes. Single mutant lines demonstrated no effect on starch content. When double mutant lines (*be1*/*be2*, *be2*/*be3* and *be1*/*be3*) were produced the

results indicated that *BE1* played no apparent function in leaf starch synthesis as there was no additive effect in *be1/be2* or *be1/be3* double mutants compared to the *be2* or *be3* single mutant plants. The *be2/be3* double mutant, however, resulted in the inhibition of starch accumulation and a reduction in growth, which was hypothesized to be due to the accumulation of maltose within the cells. This is thought to disturb the osmotic potential of the cell, leading to feedback regulation which may affect processes such as photosynthesis, carbon metabolism and ultimately cell growth (Dumez *et al.*, 2006).

Two conserved types of α-1,6-glucan hydrolase debranching enzymes (DBE) are known to occur in all plants. These are defined as pullulanase-type DBE's (also known as R-enzyme and limit-dextrinase) and isoamylase-type DBE's, being differentiated on the basis of the preferred substrate and sequence similarity (Burton *et al.*, 2002; Bustos *et al.*, 2004; Lloyd *et al.*, 2005). The differences in substrate specificities may be evidence of the different function played by these enzymes within starch metabolism, with LDA and ISA3 preferring short β-limit dextrins, whereas *ISA1*/*ISA2* prefers glycogen-like amylopectin (Doehlert and Knutson, 1991; Wu *et al.*, 2002; Hussain *et al.*, 2003; Takashima *et al.*, 2007).

Some isoforms of the isoamylase type debranching enzymes are known to be involved in starch metabolism as mutations in them lead to plants which accumulate a glycogen like molecule (phytoglycogen, PG) in addition to starch. PG closely resembles amylopectin in that it contains α-1,4 chains which are linked to α-1,6 branch points, however the proportion of α-1,4 linkages are significantly higher than the amount of α-1,6 branch points within this water soluble polysaccharide than in amylopectin (Manners, 1985). The maize *sugary1* (*su1*) mutation was one of the first to be identified as being involved in starch metabolism in higher plants (James *et al.*, 1995). The *su1* mutant plants accumulate PG and the mutant allele was shown to encode an isoamylase type debranching enzyme. Since then similar phenotypes have been shown to occur in mutant and transgenic plants lacking isoamylase activity in

many different species (James *et al.*, 1995; Mouille *et al.*, 1996; Fujita *et al.*, 1999; Zeeman *et al.*, 1998b; Burton *et al.*, 2002; Bustos *et al.*, 2006). There are multiple isoforms of ISA and two of them form a heteromultimeric complex which is necessary for proper starch synthesis (Bustos *et al.*, 2004; Delatte *et al.*, 2005; Wattebled *et al.*, 2005). If the expression of either of the subunits of the complex is inhibited, the other subunit does not accumulate, probably as it is unstable outside of the complex (Bustos *et al.*, 2004; Delatte *et al.*, 2005; Wattebled *et al.*, 2005).

The second LDA type of DBE has been shown to be involved in starch degradation in plants (Doehlert and Knutson, 1991; Wu *et al.*, 2002; Hussain *et al.*, 2003; Wattebled *et al.*, 2005; Delatte *et al.*, 2006; Takashima *et al.*, 2007). There are tantalising hints though, that it may also play a role in starch synthesis (Dinges *et al.*, 2003). When a mutant *zpu* allele (that encodes pullulanase in maize) was combined with different *sul* alleles which reduce, but do not completely inhibit isoamylase-type DBE activity, increased amounts of phytoglycogen were found (Dinges *et al.*, 2003). This implies that there is some overlap in these enzyme functions, with LDA being able to compensate somewhat for a reduction in the ISA1/ISA2 heteromultimeric enzyme complex. In addition, Single nucleotide polymorphisms (SNP's) within a pullulanase gene in sorghum were associated with alterations in digestibility, most likely due to an alteration in starch structure (Gilding *et al.*, 2013). This example of the duality of the role that these enzymes play within the synthesis of starch as well as its degradation highlights the complexity of starch metabolism, with the varying functions between respective enzyme isoforms adding to the challenge of trying to understand the underlying mechanisms.

This project involves the examination of enzymes involved in adding and removing phosphate groups from glucose residues within the amylopectin molecule, thus the enzymes involved in starch synthesis will not be further discussed. The amount of phosphate in starch

has been shown to influence starch degradation (described below) and, therefore, the next section will concentrate on what is known about starch degradation in leaves and on the role(s) that starch phosphate metabolism plays within this process.

1.4 Leaf Starch Degradation

Over the past decade many enzymes involved in starch degradation have been investigated with the roles that they play within the pathway being described. *Arabidopsis* mutants exhibiting a starch excess (sex) phenotype have greatly aided in the identification of enzymes involved in the process of starch degradation (Caspar *et al.*, 1991 and later papers). The enzymes that have been identified as being important through analysis of these mutants will be outlined in the following paragraph.

The first *Arabidopsis* mutant identified that exhibited a starch excess phenotype (*sex1*; Caspar *et al.*, 1991) demonstrated the essential nature of reversible starch phosphorylation in its turnover (Zeeman *et al.*, 2010). The *SEX1* gene was identified as encoding a starch binding protein (Yu *et al.*, 2001), which had previously been shown to be essential for starch phosphorylation and degradation in potato (Lorberth *et al.*, 1998). It was later shown to be able to directly phosphorylate starch in a dikinase reaction (Ritte *et al.*, 2002) which is controlled by the redox status of the chloroplast (Mikkelsen *et al.*, 2004). This enzyme was named glucan water dikinase (GWD; Ritte *et al.*, 2002). Although leaf starch is phosphorylated by this enzyme throughout its synthesis during the light period, it becomes highly phosphorylated at the onset of dark where the GWD protein binds to the surface of the granule (Ritte *et al.*, 2000). The process of starch granule phosphorylation does not degrade the granule itself, but it is critical as it allows other degradative enzymes access to polyglucans. The starch granule surface is resistant to the activity of enzymes such as α-amylases and β-amylases, with the same being speculated to be the case with the DBEs

(Santelia *et al.*, 2011). Because of the transient phosphorylation at the surface, the crystallinity of the starch is disrupted rendering it soluble which aids in the activity of the glucan-hydrolysing enzymes (Edner *et al.*, 2007). As mentioned before, glucose moieties in starch are phosphorylated at the C-6 or C-3 positions. GWD activity is specific for the C-6, while the activity of a second protein, the phosphoglucan, water dikinase (PWD) is directed towards the C-3 position (Ritte *et al.*, 2006). Phosphorylation of starch by GWD is a prerequisite for PWD activity (Baunsgaard *et al.*, 2005; Kötting *et al.*, 2005; Ritte *et al.*, 2006). This is shown both by the activities of the recombinant proteins and because phosphate free starch is obtained in *Arabidopsis* thaliana *sex1* (*gwd*) null mutants while it is only phosphorylated at the C-6 position in *pwd* mutants (Ritte *et al.*, 2006). The repression of these enzymes results in a severe starch-excess phenotype in *gwd* mutants, with only a modest one in *pwd* mutants (Yu *et al.*, 2001; Baunsgaard *et al.*, 2005; Kötting *et al.*, 2005).

Following degradation of the granule surface, soluble phosphorylated glucans are produced. It is necessary to remove phosphate from these before they are further degraded because β-amylases cannot degrade past phosphorylated residues (Takeda and Hizukuri, 1981; Fulton *et al.*, 2008). The first enzyme discovered that accomplishes this is encoded at a locus known by several names, *SEX4*, *PTPKIS1* or *DSP4* (Zeeman *et al.*, 1998a; Fordham-Skelton *et al.*, 2002; Niittylä *et al.*, 2006; Kerk *et al.*, 2006; Sokolov *et al.*, 2006; Kötting *et al.*, 2009). In *sex4* mutants soluble phospho-oligosaccharides accumulate after being released from the surface of the starch granule through the action of α-amylase 3 (AMY3) and the debranching enzyme isoamylase 3 (*ISA3*; Kötting *et al.*, 2009).

Two similar SEX4-like phosphatase proteins have also been identified within *Arabidopsis*, known as Like Sex Four-1 (*LSF1)* and *LSF2*, respectively. Although mutant *lsf1* plants exhibit a starch excess phenotype there is no compelling evidence to demonstrate that the protein acts as a glucan phosphatase, as no decrease in glucan-dephosphorylation activity

as well as no accumulation of phospho-oligosaccharides was found in *lsf1* plants. This has led to the hypothesis that *LSF1* plays a regulatory role in starch degradation (Comparot-Moss *et al.*, 2010). The other *Arabidopsis* homolog *LSF2*, however, has been characterized as a phosphoglucan phosphatase that shows specificity for the C-3 bound phosphate. Starch from *lsf2* plants show an increase in C-3 bound phosphate, which is not seen in the *sex4* silenced plants (Santelia *et al.*, 2011). Previous studies have elucidated that phosphate located at the C-6 position elicits only slight changes, whereas C-3 phosphorylation enforces substantial steric effects foreseen to disrupt the normal glucan backbone conformation (Hansen *et al.*, 2009). The major effect that the amount of C-3 phosphate has on starch conformation and structure in comparison to the effect elicited by the phosphate proportion located on C-6 position is quite interesting, as the inhibition of starch degradation is far greater in *gwd* than *pwd* mutants indicating the C-3 bound phosphate is not as important as C-6 in allowing access to starch degradative enzymes.

It appears that β-amylase isoforms are the main enzymes that degrade the linear chains of the released soluble glucans from the starch granules. They are exoamylases, producing maltose from the non-reducing ends of glucans by hydrolysis of α-1,4 bonds. Nine genes encode putative β-amylase isoforms in the *Arabidopsis* genome of which four (*BAM1-4*) encode proteins which are targeted to the chloroplast (Fulton *et al.*, 2008), several of which have been demonstrated to be involved in starch degradation. Transgenic plants repressed in the activity of the plastidial β-amylase 3 (PCTBMY1; BAM3; BMY8) demonstrated inhibited leaf starch degradation in both potato and *Arabidopsis* (Scheidig *et al.*, 2002; Kaplan and Guy, 2005). In *Arabidopsis bam1* mutants starch degradation is unaffected; however, a *bam1/bam3* double mutant disrupts normal starch degradation effect to a greater degree than seen in *bam3* plants, suggesting that some redundancy exists between these two isoforms (Fulton *et al.*, 2008). The BAM4 mutants also have impaired starch degradation;

however the isoform demonstrates no catalytic activity (Fulton *et al.*, 2008). Thus, the mechanism by which this occurs is unclear.

As mentioned previously, a role for DBEs in starch degradation has been described. Knockout mutants of *isa3* exhibited a starch excess phenotype (Wattebled *et al.*, 2005). No effect on starch turnover was seen in single *lda* mutants, however when *lda* was repressed together with *isa3* simultaneously an even greater starch excess phenotype was obtained indicating a level of redundancy between the function of these two enzymes (Delatte *et al.*, 2006). Interestingly the role of pullulanase in starch degradation in maize appears to be more important than in Arabidopsis. Complete repression of pullulanase-type DBE activity, through a mutation in the *zpul*-204 allele alone resulted in inhibited degradation of leaf starch (Dinges et al., 2003), whereas mutant Arabidopsis plants repressed in both *isa3* and *lda* could mobilize some starch at night and accumulated small soluble branched glucans suggested to be starch degradation products liberated by *AMY3* (Delatte *et al.*, 2006).

Plants lacking the sole chloroplastic α-amylase (AMY3) have starch degradation rates similar to wild-type plants and do not demonstrate a starch excess phenotype (Yu *et al.*, 2005). *AMY3* is the source of the small branched glucans degraded from the surface of the starch granule and its removal in *isa3* or *isa3/lda* backgrounds reduces the capacity of the plants to degrade starch further. Plants lacking *ISA3*, *LDA* and *AMY3* exhibit starch degradation which is completely inhibited and which leads to a highly reduced growth phenotype (Streb *et al.*, 2012).

The enzymes above lead to the production of linear glucan chains, which have to be mobilised to mono-saccharides. There are two pathways that achieve this in *Arabidopsis* leaves. The first involves a disproportionating enzyme (*DPE1*), present in different plant organs containing starch (Kakefuda *et al.*, 1986; Lin and Preiss, 1988; Takaha *et al.*, 1993). DPE1 transfers glucan chains from α-1,4 glucans with a degree of polymerisation of at least

three (dp3), to another α1-4 polyglucan ultimately leading to the production of glucose which can be exported to the cytosol, (Lin and Preiss, 1988; Okita *et al.*, 1979). This is exported by a glucose transporter located on the plastid inner membrane, (Weber *et al.*, 2000; Servaites and Geiger, 2002; Niittylä *et al.*, 2004). Plants repressed in *dpe1* accumulate a series of malto-oligosaccharidesc (MOS), most prominently maltotriose, but also maltotetraose, maltopentaose, maltohexaose and maltoheptaose (Critchley *et al.*, 2001; Lütken *et al.*, 2010). These plants demonstrate a mild inhibition of starch degradation, indicating that this pathway has a minor influence on starch mobilisation.

Other than MOS, maltose is one of the main products of starch degradation. It is exported from the chloroplast into the cytosol by a maltose transporter (MEX1; Niittylä *et al.*, 2004) where it is acted upon by a second disproportionating enzyme, DPE2, which is distinct from DPE1 in terms of its activity (Chia *et al.*, 2004; Lloyd *et al.*, 2004; Lu and Sharkey *et al.*, 2004; George *et al.*, 2012). DPE2 is a transglucosidase that metabolises cytosolic maltose by transferring a single glucose molecule to a polymer leading to the liberation of the other glucose molecule (Chia *et al.*, 2004; Lloyd *et al.*, 2004; Lu and Sharkey, 2004). Repression or mutation of *DPE2* results a reduced net rate of leaf starch degradation (Chia *et al.*, 2004; Lloyd *et al.*, 2004; Lu and Sharkey, 2002; George *et al.*, 2012) and these plants also accumulate increased maltose levels similar to *mex1* mutants (Niittylä *et al.*, 2004; Lloyd *et al.*, 2004; Chia *et al.*, 2004; Lu and Sharkey, 2004). During the night a decrease in cytosolic sucrose levels is observed in plants lacking DPE2, providing evidence that the conversion of maltose to utilizable sugars is achieved by its action (Chia *et al.*, 2004; Lu and Sharkey, 2004). The impairment of starch degradation is much greater in plants where maltose metabolism is repressed (Niittylä *et al.*, 2004; Lloyd *et al.*, 2004; Chia *et al.*, 2004; Lu and Sharkey, 2004) than when the plants are unable to produce glucose via DPE1 (Takaha *et al.*,

1993; Critchley *et al.*, 2001). This demonstrates that maltose production is more important than glucose production in terms of flux from starch degradation.

Clearly most of the recent advances in understanding starch degradation have emanated from examining Arabidopsis. When the pathway established in this model species has been compared directly with the lesser amount known in other species, then it is clear that a great number of the steps are conserved. This is demonstrated by the strong starch excess phenotypes shown in *gwd, bam3* and *dpe2 Arabidopsis* mutants (Lao *et al.*, 1999; Yu *et al.*, 2001; Chia *et al.*, 2004; Lu and Sharkey, 2004; Kaplan and Guy, 2005), which are mirrored by phenotypes shown in transgenic potatoes lacking these proteins (Lorberth *et al.*, 1998; Scheidig *et al.*, 2002; Lloyd *et al.*, 2004). In addition the *dpe1* of *Arabidopsis* mutant and transgenic potato plants show only a mild decrease in the degradation of leaf starch (Critchley *et al.*, 2001; Lütken *et al.*, 2010). Some differences can be seen; however, for example the more important role of pullulanase in maize leaf starch degradation noted earlier (Dinges *et al.*, 2003). The general conservation of this pathway implies that control of starch degradation must be important for the plant's survival, something demonstrated by recent experiments showing the importance of starch in determining biomass (Sulpice *et al.*, 2010). Not all the enzymes elucidated in the Arabidopsis pathway have thus far been assessed in other species though, so it remains to be seen how universal it is.

1.5 Starch degradation in other organs

All the work on starch degradation described so far has involved analysis of the pathway in leaves. Starch is also present in other organs and it is possible that the pathway of starch degradation in these may differ to that discovered in leaves. In some cases it is clear that there must be some differences. Seed germination in cereals, for example, is characterized by disruption of the cellular structure within the endosperm leading to extra-

plastidial enzymes gaining access to the starch granule (Zhu *et al.*, 1998) which is very different to the maintenance of cell integrity in leaves during the day/night cycle. In my dissertation I have examined some aspects of starch degradation in potato tubers and tomato fruits, thus these organs will be discussed within the rest of this section.

1.5.1 Starch Degradation in Potato Tubers

One undesirable quality in the potato processing industry which is a direct result of starch degradation is cold-induced sweetening, where there is an increase in levels of reducing sugars (mainly glucose and fructose) in potato tubers stored between 0-6ºC (Müller-Thurgau, 1882). This trait has great economic significance since, during the frying process these reducing sugars react with amino acids leading to the formation of discoloured, inedible products (Dale and Bradshaw, 2003). Further attention to this process has come about since the discovery that acrylamide, a potent neurotoxin is formed by the reaction of asparagine with reducing sugars from an N-glycoside intermediate of the Maillard reaction (Stadler *et al.*, 2002; Mottram *et al.*, 2002). The level of acrylamide formed is correlated with the amount of sugars present in the tuber (Olsson *et al.*, 2004; Williams, 2005; De Wilde *et al.*, 2005). Some insight has been gained into the process of cold-sweetening and there are a number of approaches which have utilised biotechnological tools that have successfully inhibited cold-induced sweetening (CIS).

Some enzymes involved in CIS have been identified and characterized (Sowokinos, 2001; Kumar *et al.*, 2004). Changes in carbon fluxes occur during CIS where starch degradation is correlated with an increase in sucrose synthesis (Isherwood, 1973). This has been demonstrated to occur through a combination of the enzymes UDP-Glc pyrophosphorylase, sucrose phosphate synthase and sucrose phosphate phosphatase (Sowokinos, 2001). Sucrose is transported into the vacuole before being hydrolyzed to glucose and fructose by vacuolar acid invertase (VINV; Isla *et al.*, 1998), with a correlation

between reducing sugar accumulation and VINV activity being demonstrated during storage (Matsuura-Endo *et al.*, 2004). Several attempts aimed at repressing VINV activity have been employed (Greiner *et al.*, 1999; Agarwal *et al.*, 2003) such as reducing gene transcription (Zrenner *et al.*, 1996; Zhang *et al.*, 2008; Bhaskar *et al.*, 2010) or the expression of a tobacco invertase inhibitor in potato (Greiner *et al.*, 1999), both of which led to repression of CIS.

One other attempt aimed at inhibiting cold-sweetening included the genetic modification of potato where an enzyme was shown to be involved in starch degradation. As was said above GWD is important in leaf starch degradation. When it is repressed in potato tubers starch degradation is also impaired at low temperatures (Lorberth *et al.*, 1998). This is, however, the only protein demonstrated to be involved in both potato leaf starch degradation and CIS. For example, *DPE2* is essential for normal starch degradation in potato leaves, however when this enzyme is repressed no effect was seen in terms of inhibiting CIS (Lloyd *et al.*, 2004). *BAM3* expression is induced by cold temperatures as a mechanism to begin starch degradation, thereby producing soluble sugars aiding the plant to tolerate cold stress through maintaining appropriate osmotic potential (Kaplan and Guy, 2005; Kaplan *et al.*, 2006, and references therein). When BAM3 expression was repressed in potato however, it led to a repression of starch degradation in leaves (Scheidig *et al.*, 2002), but not cold-stored tubers (Prof. Jens Kossmann, Stellenbosch University, Pers. Comm.).

1.5.2 Tomato Fruit Metabolism

Compared to leaves or other storage organs such as maize seeds or potato tubers, carbohydrate metabolism in fruits has not been studied as extensively. The main model plant that people have used to study fruit metabolism is tomato however, due to its relatively large genome size (approximately 950 Mb; Asamizu, 2007) it is more difficult to isolate mutants in this species than in *Arabidopsis*. This helps to explain the relatively poor understanding of

this process. More consideration is now being paid to tomato fruit, owing to the importance of fruits in the human diet (Carrari and Fernie, 2006). Tomato fruit metabolism is interesting due to the intense metabolic alterations occurring during its development, where there is a shift from partially photosynthetic to wholly heterotrophic metabolism during ripening. This is accompanied by a conversion of chloroplasts into chromoplasts alongside accumulation of carotenoids and lycopene (Carrari and Fernie, 2006). Several studies have determined the role of specific enzymes on fruit metabolism, which include invertase (Fridman *et al.*, 2004) and plastidial fructose 1,6-bisphosphatase (Obiadalla-Ali *et al.*, 2004a) amongst several others.

One advance that has helped to study tomato fruits has been the development of the dwarf Micro-Tom cultivar (Scott and Harbaugh, 1989) which is used as a model (Meissner *et al.*, 1997). In the study by Obiadialla-Ali *et al.*, (2004b) carbohydrate metabolism was examined during fruit development of this cultivar and demonstrated that starch accumulates very early in development and is then degraded as the fruit ripens, something also seen in other varieties (Ohyama *et al.*, 1995; Klann *et al.*, 1996; Chengappa *et al.*, 1999; D'Aoust *et al.*, 1999; Nguyen-Quoc *et al.*, 1999). That study also demonstrated that the metabolism of the pericarp (outer tissue of the fruit) was different to that of the placenta (inner tissue of the fruit). It is not clear whether or not the starch in tomato fruits is of physiological relevance, however one hypothesis made more than 30 years ago is that the transient starch functions as a carbohydrate reserve for the developing fruit contributing to the soluble hexose levels as the fruit matures (Dinar and Stevens, 1981).

1.6 Aims and Scope

In this dissertation analysis was done to determine the effect of a number of genes which have been shown to be involved in starch phosphate metabolism in different Solanaceae species, namely tomato, *N. benthamiana* and potato.

A tomato *gwd* conditional mutant was obtained which demonstrates a starch excess phenotype in the leaves. I wish to study this to see the effect of inhibited starch degradation in the fruit has on normal fruit development. The project involves analysing both wild type and conditional mutant tomato plants to investigate if any differences occur in regards to fruit starch and sugar content and if these differences elicit any effects on its development.

Secondly, this dissertation will investigate the role of *SEX4*, *LSF1* and *LSF2* in *Nicotiana benthamiana* and *Solanum tuberosum*. These enzymes have been demonstrated to be involved in starch degradation in *Arabidopsis*, but their roles in other species are yet to be determined. The function of these enzymes was investigated in *N. benthamiana* and potato by employing both a transient and a stable gene silencing technique, namely VIGS (virus induced gene silencing) and RNAi (ribonucleic acid interference).

The experimentation involves silencing the respective genes and elucidating if any effect is elicited in terms of starch levels as well as starch phosphate content. The effects of repressing these genes had been investigated in both leaves and cold-stored potato tubers to determine if CIS is inhibited.

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Chapter 2: Analysis of the effect of a tomato *gwd* conditional mutant on fruit metabolism

2.1 Introduction

Carbohydrate metabolism in fruits has not been studied as extensively as in leaves or storage organs of other plants. It is, however, beginning to be examined in detail in tomato since fruit are an integral part of human nutrition (Carrari and Fernie, 2006). The development of the dwarf tomato cultivar Micro-Tom (Scott and Harbaugh, 1989) that is used as a model system (Meissner *et al.*, 1997) has helped in this regard. In one study by Obiadialla-Ali *et al.*, (2004) carbohydrate metabolism was analysed during fruit development in this cultivar and it was shown that the main carbohydrates (starch, soluble sugars) were similar to those in fruits from other varieties (Ohyama *et al.*, 1995; Klann *et al.*, 1996; Nguyen-Quoc *et al.*, 1999; D'Aoust *et al.*, 1999; Chengappa *et al.*, 1999) indicating that analysis of this variety could provide insight into tomato fruit metabolism generally.

The physiological role which starch plays in tomato fruits is unclear and to determine its importance tomato plants which are impaired in starch degradation could be analysed. Recently a *gwd* mutant in tomato was identified (Nashilevitz *et al.*, 2009) in which starch degradation was inhibited in its leaves. The mutation led to pollen lethality which was overcome through the production of a conditional mutant (CM) where *GWD* expression was driven by a LAT52 pollen specific promoter (Twell *et al.*, 1990; Nashilevitz *et al.*, 2009). Due to the impairment in starch degradation observed in other plants species lacking *GWD* (Lorberth *et al.* 1998; Yu *et al.*, 2001; Vriet *et al.*, 2010; Hirose *et al.*, 2013) it is reasonable to assume that this may also be the case in tomato fruit. In this project, I analyse the CM described by Nashilewitz *et al.* (2009) to see if it affects starch degradation in fruits and, if so, how that affects fruit carbohydrate metabolism.

2.2 Material and methods

2.2.1 Plant growth

Wild type (WT, cv Microtom) and isogenic conditional mutant (CM) seeds (Nashilevitz *et al.*, 2009; a kind gift of Prof. Avraham Levy, Weizmann Institute of Sciences, Rehovot, Israel) were planted into 4:1 vermiculite: sand mixture which had been sterilized by autoclaving prior to use. The plants were grown under glasshouse conditions using 0.15% (w/v) solution of hydrophonic solution (Chemicult) and 0.1% (w/v) calcium nitrate.

2.2.2 Soluble sugar and starch measurements

Twenty-five mg samples were taken from the inner (placenta) or outer (pericarp) tissue of the tomato fruit at 25, 30, 35, 45, 55 and 65 days after flowering (DAF). 1 ml of 80% (v/v) ethanol was added and heated at 80 °C for 1 h to remove soluble sugars. The ethanol solution was removed and this was used to determine the major soluble sugar amounts by the following method. 50 µl of the sample was combined with 250 µl of assay buffer (10 mM MOPS-KOH (pH 6.9), 5 mM $MgCl_2$, 1 mM ATP, 1 mM NAD, 1 U/ml glucose-6-phosphate dehydrogenase from *Leuconostoc*). Glucose, sucrose and fructose were determined by measuring the increase in absorbance at 340 nm following the sequential addition of 1 U/ml hexokinase, 1 U/ml phosphoglucoisomerase and excess β-fructosidase.

For determination of starch the ethanol insoluble sample was further washed with 1 ml 80% (v/v) ethanol. The liquid was removed and 0.4 ml 0.2 M KOH was added and heated at 95°C for 1 h to solubilise the starch. This was followed by sample neutralisation through the addition of 70 µl of 1 M acetic acid. 10 µl of this was mixed with 10 µl of 50 mM NaAC pH 5.6 and 10 U/ml amyloglucosidase and incubated at 37 °C for 2 – 3 h. Two hundred and fifty µl of assay buffer containing 10 mM MOPS-KOH (pH 6.9), 5 mM $MgCl_2$, 1 mM ATP, 1 mM NAD was added to the sample and the amount of glucose was determined by addition of 1

U/ml glucose 6-phosphate dehydrogenase from *Leuconostoc* and 1 U/ml hexokinase from yeast and following the change in absorbance at 340 nm.

2.2.3 Protein extraction

One ml extraction buffer containing 50 mM Tris-HCL pH 7.5; 0.1% Triton X-100; 5 mM DTT; 2 mM EDTA; 1 mM β-mercaptoethanol was added to 200 mg of tissues. Samples were homogenised using a mechanical grinder and centrifuged at 20000×g at 4 °C. Protein concentration in the extracts were determined using a commercially available kit (Biorad) based on the method of Bradford (1976).

2.2.4 Immunoblotting

Fifty µg of total protein was separated by 8% (w/v) SDS-PAGE and blotted onto a PVDF membrane using a semi-dry blotting system (Biorad). The membrane was removed and blocked in 4% (w/v) fat free milk powder and 2% (w/v) Bovine Serum Albumin Fraction V (Roche) in TBS-T overnight. Primary antibody (1:1000 dilution) was added to each of the membranes in TBS-T (20 mM Tris, pH 7.6; 137mM NaCl2; 0.1% (v/v) Tween-20) and incubated for 1 h, followed by the removal of primary antibody and a further three washes in TBS-T for 5 minutes. The blot was then incubated in TBS-T containing alkaline phosphatase labeled goat anti rabbit IgG (1:10000 dilution; KPL, Gaithersburg, MD 20878 USA) and incubated for 1 h. Upon removal of the secondary antibody, another three 5 minute washes were performed. The membranes were rinsed in H_2O, before substrates (5-Bromo-4-Chloro-3'- Indolyl Phosphate p-Toluidine/ Nitro-Blue Tetrazolium Chloride) were added. Once the desired staining was obtained the reaction was stopped by rinsing the membranes with H_2O.

2.3 Results and Discussion

2.3.1 Starch degradation is uninhibited in fruits of the conditional mutant

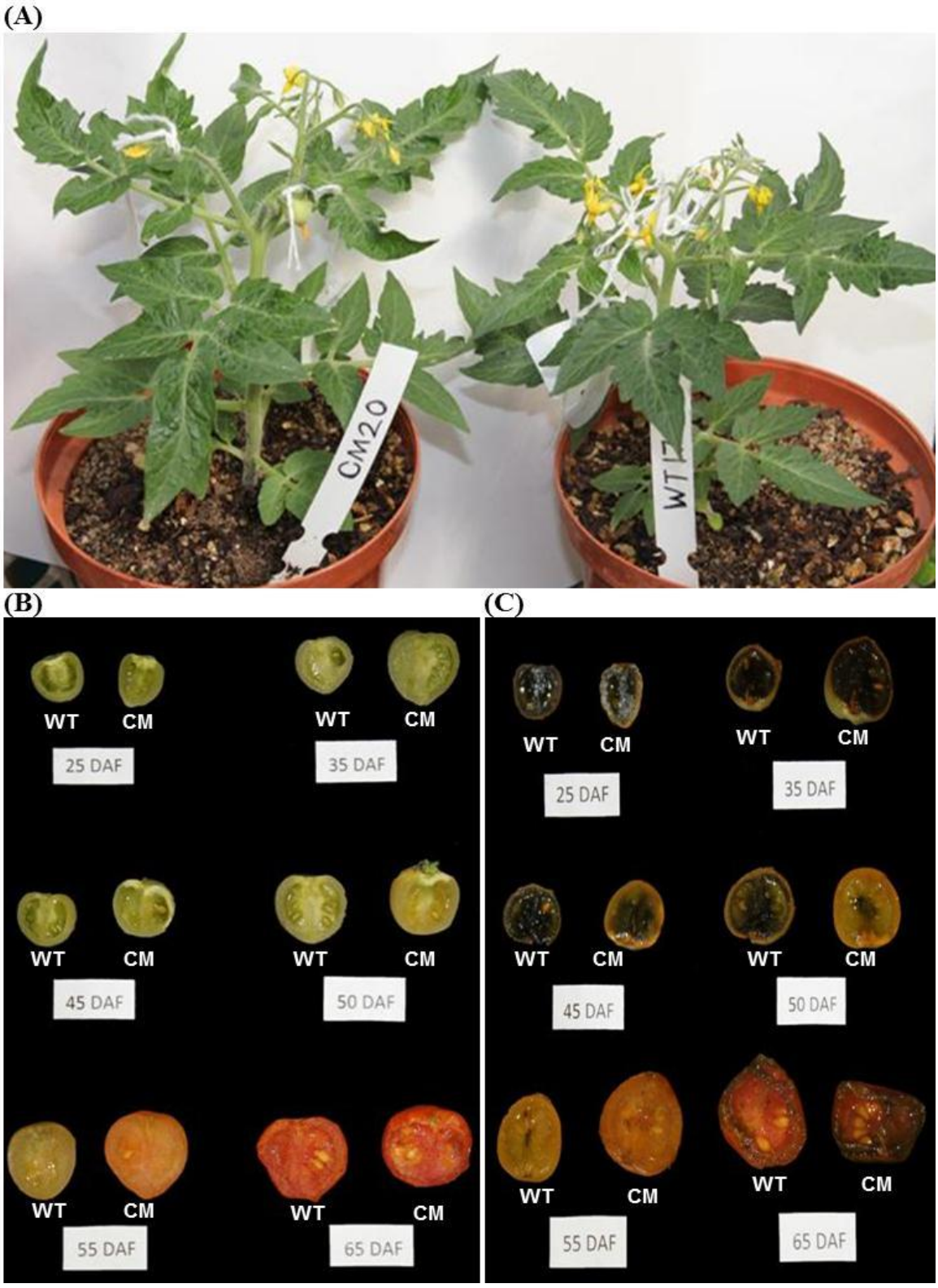

Figure 2.1 (A) the conditional mutant (CM) appears phenotypically similar to wild type (WT) plants. (B) WT and CM tomato fruit harvested at respective time points. (C) WT and CM tomato fruit harvested at respective time points stained with iodine solution.

The conditional mutant was phenotypically similar to the control (Fig. 2.1A) and so a time course, examining the major carbon pools in fruits, was performed. The highest starch amounts were observed at 25 and 35 DAF at the developmental stage when the fruit were still green (Fig 2.1B) before decreasing gradually as the fruit ripened. The starch was retained longer in the placenta than in the pericarp as found previously (Obiadalla-ali *et al.*, 2004), presumably because the placenta functions to provide nutrition for the fruit as it develops. A few differences were noted between the WT and CM in both pericarp and placental tissue, with the starch contents within the mutant decreasing faster in the pericarp, while in the placenta from the mutant the onset of degradation was delayed. Generally the starch content within the mutant fruit was degraded earlier than WT (Fig 2.2). This was also observed visually when the fruit were stained with an iodine solution (Fig 2.1C)

The earlier starch degradation within the mutant might be explained as a response to reduced carbon export from the leaves meaning that the fruit relies on its own stores to support its development. Irrespective of the reason, the data demonstrate that starch degradation in fruit from the CM is, surprisingly, not impaired. One possibility for this is that starch catabolism in tomato fruit is different from leaves of many species and does not need a functional GWD protein or, that the pollen specific promoter used to manufacture the CM also allows expression in fruits. These possibilities are examined later in the chapter.

Soluble sugar contents were altered in the pericarp with the CM containing consistently lower glucose, but higher fructose and sucrose concentrations compared to the WT. The lowered glucose might be due to the inhibited leaf starch degradation, thus less sucrose export to the fruit, which did not seem to affect the development of the fruit probably as the fruit starch could be utilized. Although the pericarp glucose concentration of the CM is consistently decreased, the fructose and sucrose contents are generally higher than WT which may be a response in order to compensate for the lower glucose levels.

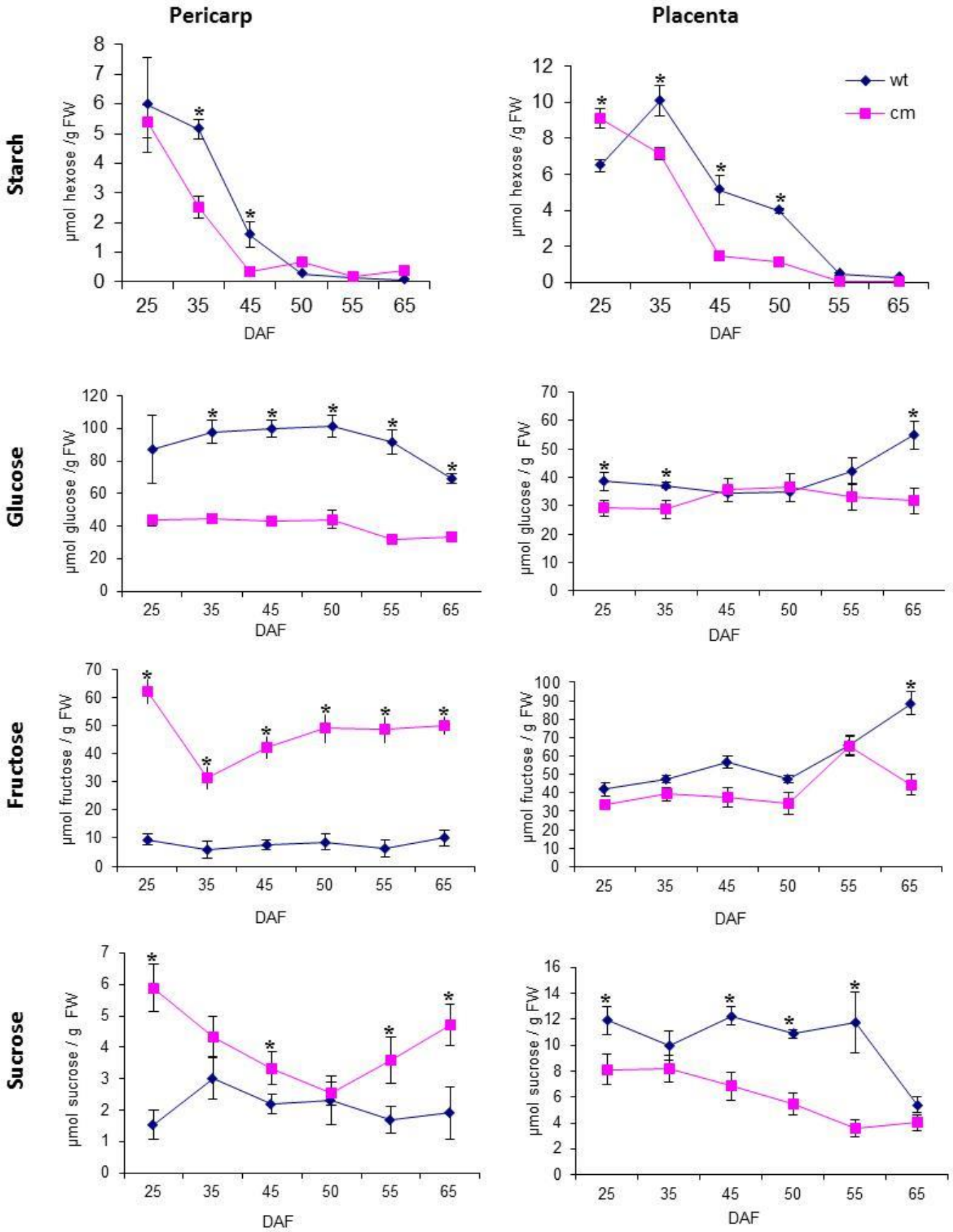

Figure 2.2: Starch and soluble sugars: starch, glucose, fructose and sucrose content in pericarp (left) and placenta (right) of WT (blue diamond) and CM (pink square) tomato fruits. Values are presented as mean ± SE of five individual samples per line and values with an * were determined by *Students t*-test to be significantly different ($P < 0.05$) from the WT.

Within placental tissue, glucose was found to be higher in the WT than the CM while fructose concentrations of both lines were similar, except at 65 DAF. Finally, the WT contained generally higher sucrose compared to the CM. The lowered placental sucrose content of the CM can be attributed to the impaired starch degradation in the leaves as previously mentioned. Overall the sucrose contents measured in the pericarp and placenta of WT and CM are lower than that of both glucose and fructose content and this is consistent with previous findings in tomato fruit studies (Damon *et al.*, 1988; Klann *et al.*, 1996; Schaffer and Petreikov 1997a).

2.3.2 GWD is expressed in the young fruit of tomato tissues

Since no effect was seen on starch degradation, it became necessary to evaluate the tomato fruit of the CM to elucidate whether they lacked GWD. For this reason, I used immunoblots to detect GWD in fruits harvested at 25 and 35 DAF. This revealed that a 160 kDa band could be detected for GWD in the pericarp and placenta from both the WT and CM (Figure 2.3). Presence of the GWD within the CM can, therefore, explain the unaltered starch degradation seen in the fruit. In the study of Twell *et al.*, (1990) it was stated that low level expression of the LAT52 promoter could be detected in both seeds and roots indicating a lack of pollen specificity. From my data young fruit can now be included in addition. This tomato mutant cannot, therefore, be used to study the role of impaired starch degradation on tomato fruit carbohydrate metabolism. Perhaps a different approach could be employed, for example an anti-sense or RNAi silencing construct could be used to produce transgenic lines where GWD is placed under the control of fruit specific promoter such as from the tomato E8 (Deikman *et al.*, 1992) or 2A11 genes (Van Haaren and Houck, 1993). This would hopefully result in more specific repression of the gene and may prove to be a more fruitful alternative.

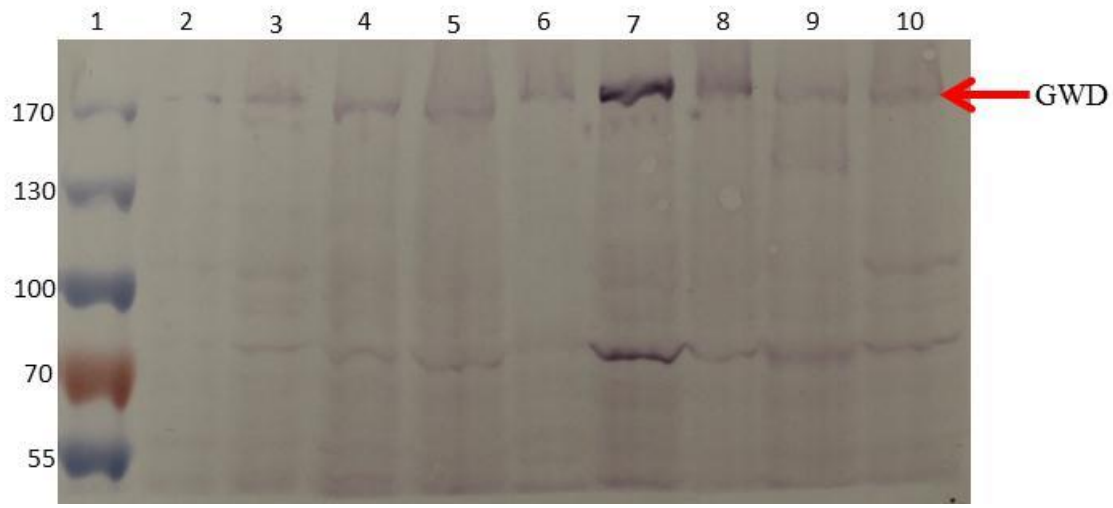

Figure 2.3; Immunoblot analysis of WT and CM pericarp and placenta harvested at 25 and 35 DAF tested against GWD-antibody. Lane 1: Fermentas pre-stained protein marker; Lane 2: potato extract (positive control); Lane 3-4 WT pericarp and placenta at 25DAF; Lane 5-6 CM pericarp and placenta at 25DAF; Lane 7-8 WT pericarp and placenta at 35 DAF; Lane 9-10 CM pericarp and placenta at 35DAF. (50 µg of protein was loaded for all samples).

2.4 Conclusion

Unfortunately the presence of the GWD protein within fruits of the CM meant this study was unable to determine the physiological importance of starch during fruit development. Until a tomato mutant or a transgenic line which effectively inhibits starch degradation in the fruit is obtained, the relevance of starch as well as the effect this could have of fruit development will remain a mystery.

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Chapter 3: Research Article

The following chapter has been written to be submitted to BMC Plant Biology as it is intended to submit it there for publication. Several authors are listed. The first author (ES) performed most of the experimental work within the chapter and wrote the first draft of the manuscript. He was advised by JK and JL who also corrected the first draft. The only experimental work not performed by ES was the measurement of phosphorylated malto-oligosaccharides which was performed by GG in the lab of SZ on samples prepared by ES.

Virus-induced gene silencing of SEX4, LSF1 and LSF2 leads to starch excess in leaves of *Nicotiana benthamiana*

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Abstract

Background

The *SEX4* gene was discovered through analysis of mutant *Arabidopsis* plants impaired in starch degradation. SEX4 was shown to catalyse the dephosphorylate starch at the C-6 and C-3 position and repression of its activity resulted in the accumulation of phosphorylated oligosaccharides. Two additional, similar genes known as *Like Sex Four–1* and *2* (*LSF1* and *LSF2*) respectively are present in the *Arabidopsis* genome. These have also been shown to be involved in the degradation of starch as mutations in them lead to a starch excess phenotype. As with SEX4, LSF2 has been shown to dephosphorylate starch, specifically at the C-3 position of the starch molecule, whilst LSF1 does not display the same activity. The role of these proteins have, thus far, only been studied in *Arabidopsis* so it remains to be seen if the pathway of starch degradation defined in this species is also present in other plants.

Results

SEX4, *LSF1* and *LSF2* play similar roles in starch degradation in *Nicotiana benthamiana* as in *Arabidopsis thaliana.* The repression of these enzymes leads to a starch excess phenotype, with the starch C-3 to C-6 phosphate ratio being altered in all silenced lines. An accumulation of phosphorylated malto-oligosaccharides being detected in *SEX4* and *SEX4:LSF2* silenced plants is also described.

Conclusion
Starch degradation in *N. benthamiana* leaves progresses by dephosphorylation of liberated malto-oligosaccharides by SEX4 and LSF2. LSF1 has a similar function as that described in *Arabidopsis*.

Key words
Starch phosphatase, *SEX4*, *LSF1*, *LSF2*, starch degradation, C-6 and C-3 starch bound-phosphate

Background

Starch is one of the most important plant products in the world [1]. It is produced during photosynthesis in the chloroplasts of leaves and subsequently degraded during the night [2]. Disrupting leaf starch metabolism affects plant development and growth rate [3,4,5] as, if it is depleted, it results in carbon starvation during the night leading to rapid changes in metabolism and gene expression that can inhibit growth [6,7,8,9,5]. Equally, insufficient starch turnover results in carbon that cannot be utilized for leaf and root development [10,7,11,5]. The starch granule itself is insoluble and resistant to hydrolytic enzymes. Starch phosphorylation is essential for proper starch degradation as it disrupts the crystallinity of the granule facilitating its hydrolysis [12,13] and this process is initiated by phosphorylation of the granule surface at the beginning of the light to dark transition [14,15]. The phosphate is located solely on the branched amylopectin fraction within starch with about 70-80% occurring at the C-6 and the remaining 20-30% at the C-3-positions [16,15]. It is incorporated by two enzymes, the glucan, water dikinase (GWD) [14,15] and phosphoglucan, water dikinase (PWD) [17,18,15]. Subsequent dephosphorylation of the starch, or starch breakdown products, has been shown to be crucial for its turnover [19,20] as the added phosphate group obstructs hydrolytic activity of β-amylase [21,22]. This prevents catabolism of soluble phosphoglucans that are liberated from the granule. In addition, other glucan hydrolytic enzymes, such as debranching enzymes, are speculated to be inhibited by the presence of phosphate groups located in close proximity to the targeted branch points [20].

The *Starch Excess-4 (SEX4)* locus encodes a protein that is able to dephosphorylate phosphoglucans at the C-6-position, with *sex4* mutants demonstrating impaired starch degradation resulting in a starch excess phenotype that develops over repeated diurnal cycles [23,24,25,19]. Phospho-oligosaccharides, considered to be normal starch breakdown intermediates, accumulate in the mutant but are not detected within in wild-type plants [19]. Two similar genes are present in the *Arabidopsis* genome which shows high similarity to *SEX4*, designated *Like Sex Four-1* and *Like Sex Four–2* (*LSF1* and *LSF2*). Both are necessary for normal leaf starch degradation with LSF2 showing phosphoglucan phosphatase activity [20] while the precise biochemical function of LSF1 is unknown it appears unlikely to be a polyglucan phosphatase [26].

These genes have thus far only been studied in *Arabidopsis thaliana*, it was therefore of interest to determine if these played similar roles in leaves of other plant species. It was for this reason that the roles of these genes were investigated in leaves of *N. benthamiana* using virus-induced gene silencing.

Results

Solanaceae species contain orthologs of SEX4, LSF1 and LSF2

Solanaceae sequences for *SEX4*, *LSF1* and *LSF2* were translated and aligned to the DSP (Dual specificity phosphatase) domain of species previously described [20]. Parsimony analysis (Figure 1) indicated each of the respective Solanaceae sequences have high homology to their *Arabidopsis* orthologs, with a bootstrap value of 89% for *SEX4*, and 79% and 77% for *LSF1* and *LSF2*, respectively.

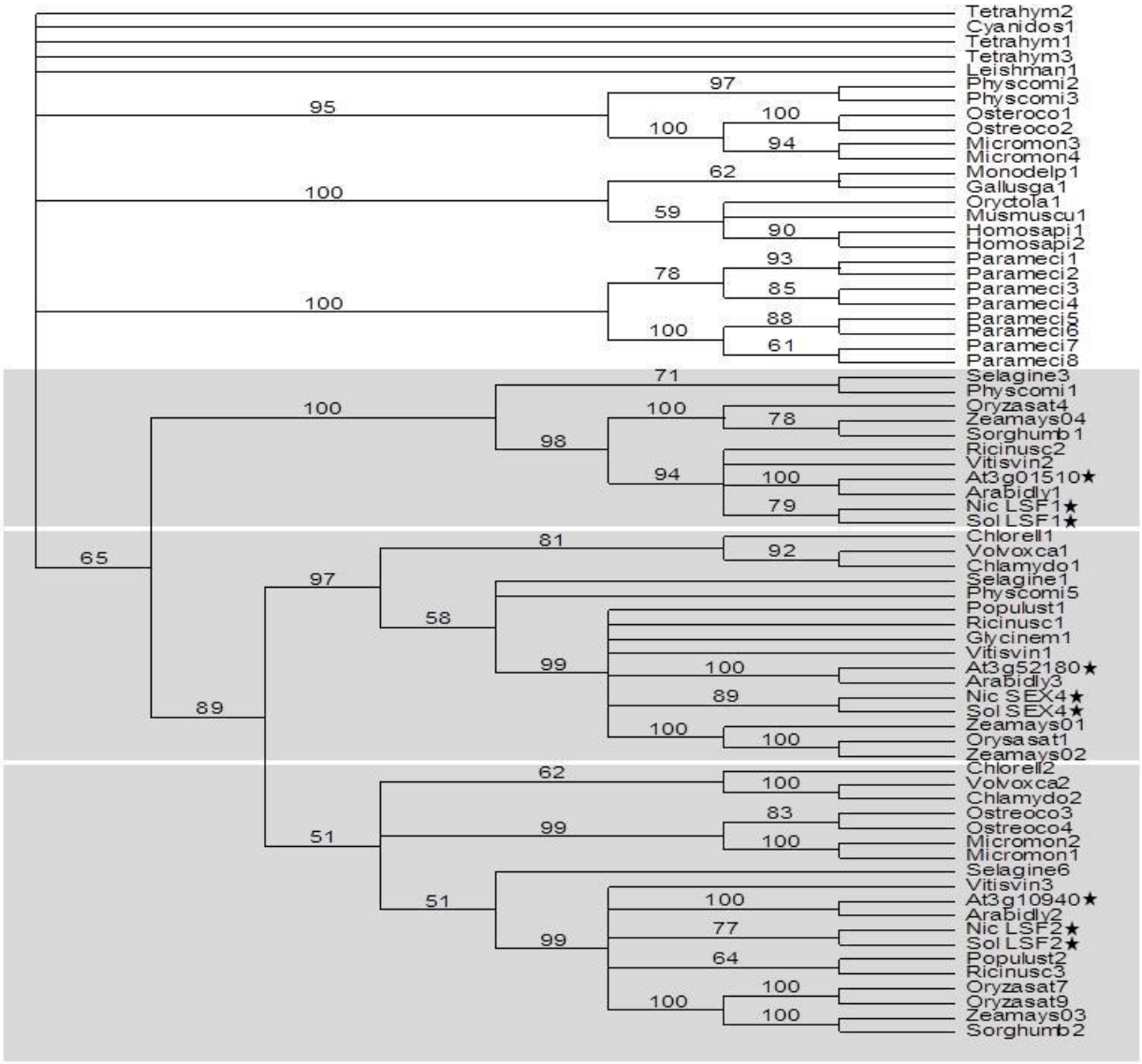

Figure 1: Phylogenetic analysis of SEX4, LSF1 and LSF2 DSP from *Solanaceae* species. Parsimony bootstrap tree using 100 Replicates. *Arabidopsis, Nicotiana benthamiana* and *Solanum tuberosum* sequences for *SEX4*, *LSF1* and *LSF2* are indicated with a star (★) within the respective groups (shaded).

Silenced plants demonstrate a starch excess phenotype

Virus-induced gene silencing (VIGS) was used to examine the roles of *SEX4*, *LSF1* and *LSF2* in *N. benthamiana*. 300bp fragments of each were amplified and used to produce vectors for transient repression of the genes individually and, as *SEX4* and *LSF2* but not *LSF1* are thought to be involved in de-phosphorylation [26,19,20], we also produced a chimeric construct designed to repress both *SEX4* and *LSF2* simultaneously. Following *Agrobacterium* infiltration silencing of the genes was examined using semi-quantitative PCR. All of the

genes were shown to be repressed in the leaves of the respective single and double construct, without adversely affecting expression of the other genes (Supplemental Figure 1).

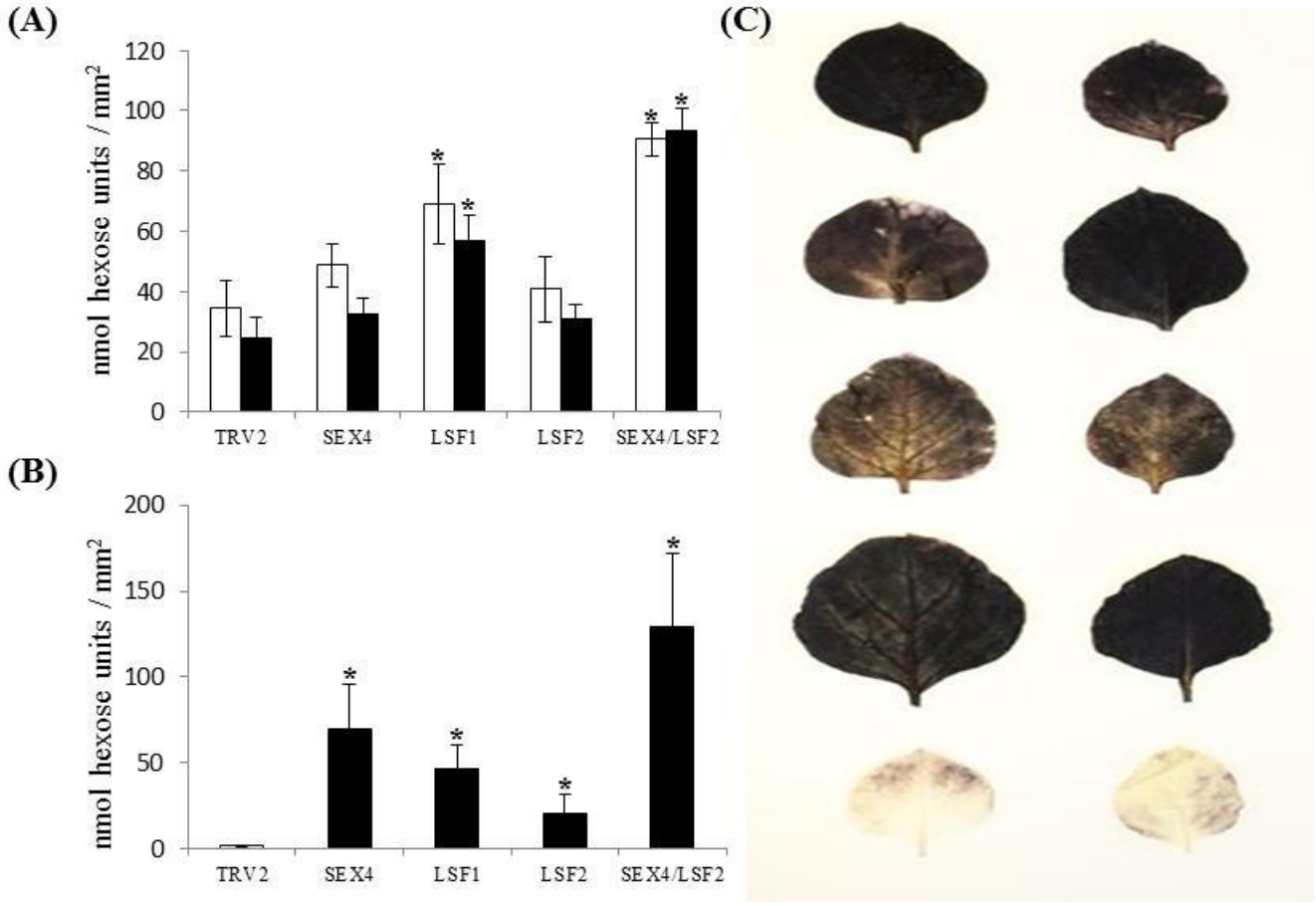

Figure 2 (A): Leaf starch contents in greenhouse grown plants. Starch contents were determined for TRV2, *SEX4*, *LSF1*, *LSF2*, and *SEX4:LSF2* plants at the end of the day (open bars) and end of the night (closed bars). (**B) Leaf starch content in darkened plants.** Starch contents were determined for TRV2, *SEX4*, *LSF1*, *LSF* and *SEX4:LSF2* after the plants had been kept in the dark for six days. All values are presented as mean ± SE of six individual plants and values with an * were determined by *Students t*-test to be significantly different (P< 0.05) from TRV2. **(C)** Visualisation of leaf starch fromSEX4, LSF1, LSF2 SEX4/LSF2 and TRV2 plants stained with iodine after being placed in the dark for three days

To quantify the effect repression of these genes had on starch metabolism the amounts were measured at the beginning and end of the day (Figure 2A). Increased starch was found in the *LSF1* and *SEX4/LSF2* silenced plants at both time points, but there was no difference in either *SEX4* or *LSF2* silenced plants.

The plants were placed in the dark for three days after which source leaves were stained with iodine. This demonstrated that all the silenced plants were impaired in starch mobilization (Figure 2C). The amounts of starch still present in leaves after the plant had been in the dark

for six days were investigated (Figure 2B). All the silenced plants contained increased starch concentrations with the highest amount being found in the SEX4/LSF2 silenced plants.

31-Phosphorous NMR

To determine the amounts and location of covalently bound phosphate 31-P NMR was used. The ratio of C-3/C-6 in the TRV2 was 1:1.5 compared to a proportion of C-3/C-6 bound phosphate occurring in equal amount (1:1) in the *SEX4*, *LSF2* and *SEX4/LSF2* silenced plants. The ratio of C-3/C-6 within the *LSF1* repressed plants was found to be 1:1.4. The NMR spectra for the various lines are illustrated in (Figure 3).

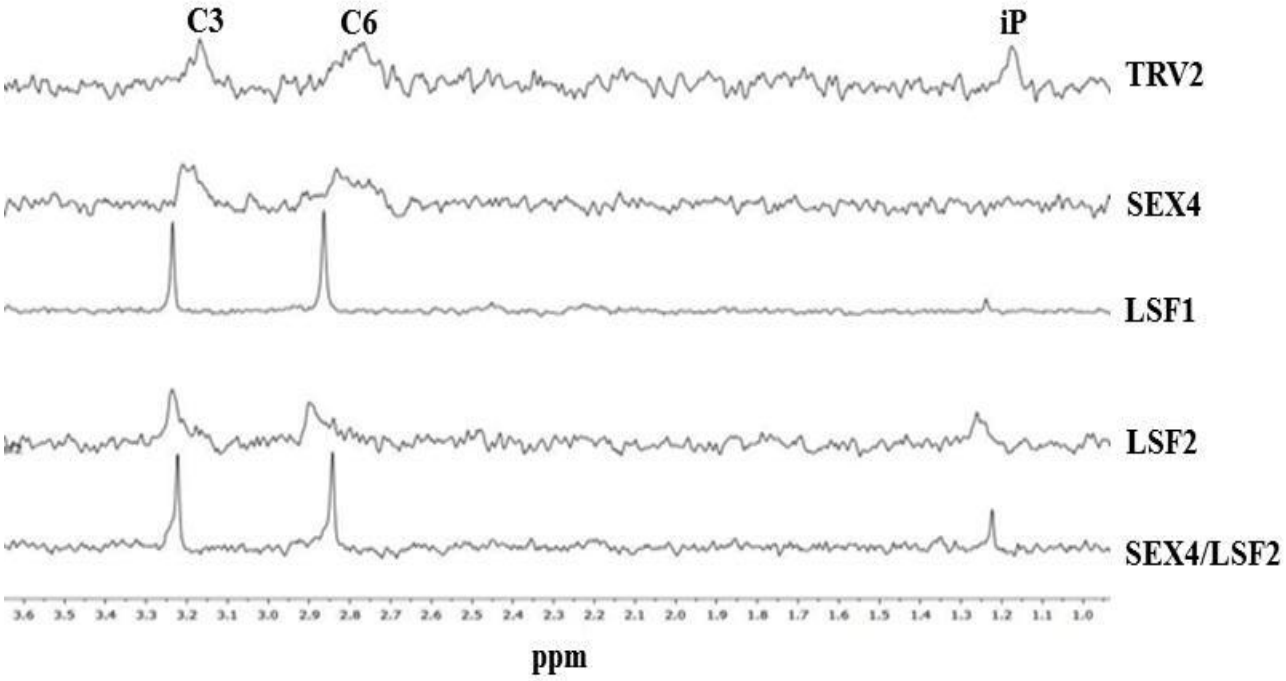

Figure 3: 31-P NMR spectra of C-3 and C-6 bound phosphate on starch. 31-P Spectra of TRV2, *SEX4*, *LSF1*, *LSF2* and SEX4/LSF2 silenced plants.

Phosphorylated malto oligosaccharide analysis using HPAEC-PAD

To examine if phosphorylated glucans accumulated in the leaves of *N. benthamiana*, HPAEC-PAD was used. Phosphorylated malto-oligosaccharides accumulate in *SEX4* and *SEX4/LSF2* repressed plants, similar to previous studies in Arabidopsis [19,20], while minimal amounts were found in the other VIGS lines (Figure 4).

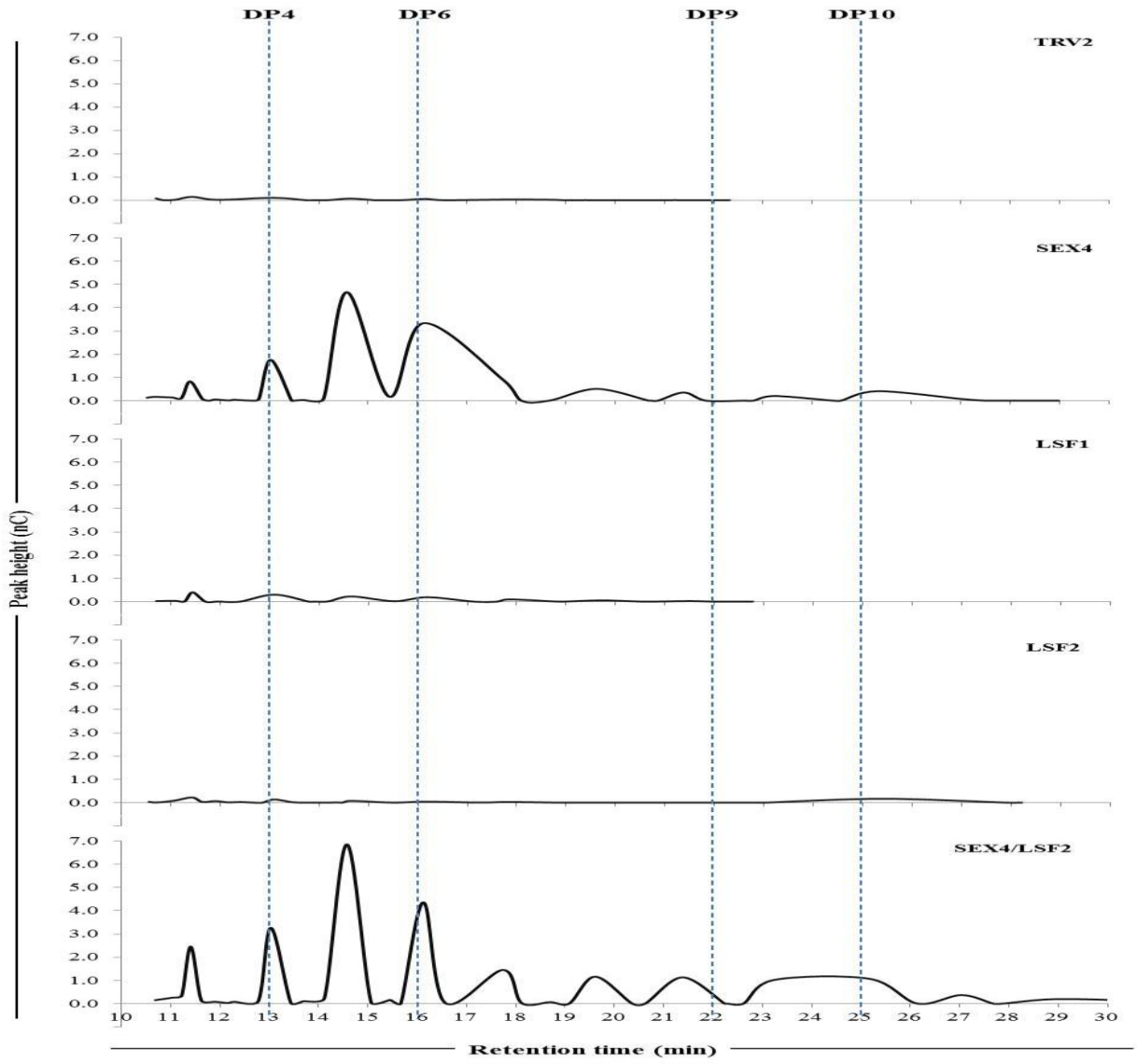

Figure 4: Phosphorylated oligosaccharides of TRV2, SEX4, LSF1, LSF2 and SEX4/LSF2. Phosphatase treated soluble extracts from TRV2, SEX4, LSF1, LSF2 and SEX4/LSF2 leaves. One representative chromatogram (from five replicates of each) is shown using the peak height vs. the retention time. Numbered dashed lines indicate the degree of polymerization (DP) of the detected oligosaccharides. Retention times of 13; 16; 22 and 25 minutes correspond to chains with a DP 4; DP 6; DP 9 and DP 10 as demonstrated by standards run.

Discussion

Much has been learned about the pathway of leaf starch degradation through analysis of *Arabidopsis* mutants, but it is of interest to see if the same pathway holds true in other species. The last common ancestor shared between *Arabidopsis* and tobacco lived approximately 125 million years ago when the Asterids and Rosids diverged [27], meaning that there has been much time since then for the pathway of leaf starch degradation to evolve

independently. Despite that, many similarities between *Arabidopsis* and *Solanaceae* leaf starch degradation have been observed. For example it was shown that the repression of similar isoforms of β-amylase [28,29], disproportionating enzyme 1 [30,31,32], disproportionating enzyme 2 [33,34,32] and glucan, water dikinase [35,36] affect starch degradation in similar ways in *Arabidopsis*, potato and tobacco.

Phylogenetic analysis of Solanaceae sequences for *SEX4*, *LSF1* and *LSF2* show a high homology to their respective *Arabidopsis* orthologs (Figure 1). Although this analysis indicates that the tobacco sequences are likely to be orthologous to the Arabidopsis genes, it does not demonstrate that they have the same function. To examine this we decided to silence their expression using a transient system.

Virus induced gene silencing allows the rapid analysis of gene function and has previously been used successfully in analysing the effect of some genes on starch degradation [32]. We amplified 300 bp fragments of each putative ortholog and used them to produce vectors designed to silence the three genes individually and, because SEX4 and LSF2 (but not LSF1) have been shown to be starch phosphatases in *Arabidopsis*, we also produced a vector that should allow simultaneous silencing of both *SEX4* and *LSF2* as both these genes are thought to encode different starch phosphatases. Following infiltration of *N. benthamiana* with Agrobacteria containing these vectors we were able to see a large decrease in mRNA accumulation of the targeted genes without an effect on the others (Supplemental Figure 1).

Silenced lines have altered starch bound phosphate

Starch isolated from leaves was analysed using 31P NMR with the spectra generated for these silenced plants being comparable to those in Arabidopsis leaf starch [20], with the C-6 and C-3 phosphate peaks seen at 2.8 and 3.2 ppm (Figure 2). The proportion of C-3 to C-6 phosphate was found to be 1:1.5 within the control, 1:1 within the *SEX4*, *LSF2* and *SEX4/LSF2* silenced plants and 1:1.4 in the *LSF1* silenced plants. The relative amounts of

leaf starch C-3-bound phosphate are much higher in *N. benthamiana* than Arabidopsis which contains approximately five fold more G6P than G3P [20]. This could indicate a difference in the relative amounts of GWD and PWD between the species although this needs to be determined experimentally. The increased proportion of C-3 bound phosphate seen in *LSF2* and *SEX4/LSF2* silenced plants correspond with what was found in *Atlsf2* single and *Atsex4/Atlsf2* double mutants [20]. On the other hand, starch from *Atsex4* plants contained a far higher ratio of C-6 compared to C-3 bound phosphate [20], but that from the *SEX4* repressed *N. benthamiana* plants did not demonstrate this. It is possible that the specificities of these enzymes may not be identical in *Nicotiana* as in *Arabidopsis*. AtSEX4 has been demonstrated to have a low efficiency of dephosphorylating glucose residues at the C-3 position. Perhaps in *Nicotiana* both SEX4 and LSF2 act on the C-3 and C-6 positions, which would help explain why the same increase in the proportion of C-3 bound phosphate is seen in the *SEX4* and *LSF2* repressed plants. If this hypothesis is correct, it would have to be assumed that more C-6 than C-3 bound phosphate would be removed by the enzymes either due to them having a higher specificity for C-6 bound phosphate, or because less C-3 phosphate becomes solubilised by amylolytic enzymes. The *LSF1* silenced plants had a C-3/C-6 ratio which more closely resembled to the TRV2 control. This supports the previous hypothesis that LSF1 is not a polyglucan phosphatase [26], with the mechanism by which this enzyme affects starch degradation yet to be elucidated.

Starch degradation is repressed in the VIGS lines

Measurement of starch showed that only the plants silenced for *LSF1*, or both *SEX4* and *LSF2* contained increased amounts during a day/night cycle (Figure 2A). *Arabidopsis sex4* mutants accumulate increased starch [19], so it is unclear why the same does not occur in the *N. benthamiana SEX4* silenced plants. One explanation could be that this is because VIGS leads only to a transient repression of gene expression and there has not been enough time for

the starch excess phenotype to develop in these lines. Often the amount of starch degraded at night in plants repressed in starch degradative genes, and which show a starch excess phenotype, is similar to wild-type plants [35,28,34,32]. This means that small differences in the amount of starch degradation, within the measuring error of the experiment, lead to the development of a starch excess phenotype over time. Although we could not see a difference in starch over a day/night cycle, all the plants were clearly impaired in their ability to eliminate starch when leaves were darkened for several days (Figure 2B,C). Similar to the experiments described here, no phenotype on starch degradation was seen in *Atlsf2* mutant plants over a day/night cycle, however a *Atsex4/Atlsf2* double mutant was impaired in starch degradation to a greater extent that the in *Atsex4* [20]. The data in this study indicates, interestingly, that *LSF2* has a more important role in *N. benthamiana* than in Arabidopsis as silenced plants still contained starch after at least three days of darkness (Figure 2B,C) while Arabidopsis *lsf2* mutants were devoid of starch after just one night period [20]. In Arabidopsis there appears to be a difference in the specificities of SEX4 and LSF2 towards the C-6 and C-3 bound phosphate [19,20], with AtSEX4 removing mainly C-6 and AtLSF2 mainly C-3 bound moieties. As there is a considerably higher amount of C-6 than C-3 bound phosphate in Arabidopsis leaf starch [19], it is likely that mutations affecting *AtSEX4* would have a greater effect than ones affecting *AtLSF2*. *N. benthamiana* leaf starch contains more phosphate bound at the C-3 position than Arabidopsis (Figure 3). If NbSEX4 and NbLSF2 play the same roles as hypothesized for their Arabidopsis orthologs, then it is unsurprising that LSF2 plays a greater role in *N. benthamiana* as removal of C-3 bound phosphate would be more important for starch degradation in this species. On the other hand if, as hypothesized above, both NbSEX4 and NbLSF2 are able to remove phosphate at the C-6 and C-3 positions, then it would indicate simply that NbLSF2 is more important than AtLSF2. Repression of *LSF1* or the simultaneous silencing of *SEX4* and *LSF2* resulted in a severe starch excess phenotype in the leaves over a diurnal cycle, underlying the importance of these

enzymes for normal starch degradation in *N. benthamiana*, consistent with previous findings for these specific mutant *Arabidopsis* plants [26,19,20].

P-Oligos accumulate in SEX4 and SEX4/LSF2 silenced plants

Atsex4 and *Atsex4/lsf2* mutant plants were shown to accumulate phosphorylated malto oligosaccharides (P-Oligos) [19,20], thus it was of interest to determine if the same was true for leaves of *N. benthamiana*. When these enzymes are repressed P-Oligos were detected in *NbSEX4* and *NbSEX4/LSF2* silenced plants (Figure 4). Based on the retention times of the standards the degree of polymerisation (DP) for the P-Oligos within the respective samples in the chromatograms could be determined. The chains length of the P-Oligos seen in the SEX4 and SEX4/LSF2 silenced plants vary from DP 4 to DP 10, being mostly chains with a DP of 5-6 similar to what was found in Arabidopsis studies [19,20]. These data demonstrate that NbSEX4 is a starch phosphatase that acts on liberated P-Oligos during the dark period. The role of LSF2 is less clear as, although its repression alone led to an increase in the proportion of C-3 bound phosphate in the starch granules (Figure 3), there was no concomitant accumulation of P-Oligos (Figure 4). This is most likely due to NbSEX4 partially compensating for its action, a theory supported by the increased amounts of P-Oligos found in plants where both *NbSEX4* and *NbLSF2* were repressed compared with *NbSEX4* plants (Figure 4). NbLSF1 does not seem to be a starch phosphatase as its repression leads to neither an alteration in starch phosphate nor an accumulation of P-Oligos (Figures 3,4).

Conclusion

The data presented provide evidence that the role played by SEX4, LSF1 and LSF2 is similar in *N.benthamiana* compared to *Arabidopsis*, although some differences are apparent. Unlike *Atsex4* mutants, the *SEX4* silenced plants do not exhibit a starch excess at the end of the night, which could be as a result of the transient nature of VIGS silencing. As in Arabidopsis,

LSF2 repression did not show a starch excess at the end of the night, however all the lines led to inhibition of starch degradation after an extended dark period demonstrating that all the proteins studied are involved in the starch breakdown pathway. A combination of alterations in starch bound phosphate and accumulation of P-oligos indicates that NbSEX4 and NbLSF2 are starch phosphatases, while NbLSF1 is not. The main difference between the roles of these enzymes in Arabidopsis and *N. benthamiana* is that NbLSF2 seems to play a more important role than AtLSF2, which may be due to the increased C-3 bound phosphate found in *N. benthamiana* leaf starch compared with Arabidopsis.

Given what is known about these genes from previous *Arabidopsis* and now *N. benthamiana* studies, it will be interesting to determine if these elicit similar effects in starch rich organs such as potato tubers. This might be useful as the starch from this crop contains relatively high amounts of phosphate compared to starches from cereal crops [37,38,36,39]. Changes in the amount or proportion of phosphate located at the C-3 position may cause major conformational changes in the starch which could potentially lead to certain desirable characteristics. Further biochemical testing on the starch would be required to determine if the changes in the ratio of starch bound phosphate from these silenced plants leads to desirable qualities and if these can be useful in industrial application.

Methods

Plasmid construction, plant transfection and generation

N. benthamiana seeds used in these experiments were treated and grown under sterile conditions as those described by [32]. The tobacco rattle virus (pTRV1, pTRV2, and pTRV2-PDS) construct design and principles which are required for virus induced gene silencing (VIGS) were previously described by [40].

The 300 bp fragments for *SEX4*, *LSF1* and *LSF2* were produced by PCR using tobacco EST's obtained from the French Plant Genomic Resource Center [http://cnrgv.toulouse.inra.fr/en; *SEX4*, KG9B.002H08F; *LSF1*, KT7B.107M01F and *LSF2*, KP1B.110M02F] as templates.

The *SEX4* gene was ligated into the *ApaI* and *SalI* restriction sites within the pBluescriptSK vector, then restricted using *KpnI* and *XbaI* and ligated into the same sites within TRV2. *LSF1* was ligated into the *Bam*HI and *Sma*I sites of TRV2. *LSF2* was ligated into pGEM-T-Easy (Fermentas) then restricted using *Eco*RI and ligated into the same site in TRV2. The *SEX4*::*LSF2* double construct was produced by ligation of the *LSF2* fragment into the *EcoRI* site of the TRV2::SEX4 vector. All ligations were performed using T4 DNA Ligase (Fermentas).

The *Agrobacterium tumefaciens* strain GV2206 was transformed using the freeze/thaw method [41] with either the pTRV2 vector alone or containing sequence coding for *SEX4*, *LSF1*, *LSF2* or a combination of both *SEX4* and *LSF2.* Vacuum infiltration of the constructed VIGS plasmids into the plants as well as subsequent plant generation was done according to [32]**.** The 300 bp sequences used in the repression of the respective target genes were as follows.

SEX4

CGTGCCGAAATAAGGGATTTTGATGCATTTGATTTGAGGTTGCGGCTTCCAGCTGTCGTAAGCAAACTGAGCAAGGCCGTCA
ATAGAAATGGGGGTGTGACTTACATACATTGCACCGCTGGACTTGGCAGAGCTCCGGCAGTTGCGTTGACATATATGTTCTG
GGTTCAAGGCTATAAGCTTGGTGAAGCTTTCAATTTACTCATGAGCAAGCGCTCCTGCTTTCCAAAACTAGATGCCATCAAAA
GTGCCACAGCAGACATTCTTACAGGCCTTAAAAGGAAGCCTGCCACATTGACA

LSF1

ATGATCTTGGTATCCGTTACAGCAAGATTACGGAGCAAATATATGTGGGATCGTGCATACAAAAGGAAGCTGATGTAGAAAT
GCTGTCAGATGTTATGGGGATCTCCGCTGTACTGAATTTTCAGAGTGGGATCGAGGCTGAAAATTGGGGAATTAGTGTCAAC
ATAATCAATGAATCGTGCCAAAGGTTTAACATCCTTATGATCAACTATCCCATAAGGGAAGGTGATTCATTTGACATGAGAA
AGAAACTTCCGTTTTGCGTTGGTCTTCTTCTACGTCTATTGAAAAAGAACCACC

LSF2

TCACATGAGAAGGCCTGCAAGAGATTTTGATCCAGATTCCCTGAGGAGTGTATTACCTAAAGCTGTTTCATCACTGGAGTGG
GCGATTTCAGAAGGAAAAGGAAGAGTGTATGTACATTGCACTGCTGGATTGGGAAGGGCCCCTGCTGTTTCAATTGCTTATA
TGTTCTGGTTCTGTGGGATGGATCTAAATACAGCTTATGATACACTCGTTTCAAAGAGACCCTGTGGGCCCAACAAAAGGTC
AATACAGGGAGCTACTTATGATTTGGCTAAAAATGATCAGTGGAAGGAGCCGTT

RNA isolation and reverse transcription-PCR

RNA was isolated from ground leaf material using the RNeasy kit (Qiagen) following the manufacturer instructions. RNA quality and integrity was assessed using agarose gel electrophoresis. This was followed by DNase treatment, where 10 units of RNase-free DNAase1 (Fermentas) was added to the RNA samples and incubated at 37°C for 30 min. One µg Total RNA was converted to cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) according to manufacturer instructions.

Semi-quantitative PCR

Primers were designed against the potato cDNA sequences from Potato Genome Sequencing Consortium [http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml]. The respective sequences for each of the enzymes were as follows; *SEX4* - PGSC0003DMT400070294 CDS; *LSF1*- PGSC0003DMT400077364 CDS; *LSF2* - PGSC0003DMT400074765 CDS and the Potato *ACTIN* gene. The primers used included; *SEX4* - Forward primer 5-TGTTGTTTCACTGGGGATGA-3, Reverse Primer 5-CTCCGAAGCTTGTCCACATC-3; *LSF1* - Forward primer 5-TGTCAATGGCCTCTCAATCA-3, Reverse Primer 5-AACCAATTCAGGCAGTCCAC-3; *LSF2* - Forward primer 5-CCTGCAAGGTCGTCTGTCTT-3, Reverse Primer 5-TTCCCCACATCAAGGACTTC-3; *ACTIN* - Forward primer 5-AGATCCTCACTGAGCGTGGT-3, Reverse primer 5-GATTCCAGCTGCTTCCATTC-3.

Determination of the major carbohydrate pools

Starch and soluble sugar were quantified based on the method of [42]. Fifty mm^2 leaf discs were incubated in 1 ml of 80% (v/v) ethanol at 80°C for 1 h. The supernatant was removed

and used used to determine soluble sugars. The leaf discs were washed with 80% (v/v) ethanol and the supernatant was removed after which 0.4 ml of 0.2 M KOH was added and the samples incubated at 95°C for 1 h then 70 µl of 1 M acetic acid was added to neutralise the solution.

To measure soluble sugars, 50 µl of ethanol extract was added to 250 µl assay buffer containing 10 mM Hepes (pH 6.9), 5 mM $MgCl_2$, 1 mM ATP, 1 mM NAD. Glucose was determined by addition of 1 U/ml hexokinase from yeast and 1 U/ml glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Roche). Fructose was determined by subsequently adding 1 U/mL phosphoglucose isomerase from *Saccharomyces cerevisiae* (Sigma). To measure sucrose, 1 U/ml of invertase (β-fructosidase) from *Candida utilis* (Sigma) was added following completion of the fructose reaction. After addition of each enzyme the absorbance increase was followed at 340 nm and the change in absorbance used to calculate the amounts.

To measure the starch, 100 µL of the solubilised starch solution was mixed with 100 µL of 50 mM NaAC pH 5.6 containing 10 U/ml amyloglucosidase (from *Aspergillus niger*) and 10 U/ml α-amylase from (Sigma) and incubated at 37°C for 2 h. 250 µL of assay buffer 10 mM Hepes (pH 6.9), 5 mM $MgCl_2$, 1 mM ATP, 1 mM NAD was then added to 10 µl of digested starch solution in micro titre plate. Glucose was determined by the addition of hexokinase and glucose 6-phosphase dehydrogenase and following the change in absorbance at 340nm.

Starch isolation

Half a gram of leaves was homogenized in 5ml extraction buffer which contained 30 mM Hepes-KOH, (pH 7.5) 1 mM EDTA, 1.5 mM DTT. The homogenate was passed through a 100µm filter and centrifuged at 20000 x g at 4°C for 5 min. The pellet was washed once with extraction buffer before being washed three times with a solution containing 30 mM Hepes-KOH (pH 7.5), 1 mM EDTA, 1.5 mM DTT, 0.1% SDS. Ice cold acetone was added to the

starch and the solution mixed before the starch was allowed to settle under gravity. This was repeated six times, before allowing the acetone to evaporate.

Starch iodine staining

Leaves were harvested from plants before being bleached in 80% (v/v) ethanol at 80°C for 12 h. The leaves were washed with water several times then stained for 10 min with Lugol's solution [4% (w/v) Potassium Iodide, 2% (w/v) Iodine]

Extraction and measurement of total malto-oligosaccharide content

Malto-oligosaccharide and phosphorylated malto-oligosaccharide contents were determined using methods described by [30,19]. Two ml of 1 M perchloric acid was added to 50 mg of leaf sample, which was ground using a mortar and pestle under liquid nitrogen. The samples were incubated at 4°C for 30 min and centrifuged at 3890*g* for 5 min at 4°C. Two hundred µl of the supernatant was removed and added to 160 µl 2 M KOH, 0.4 M MES pH 5.6, 0.4 M KCl. The potassium perchlorate precipitate was removed by centrifugation at 3500*g* for 5 min at 4°C. 50 µl of the neutralized soluble fraction was then added to 66 µl sugar removal buffer [0.1 M MES pH 5.6, 0.75 U β-fructosidase, 0.1 U glucose oxidase, 65 U catalase] and incubated for 2 h at 32°C followed by a further incubation for 40 min at 70°C. The malto-oligosaccharide content was determined by adding 50 µl to an equal volume of assay buffer (50 mM sodium acetate (pH 5.6) containing 0.25 U maltase and 2 U amyloglucosidase), incubated for 2 h at 37°C. Glucose units were determined as described in starch analysis above.

Phospho-oligosaccharide samples for HPAEC-PAD analysis were prepared as described [19] where 100 µl aliquot of the neutralized soluble fraction was incubated in 100 µl antarctic phosphatase buffer (New England Biolabs) with or without 15 units of antarctic phosphatase (New England Biolabs) for 3 hrs at 37°C. The samples were purified by ion exchange on

sequential columns of DOWEX 50W and DOWEX 1 (Sigma-Aldrich) with a 2 mL bed volume each as follows: sample volume was made to 0.5 mL with water, applied to the column; the neutral compounds were eluted with 5 mL water. Eluted samples as well as control samples (mock phosphatase treated samples) were then lyophilized and analyzed by HPAEC-PAD analysis. Chromeleon software for DIONEX was used in analysis of the samples with the peak integration data and retention time used to illustrate the phosphorylated chains within the respective silenced and control plants. The standards used were phosphorylated oligosaccharides with known chain length.

P-31 NMR analysis of starch-bound phosphate

Purified starch was suspended in 900 µl deuterium oxide (Aldrich) which contained 0.1 M MES at pH 6.85. The solution was heated at 95˚C for 10 min with shaking to solubilise the starch. The solution is allowed to cool and 1 U/ml of α-amylase from wheat (Megazyme) was added and incubated at 50˚C for 2 h. This solution was heated to 95˚C to denature the enzyme. A further 100 µl of a solution of 2mm EDTA containing 1 mg NAD was added. This was then placed into a 5 mm NMR tube and analysed on a Varian Inova 600 MHz NMR spectrometer overnight, following the parameters described [20]. The ratio of C-3-P to C-6-P was determined from peak integration data generated using the MestReNova computer program (www.mestrelab.com).

Phylogenetic Analysis

Parsimony bootstrap phylogenetic analysis was performed using the PAUP V 4.0 10 computer program. The respective tobacco ESTs and potato sequences were translated into protein sequences and aligned with protein DSP domain sequences shown [20], using the method described by [43]. Expressed sequence tags (ESTs) for tobacco were obtained from the French Plant Genomic Resource Center (http://cnrgv.toulouse.inra.fr/en) [*SEX4*

KG9B.002H08F; *LSF1*, KT7B.107M01F and *LSF2* KP1B.110M02F]. Potato sequences were obtained from the Potato Genome Sequencing Consortium (http://solanaceae.plantbiology.msu.edu/pgsc) [*SEX4* - PGSC0003DMT400070294 CDS*; LSF1*- PGSC0003DMT400077364 CDS; *LSF2* - PGSC0003DMT400074765 CDS]. All other sequences came from the analysis in [20].

Abbreviations

SEX4 Starch Excess-4; LSF1 Like Sex Four–1; LSF2 Like Sex Four–2; C-3 carbon-3; C-6 carbon-6; TRV2 tobacco rattle virus2; *PDS* phytoene desaturase; P-Oligos phosphorylated-Oligosaccharide; VIGS virus-induced gene silencing; mg milligram; µl microliter; NAD Nicotinamide adenine dinucleotide; DSP Dual specificity phosphatase, EDTA Ethylene diamine tetra acetic acid; EST Expressed sequence tag, NMR nuclear magnetic resonance; U/ml unit per milliliter; MES 2-*N*-morpholino ethane sulfonic acid; HPAEC-PAD High-performance anion-exchange chromatography coupled with pulsed electrochemical detection; M Molar; Hepes 4-2-hydroxyethyl-1-piperazineethanesulfonic acid; KOH Potassium hydroxide; $MgCl_2$ Magnesium Chloride; ATP Adenosine 5-tri-phosphate; NaAC Sodium acetate; cDNA complementary deoxyribonucleic acid; RNA ribonucleic acid; kPa Kilopascal; bp base pair; PCR Polymerase chain reaction; MS Murashige and Skoog

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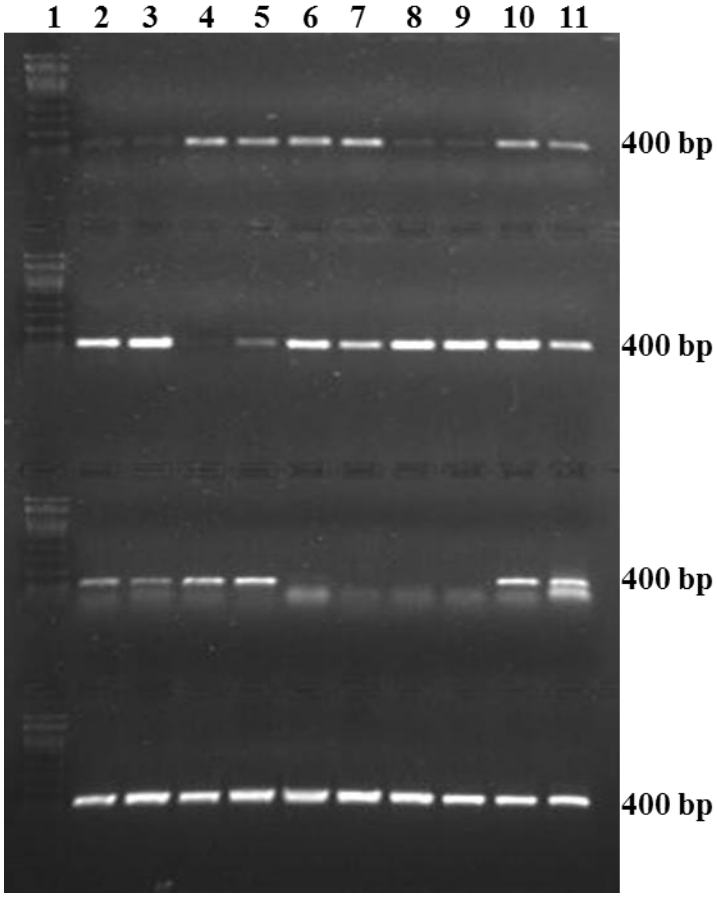
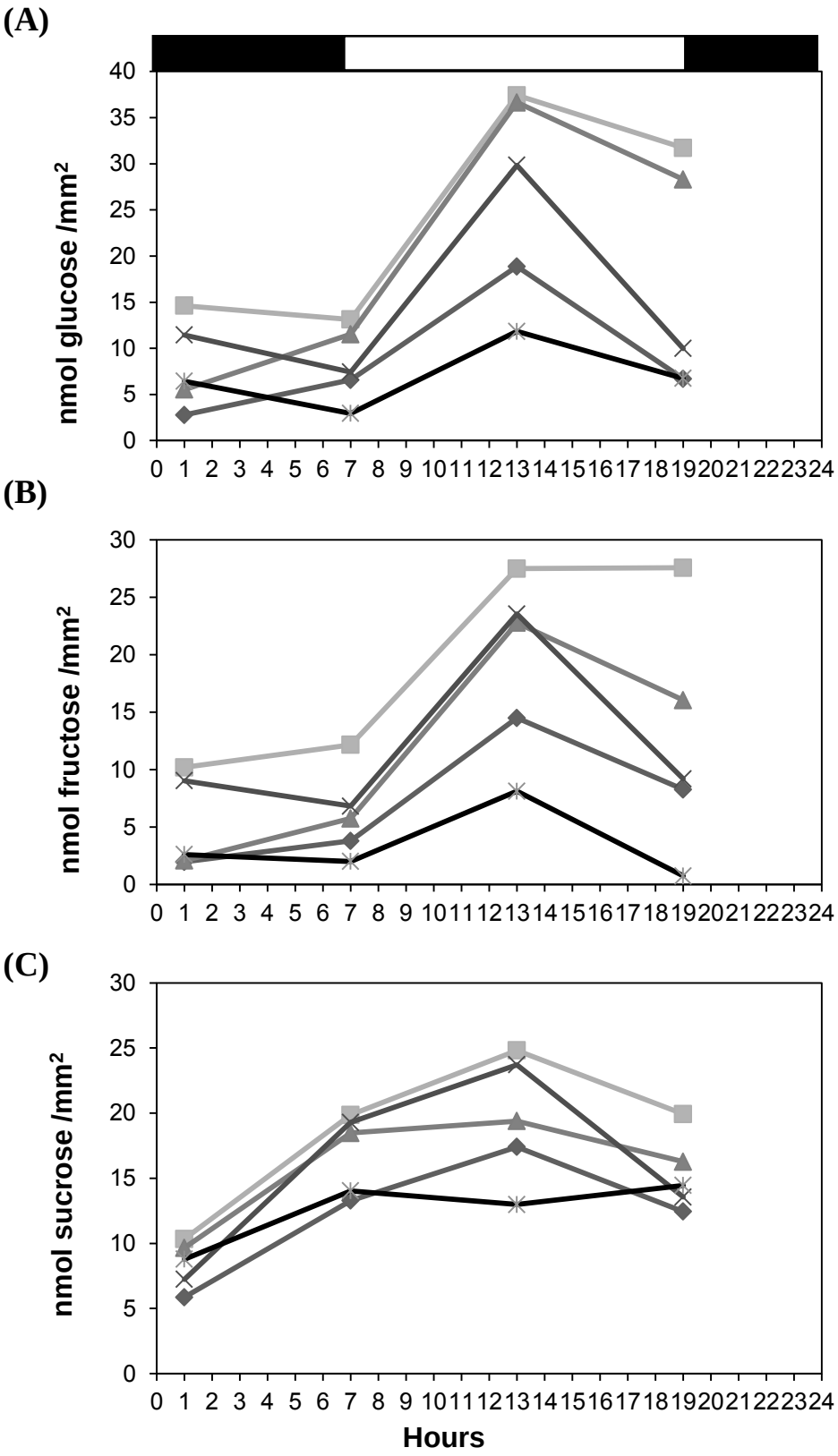
Supplemental data



Supplemental Figure 1: Semi-quantitative PCR on Leaves of silenced plants. A 1% (w/v) Agarose gel showing 4 rows from top to bottom i.e. Sex4 primers; Lsf1 primers; LSF2 primers and Actin primers. Each row shows the respective plants tested against the above mentioned primers. Lane 1 Lambda PST DNA marker, Lane 2-3 Sex4 silenced plants, Lane 4-5 Lsf1 silenced plants, Lane 6-7 Lsf2 silenced plants, Lane 8-9 sex4/lsf2 silenced plants and Lane 10-11 TRV2 control plants.

Supplemental Figure 2: *Nicotiana benthamiana* plants infiltrated with respective constructs. Shown from left to right: *PDS* (phytoene desaturase) (positive control); empty *TRV2* (negative control); *SEX4*; *LSF1*, *LSF2*, *SEX4:LSF2*



Supplemental Figure 3: Soluble sugar content over twenty four h. Glucose (a); fructose (b) and sucrose (c) from *SEX4* (dark square), *LSF1* (light square), *LSF2* (tri-angle), *SEX4:LSF2* (X) and TRV2 (*) plants measured over twenty four h with the day (white bar) and night (black bar) shown. Values are presented as mean ± SE of six individual samples per line.

Chapter 4: The roles of *SEX4* and *LSF2* in starch degradation in potato

4.1 Introduction

The level of covalently bound phosphate that occurs on starch is extremely important for industry. Potato starch, for example, contains a relatively high degree of phosphorylation compared to starch from other plants. This makes it useful in industries such as paper making as the phosphate allows it to form bonds with the hydroxyl groups on cellulose microfibrils (Ly and Brouillette, 2013). Phosphate is located on the C-6 or C-3 position of the glucose moieties within starch (Blennow *et al.*, 2002) through the actions of GWD and PWD (Ritte *et al.*, 2002; Kötting *et al.*, 2005). Increased phosphate alters the functionality of the starch giving it a higher swelling power when heated in water (Lorberth *et al.*, 1998; Santelia and Zeeman, 2011) while decreased phosphate leads to starch with a low paste viscosity (Lorberth *et al.*, 1998).

There are several examples where the phosphate content of potato starch has been altered through manipulation of starch biosynthetic enzymes (Abel *et al.*, 1996; Kossmann *et al.*, 1999; Lorberth *et al.*, 1998; Safford *et al.*, 1998; Jobling *et al.*, 1999; Schwall *et al.*, 2000). However, enzymes involved in removing phosphate from starch, known as SEX4 and LSF2, have been described recently in Arabidopsis (Kötting *et al.*, 2009; Santelia *et al.*, 2011) and mutations in them led to leaf starch with altered phosphate contents. These enzymes, therefore, represent potentially a novel method to alter starch phosphate contents in storage organ starch.

Not only did mutations in these enzymes affect starch phosphate, but they also repressed starch degradation in *Arabidopsis* leaves. A third Arabidopsis protein AtLSF1, which is similar to AtSEX4 and AtLSF2, is also involved in leaf starch degradation by an, as yet, undiscovered mechanism (Comparot-Moss *et al.*, 2010). Potato tubers stored at low

temperatures degrade starch during cold induced sweetening (CIS) which is deleterious to their use in the processing industry. Although several biotechnological solutions to CIS have been developed (Zrenner *et al.*, 1996; Lorberth *et al.*, 1998; Greiner *et al.*, 1999; Agarwal *et al.*, 2003; Zhang *et al.*, 2008; Bhaskar *et al.*, 2010), manipulation of the orthologous genes in potato may provide a further method to inhibit this process. This chapter, therefore, examines the effect of repressing potato genes orthologous to the Arabidopsis polyglucan phosphatases SEX4 and LSF2 as well as LSF1 on starch phosphate and CIS.

4.2 Methods and materials

4.2.1 Construct preparation

Three hundred bp fragments for Solanaceae homologs of *SEX4*, *LSF1* and *LSF2* were produced by PCR using tobacco EST's (*SEX4* KG9B.002H08F; *LSF1*, KT7B.107M01F and *LSF2* KP1B.110M02F) obtained from the French Plant Genomic Resource Center (http://cnrgv.toulouse.inra.fr/en) as a template. The sequences were identified as encoding proteins that were most similar to the Arabidopsis predicted SEX4, LSF1 and LSF2 proteins using the tBLASTn algorithm. Primers for PCR are shown within table 4.1

The *SEX4* PCR product was ligated into pGEM-T-Easy while the *LSF1* and LSF2 fragments were ligated into the *SmaI* and *BamHI* or the *EcoRV* and *BamHI* sites within pBluescriptSK+ respectively using T4 DNA ligase (fermentas). To produce the *SEX4:LSF2* double construct, *LSF2* was ligated into the *KpnI* and *XbaI* sites pBK-CMV which already contained the *SEX4* fragment ligated into the *EcoRI* site. Primers were designed against the T7, T3 and SP6 promoters which included homologous attB1 or attB2 recombination sites. These were used to amplify products of the fragments flanked by the AttB sites which were recombined into pHellsgate2 (CSIRO Plant Industries) using BP clonase II (Invitrogen) according to manufacturer instructions.

Table 4.1: Primers showing included restriction sites (lower case and italicised) to amplify 300bp fragments from *SEX4, LSF1* and LSF2 tobacco ESTs.

SEX4	**Forward primer**	**5-**CA*gggccc*CGTGCCGAAATAAGGGATTT**-3**
	Reverse Primer	**5-**GA*gtcgac*TGTCAATGTGGCAGGCTTGG **-3**
LSF1	**Forward primer**	**5-**CTA*cccggg*ATGATCTTGGTATCCG**-3**
	Reverse Primer	**5-**CGATGA*ggatcc*CGGTGGTTCTTTTTCAATAG**-3**
LSF2	**Forward primer**	**5-GA*gatatc*ATGTTCAGAGCTTGGAATTC-3**
	Reverse Primer	**5-CA*ggatcc*TTTTTAGCCTTTTCATAAGTAGCT -3**

4.2.2 *Agrobacterium* transformation

Agrobacterium tumefaciens strain CS681 was transformed with the various pHellgate2 recombinant vectors using the freeze/thaw method (An *et al.*, 1988). Cells were selected on YEP plates containing 25 µg/ml (w/v) rifampicin, 20 µg/ml (w/v) carbenicillen and 10 µg/ml (w/v) spectinomycin.

4.2.3 Plant preparation

Wild type (cv Desirée) potato plants were cultured on MS media containing 0.43% (w/v) Murashige and Skoog (MS) media with vitamin, 1.5% (w/v) sucrose and 0.6% (w/v) PlantGel (Highveld Biological, PTY, LTD).

4.2.4 Plant transformation

Agrobacterium cells were inoculated in 10 ml liquid YEP media containing antibiotics and grown until they reached an optical density (OD) of between 1.0 – 1.2. The

cells were collected by centrifugation at 3000×g for 5 min, followed by re-suspension in MS30 media containing 0.43% (w/v) Murashige and Skoog media with vitamin (MS), 3% (w/v) sucrose and brought to pH 5.8 using NaOH. Approximately forty 1 cm^2 potato leaf slices were placed in 20 ml of MS30 media with the adaxial side facing down. 100 µl of *Agrobacterium* solution containing the appropriate constructs were added to the potato leaf slices and the solution was shaken for 15 min at 35 rpm followed by incubation at room temperature in the dark for 2 to 3 days.

After removal of the *Agrobacterium* solution the leaf cuttings were washed seven times with distilled water containing 250 mg/L cefotaxime. These were placed onto MGC media containing 0.43% (w/v) MS, 16% (w/v) glucose, 5 mg/L (w/v) NAA, 0.1 mg/L (w/v) BAP, 250 mg/L (w/v) cefotaxime and 50 mg/L (w/v) kanamycin with the adaxial side facing down. The samples were kept for seven days under lights and then transferred onto MGS media containing 0.43% MS, 16% (w/v) glucose, 2 µg/L (w/v) zeatin riboside, 20 µg/L NAA, 20 µg/L GA_3, 250 mg/L cefotaxime, 50 mg/L kanamycin, 0.22% (w/v) gelrite and brought to pH 5.6 using NaOH. The samples were subsequently transferred every two weeks onto fresh medium whilst shoot development occured. When the shoots were 2-3 cm long they were placed onto rooting media containing 0.43% (w/v) MS, 20% (w/v) sucrose, 250 mg/L cefotaxime and 50 mg/L kanamycin. They were allowed to grow and develop into plants, which were planted in soil before being grown either in a glasshouse or growth room where plants were grown with 16 hours light and 8 hours dark.

4.2.5 RNA isolation and reverse transcription-PCR

Total RNA was isolated using the CTAB method (White *et al.*, 2008) and then treated with 10 units of RNase-free DNAase1 (Fermentas) at 37°C for 30 min. Total RNA (1 ug) was

converted to cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) according to manufacturer instructions.

4.2.6 Semi-quantitative PCR

Primers were designed against the potato cDNA sequences for the respective enzymes (obtained from the Potato Genome Sequencing Consortium http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml). The sequences used included *SEX4* - PGSC0003DMT400070294 CDS*; LSF1*- PGSC0003DMT400077364 CDS; *LSF2* - PGSC0003DMT400074765 CDS; *ACTIN* (*S. tuberosum*). The primers designed are listed in the table 4.2.

Table 4.2: Primers for semi-quantitative PCR to examine expression of *SEX4*, *LSF1* and *LSF2* in potato.

SEX4	**Forward primer**	**5-TGTTGTTTCACTGGGGATGA-3**
	Reverse Primer	**5-CTCCGAAGCTTGTCCACATC-3**
LSF1	**Forward primer**	**5-AACCAATTCAGGCAGTCCAC-3**
	Reverse Primer	**5-TGTCAATGGCCTCTCAATCA-3**
LSF2	**Forward primer**	**5-CCTGCAAGGTCGTCTGTCTT-3**
	Reverse Primer	**5-TTCCCCACATCAAGGACTTC-3**
ACTIN	**Forward primer**	**5-AGATCCTCACTGAGCGTGGT-3**
	Reverse primer	**5-GATTCCAGCTGCTTCCATTC-3**

Amplification conditions were as follows: SEX4 primers: 94 ˚C for 5 min, 28 X (94˚C for 30 s, 56˚C for 30 s, 72˚C for 30 s), 72˚C for min. LSF1 primers: 94 ˚C for 5 min, 33 X (94˚C for 30 s, 53˚C for 30 s, 72˚C for 30 s), 72˚C for min. LSF2 primers: 94 ˚C for 5 min, 42 X (94˚C for 30 s, 52˚C for 30 s, 72˚C for 30 s), 72˚C for min. ACTIN primers: 94 ˚C for 5 min, 35 X (94˚C for 30 s, 53˚C for 30 s, 72˚C for 30 s), 72˚C for min.

4.2.7 Determination of soluble sugars and starch

Ten mg of tuber or 50 mm^2 of leaf material was incubated with 1 ml of 80% (v/v) ethanol at 80°C for 1 h. The supernatant was transferred to a new tube for further analysis. The remaining material was washed with 80% (v/v) ethanol and the supernatant discarded. To the remaining material 0.4 ml of 0.2 M KOH was added and the samples incubated at 95°C for 1 h before 70 µl of 1 M acetic acid was added.

To measure the sugars, 50 µl of the aqueous ethanol supernatant was combined with 250 µl assay buffer [10 mM Hepes (pH 6.9), 5 mM $MgCl_2$, 1 mM ATP, 1 mM NAD]. Glucose concentration was determined by addition of 1 U/ml hexokinase from yeast and 1 U/ml glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Roche) and following the increase in absorbance at 340 nm. After completion of the reaction fructose concentration was determined by adding 1 U/mL phosphoglucose isomerase from *Saccharomyces cerevisiae* (Sigma) and following the increase in absorbance at 340 nm. Following completion of that reaction sucrose concentrations were determined by addition of 1 U/ml invertase (β-fructosidase) from *Candida utilis* (Sigma) and following the increase in absorbance at 340 nm. The change in absorbance following addition of each coupling enzyme was recorded used to calculate the amount of glucose equivalents present in the sample.

For starch measurements, 100 µL of the neutralised KOH solution was mixed with 100 µL of 50 mM NaAC pH 5.6 containing 10 U/ml amyloglucosidase (from *Aspergillus*

niger) and 10 U/ml α-amylase from (Sigma) incubated at 37°C for 2 h. 10 µl of leaf and a 10 times dilution of the tuber solution was added to 250 µL of assay buffer [10 mM Hepes (pH 6.9), 5 mM $MgCl_2$, 1 mM ATP, 1 mM NAD]. Glucose was determined by addition of 1 U/ml hexokinase from yeast and 1 U/ml glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and following the increase in absorbance at 340 nm. The change in absorbance recorded was used to calculate the amount of hexose equivalents present in the sample.

4.2.8 Starch isolation from leaves and tubers

Half a gram of leaf material was homogenized in 2 ml extraction buffer which contained 30 mM Hepes-KOH (pH7.5), 1 mM EDTA and 1.5 mM DTT. The homogenate was passed through a 100 µm filter and centrifuged at 20 000×g and 4°C for 5 min. The supernatant was discarded and the pellet washed with extraction buffer before being washed three further times with a solution containing 30 mM Hepes-KOH (pH 7.5), 1 mM EDTA, 1.5 mM DTT and 0.1% (w/v) SDS. Ice cold acetone is then added to the starch, the solution is mixed with the starch being allowed to settle under gravity. This was repeated six times, before allowing the acetone to evaporate.

Approximately 2 g of tuber material was ground using a mechanical blender to which 50 ml of extraction buffer containing 30 mM Hepes-KOH, (pH7.5) 1 mM EDTA, 1.5 mM DTT was added. Starch was allowed to settle under gravity and the supernatant removed. Fifty ml extraction buffer was added to the sediment and re-suspended before being passed through two layers of Miracloth (Calbiochem) and centrifuged at 20 000×g and 4°C for 5 min. The starch was then treated as for the leaf starch following the centrifugation step.

4.2.9 Visualization of starch in leaves

Leaves were covered with aluminium foil and left for three days. After harvesting they were incubated in 80% (v/v) ethanol at 80°C for 12 h. After washing with water several times they were stained for 10 min with Lugol's solution (4% (w/v) Potassium iodide, 2% (w/v) iodine).

4.2.10 Determination glucose-6-phosphate content in tuber starch

Ten mg of powdered tuber material was heated in 80% (v/v) ethanol for 1 h after which the ethanol was removed. 0.5 ml 0.7 M HCL (from Nielson *et al.*, 1994) was added and heated at 95°C for 4 h before 400 µl of the acid-hydrolyzed sample was mixed with 400 µl 0.7 M KOH.

To measure glucose, 10 µl of a 10 times dilution of the neutralized solution was analyzed as described in section was added to 250 µL of assay buffer [10 mM Hepes (pH 6.9), 5 mM $MgCl_2$, 1 mM ATP, 1 mM NAD]. Glucose was determined as discussed in section 4.2.7.

To measure glucose-6-phosphate, 30 µl of the neutralized sample was added to 230 µl of assay buffer [100 mM Hepes-KOH pH 7.5, 10 mM $MgCl_2$, 2 mM EDTA, 2 mM NAD]. The absorbance reading was monitored at 340 nm and the reaction was started by adding 1 unit of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*. The change in absorbance was then used to calculate the glucose-6-phosphate amount and this was related to the amount of glucose determined.

4.2.11 Amylose determination

Amylose concentrations were determined according to (Hovenkamp-Hermelink *et al.*, 1988) where 500 µl of a 45% (v/v) perchloric acid solution was added to 20 mg of starch. The sample was left at room temperature for 5 minutes before 50 µl was added to 450 µl of water. A further 500 µl of diluted Lugol's solution [19.8 mM Potassium Iodide, 6.55 mM Iodine] was then added. Absorbances were measured at 618 nm and 550 nm and the percentage amylose determined using the following formula: Amylose (%) = 3.5-5.1xR)/(10.4R-19.9) R= OD618nm/OD550nm.

4.2.12 31-P NMR analysis of starch bound phosphate

Ten mg starch was mixed thoroughly in 900 µl deuterium oxide which contained 0.1 M MES at pH 6.85 using a vortex mixer. The solution was heated at 95˚C for 10 min with shaking and allowed to cool before 5 units of α-amylase, (from cereal, Megazyme) were added and incubated at 50˚C for 2 h. The solution was heated to 95˚C for 5 min, allowed to cool before a further 100 µl of a solution containing 1 mg NAD (Internal reference) and 2 mM EDTA was added to the sample. This was then placed into a 5 mm NMR tube and analysed on a Varian Inova 600 MHz NMR spectrometer overnight, following the parameters described by Santelia *et al.*, (2011).

4.3 Results and discussion

4.3.1 Production of potato plants repressed in expression of genes encoding orthologs of At*SEX4, AtLSF1* or *AtLSF2*

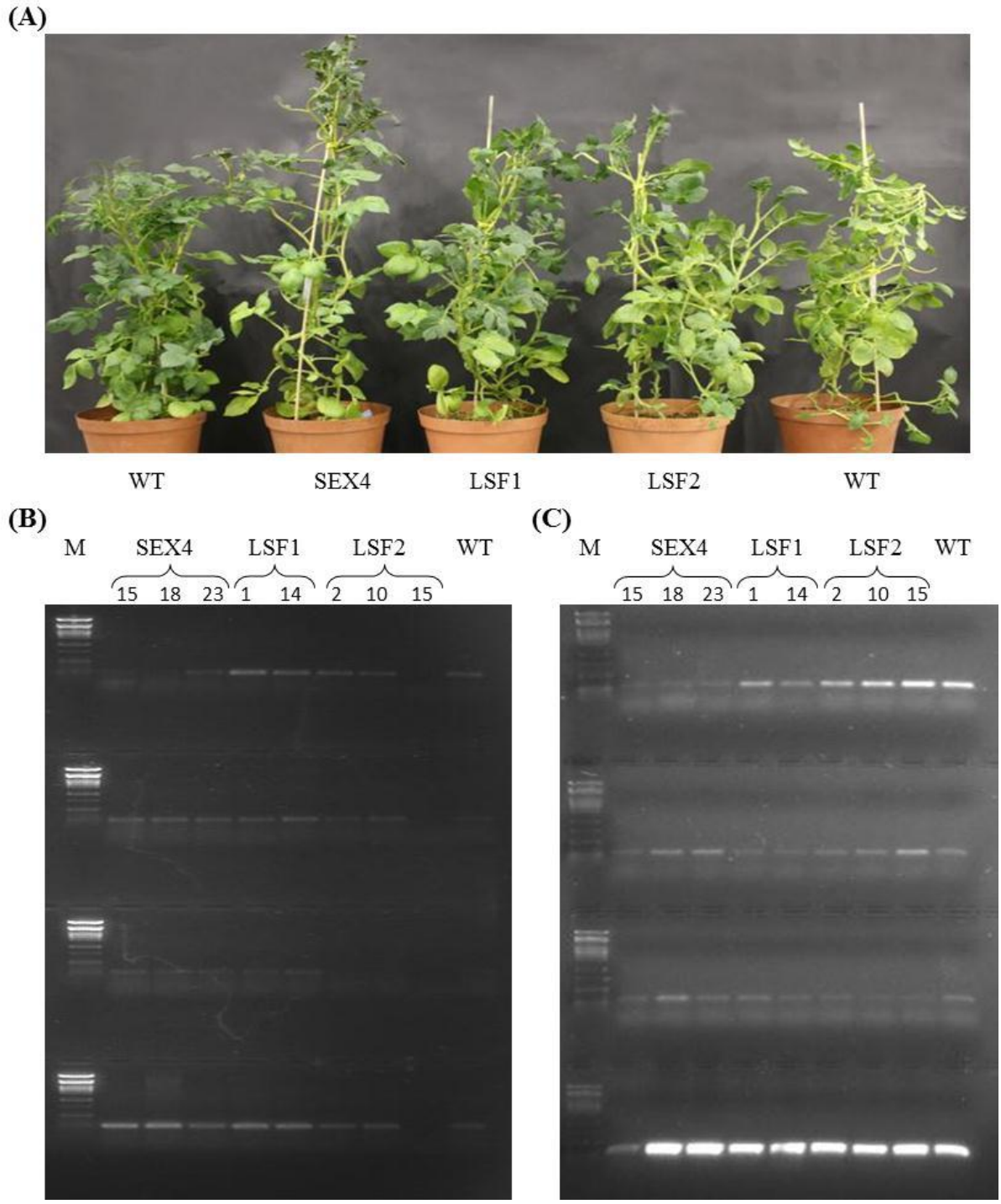


Figure 4.1: Silenced plants and semi-quantitative PCR (A) Potato plants repressed in expression of *SEX4*, *LSF1* or *LSF2*. (B) Semi-quantitative PCR of RNAi potato leaf and (C) RNAi potato tuber tested against SEX4; LSF1; LSF2 and Actin primers.

To test whether the genes targeted by the RNAi silencing constructs were successfully repressed in transgenic potato plants, transcriptional analysis was performed (Fig 4.1B,C). The amount of transcript of both *SEX4* and *LSF2* was decreased in leaves and tubers of several lines (Fig. 4.1B,C), and the transgene appeared to only affect expression of the targeted gene. The LSF1 construct was not successful in repressing *LSF1* expression in both tissues tested (Fig. 4.1B,C). As no lines were recovered with *LSF1* transcription, these were not evaluated in the rest of the analysis. Plants grown in soil were phenotypically indistinct from each other and the control (Fig. 4.1A).

4.3.2 Repression of *SEX4* and *LSF2* results in a starch excess phenotype in leaves

A qualitative approach was taken to examine if the genes were involved in starch degradation. Source leaves were covered in aluminium foil for three days before being bleached and stained with iodine solution (Fig. 4.2). It is clear that the transgenic lines are inhibited in starch degradation as they still contained large amounts of starch.

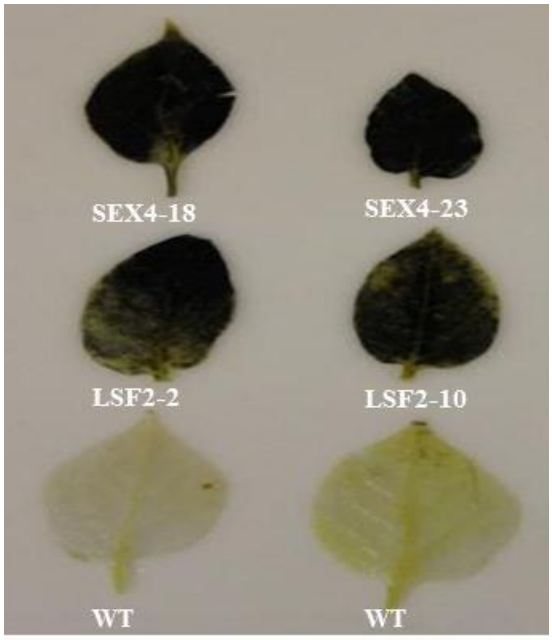
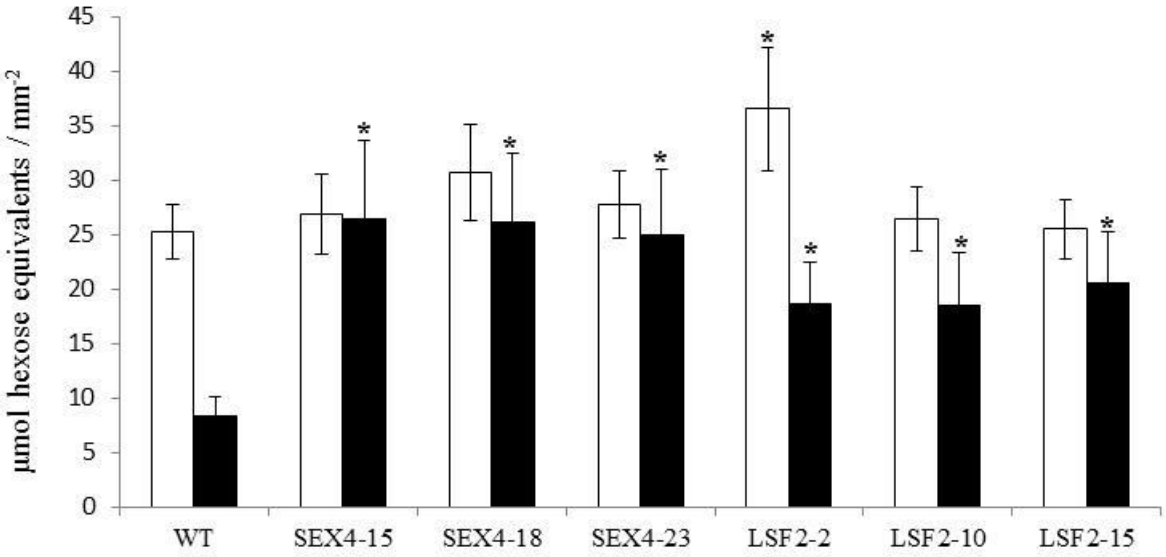


Figure 4.2: Iodine staining potato leaves after incubation in the dark for 3 days. Leaves from two separate lines where either *SEX4* or *LSF2* were repressed are compared with an untransformed control

To quantify the effect of repressing *SEX4* or *LSF2*, starch contents were determined at the end of the day and the end of the night. A significant increase in the amount of starch at the end of the day was found in only one line (LSF2-2). However, at the end of the night there is significantly higher starch in all of the *SEX4* and *LSF2* silenced plants. These data clearly show a function in potato leaves for *SEX4* and *LSF2* in starch degradation, although it appears that their relative importance differ slightly to those of the orthologous *Arabidopsis* genes. Although *SEX4* has been shown to be necessary for starch degradation in *Arabidopsis*, mutational analysis indicated that *LSF2* was not required for normal starch turnover. In this study it is clear that repression of *LSF2* alone had a major effect on starch degradation and it is, therefore, more important for starch turnover in potato leaves than in *Arabidopsis*. This is similar to the effect of transient *LSF2* repression in *Nicotiana benthamiana* (Chapter 3), which indicates that the more important role of LSF2 is conserved in the *Solanaceae* species.

Figure 4.3: Leaf starch content in leaves of WT, and lines where *SEX4* and *LSF2* transcription is repressed at the



end of the day (open bars) and end of the night (closed bars). Values are presented as mean ± SE of six individual samples per line and values with an * were determined by *Students t*-test to be significantly different ($P < 0.05$) from the WT.

4.3.4 Tuber starch content and amylose content

As repression of these genes was shown to influence starch metabolism in the leaves of both *Arabidopsis* (Kötting *et al.*, 2009; Comparot-Moss *et al.*, 2010; Santelia *et al.*, 2011) and *N. benthamiana* (Chapter 3), I decided to measure the starch contents within actively growing tubers of the transgenic plants (Fig. 4.4A). Overall no differences were seen in the starch contents of the *SEX4* and *LSF2* silenced plants when compared to the WT.

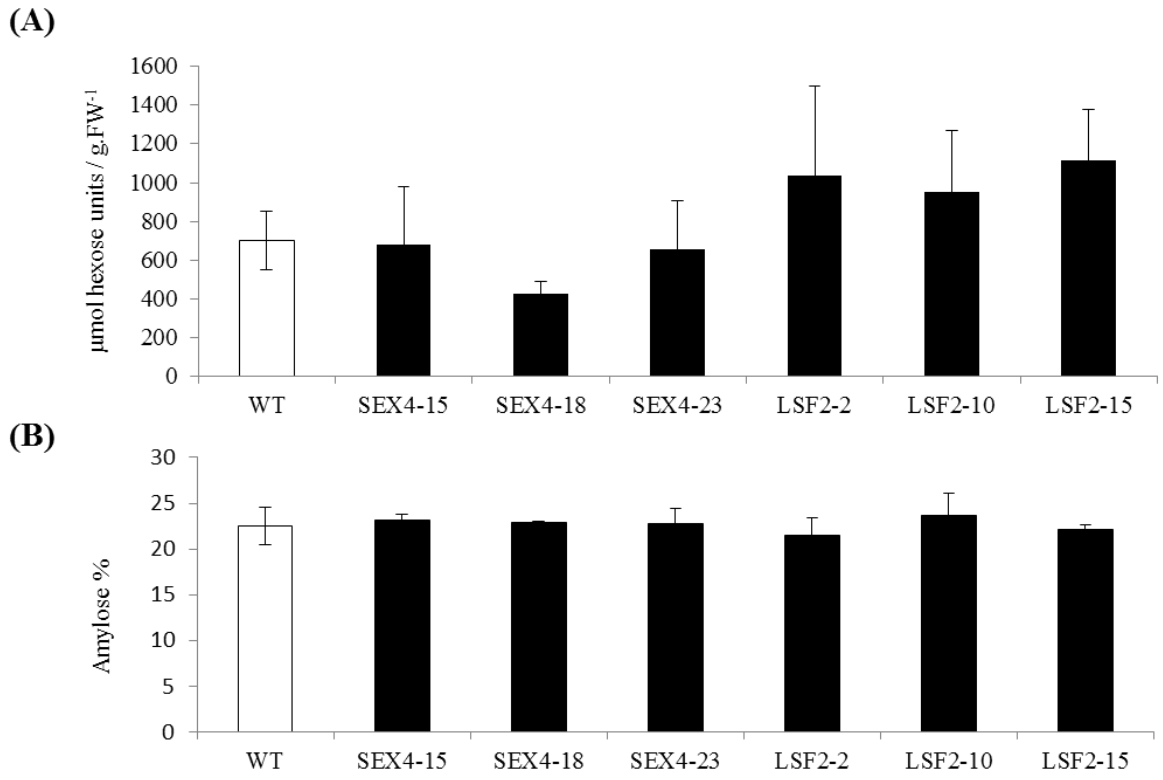


Figure 4.4: (A) Starch content of WT, SEX4 and LSF2 from actively growing tubers.Values are presented as mean ± SE of five individual samples per line. (B) Proportion of starch that is amylose determined using an iodine binding assay. Values are presented as mean ± SE of three individual samples per line.

The amylose content within the tuber starch was also measured (Fig 4.4B). Previous results showed that *sex4* mutants had increased amylose amounts within the leaf starch of *Arabidopsis* (Zeeman *et al.*, 2002b). However no significant differences were found in regards to the amount of amylose present within the tuber starch. The difference between Arabidopsis leaves and potato tubers is likely due to the increased starch levels in the Arabidopsis leaves leading to an increase in the amount of the amylose synthesizing granule

bound starch synthase (GBSS; Zeeman *et al*,. 2002). As starch levels were unaltered in the potato tubers of the transgenic lines in this study, it is reasonable to assume that GBSS amounts remain the same.

4.3.6 Tuber starch phosphate determination

Mutations in *Arabidopsis SEX4* and *LSF2* resulted in leaf starch with altered phosphate contents (Kotting *et al.*, 2009; Santelia *et al.*, 2011). I decided, therefore, to examine this in potato tubers and initially quantified the amounts of glucose-6-phosphate in starch that had been isolated from actively growing tubers (Table 4.3). The results indicate that both SEX4-15 and SEX4-18 had a higher amount of phosphate at the C-6 position. As AtSEX4 is thought to dephosphorylate starch at the C-6 position (Kötting *et al.*, 2009), it is reasonable to assume that potato plants in which SEX4 is repressed would contain increased amounts of phosphate at that position.

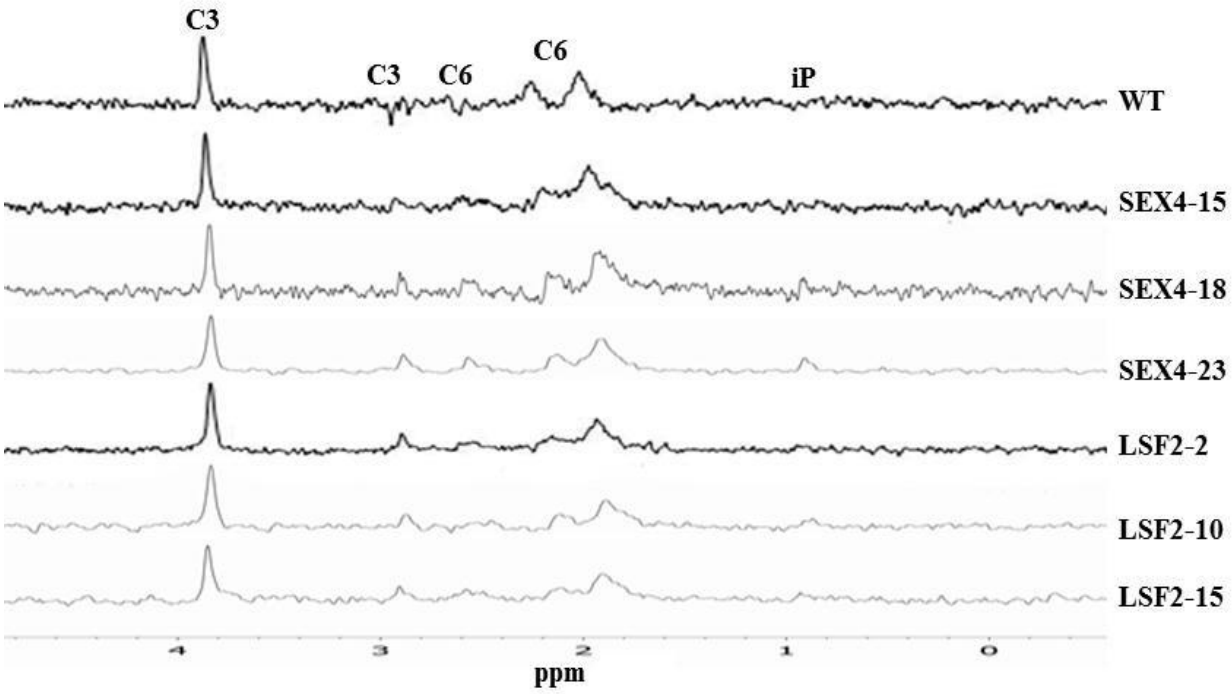


Figure 4.5: 31-P NMR spectra of α-amylase digested starch samples from WT, SEX4 and LSF2 tubers

No significant change was found in the amount of phosphate at the C-6 position in plants repressed in LSF2 expression (Table 4.2), however, as AtLSF2 was shown to dephosphorylates the C-3 position (Santelia *et al.*, 2011) it might be expected that the amount of phosphate at that position would increase instead. To test this it was decided to examine the ratio between C-3 and C-6 starch bound phosphate in the transgenic potato plants using 31-P NMR (Fig 4.5) and used these ratios to calculate C-3 phosphate amounts using the glucose-6-phosphate data. This data indicated that the proportion of C-3 phosphate was indeed significantly increased in starch from LSF2-2 and LSF2-10 silenced plants and that, as a result, amounts of C-3 bound phosphate were increased (Table 4.3).

Table 4.3: C-6 and C-3 bound phosphate on WT, SEX4 and LSF2 silenced plants. G6P was determined enzymatically and the G3P estimated using the ratio of C-3 and C-6 bound phosphate based on the peak areas of 31P-NMR spectra Values are presented as mean ± SE of five individual plants per line and values with an * were determined by *Students t*-test to be significantly different ($P < 0.05$) from the WT.

Genotype	C-6 Bound nmol G6P µmol^{-1} hexose equivalents	C-3 Bound Glc Equivalents	C-3/C-6 Bound
WT	6.41 ± 2.03	3.65 ± 1.57	1 : 1.7
SEX4-15	14.1 ± 1.51 *	7.75 ± 0.83 *	1 : 1.8
SEX4-18	29.68 ± 9.66 *	14.24 ± 4.63 *	1 : 2.1
SEX4-23	16.69 ± 5.43	8.67 ± 2.82	1 : 1.9
LSF2-2	9.99 ± 3.34	11.18 ± 3.74 *	1 : 0.9
LSF2-10	10.25 ± 1.74	7.48 ± 1.27 *	1 : 1.4
LSF2-15	9.37 ± 1.8	6.65 ± 1.27	1 : 1.4

The NMR data also indicates that in the *SEX4* silenced lines the absolute amounts of phosphate bound at the C-3 position were also increased significantly as shown by the significant increase in starch isolated from the SEX4-15 and SEX4-18 lines. This adds weight

to the argument put forward in Chapter 3 that the specificities of SEX4 and LSF2 in solanaceous species are not the same as those demonstrated in Arabidopsis and they may be involved in dephosphorylating starch at both the C-6 and C-3 positions. Changes in the amounts and/or proportions of the glucose-6 and glucose-3 phosphate can elicit conformational changes in the starch that could give it altered functionalities which might be useful in industry. Unfortunately, this initial experiment did not yield enough sample to test for starch functionality, for example by using a rapid visco-analyzer, however, work is ongoing to achieve this.

4.3.8 *SEX4* or *LSF2* repression do not influence cold-induced sweetening in tubers

Cold-sweetening is a process which negatively affects the potato processing industry. Repression of GWD, an enzyme shown to phosphorylate starch at the C-6 position, resulted in the inhibition of starch degradation in the leaves and was also shown to block cold sweetening (Lorberth *et al.*, 1998), however, another enzyme involved in starch degradation (DPE2) affected leaf, but not tuber starch degradation (Lloyd *et al.*, 2004) Since starch degradation was inhibited within leaves of the *SEX4* and *LSF2* repressed plants I examined the effects of repression of these genes in cold stored tubers.

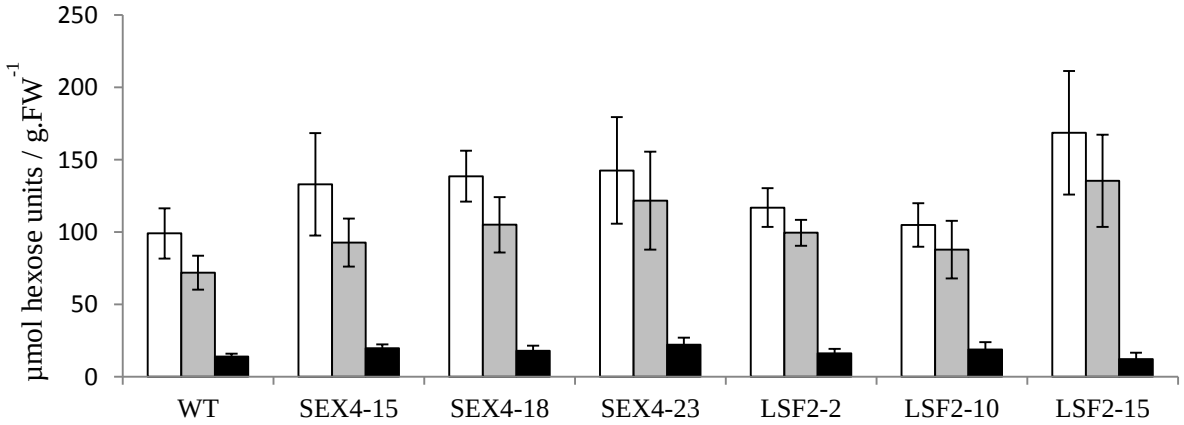


Figure 4.6: Soluble sugar contents in tubers stored at 4°C for 8 weeks. Glucose (white bars), fructose (grey bars), sucrose (black bars) were determined enzymatically. Values are presented as mean ± SE of tubers isolated from six individual plants per line.

Soluble sugar contents were measured in WT, *SEX4* and *LSF2* in tubers incubated at 4˚C for 2 month (Fig 4.6). The results indicate that silencing of *SEX4* and *LSF2* had no effect in terms of repressing cold sweetening. It could be that starch degradation in potato tubers is different than in leaves, so that SEX4 and LSF2 do not play such an important role. However, it is also possible that there is some functional redundancy between them that is more pronounced in tubers than leaves. It may, therefore, be necessary to repress both *SEX4* and *LSF2* simultaneously in order to achieve good repression of CIS. Experiments aimed at determining this are ongoing.

4.4 Conclusion

The study was successful in repressing SEX4 and LSF2, which led to the elucidation of the roles of these enzymes, demonstrating that they function in a similar manner as been described in Arabidopsis (Kötting *et al.*, 2009; Santelia *et al.*, 2011). One major difference in potato compared with Arabidopsis is that *LSF2* alone was shown to be necessary for normal starch degradation in leaves. This may be a more general phenomenon in the *Solanaceae* as it also appears to be important in *N. benthamiana* (Chapter 3). The manipulation of these enzymes allows the alteration of the ratio of phosphate at the C-3 and C-6 positions of the starch which may hopefully lead to increased functionality and industrial use.

4.5 References

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Chapter 5: General Discussion

Starch is an important plant product that is used in very many industries. Not only is it a valuable commodity to industry but it also forms a large part of the human diet. Based upon market research (Modified starch market by raw materials, applications & geography - Global forecasts to 2017: www.marketsandmarkets.com), the estimated value of the starch market sits at $12.7 billion in 2012. With worldwide demand for starch in food and non-food areas continuing to increase this value is expected to rise to about $15.2 billion by 2017. Starch usage is currently estimated at about 51% for food applications with the remainder being used for non-food applications, such as paper making and the production of biodegradable plastics.

Because of its importance, extensive research has been performed on starch metabolism with the process being investigated in a number of plant species. These studies, particularly recent ones in *Arabidopsis*, have explained many of the mechanisms and enzymes involved in both its synthesis and degradation. Whether the model produced using *Arabidopsis* data holds true for all the enzymes characterized in the pathway in other plant species still needs to be investigated. If it does then this should help greatly in the rational manipulation of starch structure for use by industry which would be extremely useful as it would allow for cost minimization in that the desired type of starch can be produced in the plant, negating subsequent downstream modifications. These modifications involve physical and chemical treatment and usually require the incorporation of a specific functional group which helps stabilize the glucan. An example of this is the paper making industry where it was shown that adding phosphate esters to the pulp allowed for the manufacturing of a higher quality paper which is not susceptible to linting, a problem whereby paper fibres become detached and settle on parts of the printing press. In this particular instance it is advantageous for the starch to be phosphorylated and this is done by chemical methods where phosphate

esters are added to the pulp suspension (Ly and Brouillette, 2013), a process that is both labour and cost intensive. If plants with high amount of starch can be modified so that their starch phosphate contents are increased, then it would decrease this cost to industry.

As mentioned above, starch which contains a high amount of phosphate is extremely valuable to industry owing to solutions of it being both viscous and forming a stable paste. Manipulating genes involved in starch phosphate metabolism, therefore, might aid in obtaining a more highly phosphorylated starch molecule. Although this has been achieved in potato where BE or SS enzymes were repressed (Abel *et al.*, 1996; Safford *et al.*, 1998; Kossmann *et al.*, 1998; Jobling *et al.*, 1999; Schwall *et al.*, 2000) this was accompanied by alterations in amylopectin and/or amylose, meaning that the effect of alterations in starch phosphate alone in these plants could not be assessed.

In this project a number of genes which have been shown to be involved in starch phosphate metabolism were analysed. The importance of starch in developing tomato fruit was investigated in a *gwd* conditional tomato mutant. This mutant contains GWD expressed under a pollen specific promoter enabling it to overcome the pollen lethality that was found in the mutant. GWD repression normally results in starch which cannot be degraded. This is most likely due to the contribution the phosphate incorporated has on altering the starch granules structure and increasing the accessibility to glucan hydrolysing enzymes. Starch serves as a long term carbohydrate reserve and accumulates transiently in tomato fruit being subsequently degraded as the fruits develop. It was therefore of interest to observe what kind of effects the developing fruit would demonstrate if the starch within them were unable to be degraded. No such effects were seen on the fruit as these developed as the WT and, more importantly, without any difference being seen in terms of starch degradation. Using immunoblot analysis the presence of GWD was detected within young tomato fruit of the mutant. Thus the objective of the study was not met and the physical relevance that starch

plays within tomato fruit development was not determined. A system which effectively blocks starch degradation in the fruit is needed to answer this question. If starch degradation were blocked in fruits, the lack of carbon from starch would not necessarily lead to any adverse effects on tomato fruits as starch comprises a very small portion of the carbon pool. In my study, at most only about 5% of the total sugars were present in starch (Chapter 2: Figure 2.2). Also tomato fruits both import soluble sugars from the leaves and have the ability to photosynthesize, so if the starch is unable to be degraded the amount of carbon fixed in it can be easily compensated for. However the physical presence of starch within fruit chloroplasts might disrupt the normal transition of chloroplast to chromoplast thereby affecting normal fruit development and processes such as ripening. Until a biological system is available to examine this, this possibility will remain unanswered.

Phosphorylation of starch is important as the phosphate renders the starch molecule susceptible to attack by hydrolytic enzymes; however this phosphate also hinders the eventual degradation of the granule. It is, thus necessary for the phosphate groups to be removed. *SEX4*, *LSF1* and *LSF2* have been shown to be involved in starch degradation with SEX4 and LSF2 particularly involved in the dephosphorylation of starch at the C-6 and C-3 positions respectively. Until now the role of these enzymes has not been studied outside of *Arabidopsis*. In this dissertation they were functionally analysed in both *N. benthamiana* and *Solanum tuberosum*, by employing two gene silencing mechanisms VIGS and RNAi which were shown to lead to the successful repression of these enzymes in both plant species. VIGS is a technique which allows for transient silencing of genes mainly within leaves of plants and, in this study, allowed for the role of these enzymes to be studied in transitory starch degradation in *N. benthamiana*. This method is rapid, allowing the analysis of gene function in a relatively short timeframe. Although the RNAi approach is not as rapid as the VIGS, it allows for stable transformation, where the role of the enzymes could be investigated both

within leaves and storage organs of potato. It also allows for the study of leaf starch degradation over a longer period than VIGS.

This study validates the already described function of these enzymes in starch degradation, with some similarities and potential differences being noted in the specificities of the enzymes between the respective species. The analysis of *sex4* and *lsf2* Arabidopsis mutants, led to the genes being described as encoding starch phosphatases having the ability to dephosphorylate starch at the C-6 and C-3 positions respectively, with the lack of these enzymes leading to alterations in the proportion of phosphate bound on the respective positions of the starch. The same is true, in both *N. benthamiana* leaf starch as well as potato tuber starch as when either *SEX4* or *LSF2* are repressed the ratio of bound phosphate is altered, however the distributions obtained for the solanaceae species are not exactly the same as those described in Arabidopsis. Mutations eliminating *AtSEX4* or *AtLSF2* increased the proportion of C-6 or C-3 starch bound phosphate respectively. In *N. benthamiana* when *SEX4* and *LSF2* were repressed either individually or in combination with each other the relative proportion of C3 bound phosphate increased (Chapter 3: Figure 3). *SEX4* silenced potato plants contained tuber starch with increased amounts C-6 and C-3 bound phosphate, as well a change in the ratio in which the phosphate is distributed. Tuber starch from *LSF2* silenced plants contained an increase in amount and proportion of C-3 bound phosphate, while that from *SEX4* repressed lines contained an increase in both C-6 and C-3 phosphate, with the C-6 increasing to a greater extent than the C-3 (Chapter 4: Figure 4.5 and Table 4.3). Leaf and tuber starches are manufactured for different purposes (short term vs long term storage) over different time periods (one day vs several month) so it might be expected that there would be differences in the effects that repressing SEX4 or LSF2 have on them. Generally though it can be seen that the proportion of C-3 bound phosphate is increased in all the lines where either gene was repressed. This is clearly different from Arabidopsis as C-3 bound starch

phosphate in *Atsex4* plants was greatly decreased. I think that this is indicative of a difference in specificity of the Solanum proteins compared with Arabidopsis (discussed in greater depth below).

In terms of starch degradation, VIGS repression of neither *SEX4* nor *LSF2* led to a starch excess phenotype during a day/night cycle (Chapter 3: Figure 2A), however RNAi silenced *SEX4* and *LSF2* potato plants did (Chapter 4: Figure 4.3). This is most likely an effect caused by the stable silencing nature of RNAi over transiently silenced VIGS plants, with a greater amount of time occurring in the potato plants for the starch excess phenotype to develop. Unlike *SEX4* and *LSF2*, transient repression of *LSF1* on its own or the simultaneous silencing of both *SEX4* and *LSF2* led to inhibited starch degradation in *N. benthamiana* leaves (Chapter 3: Figure 2A). Unfortunately, an insufficient number of RNAi repressed *LSF1* plants and double repressed *SEX4/LSF2* plants were obtained so no comparison between solanum species on the role of these enzymes can be attempted.

When each of the repressed *N. benthamiana* and potato plants were placed in the dark for several days a substantial amount of starch remained in the leaves of all the silenced lines Chapter 3: Figure 2C; Chapter 4: Figure 4.2), similar to Arabidopsis *sex4* and *lsf1* mutants, butnot *Atlsf2* mutant plants. This indicates that *LSF2* in potato and *N. benthamiana* has a more important function than in Arabidopsis. Although starch degradation was inhibited in leaves of *SEX4* or *LSF2* repressed potato plants this was not the case in cold stored tubers, meaning either that the pathway of starch degradation differs between leaves and tubers, or that there is functional redundancy between the two enzymes. This attempt was to solve a problem of the potato industry, therefore, failed. There is still hope that simultaneous repression of both the starch phosphatases together may solve this problem and such plants are currently being generated.

I propose that the activity of SEX4 and LSF2 differs between Solanum species and Arabidopsis in that the potato and *N. benthamiana* enzymes are able to dephosphorylate both the C-6 and C-3 positions while the Arabidopsis proteins act on either C-6 or C-3. The route via SEX4 seems to be more important in all species as there is more starch degradation in plants repressed in LSF2 than in SEX4 (Chapter 3: Figure 2B,C; Chapter 4: Figure 4.3; Santelia *et al.,* 2011). The differences seen in the phosphate distribution on starch of the various mutants, VIGS and transgenic plants could be due to the relative amounts of these proteins present between the species. We attempted to examine protein levels using antibodies that recognise AtSEX4 or AtLSF2 (Kind gift of Prof. Sam Zeeman and Dr Oliver Kötting, ETH, Zurich, Switzerland), however this failed as the antibodies were too non-specific in *N. benthamiana* and potato (data not shown). Another possibility could be that these enzymes act in different parts of the plastid. It has been demonstrated that there is a difference in the starch binding capacity between the two Arabidopsis enzymes, with SEX4 having the higher affinity for starch (Santelia *et al.,* 2011). AtSEX4 contains a CBM20 starch binding domain (Niittylä *et al.,* 2006) while no carbohydrate binding motifs are found in the AtLSF2 protein (Santelia *et al.,* 2011). It could be that SEX4 binds to the starch granule and removes phosphate from glucans almost as soon as they are released from the surface, while LSF2 is present more in the stroma and acts on the minority of molecules that escape the action of SEX4.

The two enzymes clearly act in the same pathway as transient repression of both genes lead to a greater inhibition of starch mobilization than when either was targeted alone. This was accompanied by an increase in P-Oligo accumulation, presumably as these are the substrate for both enzymes so repressing them simultaneously would lead to their increased accumulation. Using VIGS to repress both enzymes in *N. benthamiana* demonstrates one advantage of this system over studying mutants as *Atsex4/Atlsf2* double mutants show

reduced growth rates (Santelia *et al.*, 2011), meaning that any analysis of them might be affected by them being in different developmental stages. This problem is overcome using VIGS and the plants appeared unaffected by the repression of both *NbSEX4* and *NbLSF2*, presumably as the reduced growth phenotype in Arabidopsis is the product of a long period of altered starch degradation.

This project was successful in analysing the roles of the dephosphorylating enzymes in *N. benthamiana* and potato. The knowledge gained corresponds to previous findings in *Arabidopsis* with these enzymes shown to play similar roles in these species although some of the activities of the enzymes differ and seem to be more important in one species compared with another. To further understand the exact specificities of the enzymes from the respective solanaceae species, these proteins have to be isolated and purified using a recombinant expression system. These would then have to be incubated with polyglucans which had been phosphorylated at the C-6 and/or C-3 positions by GWD and PWD. Such experimentation may hopefully uncover the full role played by these dephosphorylating enzymes and how the activity of these may differ between the respective plant species.

Unfortunately, nothing new could be added to the understanding to the relevance of starch metabolism in tomato fruit as well as in finding a solution to alleviate a problem for the potato processing industry. What is now known is that *SEX4* and *LSF2* enzymes can be repressed producing plants which contain increased starch in the leaves, as well as tuber starch which has higher phosphate as well as an altered distribution of that phosphate. These starches need to be tested to determine if they have increased or improved functionality, or if these exhibit characteristics which may make it useful in entirely new functions in industry. Efforts are on-going to achieve this.

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Abbreviations

AMY	α-amylase
ATP	Adenosine 5-tri-phosphate;
BAM	β-amylase
BCIP/NBT	5-Bromo-4-Chloro-3-Indolyl Phosphate p-Toluidine/ Nitro-Blue Tetrazolium Chloride
BE	Branching enzyme
Bp	Base pairs
C-3	Carbon-3;
C-6	Carbon-6;
cDNA	Complementary deoxyribonucleic acid
CIS	Cold-induced sweetening
Cm	Centimeter;
CM	Conditional Mutant
CTAB	Cetyltrimethylammonium bromide
DAF	Days after flowering
DBE	Debranching enzyme
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
DPE1-2	Disproportionating enzyme 1-2
DSP	Dual specificity phosphatase,
DTT	Dithiotreitol
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
ETOH	Ethanol
FW	Fresh Weight
G	Gram
GBSS	Granule-bound starch synthase
GWD	Glucan, water dikinase
Hepes	4-2-hydroxyethyl-1-piperazineethanesulfonic acid;
HPAEC-PAD	High-performance anion-exchange chromatography coupled with pulsed electrochemical detection
h	Hours
ISA	*Isoamylase*
kDa	Kilodalton
KOH	Potassium Hydroxide
kPa	Kilopascal
L	litre
LDA	*Limit Dextrinase*
LSF1	*Like Sex Four–1*

LSF2	*Like Sex Four–2*
M	Molar
MES	2-*N*-morpholino ethane sulfonic acid
MEX	Maltose exporter
µl	microliter
Mg	milligram
$MgCl_2$	Magnesium Chloride
Min	minutes
mL	Millilitre
mM	millimolar
MS	Murashige and Skoog
NaAC	Sodium acetate
NAD	Nicotinamide adenine dinucleotide
Nm	Nanometer
NMR	Nuclear magnetic resonance
OD	Optical Density
PCR	Polymerase chain reaction
PDS	Phytoene desaturase
PG	Phytoglycogen
pH	measure of the acidity or basicity
P-Oligos	Phosphorylated-Oligosaccharide
PWD	Phosphoglucan, Water dikinase
RNA	Ribonucleic acid;
RNAi	Ribonucleic acid interference
SBE	Starch branching enzyme
SDS	Sodium dodecyl sulfate
SEX4	*Starch Excess-4;*
SS	Starch synthase
Tris-HCl	Tris(Hydroxymethyl)-aminomethane
TRV1-2	Tobacco Rattle Virus 1-2
U	Units
U/ml	unit per milliliter;
VIGS	Virus-induced gene silencing;
VINV	Vacuolar invertase
(v/v)	(volume/volume)
(w/v)	(weight/volume)
WT	Wild-type