Analysis of enzymes involved in Starch Phosphate Metabolism

by

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Declaration

The experimental work in this thesis was supervised by Dr. J.R. Lloyd and was conducted in 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

Signed: ……. Date: …….

Abstract

This project examined the role of proteins in starch phosphate metabolism. The first part was aimed at the functional characterization of the *SEX4*, *LSF1* and *LSF2* genes in both plants and bacteria. Constructs were produced to allow for expression of the three proteins in *E. coli* with the SEX4 and LSF2 proteins being successfully purified and used to produce antibodies. Immunoblot analysis indicated that the antibodies recognised the repective proteins in extracts, but it was not clear if they actually recognised the proteins or the GST tags they were fused to.

Virus induced gene silencing constructs were also produced to allow repression of these three genes in *Nicotiana benthamiana*. This resulted in a starch excess phenotype being observed in the leaves of silenced plants which is consistent with the known or presumed roles for the genes. The antibodies produced were not specific enough to confirm that the respective protein were actually repressed, but it is likely that this was the case as plants infiltrated at the same time with a VIGS vector designed to repress *phytoene desaturase* exhibited a chlorophyll bleaching phenotype. These data confirm that *SEX4* and *LSF1* probable play the same role in *N. benthamiana* as in Arabidopsis, and provide evidence that *LSF2* is also necessary for starch degradation.

It was also attempted to characterise these proteins with respect to their substrate utilization by setting up a glyco-array experiment. Various potato starches from genetically modified plants were subjected to hydrolytic attack by starch degrading enzymes and fractionated by anion exchange chromatography to produce a multitude of glucans. These will be spotted onto glass filters and probed with the purified proteins to see if they bind to specific starch breakdown products preferentially.

The project also involved investigating the effect the SEX4 protein has on *E. coli* glycogen contents. SEX4 was expressed in wild type and *glgX* mutant *E. coli* strains as it has been shown that this stops glycogen accumulation in the wild type, but not the *glgX* mutant. The cells were grown in liquid culture and glycogen contents measured. In liquid cultures *SEX4* had no effect on glycogen contents in the wild type, possible because of problems with plasmid stability in the strain used.

This final part of the project investigated the effect that a *gwd* mutation has on carbohydrate metabolism in leaves and fruits of the Micro-tom tomato cultivar. Starch and soluble sugar contents were measured in leaves and ripening fruits. A starch excess phenotype was found in the leaves, but no change in starch contents was determined in either the placenta or pericarp of the fruit. Soluble sugar contents were reduced in the fruit tissues, although the reason for this in unclear.

Opsomming

Hierdie projek het die rol van proteine in stysel-fosfaat metabolisme ondersoek. Die eerste deel handel oor die funksionele karaktiseering van die *SEX4*, *LSF1* en *LSF2* gene in beide plante en bakteriee. Vektore is gekonstrueer om die uitdrukking van die drie proteine in *E.coli* toe te laat terwyl die SEX4 en LSF2 proteine suksesvol gesuiwer is vir die gebruik vir teenliggaam produksie. Immunoklad analises het getoon dat die teenligame die spesifieke proteine in die ekstrak herken het, maar dit was nie duidelik of dit die onderskeie proteine was of die GST-verklikker waaraan die onderskeie proteine verbind was nie.

Virus geindiseerde geen onderdrukking konstrukte is ook geproduseer om toe te laat vir die onderdrukking van hierdie drie gene in *Nicotiana benthamiana*. Dit het ‘n stysel oorskot fenotipe tot gevolg gehad in die blare van onderdrukte plante wat konstant is met die bekende of voorgestelde rolle van die gene. Die teenliggame wat geproduseer is was nie spesifiek genoeg om te bewys dat die onderskeie proteine wel onderdrukis nie. Dit kon wel die geval gewees het want plante geinfiltreer op dieselfde tyd met ‘n VIGS vektor wat ontwerp is om *phytoene desaturase* te onderdruk het ‘n chlorofil bleikings fenotipe getoon. Hierdie data bevestig dus dat *SEX4* en *LSF1* moontlik dieselfde rol speel in *N. benthamiana* as in Arabidopsis, en toon bewyse dat *LSF2* ook nodig is vir stysel afbreek.

Karakterisasie van die onderskeie proteine met respek tot hul substraat gebruik is ondersoek deur ‘n gliko-array eksperiment. Verskillende aartappel stysels van genetiese gemodifiseerde plante was geonderwerp aan hydrolitiese afbreek deur stysel afbrekende ensieme en geskei deur anioon uitruilings chromotografie om veelvuldige glukans te

vervaardig. Dit is geplaas op glas filters en is ondersoek saam met die gesuiwerde proteine om te sien of dit mag bind aan spesifieke stysel afbreek produkte.

'n Verdere ondersoek is onderneem na die effek van die SEX4 protein op *E. coli* glikogeen inhoud. SEX4 was uitgedruk in die *E .coli* wildetipe en *glgX* mutant omdat dit reeds bewys is dat SEX4 glikogeen ophoping veroorsaak in die wildetipe maar nie in die *glgX* mutant. Die selle is opgegroei in vloeibare media en glikogeen inhoud is gemeet. In vloeibare media het SEX4 geen effek op die wildetipe se glikogeen inhoud nie wat moontlik kan wees as gevolg van plasmied stabiliteit in die *E. coli* ras wat gebruik is.

Die finale deel van die projek was om die effek van 'n *gwd* mutasie op koolhidraat metabolisme in blare en vrugte van die Micro-tom tamatie kultivar te ondersoek. Stysel en oplosbare suikers is gemeet in blare en rypwordende vrugte. 'n Oortollige stysel fenotipe is in die blare gevind maar geen verandering in stysel inhoud is waargeneem in die plasenta of perikarp van die vrug nie. Oplosbare suiker inhoud het afgeneem in die vrugweefsel dog is die rede hiervoor nie te verstane.

Acknowledgments

I would like to thank Prof Jens Kossmann and Dr James Lloyd for providing me with the opportunity to conduct this research under their supervision at the Institute for Plant Biotechnology.

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To my family and friends, especially Farida Allie, whose love and support has seen me through some trying times, Thank you!

I would also like to say a special thanks to my parents Ridwan and Shereen Samodien. Thanks for all the continued support and conditional love over the years. I love you both very much and this thesis is dedicated to you.

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ATP	Adenosine 5-tri-phosphate
BAM1-4	β-amylase 1-4
BCIP/NBT	5-Bromo-4-Chloro-3'- Indolyl Phosphate p-Toluidine/ Nitro-Blue Tetrazolium Chloride
Bp	Base pairs
CaCl	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
CM	Conditional mutant
CTAB	Cetyltrimethylammonium bromide
CV	Column volume
dsRNAs	Double stranded Ribonucleic acids
DTT	Dithiotreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
ETOH	Ethanol
FMS2	Fluorescence monitoring system
FPLC	Fast protein liquid chromatography
FW	Fresh weight
g	Gram
gDNA	Genomic DNA

GWD	Glucan, water dikinase
GST	Glutathione S Transferase
Hrs	Hours
IPTG	Isopropyl ß-D-thiogalactopyranoside
ISA1-3	Isoamylase 1-3
KCl	Potassium chloride
kDa	Kilodalton
KOH	Potassium hydroxide
kPa	Kilopascal
L	Litre
LDA	Limit dextrinase
LiCl	Lithium chloride
LSF1	Like sex four 1
LSF2	Like sex four 2
PCR	Polymerase chain reaction
PWD	Phosphoglucan, water dikinase
M	Molar
$MgCl_2$	Magnesium chloride
mg	Milligram
Min	Minutes
Ml	Millilitre
mM	millimolar

MOPS-KOH	3-(N-morpholino)propanesulfonic acid- potassium hydroxide
MS	Mass spectroscopy
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
Nm	Wavelength
OD	Optical density
PDS	Phytoene desaturase
pH	Acidity/alkalinity
PSII	Photo-system II
SDS	Sodium dodecyl sulfate
SEX4	Starch excess four
siRNA	Small interfering ribonucleic acids
SSIII	Starch synthase III
SuSy	Sucrose synthase
RNAi	Ribonucleic acid interference
Tris-HCl	Tris(Hydroxymethyl)-aminomethane
TRV1-2	Tobacco rattle virus 1-2
U	Units
(v/v)	(Volume/volume)
VIGS	Virus-Induced Gene Silencing
(w/v)	(Weight/volume)
WT	Wild type

Chapter 1

Literature Overview

1. Literature Overview

1.1 The importance of starch

Starch is the main storage carbohydrate of most plants. In leaves it is a product of photosynthesis in chloroplasts, accumulating during the day and being degraded at night to form sucrose. This is exported to storage organs, such as tubers and seeds, where it is re-synthesized to starch which accumulates as a long-term carbon store. This conversion of starch to sucrose in leaves is regarded as one of the largest carbon fluxes, which occurs daily on the planet (Niittyla et al, 2006). Starch is also a renewable resource that is being used in many industrial applications (Kossmann and Lloyd, 2000) and because of its role both in industry and as a plant storage product it is arguably one of the most important carbohydrates in plants (Zeeman *et al*, 2007).

1.2 Starch structure

Much work has been done over the past decades to examine the structure of starch granules. Starch consists of two glucose polymers amylose and amylopectin, where amylose is an essentially unbranched α1,4-polyglucan while amylopectin contains both α1,4 bonds and α1,6 branch points. The chains within amylopectin are arranged in an ordered manner probably according to the 'cluster' model (Hizukuri, 1970) where short chains cluster together in ordered arrays of densely packed double helices and the clusters are linked by longer chains. This structured formation of chains within the amylopectin molecule means that it is semi-crystalline and, as amylopectin makes up 70-90% of starch, it means that the starch is also semi-crystalline. Small angle X-ray scattering has

demonstrated that there is a 9nm repeat structure in all tested starches, composed of crystalline and amorphous layers (Waigh et al, 1998). This is thought to contain one layer of double helical clusters forming the crystalline layer interspersed with amorphous amylose. Different starch granules also show different types of crystallinity based on different arrangements of the double helices. Cereal seed starches for example contain what is known as the A type allomorph, while potato tuber starch contains the B type (Gallant et al, 1997; Gerard et al, 2001). The difference between the two is that the A type is more compact, than the B type (Hejazi et al, 2008).

Starch granules also often contain 'growth rings' which were once considered to be produced during the day night cycle, however most recent evidence indicates that this is not the case (Pilling and Smith, 2003). A circadian rhythm could bring about growth ring formation through periodic changes in the activities of isoforms of starch synthases. However the study of Pilling et al (2003) concluded that circadian rhythms, physical mechanisms, and perhaps diurnal rhythms could all be contributing factors which control growth ring formation in starch granules of potato tubers, and the data produced during that study suggest that a complex interplay exists between several of these factors.

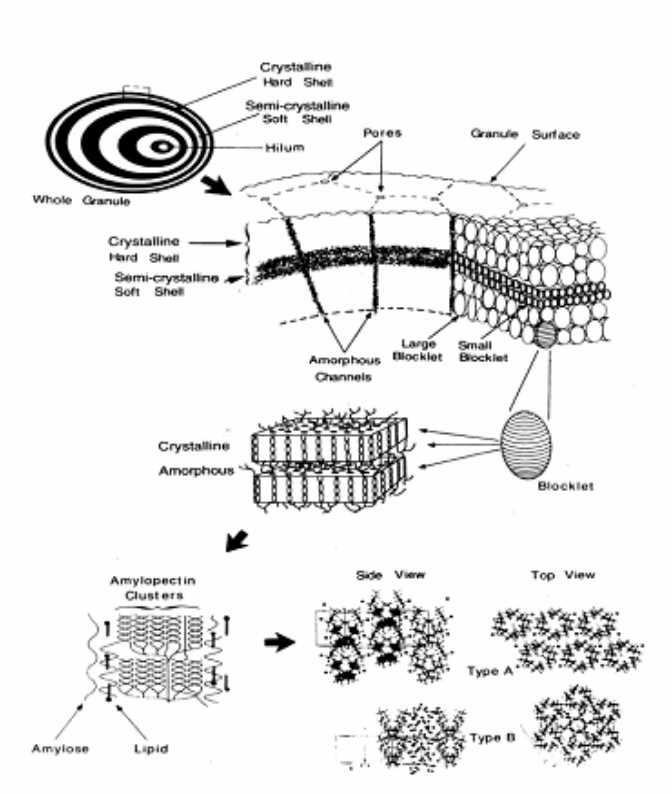


Figure 1.1: Overview of Starch granule at different organisational levels. It shows how the various compartments are arranged in a very structured manner which makes up the starch granule (Gallant et, al 1997).

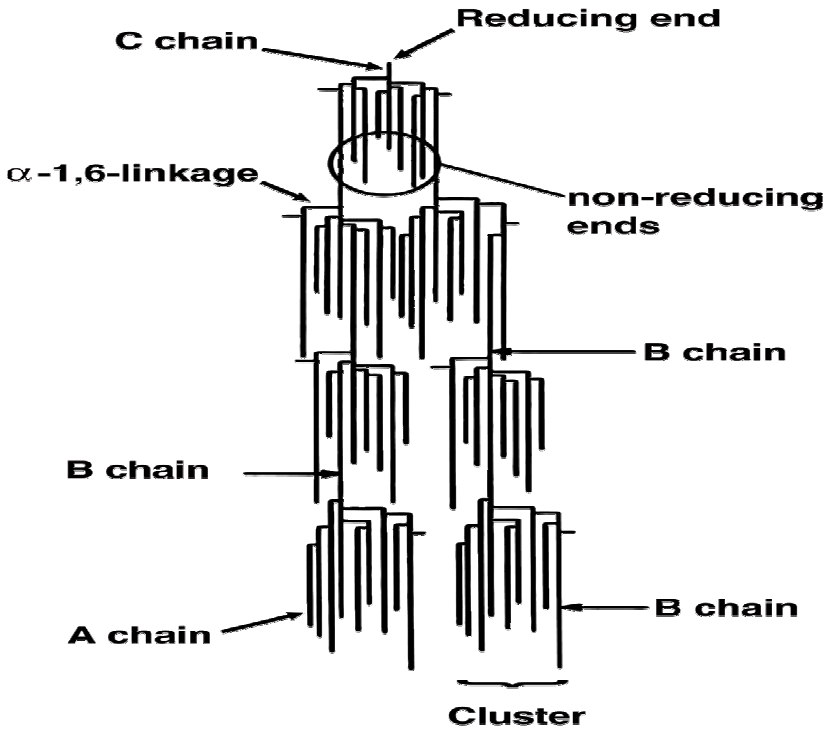


Figure 1.2: Branched nature of the starch molecule. Reproduced from http://www.jic.ac.uk/STAFF/cliff-hedley/Starch.html

1.3 Starch metabolism

In recent years great insight has been gained into the pathways of starch synthesis (Kossmann & Lloyd, 2000; Smith et al, 2003; Ball and Morell, 2003) as well as its degradation (Zeeman *et al,* 2006, 2007, Lloyd *et al.*, 2004) through the analysis of transgenic plants and Arabidopsis mutants. In terms of synthesis a number of starch synthase, starch branching and debranching enzyme isoforms have been studied and their contributions to the synthesis of the starch molecule revealed. Many of the enzymes involved in the degradation of starch have now also been examined with their functions being elucidated. This project involves the examination of enzymes involved in adding and removing phosphate groups from glucose residues within the amylopectin molecule, especially in regard to their role in starch degradation. As such the roles of enzymes

involved in forming the starch molecule will not be further considered. The rest of this section will be taken up considering the evidence for the roles of various enzymes involved in degrading starch as well as in its phosphorylation.

1.3.1 Starch degradation

Many enzymes are known to be able to degrade starch *in vitro*, and recently it has become clear which ones are responsible *in vivo*. Most recent work has examined leaf starch degradation, and revealed some differences between this process in chloroplasts and in storage organs. For example α-amylases, which cleave α-1,4 bonds within the polyglucan, play an important role in the degradation of cereal endosperm starch (Smith et al., 2005), however, this enzyme has been shown not to be essential for normal starch breakdown in Arabidopsis leaves (Yu et al., 2005). In leaves it appears that β-amylase is the main enzyme that degrades the linear chains within the starch molecule. β-amylases are characterized as exoamylases which release maltose from non-reducing ends of glucans or dextrins through hydrolysis of α-1,4 linkages. α-1,6 linkages are hydrolyzed through the action of the debranching enzymes. The Arabidopsis genome contains nine genes that code for putative β-amylase isoforms of which four (BAM1, -2, -3, and -4) are chloroplastic (Fulton et al 2008). Several of these isoforms have been demonstrated to be involved in starch degradation. In both potato and Arabidopsis plants in which the plastidial β-AMYLASE3 (PCTBMY1; BAM3; BMY8) is repressed through transgenic techniques, a starch excess phenotype in their leaves is observed (Scheidig et al., 2002; Kaplan and Guy, 2005). In addition in Arabidopsis when the BAM1 isoform is mutated no effect can be seen on starch degradation; however a *bam1/bam3* double mutant

demonstrates a greater effect on starch degradation than the *bam3* mutant exhibits on its own, indicating some redundancy between these two isoforms (Fulton et al. 2008). Finally a mutation in the BAM4 gene also leads to repression of starch degradation, even though no catalytic activity from this isoform has been measured (Fulton et al., 2008).

The final group of enzymes involved in cleaving bonds between the glucan monomers of the starch polymer are debranching enzymes which cleave the α-1,6 branchpoints. Higher plants contain four different debranching enzymes: three isoforms of isoamylase (ISA1-3) and one limit dextrinase (LDA) (Lloyd et al., 2005; Burton et al, 2002; Bustos et al, 2004). It has been shown that the debranching enzyme ISA3 is required for normal rates of starch breakdown in leaves from Arabidopsis (Wattebled et al., 2005; Delatte et al., 2006) and potato (Hussain, 2002). ISA3 from either potato (Hussain et al., 2003) or Arabidopsis (Delatte et al., 2006) displays high activity with β-limit dextrins (glucans that are produced as a result of β-amylase activity during starch breakdown) consistent with the proposed important role of β-amylase in leaf starch degradation. Limit dextrinases are said to play a role in the degradation of starch in cereal endosperm. Mutant *lda* plants have normal starch metabolism and the loss of LDA in the *isa3* background enhances the severity of the starch-excess phenotype, showing that LDA contributes to starch degradation when ISA3 is missing (Delatte et al., 2006). Maize *lda* mutants (*zpu1*) display slightly elevated starch levels in leaves and a reduced rate of endosperm starch mobilization during seedling establishment (Dinges et al., 2003). These results suggest that ISA3 and LDA function primarily in starch breakdown.

The ISA1 and ISA2 isoforms, however, appear to be involved in the synthesis (Zeeman et al 1998; Bustos et al, 2003; Delatte et al 2005; Wattebled et al, 2008) rather

than the degradion of starch. Mutation in these genes not only causes the loss of detectable isoamylase activity, but also leads to disruption of the normal starch structure (Bustos et al, 2003; Delatte et al 2005; Wattebled et al, 2008)

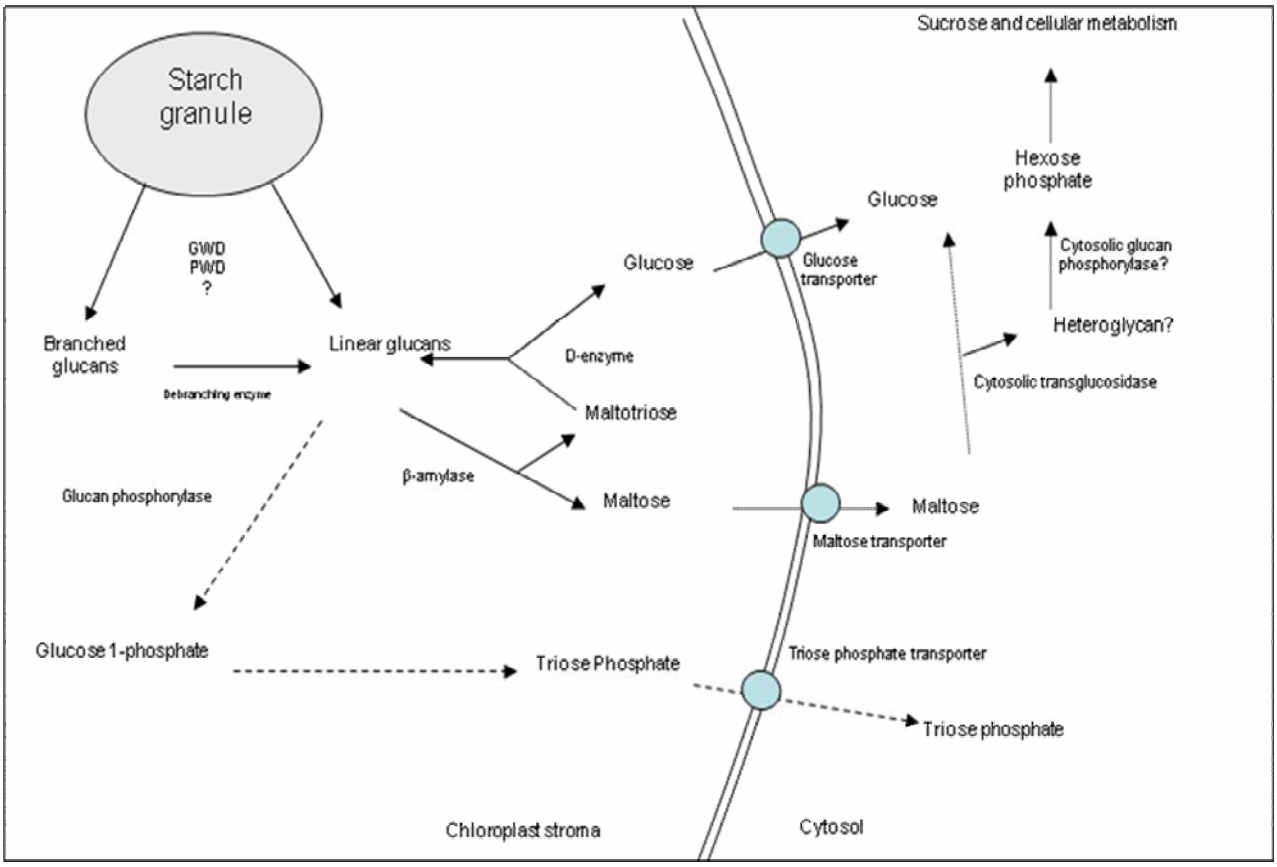


Figure 1.3: The proposed starch degradation pathway (Smith et al, 2005)

1.3.2 The incorporation of phosphate into starch and its importance in influencing starch degradation

It has become clear over the past decade that one important process in starch degradation involves the incorporation and removal of phosphate that is covalently linked to the glucose monomers within amylopectin. Phosphate is present on both the C6 and C3 positions of a small proportion the glucose monomers in starch depending on botanical

source (Hizukuri et al., 1970; Tabata et al, 1975; Baldwin et al, 1997; Jane et al, 1999; Blennow et al 2000, 2002). Generally starches from tubers contain relatively high levels of phosphate, while those isolated from leaves less so. Those from cereal seeds contain almost no covalently bound phosphate. For many years the biochemical mechanism for incorporation of starch phosphate was unknown, however it was recently demonstrated that the reaction is catalyzed by the enzyme glucan water dikinase (GWD) (Ritte et al., 2002, Mikkelsen et al, 2004). This enzyme takes ATP as the phosphate donor and transfers the β-phosphate to amylopectin, releasing the γ-phosphate into solution. It was originally thought that the GWD incorporated phosphate at both the C6 and C3 positions (Ritte et al., 2002), however it was later demonstrated that it only incorporates phosphate at the C6 position (Ritte et al. 2006) and that a second enzyme, the phosphoglucan water dikinase (PWD) (Kötting et al 2005, Baunsgaard et al 2005, Ritte et al, 2006, Hejazi et al, 2009) catalyzes the incorporation of the C3 phosphate in an identical reaction. The PWD, however, can only utilise glucan chains previously phosphorylated by the GWD as a substrate (Kotting et al 2005, Hejazi et al, 2008, 2009).

Interestingly both the GWD and PWD are necessary for normal leaf starch degradation in Arabidopsis (Lu et al, 2005; Kötting et al, 2005; Baunsgaard et al 2005), and the GWD has also been demonstrated to be essential for starch degradation in leaves, tubers and pollen from other species (Lorberth et al. 1998; Nashilevits et al, 2008). The GWD phosphorylates starch both during times of net synthesis, and also during periods of starch breakdown (Ritte et al, 2004; Nielsen et al, 1994). In chloroplasts, however, the rate of glucan phosphorylation is considerably higher when granules are being actively catabolised implying that phosphorylation at this time point might be important for

initiating granule degradation (Ritte et al, 2004). It is thought that incorporation of phosphate disrupts the helical structure of the chains (Hejazi et al. 2009) allowing better access to degradative enzymes such as β-amylase and debranching enzyme. This has been tested by examining different crystallized maltodextrins which have a similar structure to the A and B type allomorph of amylopectin (Hajazi et al., 2008, 2009). Such studies demonstrated that phosphorylation of these maltodextrins by the GWD and PWD lead to them becoming solubilised which would presumably open them up to attack by amylolytic enzymes.

The action of the GWD also seems to stimulate the activity of specific amylolytic enzymes. When starch granules were incubated with purified BAM3 as well as GWD, more maltose was released in comparison to when incubated with BAM3 alone (Edner et al., 2007). Interestingly the action of BAM3 also seemed to stimulate the activity of GWD indicating a complex interplay between these enzymes (Edner et al., 2007).

1.3.3 Removal of starch phosphate

Until recently the enzymes involved in the removal of the starch phosphate remained unclear. A chloroplastic glucan-binding phosphatase has now been described that is required for normal starch degradation in Arabidopsis (Zeeman et al., 1998; Niittylä et al., 2006; Sokolov et al. 2006). The enzyme is encoded at the *Starch EXcess 4* (*SEX4*; At3g52180) locus (also designated as *PTPKIS1* and *DSP4*) (Fordham-Skeltonet al, 2002; Kerk et al, 2006; Niittylä et al, 2006; Sokolov et al, 2006). *Arabidopsis* mutants lacking the SEX4 protein are impaired in starch degradation at night, leading to a progressive accumulation of starch as the leaves age (Zeeman and Rees, 1999). The

SEX4 protein contains a dual-specificity phosphatase domain as well as a carbohydrate-binding module. Since dual specificity phosphatases are generally believed to act on phosphorylated protein kinases (Luan, 2003), it was suggested that SEX4 might dephosphorylate such a kinase, which would in turn mediate the activity of starch degrading enzymes (Niittylä et al., 2006; Kerk et al., 2006). Recent evidence demonstrates however that the SEX4 protein is responsible for removing phosphate from starch. This comes from a study that showed firstly that the SEX4 protein is able to remove phosphate from the amylopectin molecule and, secondly, that the *sex4* mutant accumulates phosphorylated malto-oligosaccharides (which are starch breakdown products) (Kötting et al., 2009).

There are two other genes in the Arabidopsis genome coding for proteins with a high degree of similarity to SEX4, known as *Like Sex Four 1* (*LSF1; At3g01510*) and *LSF2* (AT3g10940). A recent paper has demonstrated that a mutation in LSF1 also leads to an impairment of starch degradation (Comparet-Moss et al, 2009), but it isn't clear whether LSF1 acts similarly to SEX4, or has another role. There is no data for the role of LSF2 in starch metabolism.

There are still a number of open questions about SEX4 and LSF proteins. They are the focus of much work examining their functions in Arabidopsis, but their roles have not been examined in other plants. In addition the specific glucans that these enzymes bind to have not yet been studied. For example, they may bind to starch granules as a substrate, or to starch breakdown products. These questions will be examined in this project through a number of techniques. These involve the production of antibodies against the proteins, the repression of these proteins in *Nicotiana benthamiana* using

virus induced gene silencing, and the production of starch breakdown product based glyco-arrays.

1.4 Glyco-Array Technology

Glyco-arrays are a relatively new technology, and can be defined as a micro-array of carbohydrates. The technology usually involves attaching some kind of oligosaccharide to a membrane, which can then be probed with the enzymes being studied. In recent years a number of studies have been done which have employed these arrays. A study by Shipp et al, (2007) was performed where the activities glycosyltransferases involved in plant cell wall biosynthesis were assayed. That study aimed at the characterization of various enzymes based on their biochemical activities on plant cell wall polysaccharides, which were attached to a thin-coated photo-activatable glass slide. They showed that the technique was not only relatively simple, but also provided a high-throughput method to assign biochemical function to enzymes as well as to increase the understanding of the roles played by these enzymes in this complex network. Another study where glyco-arrays were used was done by Meenakshi et al (2007), where they aimed to investigate the usefulness of mucins in understanding the progression of gastric cancer and gallstone formation. Mucins are essential cytoprotective glycoproteins and changes in epithelial mucins have been shown in different pathological conditions. The study involved formalin-fixing paraffin-embedded gastric biopsy specimens as well as surgically resected gallbladder tissue samples. These were then stained with various dyes to give an indication of the pH of the samples. The study showed that in normal gastric and gallbladder mucosae, the mucins were found to be at a

neutral pH, whereas in intestinal metaplasia, gastric carcinoma and stone-containing gallbladder, showed a significant increase in acidic mucins. The two studies mentioned are quite diverse which shows that this technology is useful for the study of protein interactions with a variety of carbohydrates.

The examples that I have given above demonstrate that glyco-arrays can be used to study many aspects of metabolism. I wish to set up such arrays to examine the binding of SEX4, LSF1 and LSF2 proteins to starch breakdown products, however they will also have the potential to study the formation of starch metabolic enzyme complexes as well as opening up the possibility of identifying new proteins involved in either synthesizing or degrading starch.

1.5 Virus Induced Gene Silencing

There are several techniques for gene silencing mechanism in plants, for example antisense and RNAi. These have been used to effectively down-regulate specific genes in many plants. Most of these methods rely on the production of double-stranded RNAs (dsRNAs), which correspond to the gene of interest leading to the initiation of the homology-based RNA degradation process. One disadvantage of producing transgenic plants is the time that it takes to do so, and the necessity to study multiple transgenic lines in order to overcome the problem of somaclonal variation. This can be overcome using virus-induced gene silencing (VIGS).

VIGS allows for transient induction of gene silencing. It involves the construction of an engineered virus which contains the sequence of a target gene that is also present within the host plant. The pathway leading to RNA silencing is known to be separated

into three distinct phases, the initiation, effector and amplification stages (Hannon, 2002). The initiation of RNA silencing is triggered by double stranded RNA (Fire et al. 1998). dsRNA is recognized by a highly specific ribonuclease known as Dicer, which falls into the RNaseIII ribonuclease family (Bernstein et al. 2001). It acts to degrade long dsRNA molecules into 21 - 25 nucleotide fragments known as small interfering RNAs (siRNAs). Cleavage by the dicer enzyme produces double stranded siRNAs with a 5'-phosphate and a two nucleotide 3' overhang (Elbashir et al. 2001). The population of siRNA molecules created is then available for integration into a large multimeric nuclease complex, which can target and cleave single stranded RNA with high specificity. The assembly of enzymes with a siRNA molecule is known as the RNA-induced silencing complex (RISC) (Hammond et al. 2000). Upon integration into the RISC the siRNA acts as a guide, targeting the complex in a sequence specific manner to homologous RNA (Nykänena et al. 2001). Endonucleolytic cleavage of the target mRNA then occurs which effectively disables it.

The elucidation of plant gene function has traditionally been based on the isolation of mutations in the gene of interest, or the production of transgenic plants where the genes expression has been repressed. There are several advantages of VIGS over the production of transgenic or mutant plants. For example it abolishes the need for laborious and time-consuming transformation, as well as the selection of the transgenics. VIGS can also be used to study the role of essential genes where the isolation of knockout mutants is impossible. The experimentation is relatively easy with the cost associated with the production of VIGS knockout plants being much lower when being compared to more traditional silencing techniques. It is also more amenable to high-throughput studies

and has been used successfully in model and non-model host systems (Lu et al. 2003; Burch-Smith et al. 2004; 2006b; Constantin et al. 2004; Fofana et al. 2004; Ding et al. 2006). The main drawbacks of using VIGS are that it leads only to a temporary repression of transcription. This means that if a phenotype needs time to develop following infection it may not be noticed. In addition, VIGS does not necessarily lead to a complete repression of a genes transcription, meaning that if a phenotype is not noted it may be that the small amounts of protein produced from small amounts of residual RNA is sufficient. At present efficient VIGS systems have not been well developed for organs other than leaves, so the role of genes cannot be studied in other parts of the plant, such as storage organs, using VIGS.

Tobacco-rattle virus (TRV) has been developed as a VIGS vector. It provides robust silencing, exhibiting a broad host range, is efficient in infecting meristematic tissue and produces only mild disease symptoms (Ratcliff et al. 2001; Liu et al. 2002; Burch-Smith et al. 2006b). Using *Agrobacterium* as a tool to introduce TRV into the host also overcomes the need for *in vitro* transcription of viral RNA or biolistic delivery techniques. VIGS has been used to study the *Leafy* gene which regulates flower development. Loss-of-function *leafy* mutants produce modified flowers that are phenocopied in TRV-*leafy*-infected plants (Ratcliff et al, 2001). A TRV vector containing the cellulose synthase (*CesA*) gene was used to silence that gene in tobacco leading to a modified cell wall (Burton et al, 2000).

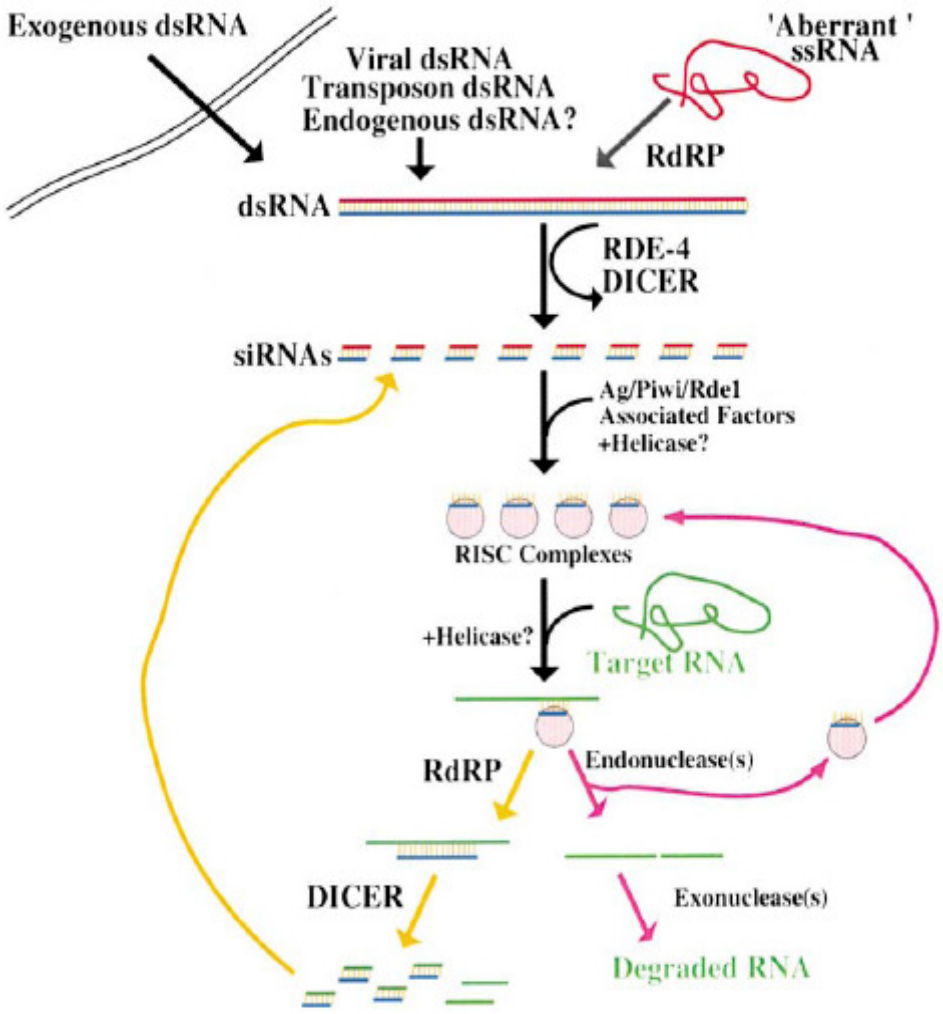


Figure 1.4: An overview of the silencing pathway and the various phases and enzymes involved. (Sijen et al, 2001)

1.6. Fruit metabolism

Carbohydrate metabolism in fruits has not been studied as extensively as it has been in leaves or other storage organs such as maize seeds or potato tubers. The main model plant that people have used to study fruit metabolism is tomato, and the reasons for the lack of progress in this area is probably because of the relatively large genome size (approximately 950 Mb; Asamizu (2007)) of tomato which means that it is more difficult

to isolate mutants in this species than in Arabidopsis. However in recent times the study of carbohydrate metabolism in developing tomato fruit has received more attention, owing to the uniqueness of the process as well as the importance of fruits in the human diet (Carrari and Fernie, 2006). Several features of the tomato fruit make it a highly interesting system to study, all of them linked to the dramatic metabolic changes that occur during development. Tomato fruit follows a transition from partially photosynthetic to true heterotrophic metabolism during development by accompanied by the differentiation of chloroplasts into chromoplasts and the dominance of carotenoids and lycopene on ripening. (Carrari and Fernie, 2006). Several studies have recently determines the role of specific enzymes on fruit metabolism, such as plastidial fructose 1,6-bisphosphatase (Obiadalla-Ali et al, 2004) and invertase (Fridman et al, 2004).

One advance that helps to study fruits has been the development of the dwarf Micro-tom tomato cultivar (Scott and Harbaugh, 1987) which is used as a model (Meissner et al, 1997). In a study by Obiadialla-Ali et al (2003) carbohydrate metabolism was analyzed during the fruit development of this cultivar. That study showed that starch accumulates very early in development and is then degraded as the fruit ripens, as has been demonstrated previously in normal tomato fruit (Ohyama et al, 1995; Klann et al, 1996; Chengappa et al, 1999; D'Aoust et al, 1999; Nguyen-Quoc et al, 1999). The 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. One way to examine this would be in tomato plants which are unable to degrade starch. Recently a GWD mutant in tomato has been identified (Nashilevitz et al, 2008) and given that plants in other species which lack

GWD are unable to degrade starch (Lorberth et al 1998; Yu et al, 2001) it is reasonable to assume that this will also be the case in tomato fruit. This will also be examined as part of this project.

1.7. Aim of the project

In this project I aim to study the function of *SEX4*, *LSF1* and *LSF2* cDNAs in tobacco using VIGS. Through the production of constructs and silencing of these genes, an indication of their function in this plant may be obtained. It will also be attempted to characterise these genes with respect to their substrate utilization by setting up a glyco-array. The glucans to be used on the array will be a series of differently phosphorylated starches which have been degraded by starch catabolytic enzymes and then fractionated by anion exchange chromatography. SEX4 and LSF1 and LSF2 proteins will be purified and used to probe the filter. Mass spectroscopy will then be used to identify which substrates they bind to best and may allow identification of the substrates which these enzymes act upon. I also aim to investigate the effect the SEX4 has on *E. coli* glycogen contents. This project also investigates the effect that a *gwd* mutation has on carbohydrate metabolism in leaves and fruits of the Micro-tom tomato cultivar variety.

Chapter 2

Protein Expression and Purification

2. Protein Expression and Purification

2.1 Introduction

As discussed in the general introduction (Section 1.3), it has been shown that SEX4 and LSF1 play a role in starch degradation in *Arabidopsis* (Zeeman and Rees, 1999; Niittylä et al., 2006; Kerk et al., 2006; Kötting et al., 2009; Comparet-Moss et al, 2009). However the same cannot be said for the LSF2 protein. In addition, the exact substrates which are utilized by the three proteins have also not been elucidated thus far. Therefore this aspect of the project is aimed at establishing a means to study these criteria through the production of antibodies. It involves the expression of the *SEX4, LSF1* and *LSF2* cDNAs in *E. coli* using the pGEX (GE Healthcare) vector system that allows the genes to be fused with the glutathione S-transferase (GST) tag. The proteins will then to be purified using GSTrap chromatographic columns together with the AKTA-prime FPLC instrument in order to produce antibodies in a rabbit host. The purified proteins generated in this part of project should allow us to study the substrates utilized by these proteins through the use of glyco-array technology. The antibodies generated can also be used to aid the functional study of these proteins in *Nicotiana bethamiana*, following their repression by virus-induced gene silencing.

2.2 Materials and Methods

2.2.1 Primers

Primers were designed against the *SEX4, LSF1* and *LSF2* gene sequences respectively. The primers include restriction sites at the beginning of the cDNAs to allow for the fragments to be ligated in-frame with the GST tag contained within the pGEX4T-

1 protein expression vector. (Table 2.1) the sequences were amplified by PCR using plasmid DNA as template and then analysed by agarose gel electrophoresis. PCR products were excised from the gel and purified using the Qiagen Gel Extraction Kit. *SEX4* and *LSF2* PCR products were ligated into the *Eco*RV site within pBluescriptSK (Stratagene, La Jolla, California) using T4 DNA Ligase (Promega) while *LSF1* was ligated into the pGEM-T-Easy vector system (Promega Corporation). This was done because *SEX4* and *LSF2* were amplified using *pfu Taq* polymerase (Fermentas), whilst the *LSF1* was amplified using Taq polymerase (Bioline). *SEX4* was excised from pBluescript using *Eco*RI and *Xho*I while LSF1 was cut with *Eco*RI alone. *LSF2* was excised by cutting with *Bam*HI and *Xho*I. All fragments were separated from the vector using agarose gel electrophoresis and purified from the gel using a commercially available kit (Qiagen gel clean up) before ligation into the pGEX-4T-1 vector using T4 DNA ligase. The genes were ligated into the same sites of the enzymes that were used to excise them from the pBluescript and pGEM-T-Easy vectors. Ligations were transformed into *E. coli* DH5α and grown on LB media containing ampicillin at 37°C overnight. Colonies obtained from the ligation were screened using colony PCR using the primers that were designed to amplify the respective genes and colonies containing inserts were transformed into *E. coli* BL21- CodonPlus cells (Stratagene).

SEX4	F'primer	5'-CGCC*gaattc***ATGAATTGTCTTCAGAATCTTCCC**-3'
		*Eco*RI
	R'primer	5'-CGCC*ctcgag***TCAAACTTCTGCCTCAGAAC**-3'
		*Xho*I
LSF1	F'primer	5'-CA*gaattc***ATGTCGTCTTCTTCTACTCCGT**-3'
		*Eco*RI
	R'primer	5'-**CTACGACTTTCCGCATAGTC**-3'
		None
LSF2	F'primer	5'-CGCG*ggatcc***ATGAGAGCTCTCTGGAAC**-'3
		*Bam*HI
	R'primer	5'-GCGG*ctcgag***TCAAGTGTCACGAAGGG**-3'
		*Xho*I

Table 2.1: Primers which had been designed for isolation of the respective genes. The underlined uppercase letters represent additional nucleotides added to protect the restriction site which is represented by the lower case *italic* letters. The **bold** UPPERCASE letters are nucleotides taken from the gene sequence.

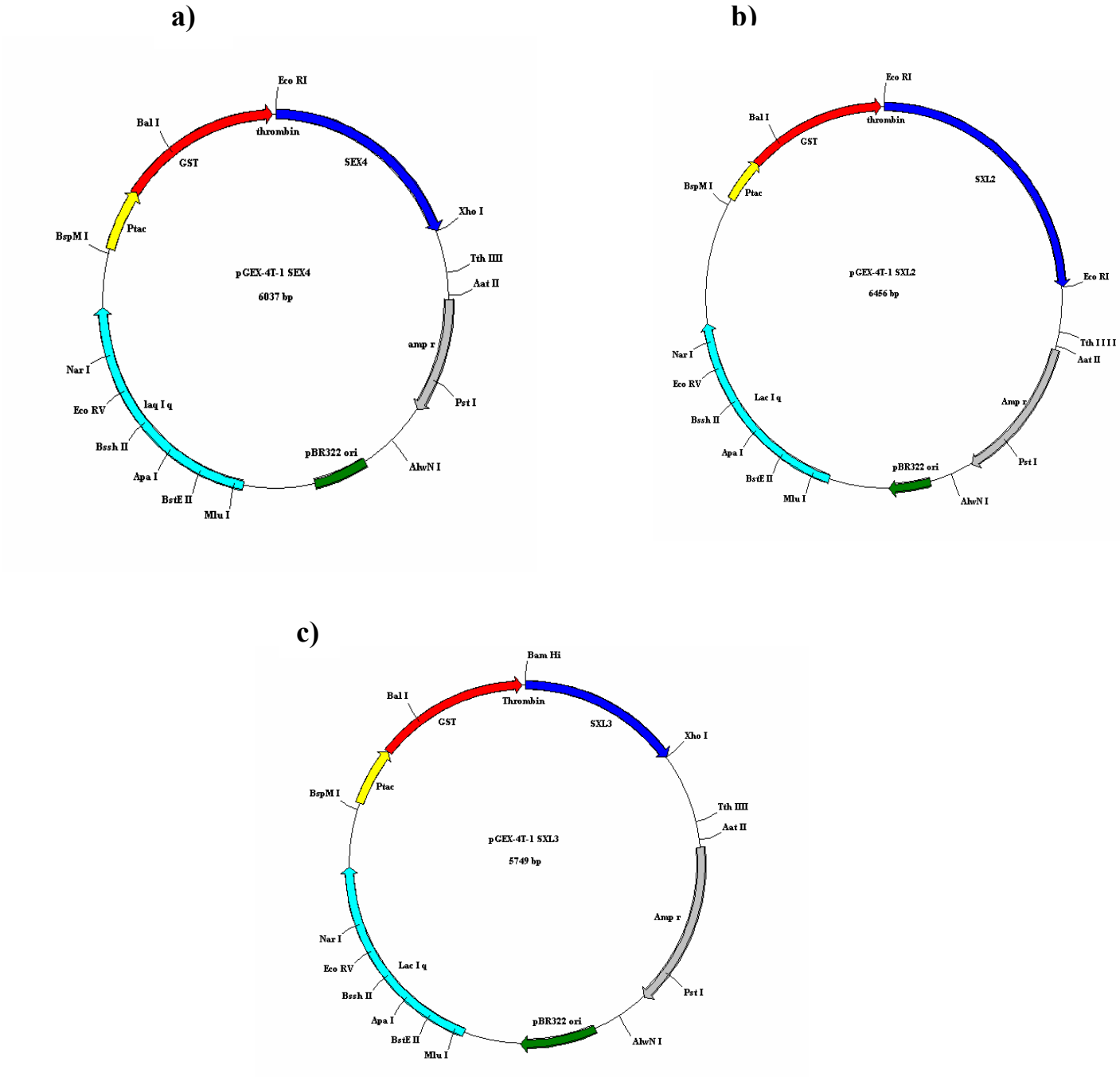


Figure 2.2: Protein expression constructs a) SEX4, b) LSF1 and c) LSF2 in pGEX4T-1

2.2 Protein Expression

Prior to protein purification the presence of the fusion protein was analysed by SDS-PAGE on proteins extracted from the various cultures containing the proteins. This involved inoculating BL21 containing the appropriate plasmid into 2ml LB media containing 20µg/ml ampicillin. The culture was incubated at 37ºC overnight and 2ml of overnight culture was inoculated into 200ml fresh LB media with the same antibiotic. This was again incubated at 37ºC with shaking until OD_{600} of 0.3 was reached. Expression of the protein was induced by the addition of IPTG to a final concentration of 2mM, 0.5mM, 1mM for SEX4, LSF1 and LSF2 respectively. The culture was then incubated at 22ºC overnight and 2ml of this was centrifuged at 20 000g for 2min and the supernatant discarded. The pellet was re-suspended in 0.4ml of SDS-reducing buffer (0.5M Tris-HCl ph 6.8; 40% (v/v) glycerol; 10% (w/v) SDS; 0.05% (w/v) Bromophenol Blue) and heated at 95ºC for 5mins. The sample was centrifuged at 20 000 g with the resulting supernatant being separated by SDS-PAGE. The gels were stained using Coomassie Colloidal Blue (Invitrogen) to examine if a protein of the correct molecular size was produced.

The remainder of the overnight culture was centrifuged at 7700g for 10 min at 4°C and the supernatant discarded. The cells were re-suspended in 10ml of ice-cold PBS (0.14M NaCl; 2.7mM KCl; 10mM Na_2PO_4; 1.8mM KH_2PO_4, pH 7.3) and frozen at -20ºC overnight. Once it was determined that the cells contained the fusion protein the sample was thawed by incubation at 37ºC for 15min then stored on ice prior to disruption by sonication. The sonicated sample was centrifuged at 7700 g for 10mins at 4ºC. The

soluble extracts were examined using SDS-PAGE to examine presence of the protein in the soluble fraction prior to further purification.

2.3 Protein Purification

The proteins were purified using 1ml GSTrap columns together with an AKTA prime fast protein chromatography instrument. The column was equilibrated using 10 column volumes (CV) of PBS (pH 7.3). The protein sample was filtered through a 0.45μM membrane and then loaded onto the column. This was again washed with 10 – 20 CV of PBS before being eluted using glutathione elution buffer (20mM reduced glutathione in 50mM Tris-HCl, pH 8.0). Fractions were collected and separated by SDS-PAGE before staining with Coomassie Colloidal Blue (Invitrogen Life Technologies).

2.4 Immunoblot Analysis

Proteins were separated by 10% (w/v) SDS-PAGE and blotted onto a PVDF transfer membrane using a semi-dry blotting system (Bio-Rad). The membrane was removed and blocked in 4% (w/v) fat free milk powder in water overnight. Primary antibody (1:1000 dilution) was added to each of the membranes in TBST-T (20mM Tris (pH 7.6, 137mM $NaCl_2$, 0.1% (v/v) Tween-20) buffer and incubated for 1 hour, followed by the removal of primary antibody before being washed three times for 5 minutes in TBS-T buffer. Secondary antibody (ReserveAPTM phophatase labelled Goat anti Rabbit IgG (KPL, Gaithersburg, MD 20878 USA) was then added (1:10000 dilution) and incubated for 1 hour in TBS-T. Upon removal of the secondary antibody, another three 5 minute washes were performed. The membranes were rinsed in H_2O, before substrates BCIP/NBT (5-Bromo-4-Chloro-3'- Indolyl Phosphate p-Toluidine/ Nitro-Blue

Tetrazolium Chloride) were added. Once the desired staining is obtained the reaction was stopped by rinsing the membranes with H_2O.

2.5 Results and Discussion

2.5.1 Construct Production

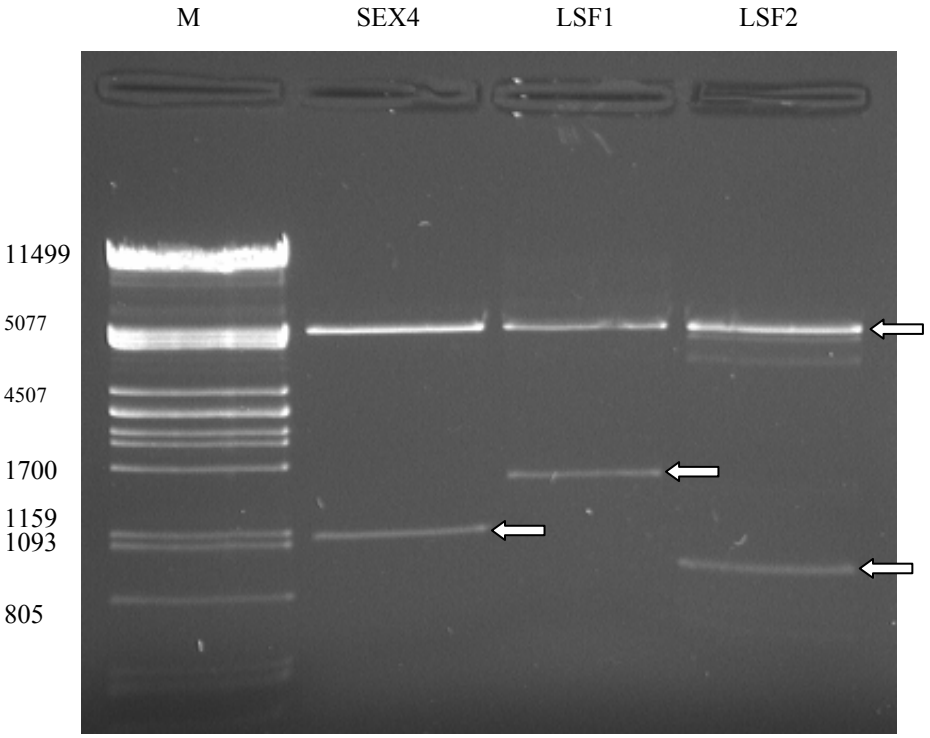


Figure 2.3: 0.8% (w/v) agarose gel showing inserts present within the pGEX-4T-1 protein expression vector. Lane 1: Lambda PST Molecular marker; Lane 2: *Sex4*; Lane 3 *Lsf1* and Lane 4: *Lsf2*.

I manufactured three constructs in pGEX4T-1 designed to allow purification of SEX4, and LSF2 from tobacco and LSF1 from Arabidopsis. The reason for the different species is that I wish to study the function of these proteins in tobacco using VIGS, so would prefer to manufacture antibody against the tobacco proteins, however I was unable to amplify a full length tobacco cDNA coding for this. I obtained a full length Arabidopsis *LSF1* cDNA from Dr Oliver Kötting and Prof. Samuel Zeeman (ETH,

Zurich). Given that antibodies against proteins from one species normally cross-react to antibodies from other species it was decided that this would be used to produce antibody against LSF1. Figure 2.3 shows a 0.8% agarose gel. Lane 1 contains the marker, while lanes 2, 3 and 4 contain restriction enzyme digests of the various constructs. These have been digested either side of the insertion site. The SEX4 construct was cut with a combination of *Eco*RI and *Xho*I, LSF1 was cut with *Eco*RI and LSF2 was cut with a combination of *Bam*HI and *Xho*I. The gel confirms that inserts of the correct size were present within the pGEX-4T-1 with lanes 2, 3 and 4 showing inserts of 1137bp for SEX4, 1520bp for LSF1 and 849bp for LSF2.

2.5.2 Protein Expression

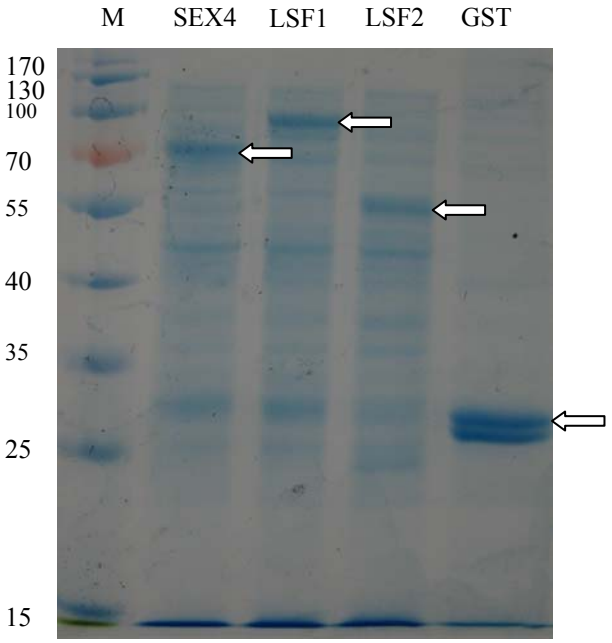


Figure 2.4: 10% (w/v) SDS-PAGE Lane M: Fermentas pre-stained protein marker; Lane 2: SEX4; Lane 3:LSF1 and Lane 4: LSF2; Lane 5: Glutathione S Transferase (GST)

Figure 2.4 shows a SDS-PAGE gel stained with colloidal Coomassie Colloidal blue where proteins extracted from pellets by heating in SDS reducing buffer have been separated SDS-PAGE. The figure shows that all the heterologously expressed plant proteins are present at the correct molecular size. Lane 2 shows a band at 70 kDa which corresponds to the SEX4 GST fusion protein, lane 3 shows a band at around 80 kDa, corresponding to the LSF1 GST fusion protein and a band at 55 kDa which represents the LSF2 GST fusion protein in present in lane 4. Lane 5 shows a band at approximately 28 kDa that corresponds to GST alone, produced by the empty pGEX-4T-1 vector. This demonstrates that the proteins are being manufactured in the bacteria and can be used for further purification.

I was unable to produce soluble extracts from containing the LSF1 protein (data not shown). This is probably because LSF1 is present in inclusion bodies, something that has been observed by other groups (Prof Samuel Zeeman, Dr Oliver Kötting, ETH Zurich Pers. Comm.). As such I was unable to continue the purification of LSF1.

2.5.3 Protein Purification

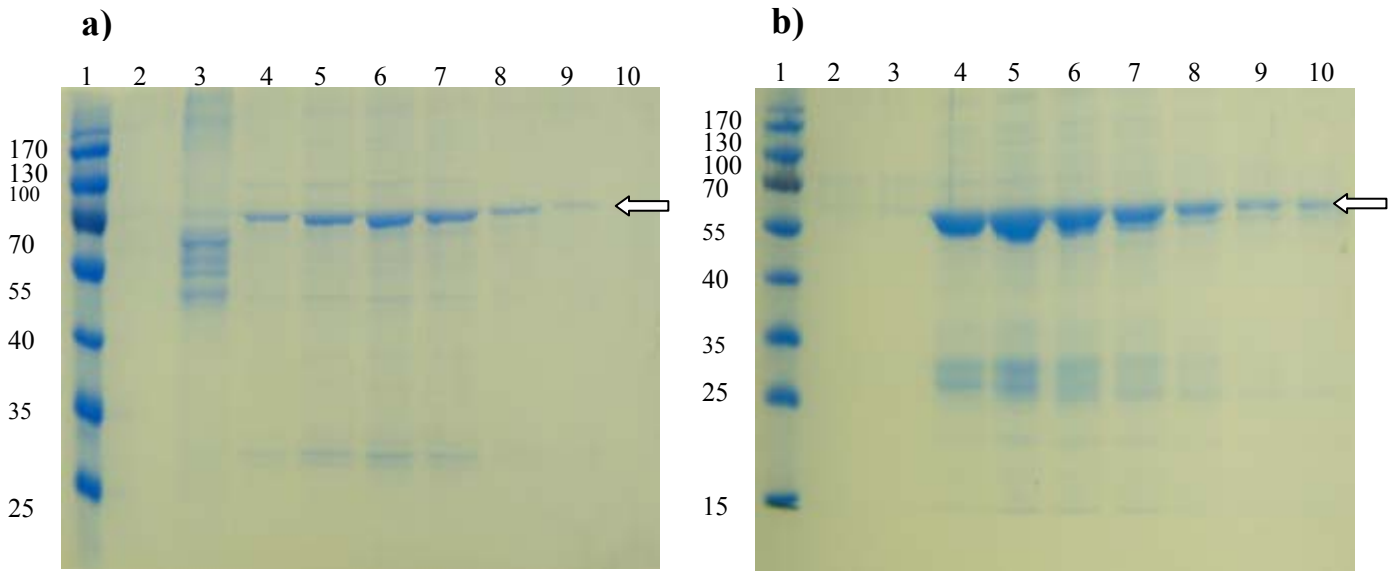


Figure 2.5 10% (w/v) SDS-PAGE gel with purified fractions of a) SEX4 and b) LSF2 proteins. Fractions of each of these were combined and an aliquot containing 1mg/ml was used to produce antibodies against the respective proteins.

I manufactured soluble extracts containing either SEX4 or LSF2 fusion proteins and purified them using a GSTrap column. Figure 2.5a shows the various fractions which eluted from the column after loading with the SEX4 fusion protein extract. These were separated by 10% SDS PAGE and stained with Coomassie Colloidal Blue (Invitrogen Life Systems). Figure 2.5b shows a similar gel containing the LSF2 fusion protein fractions. It can be seen that both proteins were fractionated successfully, with minor contamination from other proteins. The protein concentration of the various fractions were determined with a 1ml sample of each of the proteins at a concentration of 1 mg total protein/ml being submitted to Prof. Dirk Belstedt (Stellenbosch University) for antibody production. As I was unable to purify the LSF1 protein I obtained an antibody raised against the Arabidopsis protein from Prof. Samuel Zeeman and Dr Oliver Kötting

(Institute of Plant Science, ETH Zurich, Switzerland) which has previously been used to recognise LSF1 in Arabidopsis leaf extracts (Comparet-Moss et al, 2009).

2.5.4 Immunoblot Analysis

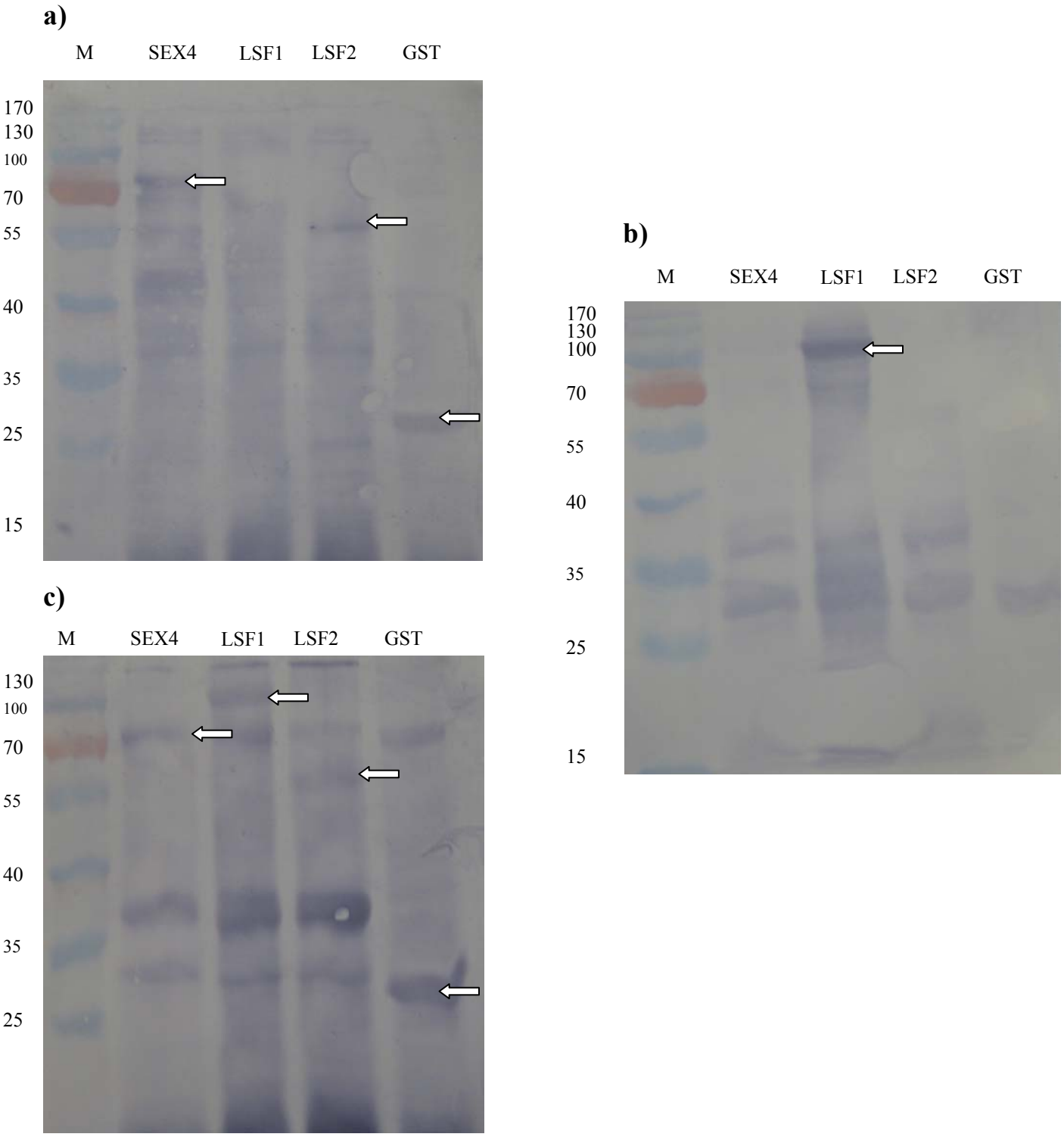


Figure 2.6a-c: Immunoblot analysis of *E.coli* extracts tested against the a) SEX4, b) LSF1 and c) LSF2 antibody.

To test the potential cross-reactivity of the antibodies I separated extracts containing the fusion proteins using SDS-PAGE and blotted them onto nylon membranes. These were then probed with the various antibodies and analysed for binding using immunoblots. Figures 2.6a, b and c show the results for the anti-SEX4, anti-LSF1 and anti-LSF2 antibodies respectively. The first thing to point out is that the anti-SEX4 and anti_LSF2 antibodies both recognise the GST tag alone (Fig 2.6a,c). Unsurprisingly both of these antibodies also recognise both SEX4:GST and LSF2:GST fusion proteins. The LSF2, but not the SEX4 antibody also recognises the LSF1:GST fusion protein (Fig 2.6a,c). It isn't clear whether the cross-reactivity is due to their attachment to the GST part of the fusion protein alone, or is genuine cross-reactivity to the plant proteins due to similarities in peptide sequences. Figure 2.6b shows that the LSF1 antibody is able to detect the LSF1:GST fusion protein, but does not recognise the other two or the GST alone.

These data demonstrate that the three antibodies recognise the heterologously expressed fusion proteins that they are designed to recognise. This does not mean, however, that they will recognise the proteins in plant extracts. It might be that the proteins are not present in high enough concentrations in plants for the antibodies to pick them up or, in the case of the anti SEX4 and anti LSF2 antibodies, that they only recognise the GST. There appears to be a high amount of background in the antibodies, which is probably due to the small amounts of native proteins that were co-purified from the GSTrap column. This is unlikely to be a problem when studying the protein in plant extracts as they will not contain these proteins, but this will be tested in Chapter 4.

Chapter 3

Production of starch breakdown products for use in glyco-arrays

3. Production of starch breakdown products for use in glyco-arrays

3.1 Introduction

As was discussed in the general introduction, the function of the *SEX4*, *LSF1* and *LSF2* genes is becoming better understood with roles being assigned for the SEX4 and LSF1 proteins (Zeeman et al., 1998; Niittylä et al., 2006; Fordham-Skeltonet al, 2002; Kerk et al, 2006; Sokolov et al, 2006; Kötting et al., 2009; Comparet-Moss et al, 2009). No studies have been performed as yet that have elucidated the exact substrates utilized by these proteins. It is known that SEX4 can dephosphoylate amylopectin (Niittylä et al., 2006), but during the starch breakdown process amylopectin is degraded by many different enzymes. It is possible that one of these breakdown products is the preferred substrate for SEX4 and LSF proteins. The aim of this part of the thesis is to try and produce a system to allow this to be studied. One way to do this would be to utilize glyco-array technology where glucans are bound to a surface and are probed with either a purified protein or a mixture of proteins. The glucan to which the protein binds preferentially can be examined using antibodies that recognize the protein, or by using a mass spectrometer. In this section I describe the production of starch degradation products which will be used in such glyco-arrays in future.

3.2 Materials and Methods

3.2.1 Analysis of starches used in the study

Starches isolated from nine different types of genetically modified potato lines were used. These starches were a kind gift of Bayer Crop Science GmBh and were isolated from transgenic potatoes where one or several enzymes within the starch metabolic pathway have been repressed, resulting in starches with different structures.

3.2.2 Determination of the glucose 6-phosphate content of the starches

0.5ml of 0.7M HCl solution was added to 150mg of starch. The mixture was heated at 95°C for 4 hours before 100µl of this solution was added to 100µl of 0.7M KOH. 30µl was combined with 230µl assay buffer containing (100mM MOPS pH 7.5; 10mM $MgCl_2$; 2mM EDTA; 2mM NAD). An absorbance reading was taken at 340nm before 1U glucose 6-phosphate dehydrogenase from *Leuconostoc* was added. The difference in absorbance is used to calculate the G 6-P amount.

3.2.3 Isolation of amylopectin using thymol

200mg of starch was dissolved in 12ml 90% DMSO overnight. 3 volumes of ethanol was added and mixed well. The samples were then centrifuged at 5000g for 5 minutes to harvest the degranularised starch. The precipitate was ground to a fine powder and ethanol was added to wash and the sample centrifuged again as before. This pellet was dissolved in 40 ml of 1% (w/v) NaCl at 80°C and the solution cooled to 30°C before 80mg thymol was added with stirring. This was incubated at 30°C for 60hr without

stirring to precipitate amylose. Amylose was harvested by centrifugation as before. This was washed twice with thymol-saturated water, re-suspended in ethanol and then again centrifuged as before followed by washing twice with acetone at -20°C.

Amylopectin was precipitated by adding 3 volumes of ethanol to the supernatant from the 60h incubation, followed by harvesting by centrifugation at 5000g for 5 min. The precipitate was then washed with ethanol, ground to a powder in a pestle and mortar and washed twice with acetone that was at -20°C.

3.2.4 Anion Exchange Chromatography

10mg of potato starch or the isolated amylopectin was placed in a 2ml microcentrifuge tube, to which 1.5ml water was added. The solution was incubated at 100ºC for 2min to dissolve the starch. The solution was then cooled to room temperature before 14U of isoamylase was added and incubated at 37ºC for 2 – 3hrs. The solution is then incubated at 100ºC to inactivate the enzyme. The same experiment was repeated using 14U β-amylase or 14U of α-amylase.

A DEAE sepharose column was prepared by placing a piece of glass wool inside a Pasteur pipette. 2ml of DEAE sepharose was added to the column. 10 column volumes (CV) of 5mM Tris-HCl (pH 7.8) solution were passed through the column to equilibrate the column. 100µl of the enzyme-digested sample was added and non-phosphorylated glucans were eluted in 1ml aliquots of water. 10µl of each eluted sample was added to 100µl of Lugols solution (4% (w/v) Potassium Iodide, 2% (w/v) Iodine reagent to monitor elution of glucans. The eluted samples were tested until no colour change

occured indicating that all the non-phosphorylated chains had been isolated. Once this happened the phosphorylated glucans were eluted using 1ml aliquots of a solution containing 100mM NaCl and 10mM HCl. The eluted samples were then again tested using Lugols reagent to test for the presence of eluted glucans.

3.3 Results and Discussion

The transgenic potato lines from which the starch was extracted are described in Table 1. All transgenic lines were in the Desirée cultivar

Enzymes repressed
Wild Type
Glucan, Water Dikinase (GWD)
Starch Synthase III (SSIII)
Branching enzyme I (BEI)
BEI and SSIII
BEI and GWD
GWD, BEI and Branching Enzyme II (BEII)
BEI, BEII and SSIII
BEII, BEI and SSIII

Table 3.1: Genetically modified potato lines from which the different starches were derived.

3.3.1 Glucose-6-Phosphate Determination

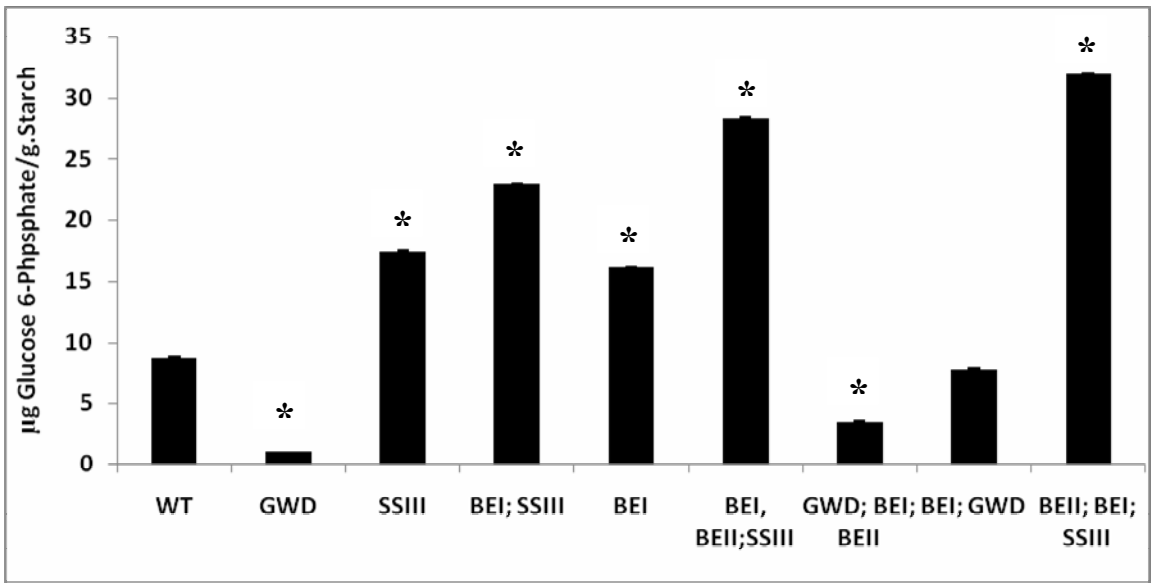


Figure 3.1: Glucose 6-phosphate content of the various gentically modified potato starch lines.Data represents means ± SEM of three independent samples. * denotes a statistically significant difference from the WT control at the 5% level (*Student's t*-test).

Figure 3.1 shows the glucose 6-phosphate contents of starches from the various lines. From the figure we can see how this differs in the different transgenic lines in comparison with that of the WT. In these lines a number of enzymes within the starch biosynthetic pathway have been repressed. It has previously been shown that repression of SSIII, BEI or BEII lead to accumulation of starch that has more covalently bound phosphate (Kossmann et al, 1999) This is the case in all these samples. In addition when more than two of these enzymes are repressed even further increases in starch phosphate were seen (Safford et al, 1998; Jobling et al, 1999, 2002; Kossmann et al 1999). The exception to this is when the GWD is repressed. In all cases this leads to a decrease in

starch phosphate, consistent with its known role in incorporating phosphate into starch (Ritte et al., 2002, Mikkelsen et al, 2004).

3.3.2 Qualitative Glucan Concentration Determination

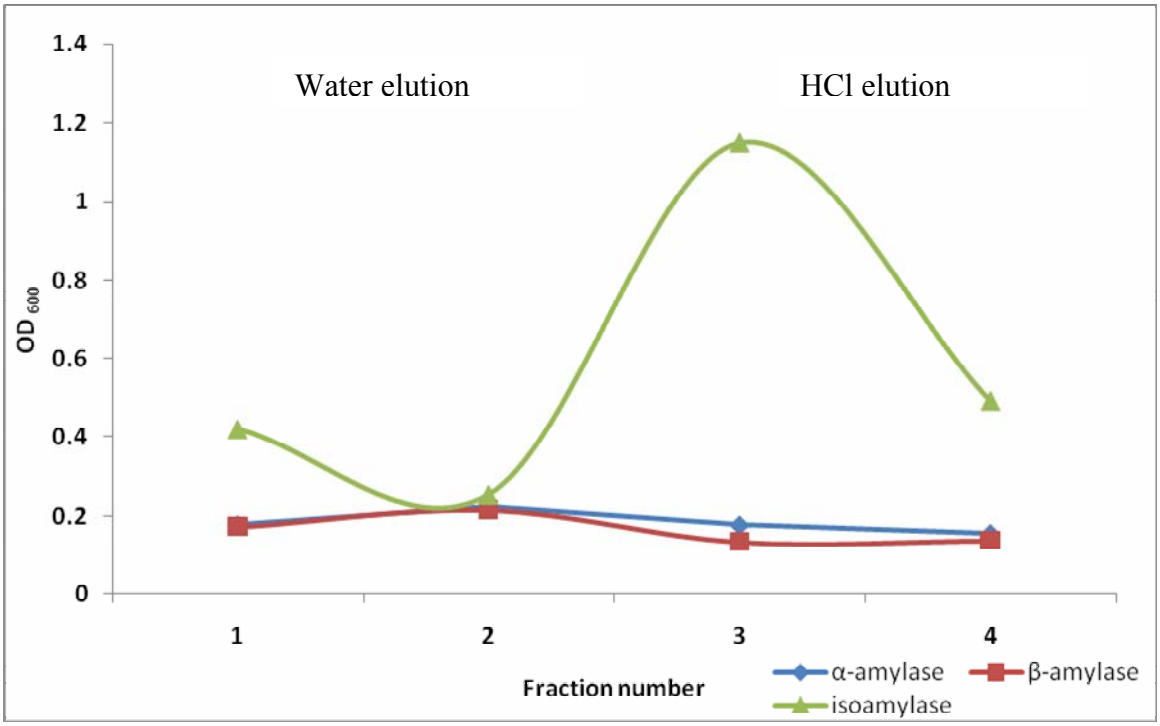


Figure 3.2: Elution pattern of glucans from DEAE sepharose column. Digested glucan samples were added to the column and eluted with water to isolate non-phospohorylated chains (Fraction 1-2) or NaCl and HCl to elute phosphorylated glucans (Fractions 3-4)The figure shows an example of starch isolated from untransformed control potatoes digested with the enzymes β-amylase, α-amylase or isoamylase. Aliquots of the fractions were added to Lugols solution and absorbance was determined at 600nm to qualitatively determine presence of glucans.

The aim of this aspect of the project was to digest starch and separate them into phosphorylates and unphosphorylated chains.. This was done for all the starches from the transgenic lines as well as amylopectin isolated from the starches. An example is shown in Figure 3.2 for a sample of WT amylopectin which had been degraded with α-amylase, β-amylase or iso-amylase before being loaded onto the anion exchange column. Fraction

1 and 2 represent the non-phosphorylated glucans which eluted with water while fractions 3 and 4 represent the phosphorylated glucan chains which eluted with an NaCl/HCl solution. The sample digested with α- or β-amylases produced relatively little glucan, although this might be because the elution of the glucans was only monitored qualitatively using an iodine solution. As the colouration of iodine by glucans depends on the chain length, it might be that small molecules were produced by digestion using α- or β-amylases which would not stain intensively. Digestion with isoamylase though produced more pronounced fractions, indicating longer chains. This would be expected as isoamylase only cleaves the α1-6 branchpoints within amylopectin, while α- and β-amylases digest α1-4 bonds, which make up the majority of the starch molecule..

These samples are the start of a longer term project. I have produced 109 fractions of starch and amylopectin breakdowbn products. What remains to be done now is to spot these onto chips and probe them with the purified SEX4, LSF1 and LSF2 proteins that I described in Chapter 2.

Chapter 4

Examination of the roles of the SEX4 and LSF proteins in *Nicotiana benthamiana* leaf starch degradation using virus induced gene silencing

4. Examination of the roles of the SEX4 and LSF proteins in *Nicotiana benthamiana* leaf starch degradation using virus induced gene silencing

4.1 Introduction

As mentioned in the general introduction much work has been done to examine leaf starch degradation in the past decade. Most of this has been performed in Arabidopsis, but it isn't clear whether the knowledge gathered from these studies is applicable in other species. In addition, although it has been demonstrated in Arabidopsis that SEX4 and LSF1 play a role in starch degradation (Zeeman and Rees, 1999; Niittylä et al., 2006; Kerk et al., 2006; Kötting et al., 2009; Comparet-Moss et al, 2009), it hasn't been examined if LSF2 is involved also. To do this in Arabidopsis would require the isolation of a knockout mutant in the *AtLSF2* gene, or production of transgenic plants lacking the protein. A quicker way to study the role of LSF2 would be to repress its activity in tobacco using virus induce gene silencing (VIGS). In addition this technique can be used to examine the roles of SEX4 and LSF1 in a species other than Arabidopsis.

For this component of the project, therefore, I aim to ascertain the function of the SEX4, LSF1 and LSF2 proteins in *Nicotiana benthamiana* by repressing their activities using VIGS and examining whether or not this impairs starch degradation. The system involves infection of the plants with TRV vector system. This system uses two vectors, derived from binary transformation plasmids, which have cDNAs encoding the TRV RNA1

(TRV1) and TRV RNA2 (TRV2) which has been inserted into the T-DNA region (Ratcliff et al, 2001). What this means essentially is that when the each vector contain different parts of the TRV genome. The two vectors can be combined by transforming them separately in *Agrobacterium tumefaciens* and then combining cultures containing the vectors. These can be infiltrated into plants and leads to the production of TRV in the plants. Both vectors contain a duplicated 35S promoter and a self-cleaving ribozyme sequence to enable rapid generation of intact viral transcripts (Gould and Kramer, 2007). Genes essential for plant to plant transmission of TRV through its nematode vector (Hernandez et al, 1997) have been deleted from TRV2 (Ratcliff et al, 2001), however, TRV2 has been engineered to contain a polylinker into which plant cDNA's can be ligated. When this is done and the vectors are used to produce TRV in plants it leads to specific down regulation of the plant gene inserted into TRV2.

4.2 Materials and Methods

4.2.1 Construct Production

Tobacco rattle virus VIGS vectors were obtained from Prof. Dinesh-Kumar (Yale University). These were TRV1, TRV2 and TRV::PDS (Dinesh-Kumar et al, 2003). The last vector contains a tobacco sequence for the *phytoene desaturase* (*PDS*) gene which is used as a positive control. TRV2 contains a polylinker which allows the insertion of DNA coding for plant genes to be repressed.

Three vectors containing *SEX4, LSF1* and *LSF2* cDNAs were obtained from Dr James Lloyd (Institute of Plant Biotechnology, Stellenbosch University). They were

originally *Nicotiana tabacum* expressed sequence tags (EST) which were obtained from the French Plant Genomic Resource Center (http://cnrgv.toulouse.inra.fr/en). The ESTs used were KL4B.111M23F (*Sex4*), KT7B.107M01F (*LSF1*) and KP1B.110M02F (*LSF2*). These sequences were present within pBluescriptSK+ (Stratagene, La Jolla, California). Inserts were excised from this plasmid using a combination of *Bam*HI and *Xho*I and ligated into the TRV2 vector cut with the same restriction enzymes.

4.2.2 Plant Preparation

Seeds of *N. benthamiana* were sterilized by suspending them in 1ml of 70% (v/v) ethanol. The tube was mixed by inversion for 2mins, after which the ethanol was decanted and the same step was repeated. Subsequently the seeds were re-suspended in 1ml 1% (w/v) sodium hypochloride. The tube was again mixed by inversion then allowed to stand for 20mins. The bleach solution was decanted and 1ml of sterile distilled water added to wash the seeds. The tube was again mixed by inversion, the water removed and the washing step repeated for a second time. The seeds were germinated on MS media containing 4.3 % (w/v) Murashige and Skoog (MS) medium with vitamins, 1.5% (w/v) sucrose and 4% (w/v) PlantGel (Highveld Biological).

The seeds were left to grow for 7 to 10 days before they were sub-cultured onto MS media. 2 weeks after this transfer the plantlets were infiltrated with an *Agrobacterium* suspension and planted into seedling mix (Master Organics).

4.2.3 *Agrobacterium* transformation

The *Agrobacterium tumefaciens* strain GV2206 was transformed with either the pTRV2 vector containing a cDNAs coding for one of *SEX4*, *LSF1*, *LSF2*, *PDS*, the empty pTRV2 vector to act as a control or the TRV1 vector (Ratcliff et al, 2001) using the freeze/thaw method (An et al, 1988).

4.2.4 Vacuum Infiltration

A. tumefaciens GV2206 containing TRV-VIGS vectors were grown at 28˚C in LB liquid media containing the antibiotics streptomycin (10µg/ml), carbenicillin (20µg/ml), kanamycin (50µg/ml) and rifampicin (25µg/ml). The cells were collected by centrifugation, re-suspended in sterile infiltration media (10mM $MgCL_{2;}$ 10mM MES-KOH pH 5.6; 150µM acetosyringone) and the OD_{600} adjusted to 0.5 using the infiltration media.

For the plant infiltration the *Agrobacterium* containing the TRV1 vector was mixed in a ratio of 1:1 with *Agrobacterium* containing TRV2, TRV2*::SEX4*, TRV2::*LSF1*, TRV2*::LSF2* or TRV2::*PDS*. The plantlets are then placed inside a plastic 60ml syringe with 20ml *Agrobacterium* solution. The plunger of the syringe was pulled out to reduce the air pressure within the syringe of approximately 50 kPa which was maintained for 30 seconds before the vacuum was broken, resulting in the media being infiltrated into the leaves of the plant. The plants were then placed into seedling mix (Master Organics) in 10cm pots. 14 – 21 days after the infiltration leaf discs of the plants were collected and used to determine starch contents when photobleaching was noticed in

the control plants infiltrated with a VIGS vector designed to repress the activity of phytoene desaturase.

4.2.5 Determination of leaf starch content

30mm^2 leaf discs were taken and incubated with 1ml of 80 % (v/v) ethanol at 80°C for 1 hour. Following removal of the supernatant, the leaf discs were washed with 80 % (v/v) ethanol. Upon removal of this, 0.4 ml of 0.2 M KOH was added. The samples were heated at 95 °C for 1 hour to solubilise the starch, after which 70µl of 1M acetic acid was added to neutralise the solution.

10µL of the solubilised starch solution was mixed with 10µL of 50 mM NaAC pH 5.6 containing 10 U/ml amyloglucosidase (from *Aspergillus niger*) and incubated at 37°C for 2 hours to digest the starch to glucose. 250µL of assay buffer (10 mM Imidazole (pH 6.9), 5 mM $MgCL_2$, 1 mM ATP, 1 mM NAD) was then added. The reaction was started by addition of 1 U/ml hexokinase from yeast and 1 U/ml glucose 6-phosphate dehydrogenase from *Leuconostoc* and the increase in absorbance at 340nm was determined. This was used to calculate the amount of hexose equivalents present in the sample.

4.2.6 Extraction of soluble protein from plant leaf material

The different plants which had been silenced with the various genes as well as the unsilenced control plants were harvested. They were ground in liquid nitrogen using a mortar and pestle. 200mg fresh weight was placed into a 2ml microcentrifuge tube to which 500µl of protein extraction buffer (50mM Tris-HCl pH 8.0; 2mM EDTA; 5mM

dithiotreitol (DTT) was added. Samples were vortexed for 1 minute and then centrifuged at 7700g for 10min with the supernatant taken and separated using SDS-PAGE electrophoresis.

4.2.7 Immunoblots

Proteins were separated as described in Section 2.2. The blots were tested using antibodies raised against the SEX4, LSF1 and LSF2 mentioned in chapter 2.

4.3. Results and Discussion:

4.3.1 Construct production

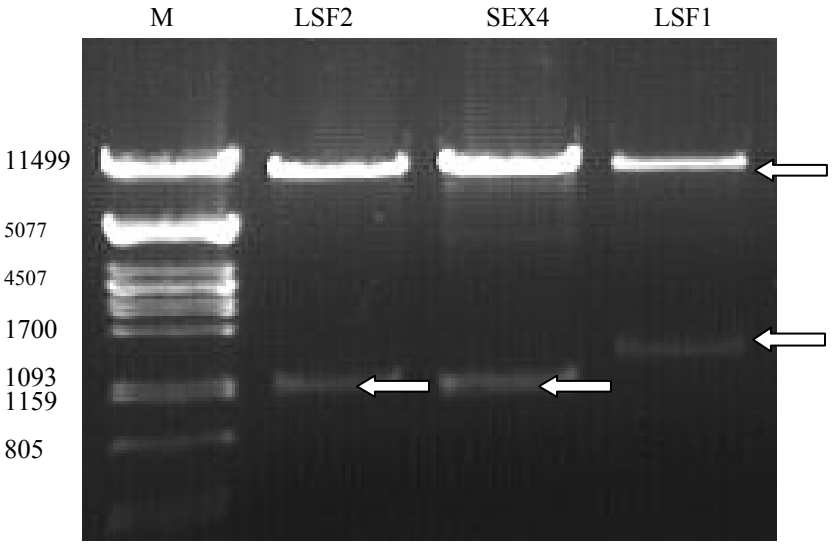


Figure 4.1: 0.8% (w/v) agarose gel showing plasmid DNA of the VIGS constructs following restriction. M denotes the molecular marker (λDNA digested with *PstI*). LSF2, SEX4 and LSF1 denote TRV2 vectors containing inserts of those cDNA's digeseted with *BamHI* and *XhoI*.

Figure 4.1 confirms that the three inserts that were required for the silencing experiment were successfully cloned into the TRV2 vector. The figure shows the three

respective cDNAs encoding LSF2, SEX4 and LSF1 were indeed present within the TRV vector. The sequences used here were full length clones for the *SEX4* and *LSF2* while a partial clone was used for *LSF1*. The figure shows a 0.8% (w/v) agarose gel with each of the constructs being subjected to restriction digest with *Bam*HI and *Xho*I. In lane M Lambda *Pst* molecular marker was used. In lane LSF2 and SEX4, a band of approximately 900bp as well as a band of 1100bp can be seen, which represents the *LSF2* and *SEX4* cDNAs present within the TRV2 vector. Lane LSF1 shows bands of approximately 1500bp and 11500bp, which correspond to the *LSF1* and TRV2 vector respectively.

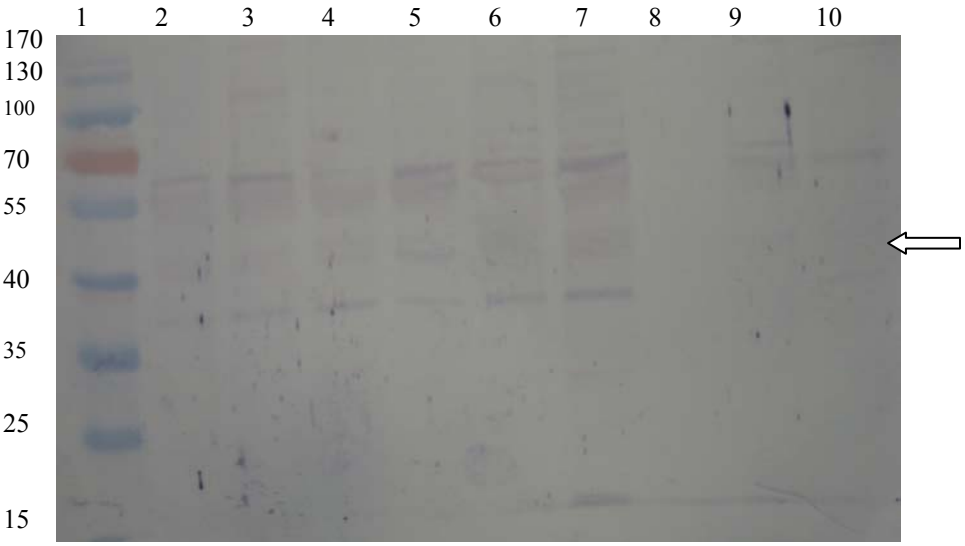
4.3.2 Virus Induced Gene Silencing

Figure 4.2: Photo bleaching of a tobacco plant that through silencing of the *PDS* gene. The plant of the left has been infiltrated with the empty TRV2 vector, while the plant on the right was infiltrated with the TRV2::PDS vector.

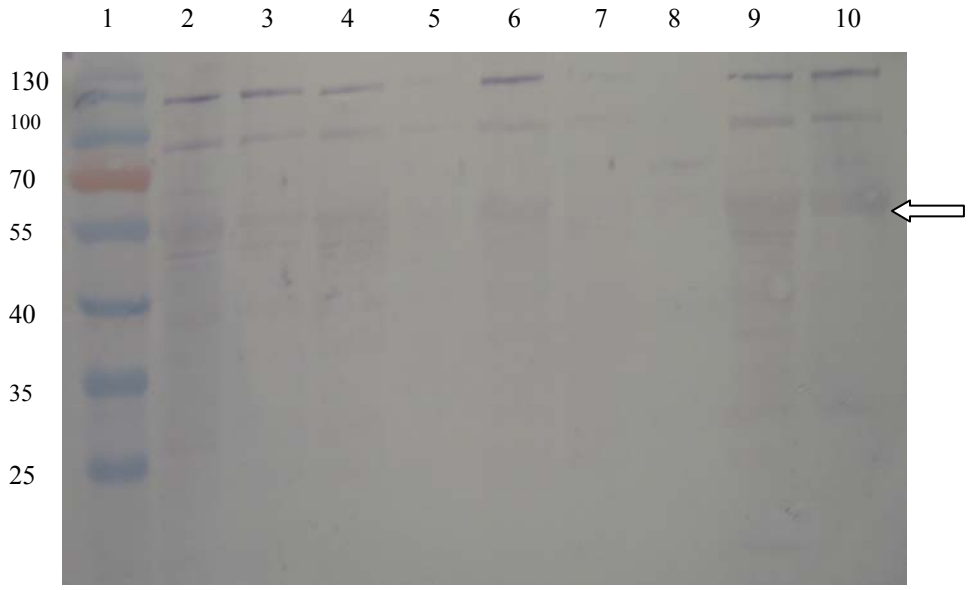
The VIGS system that I used utilises two vectors, TRV1 and TRV2, both of which have to be present within *Agrobacterium tumefaciens* for the silencing protocol to succeed. I manufactured three TRV2 constructs which should allow silencing of SEX4, LSF1 or LSF2 in *N. benthamiana*. As a positive control I used a previously manufactured construct (Kumagai et al, 1995), which allows silencing of the *PDS* gene, and leads to a bleaching phenotype in plants due to an inability to manufacture chlorophyll. These were transformed into *A. tumefaciens* which was then grown in appropriate growth media, re-suspended in infiltration media and combined with *A. tumefaciens* containing TRV1. Figure 4.2 shows plants where *PDS* has been silenced. I used this phenotype as a marker for gene repression assuming that when this phenotype was noted plants which were infiltrated at the same time, but with other constructs, would also be exhibiting gene repression.

4.3.3 Analysis of SEX4, LSF1 and LSF2 protein levels using immunoblots

a)



b)



c)

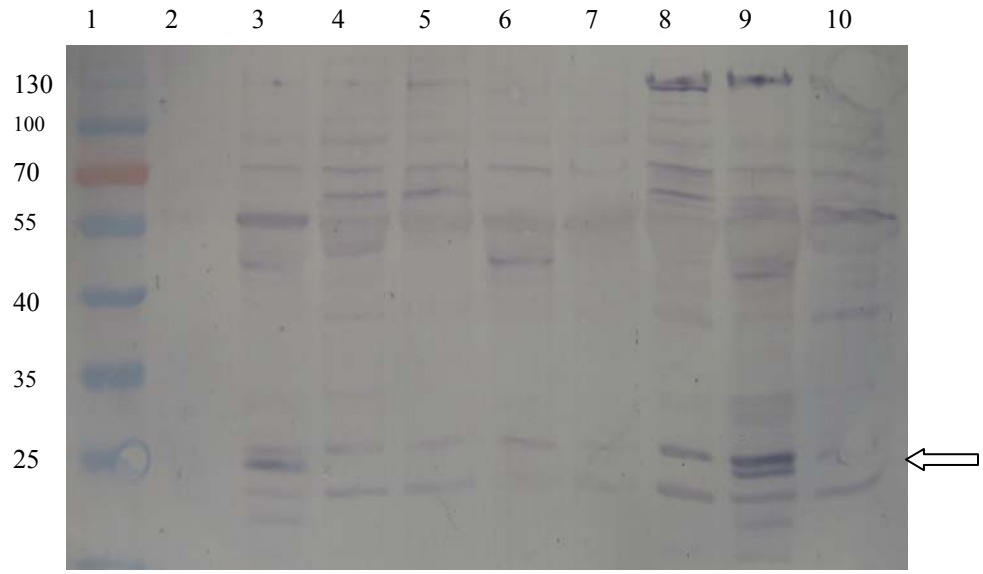


Figure 4.3a- c: Immunoblot analysis of the a) *Sex4* b) *LSF2* and c) *LSF1* silenced plants. The figure shows Fermentas pre-stained protein marker lane 1. Soluble protein extracts from silenced plants were loaded in lanes 2 – 7 and protein extracts from control plants were loaded in lanes 8 – 10. The arrows represent the location where the expected protein bands should be seen which corresponds to the MW of the protein

To elicit silencing of SEX4, LSF1 and LSF2 in *N. benthamiana* I took *A. tumefaciens* containing the appropriate TRV2 vectors that I had manufactured, combined them with TRV1 containing *A. tumefaciens* and infiltrated seedlings. As a control I used the TRV2 empty vector.

To see whether infiltration of seedlings with the constructs led to repression of SEX4, LSF1 and LSF2 proteins, immunoblots were performed. The antibodies used were manufactured as described in Chapter 2 where the SEX4 and LSF2 antibodies had been tested using recombinant protein expressed in *E. coli*. The LSF1 antibody has been demonstrated to recognise the Arabidopsis protein in plant extracts from that species (Comparet-Moss et al, 2009).

Plant samples were harvested from the various silenced and control plants. Total protein was extracted, separated by SDS-PAGE and blotted onto nylon membranes prior to being probed with antibodies. Figure 4.3a-c shows extracts from the SEX4, LSF1 and LSF2 silenced plants respectively, in lane 2 to 7 as well as those from the control in lanes 8 to 10. The results are difficult to interpret as all the antibodies appear to demonstrate much non-specific binding. This would not matter if a specific protein of the correct size was present in the control and not present in the silenced plants. However, it is not clear if this is the case in these plants.

Fig 4.3a shows results from the *Sex4* silenced plants probed with the anti-SEX4 antibody. No protein of the appropriate size can be seen in lanes from either the silenced

or control plants. The LSF1 blot (Fig 4.3b) shows a band of the appropriate size (55kDa) in control plant lanes 9 and 10. However it is difficult to say if there is repression of this protein as it still seems to be present within the silenced plants. That could mean that the silencing did not work, or that there is a protein in *N. benthamiana* of the same size as LSF1, which cross reacts with the antibody. From figure 4.3c, a clear band, probably representing LSF2, was obtained at approximately 25 kDa in the controls (lanes 8 and 9), with a clear reduction in that particular protein within the silenced plants. The antibodies were obtained relatively late into this study and due to time constraints this experiment was not able to be repeated. However to ascertain a more accurate result it this experiment will be repeated.

4.3.4 Starch contents of *Nicotiana benthamiana* leaves

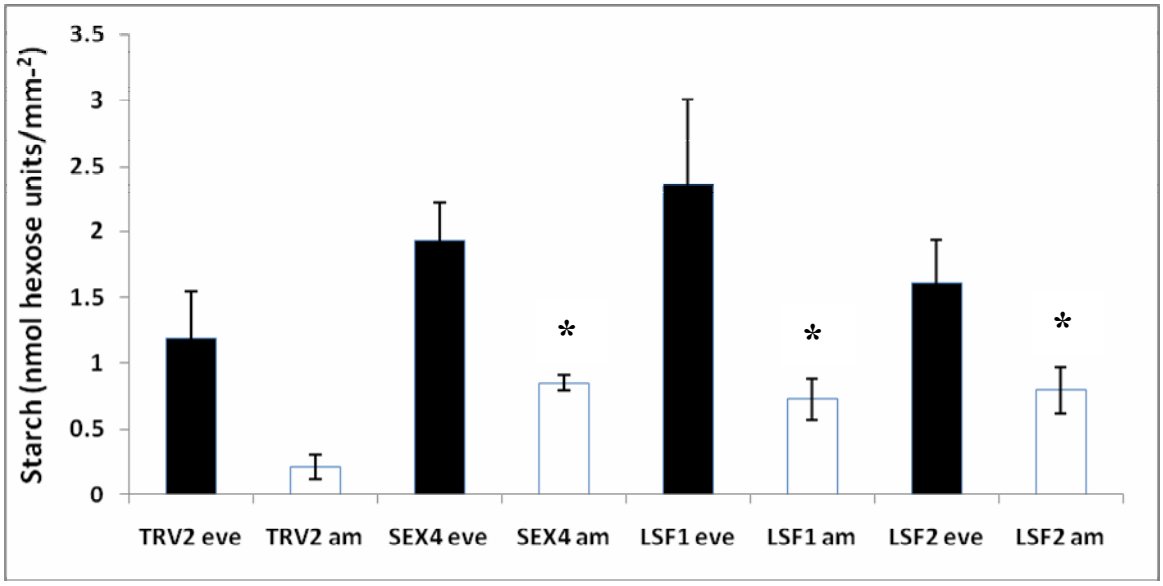


Figure 4.4: Starch contents in leaves of *N. benthamiana* plants infiltrated with *A. tumefaciens* containing VIGS vectors. The control plants were infiltrated with the empty TRV2 vector, while the SEX4, LSF1 and LSF2 plants were infiltrated with TRV2:*SEX4*, TRV2:*LSF1* and TRV2:*LSF2* respectively. Figures

represent means ± SEM of 8 independent samples. * denotes a statistically significant difference from the WT control at the 5% level (*Student's t*-test).

Despite the difficulty in confirming repression of the proteins, I measured starch contents in the leaves of the plants at the end and beginning of the light period (Figure 4.3). The results of this experiment shows that although no significant difference can be seen in the starch contents of all the lines at the end of the day, the starch contents of the silenced lines all contain significantly increased amounts of starch when compared to the control at the beginning of the day. This is an indication that these silenced plants are repressed in starch degradation. Such a result parallels the literature regarding the starch excess phenotype observed in mutant *Arabidopsis* plants lacking the Sex4 and LSF1 proteins (Zeeman and Rees, 1999; Niittylä et al., 2006; Kerk et al., 2006; Kötting et al., 2009; Comparet-Moss et al, 2009), indicating that they play the same role in tobacco as in Arabidopsis. This is the first evidence, however, that LSF2 may also play a role in starch degradation. It is not clear whether or not LSF2 is present in the chloroplast as there is evidence from computer predictions that the LSF2 protein does not contain the transit peptide as well as the carbohydrate domain that is seen in the two other isoforms, and only contains a phosphatase domain (Kötting et al., 2009). Further experimentation would be required to ascertain the true function of the LSF2 protein, perhaps the glyco-array mentioned in Chapter 3 can be used to determine the substrate for this protein could potentially point to the function.

Chapter 5

The effect of expression of *AtSEX4* in *E.coli* on glycogen contents

5. The effect of expression of *AtSEX4* on glycogen contents in *E. coli*

5.1 Introduction

This part of the project aimed at studying the effect that the SEX4 protein has on *E. coli* glycogen metabolism. The reason for this is because expression of SEX4 in has been demonstrated to lead to bacteria that are unable to accumulate glycogen (Dr James Lloyd, Institute of Plant Biotechnology, University of Stellenbosch, Pers. Comm.). Figure 5.1 shows the results of expression of such an experiment where an empty vector control and vector allowing expression of the *SEX4* gene in wild-type and *glgX* mutant strains. The *glgX* mutant lacks isoamylase, an enzyme that has been shown to be crucial in glycogen turnover as in the mutant there is decreased glycogen degradation (Dauvillee et al, 2005). The strains have been grown on rich media and then stained for glycogen production using iodine. It can be seen that WT expressing SEX4 do not accumulate glycogen, while the *glgx* mutant does. The purpose of the work in this chapter is to obtain quantitative data about how the SEX4 protein affects glycogen accumulation in the different strains. It is hoped that this will provide information to help explain why SEX4 has the effect that it does.

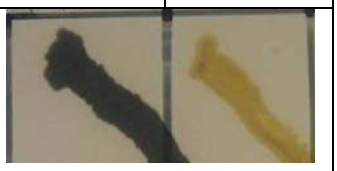
E. coli Strain	Control	SEX4
WT		
Glgx		

Table 5.1: Glycogen accumulation in WT and *glgx* mutant *E. coli* strains expressing SEX4 compared to the empty vector control. Cells were grown on media containing 2% (w/v) glucose and stained with iodine vapour to examine glycogen accumulation. Photo obtained from Dr James Lloyd.

5.2 Materials and Methods

5.2.1 *Escherichia coli* strains

The bacterial strains used in this study were obtained from the *E. coli* Genetic Resource Centre, Yale University. (http://cgsc.biology.yale.edu). The strains used were a *glgx* mutant strain from the Keio collection (Baba et al., 2006; CGSC# 10526) and its respective WT (CGSC# 7636).

5.2.2 Growth

The *AtSEX4* cDNA was present in the pBluescriptSK+ plasmid in sense orientation with respect to the LacZ promoter. This and the empty vector control were transformed into both the WT and *glgX E. coli* strains using the heat shock method. Transformed bacteria were grown in 2ml overnight cultures before being inoculated into 200ml of liquid media that contained 1.1% (w/v) K_2HPO_4, 0.85% (w/v) KH_2PO_4, 0.6% (w/v) yeast extract and 2% (w/v) glucose. 5 replicates were performed for each strain

containing each plasmid. 1ml samples were harvested at 2, 4, 6, 8, 10, 12, 24, 30, 50 and 72hrs after innoculation. The OD_{600} was determined by taking an absorbance reading at 600nm in order to compare the growth rates of the various strains. After 30 hours, once the cultures had reached stationary phase, the cells were harvested by centrifugation at 8000g for 10mins at 4°C. These were then re-suspended in M9 minimal media without a carbon source and further samples were harvested at 50 and 72 hours in order to investigate the resultant glycogen degradation.

Glycogen contents within the bacteria were determined by using the following method. 1ml samples of liquid culture were removed from the culture and the cells harvested by centrifugation at 10000g for 10 minutes and 4°C. The supernatant was discarded and the pellet re-suspended in 1 ml of 80% (v/v) ethanol by vigorous pipetting. To remove soluble sugars the samples were heated at 80ºC for 1 hour. This was followed by centrifugation at 8000g for 10 minutes after which the supernatant was again discarded. The pellet was re-suspended in 0.4ml of 0.7M HCl and heated at 95ºC for 4 hours. To this 0.4ml of 0.7M KOH was added to neutralize the reaction. The samples were then vortexed briefly and centrifuged at 10000g for 10 minutes. To measure glucose a buffer containing 300mM TRIS-HCl pH 8.1, 1mM $MgCl_2$, 1mM NAD, 1mM ATP was added to 50µl of sample. Then 1U of glucose 6-phosphate dehydrogenase/hexokinase mix (from *Leuconostoc*) was added to each of the samples with glucose being determined by monitoring the change in absorbance at 340nm.

5.3 Results and Discussion

5.3.1 *E. coli* Growth and Glycogen Determination

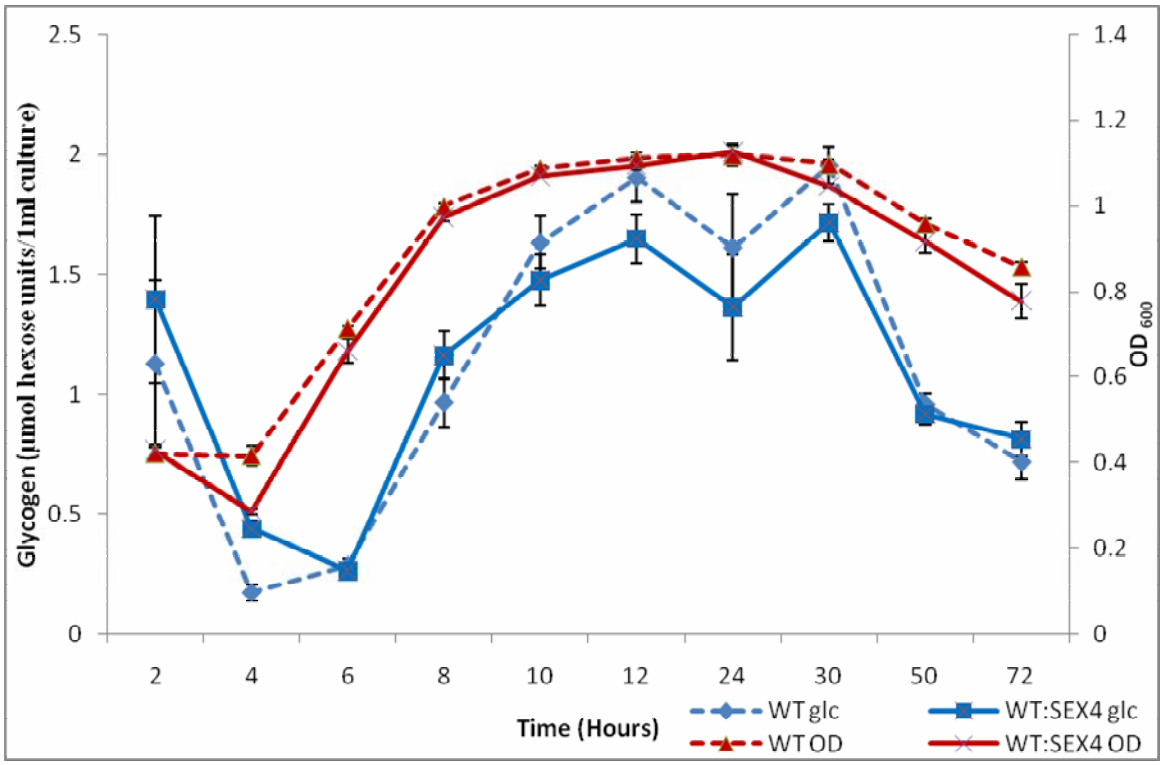


Figure 5.2: Shows the growth rate and glycogen contents of WT *E. coli* containing either the empty pBluescriptSK+ plasmid or one allowing expression of the Arabidopsis SEX4 protein. Figures represent means ± SEM of 5 samples.

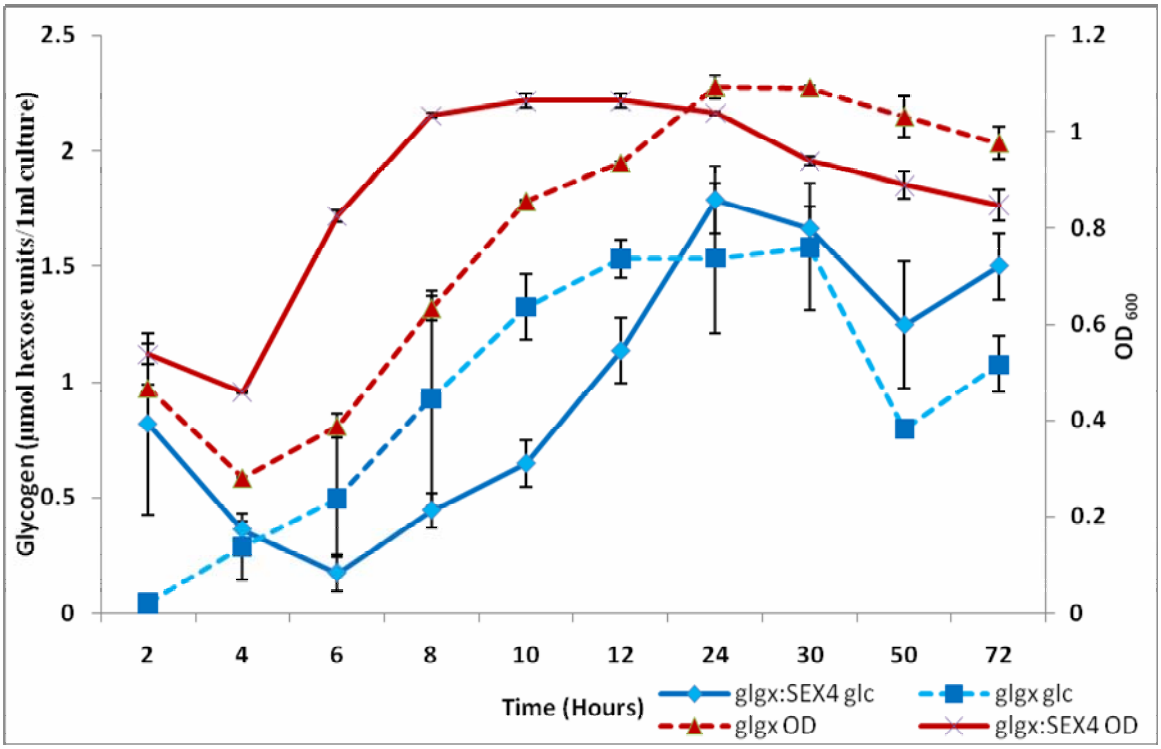


Figure 5.3: shows the growth rate and glycogen contents of glgx *E. coli* containing either the empty pBluescriptSK+ plasmid or one allowing expression of the Arabidopsis SEX4 protein. Figures represent means ± SEM of 5 samples

Glycogen contents in both WT and *glgX* mutant strains expressing the Arabidopsis SEX4 protein were determined during growth in liquid culture. The *glgX* strain lacks the debranching enzyme isoamylase and accumulates glycogen containing short external chains (Dauvillee et al, 2005). This strain was chosen as it is able to accumulate glycogen when SEX4 is expressed in it, while the WT does not (Fig 5.1). One theory for this difference between the WT and *glgx* mutant would be that the SEX4 protein affects the glycogen somehow so that it is degraded more quickly by GlgX. It is hoped that experiments such as this will help to explain this phenomonen.

The growth of the various *E. coli* cultures was monitored to see if there were differences between them. Figure 5.2 shows the rate of the WT strain either containing

the SEX4 protein, or not while Figure 5.3 shows similar data for the *glgX* mutant. Both WT lines grew at the same rate indicating that the expression of the SEX4 gene didn't influence this. Although there were significant differences in OD_{600} in the *glgX* mutant expressing SEX4 than in the empty vector control between 4 and 12 hours after inoculation, the rate of increase was not changed. This indicates that the differences in OD_{600} are due to the SEX4-expressing *glgX* coming out of lag phase later than the control.

Glycogen accumulation was then determined in the lines. The accumulation of the glycogen in the SEX4 expressing *E. coli* strain was not significantly different from the control. This is unexpected as the WT strain expressing SEX4 does not accumulate glycogen when grown on solid media (Fig. 5.1). I then plated out the cells from the liquid culture onto solid media and left them to grow overnight before staining with iodine vapour. In this case the WT expressing SEX4 appeared to accumulate as much glycogen as the control (data not shown). The most likely explanation for this is that the *SEX4* gene is no longer being expressed in the WT. The strains that this experiment was performed in contain a wild type *RecA* gene. RecA is involved in DNA recombination (Howard-Flanders et al, 1984; DiCapua et al, 1996) and is mutated in most laboratory strains as its presence leads to plasmid instability. It is likely that this has occurred in this experiment, although I did not test for the presence of the SEX4 protein using the antibody that I produced so cannot say for sure. Currently a mutant in this gene is being manufactured within the Institute of Plant Biotechnology in the TOP10 (Invitrogen) strain in order to overcome this problem. This strain is mutated in the RecA gene. Once this mutant is available the experiment will be repeated.

Chapter 6

Analysis of carbohydrate metabolism in fruit of a *gwd* tomato mutant

6. Analysis of carbohydrate metabolism in fruit of a *gwd* tomato mutant

6.1 Introduction

As was discussed in the General Introduction (Chapter 1 Section 2) an investigation into the influence of starch over tomato fruit carbohydrate metabolism would be interesting. One way to do this has become available with the production of a *gwd* mutant in tomato (Nashilevitz et al, 2008). In these lines a transposon has inserted in the *GWD* gene. The *gwd* mutation, however, caused lethality in pollen grains carrying the mutation due to their inability to degrade starch (Nashilevitz et al., 2009) and meant that the mutation from the original heterozygous line could only be propagated as a heterozygote. This problem was overcome by manufacturing a plant transformation construct where the potato *GWD* cDNA was driven in sense orientation by the pollen specific *LAT52* promoter (Twell et al., 1990). The construct was transformed into wild-type tomatoes, and lines expressing the potato cDNA were crossed into the heterozygote mutant. Plants were then identified which were homozygous for the mutation which expressed the potato *GWD* in their pollen (Nashilevitz et al., 2009).

The conditional *gwd* mutant described above demonstrates the normal starch excess phenotype in leaves (Nashilevitz et al., 2009) as described in other GWD repressed plants (Lorberth et al, 1998; Yu et al, 2001). This demonstrates that the GWD has the same effect on leaf metabolism in tomatoes as it does in potato and Arabidopsis. It is not clear whether the mutation in the *GWD* gene will also repress starch degradation in tomato

fruit although it is reasonable to assume this as presence of the GWD has been demonstrated to be essential for starch degradation in tissue other than the leaf mesophyll. Examples of this include potato tubers stored at low temperature (Lorberth et al, 1998) and tomato pollen (Nashilevitz et al., 1990). This part of the project is designed to examine whether this is true or not by measuring the levels of starch and sugars during development of fruit from the conditional tomato mutant.

6.2 Material and Methods

6.2.1 Plant Growth

Two tomato lines Wild type (WT) and conditional mutant (CM) were obtained as a gift from Prof. Avraham Levy (Weizmann Institute of Sciences, Rehovot, Israel). The WT plants contained the *LeGWD* allele, while the CM line is a homozygous *Legwd* mutant expressing the potato *GWD* in the pollen. These were planted into 4:1 vermiculite: sand mixture which had been sterilized by autoclaving prior to use. 20 seeds per tomato line were planted and the plants grown under glasshouse conditions.

6.2.2 Chlorophyll fluorescence

Chlorophyll fluorescence measurements were performed using a Fluorescence Monitoring System (FMS2), from Hansatech Instruments (Kings Lynn, United Kingdom). Plant leaves were darkened for 30 minutes prior to measurements being taken and Fv/Fm was determined.

6.2.3 Soluble sugar and starch measurements

For measurement of starch from leaf samples, discs of about 30mm^2 were harvested at the beginning and end of the day using a cork borer. Similarly starch as well as sugar measurements were also performed from fruit material where 25 mg samples were taken from the inner (placenta) or outer (pericarp) tissue of the tomato fruit at green, breaker and red stages. To these samples 1ml of 80% (v/v) ethanol was added to each disc (or fruit sample) in a micro-centrifuge tube and heated at 80°C for 1 hour to remove soluble sugars. The ethanol was removed and used to determine glucose, fructose and sucrose amounts by the following method. 50µl of the sample was combined with 250 µl of assay buffer (10mM MOPS (pH 6.9), 5mM $MgCl_2$, 1mM ATP, 1mM NAD, 1U/ml glucose-6-phosphate dehydrogenase from *Leuconostoc*. Glucose, sucrose and fructose were determined by measuring the increase in absorbance at 340nm following the sequential addition of 1U/ml hexokinase, 1U/ml phosphoglucoisomerase and excess β-fructosidase.

For determination of starch the ethanol extracted sample was further washed with 1ml 80% (v/v) ethanol. The liquid was removed and 0.4ml 0.2M KOH was added and heated at 95°C for 1 hour to solubilise the starch and then neutralised by addition of 70µl of 1M acetic acid. 10µl of this was mixed with 10µl of 50mM NaAC pH 5.6 and 10U/ml amyloglucosidase and incubated at 37°C for 2 – 3 hours. Assay buffer containing 10mM MOPS (pH 6.9), 5mM $MgCl_2$, 1mM ATP, 1mM NAD was added to the sample and the amount of glucose was determined by addition of 1U/ml glucose 6-phosphate dehydrogenase from *Leuconostoc* and following the change in absorbance at 340nm.

6.3 Results and Discussion:

a) **b)**

Figure 6.1: Micro-tom tomato plants: a) Wild Type (WT); b) Conditional Mutant (CM)

Figure 6.1 shows the two plant lines that were analysed during this study a) WT and b) CM. The plants appeared phenotypically identical.

6.3.1 Chlorophyll Fluorescence

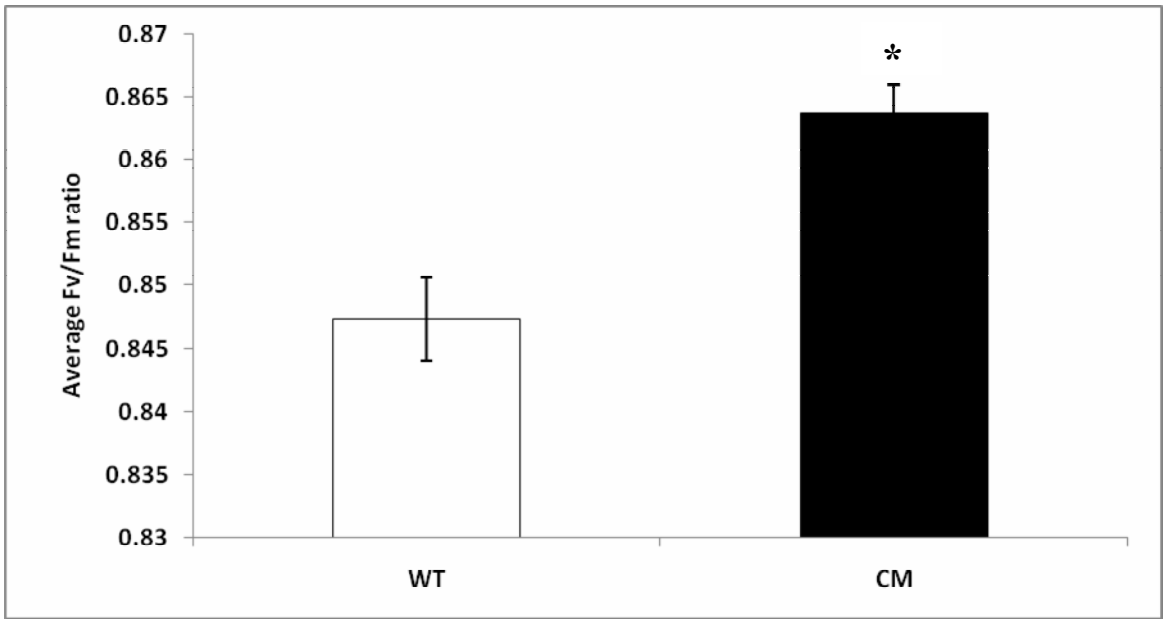


Figure 6.2: Fv/Fm in WT and CM lines. The measurements were determined following 30 minutes of darkening. Values represent means ± SEM of 7 separate measurements. * denotes a statistically significant difference from the WT control at the 5% level (*Student's t*-test).

The results of the chlorophyll fluorescence indicate that both of the WT and CM plant lines (shown in Figure 6.1) have an Fv/Fm value between 0.8 and 0.9, which is an indication that both plants have normal levels of photosynthesis. Fv/Fm is described as the quantum efficiency or yield of photosynthesis. The yield is a measure of how effectively photosynthesizing material can assimilate light or photosynthesize (Parkhill et al, 2001). It is effectively a measure of how well a plant is able to use solar energy to produce fixed carbon. Dark adapted values of Fv/Fm reflect the potential quantum efficiency of photo-system II (PSII) and are used as a sensitive indicator of plant photosynthetic performance (Maxwell and Johnson, 1999). Optimum values of 0.83 have been recorded for most plant species (Bjorkman and Demming, 1987; Johnson et al

1993). The results indicate that there is a small, but significant increase in Fv/Fm in the CM. Fv/Fm has been used as a proxy for photosynthetic carbon fixation, although some caution should be used in interpreting this without measuring that parameter also. This was not done and should be included in future experiments.

If the increase in Fv/Fm observed in the CM is indeed a result of increased carbon fixation it is interesting to speculate that this might come about to compensate for an inability to export sucrose at night in the CM. Normally starch is broken down in leaves at night and converted to sucrose for export and as starch degradation is impaired in the mutant that metabolic route cannot happen. Although I did not measure export from the leaves it is likely that this will be increased during the daytime and decreased at night as was demonstrated in the Arabidopsis *sex1* (*gwd*) mutant (Zeeman and ap Rees, 1999), which might mean that photosynthesis would increase to produce more sucrose during the day. It should be said, however, that in the study of Zeeman and ap Rees (1999) they measured carbon fixation in the mutant and could find no difference with the wild type control. Given that the difference seen in the CM was relatively small, it cannot be ruled out that this is a type 1 error, and the experiment should be repeated to examine this

6.3.2 Starch analysis in leaves

It has previously been demonstrated using iodine staining that the CM contains starch in its leaves at the end of the dark period (Nashilevitz et al., 2009). In order to obtain some quantitative data about this I took samples from the leaves at the beginning and end of the night and measured starch contents (Fig 6.3) The results obtained in this experiment shows that the starch content of leaves from the CM is significantly higher

than that of the WT both at the beginning and the end of the day. This indicates that the CM is repressed in starch degradation in its leaves and is consistent with the known role of the GWD in starch mobilisation in other plants (Lorberth et al 1998; Yu et al, 2001).

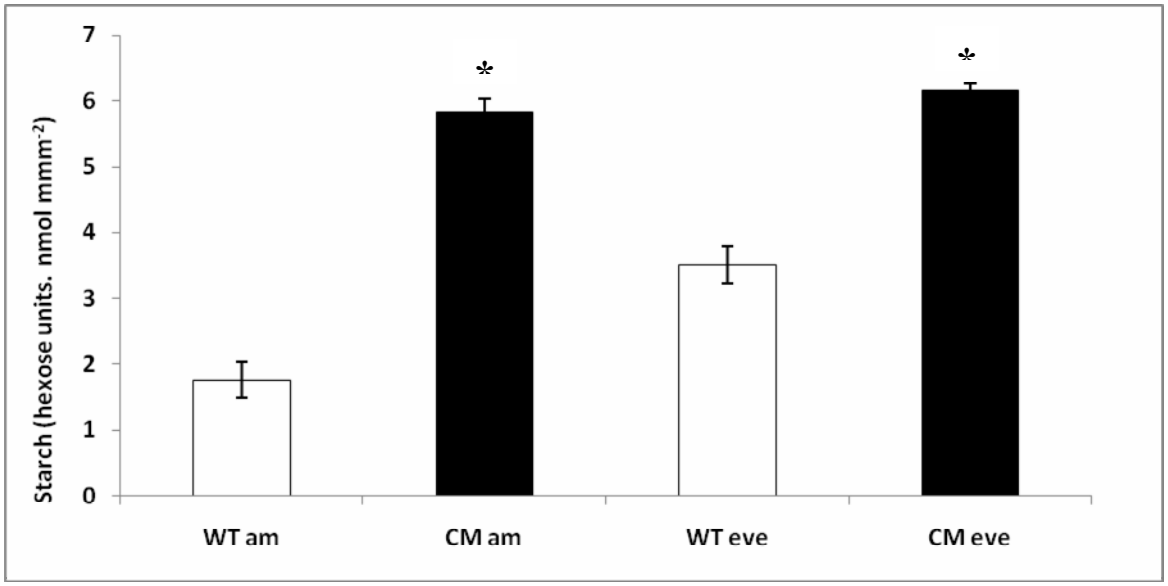


Figure 6.3: Starch contents at the beginning and end of the day in WT and CM lines. Figures represent means ± SEM of 8 individual samples. * denotes a statistically significant difference from the WT control at the 5% level (*Student's t*-test).

6.3.3 Starch and Soluble sugar analysis in developing fruits

Figure 6.4: green, breaker (orange) and red tomato fruits harvested from the various lines for analysis. Each individual fruit was separated into the pericarp, which is the outer tissues of the fruit and into the placenta tissue, which are the inner tissues.

Figure 6.4 shows the different developmental stages of the tomato fruit as it proceeds in ripening from green (left) through breaker (orange) to red fruits. In this study we aimed to establish what was happening to the carbohydrate metabolism through fruit development, specifically looking at the starch and soluble sugar content of the fruit. This was done by harvesting the fruit at these three different developmental stages.

6.3.4 Starch content in tomato fruit

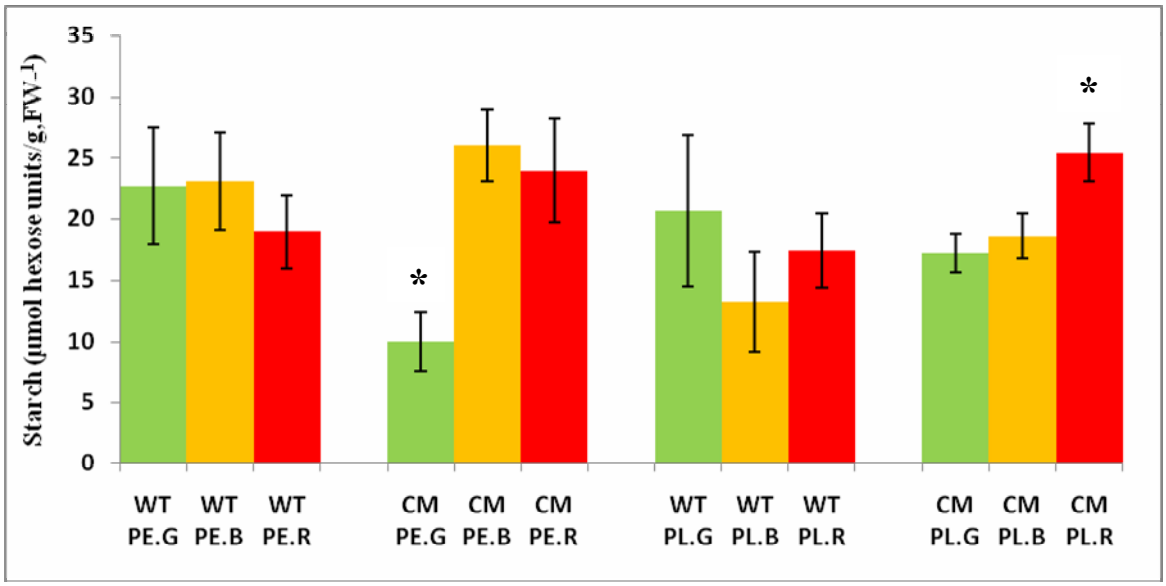


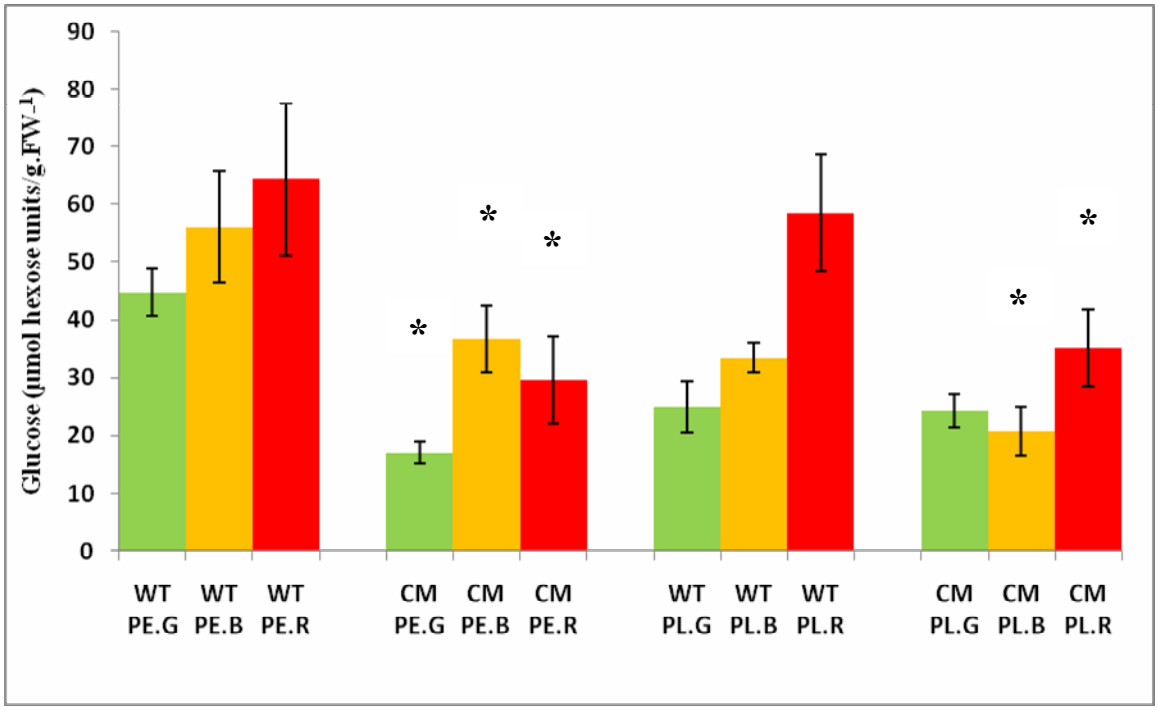
Figure 6.5: Starch content of pericarp (PE) and placenta (PL) of green (G), breaker (B) and red (R) fruit in the WT and CM lines. Figures represent means ± SEM of 5 individual samples. * denotes a statistically significant difference from the WT control at the 5% level (*Student's t*-test).

I measured starch contents in the placenta and pericarp in green, breaker and red fruit. The data for starch contents in the fruits indicates that there is little difference between the WT and CM. The fruits were harvested from both plant lines at different developmental stages and are shown in Figure 6.4. The only significant changes were in

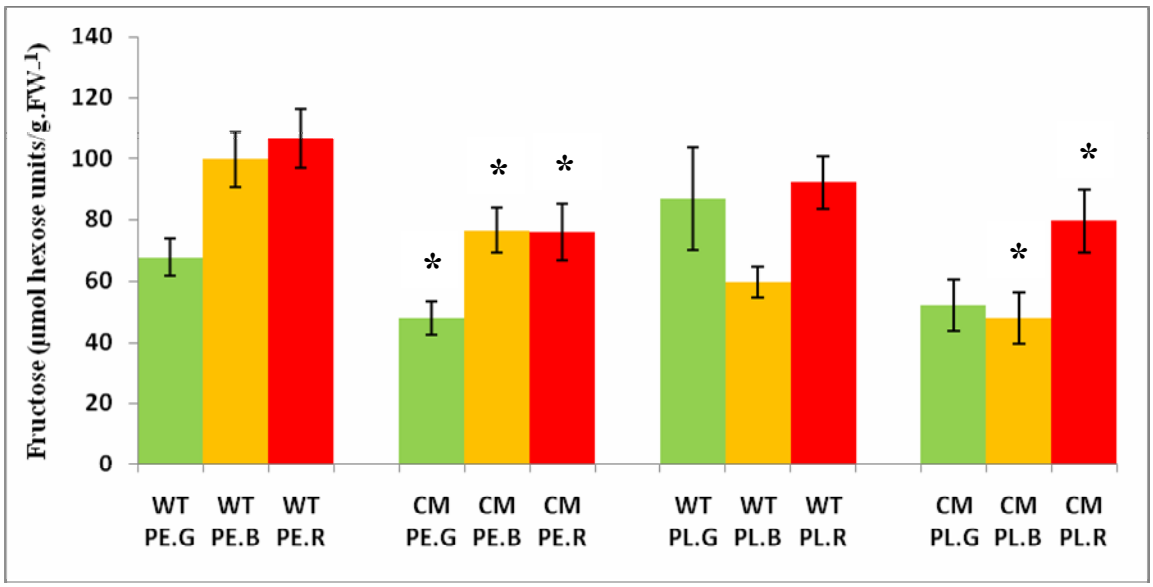
the pericarp in green fruit (where the CM contained less starch) and in the placenta in red fruit (where the CM contained more starch) (Fig 6.5). This data differs from that of Obiadalla-Ali et al (2003) in that there is no significant decrease in starch contents during fruit development. In that study it was shown that starch decreases in both the placenta and pericarp during development, and that in red fruit it is almost non-existent. The most likely reason for this is that differences in growth conditions between the studies may influence fruit metabolism. The plants in this study were grown under natural light conditions during the spring which meant that the plants were grown with 13 hours light and 11 hours of darkness. In the study of Obiadalla-Ali et al (2003) in contrast the plants were grown with 16 hours light and 8 hours dark.

6.3.5 Soluble sugar content in tomato fruit

a)



b)



c)

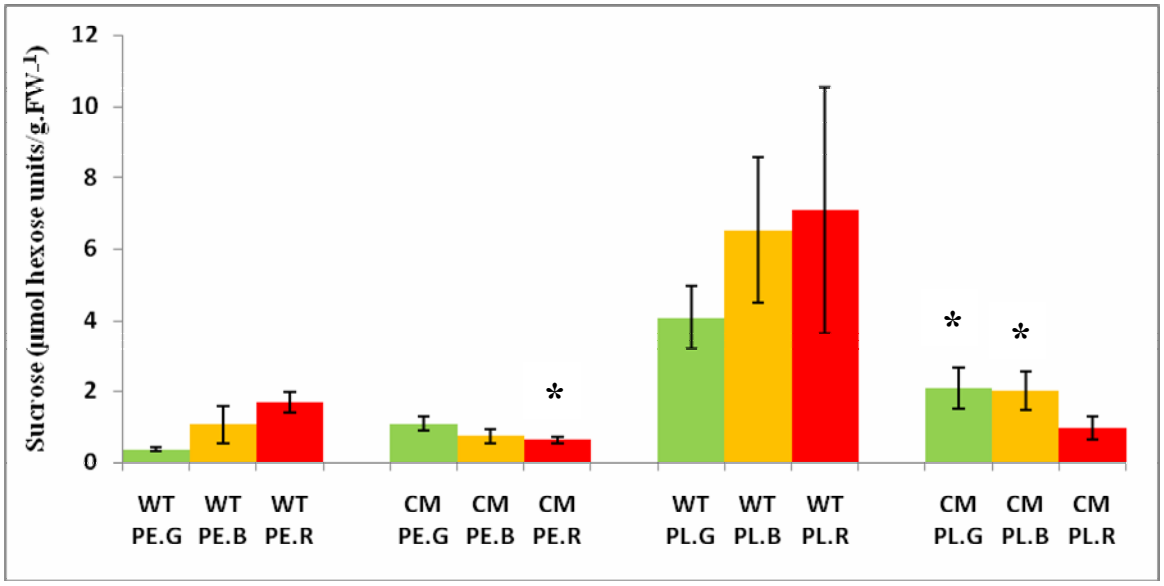


Figure 6.6 a) Glucose, b) Fructose and c) Sucrose content of placenta (PL) and pericarp (PE) of green (G), breaker (B) and red (R) fruit in the WT and CM lines. Figures represent means ± SEM of 5 individual samples. * denotes a statistically significant difference from the WT control at the 5% level (*Student's t*-test).

When examining soluble sugar levels, it seems clear that there are significant differences between the CM and WT (Fig 6.6a-c). Glucose and fructose are reduced in

the CM in both the pericarp and placenta at all stages, while sucrose is reduced in the pericarp in red fruit, and in the placenta at the green and breaker stages. Taken together this indicates that soluble sugar levels are reduced in all tissue types in the fruit at all stages. Given that the starch content in the fruit was not changed in the CM compared with the WT, it cannot be that these changes are due to a decrease in starch degradation in the CM. The more likely explanation would be that it is due to a decrease in soluble sugar export from the leaves to the fruits. This theory is contradicted somewhat by the chlorophyll fluorescence measurements which suggest an increased rate of photosynthesis in the CM. As carbon fixation and export wasn't measured, it isn't possible to show conclusively that the plants export more sugar to the fruits. In addition

I was not able to see if the GWD protein was present within the fruit. I investigated this by performing an immunoblot, where total protein extracted from the fruit were tested against the potato GWD antibody (data not shown). In the CM, GWD expression is driven by a pollen specific promoter. It is highly unlikely that the protein is expressed in the fruit; however since I could not establish the presence of the GWD protein I assume it was being repressed as in the study by Twell et al, 1990. In that study the activity of the GUS: *Lat52* fusion protein was tested by performing a GUS assay, which indicated that tomato plants exhibited high levels of GUS activity in extracts from mature anthers, with essentially no GUS activity in other vegetative or floral organs, including fruit pericarp and seeds (Twell et al, 1990).

The study was designed to examine carbohydrate metabolism in a conditional tomato *gwd* mutant. I used a chlorophyll fluorometer to measure Fv/Fm in the leaves, as well as measuring starch in leaves and fruit and soluble sugars in the fruit. Although Fv/Fm was significantly increased in leaves of the CM, this was a very small increase and is inconsistent with data from another *gwd* mutant (Zeeman and Rees, 1999) where no change in photosynthesis was noted. The data in fruit is also puzzling as there is a clear and consistent decrease in soluble sugars in the fruits of the CM, but no change in starch contents. One of the problems with this study is that the fruits were only taken in the green, breaker and red fruit stages, and no account was made for age. In a previous study on sugar and starch levels in fruits of the Microtom variety flowers were tagged at anthesis and only one fruit per bunch was allowed to develop (Obiadalla-Ali et al 2003). In that study it was shown that fruits stay different colours for relatively long periods of time, during which metabolite levels change. This is especially true for green fruit. By not taking such developmental changes into account the data from this study become very difficult to interpret. As such I would recommend that the study is repeated in the same manner as was performed by Obiadalla-Ali et al (2003).

Chapter 7

Conclusions

7. Conclusions

The project required that constructs be built to allow SEX4, LSF1 and LSF2 to be expressed in *E.coli* as well as to be silenced in *Nicotiana bethamiana*. The proteins were expressed in bacteria with the SEX4 and LSF2 fusion proteins being successfully purified and used to raise antibodies against the respective proteins. The antibodies were required to ascertain whether or not the starch excess phenotype I obtained in the VIGS silencing experiment could be attributed to the result of these proteins being repressed. Unfortunately the antibodies were not very specific with a large amount of non-specific binding being observed. This resulted in not being able to determine whether the starch excess phenotype noticed in leaves was due to the silencing of these specific three genes. Starch degradation products were also produced which will be used in glyco-arrays to help establish the substrate for the various SEX4 and LSF proteins. Finally the role of starch phosphate in tomato fruit was assessed. A conditional *gwd* mutant was examined for starch and soluble sugar contents during fruit development. Although soluble sugar levels were reduced in all fruit stages tested, starch contents were not. This is most likely due to green fruits being harvested too late at times when they did not contain starch.

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