Investigation of starch metabolism genes and their interactions

by

Adrianus Petrus Claassens

Thesis presented in partial fulfillment

of the requirements for the degree of Master of Science at Stellenbosch University

Supervisor: Dr. James Richard Lloyd

Co-supervisor: Prof. Jens Kossmann

Faculty of Natural Sciences

Department Genetics

Institute for Plant Biotechnology

December 2013

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: Signature:

Abstract

Starch is widely used in industries around the word, some of these are food, oil drilling, paper milling and cosmetics. It is a polymer which has two components, amylose and amylopectin. The production and degradation if starch in plants is fairly well studied and a sizable number of enzymes have been identified which play critical roles in its metabolism. There are still remaining questions, namely if there are more unidentified enzymes that play roles and how the enzymes interact with each another.

To study the effect on starch metabolism possible novel starch metabolic genes were studied by analysing Arabidopsis T-DNA insertion mutants for two genes, designated *SP1* (At5g39790) and *CBD1* (At5g01260). cDNAs for these two was used to produce recombinant protein and investigated potential activities. The *cbd1* mutant plants had a starch excess phenotype with iodine staining but this could not be confirmed with quantitative starch measurements. The *sp1* mutants did not have a significant difference in all the lines and time points when compared to the Wt plants. No link could be established between the SP1 kinase domain and glucan phosphorylation. From my data a clear involvement of these two genes could not yet be elucidated.

To study the interactions of starch metabolic proteins (BEI, BEII, GWD and ISA2) chimeric RNAi constructs was built and transformed into potato. Only StBEI and StBEII lines could be analysed and it was found that the G6P content was increased in both StBEI and StBEII. The BEII leaves and tubers had increased amylose contents. Intriguingly it would appear that starch isolated from both the tubers and leaves of StBEI lines demonstrated a reduction in amylose, with the leaves showing a much bigger decease than the tubers. This needs to be confirmed and the remaining lines need to be analysed.

Gaining knowledge about starch metabolism is critical in producing engineered crops that can produce more starch in a smaller agricultural area. With the population growing beyond 8 billion individuals it will be one of the best routes to enhance cop yields through biotechnology.

Opsomming

Stysel word reg oor die wereld benut in ‘n verskyndenheid van industiee. Dit is divers en sluit die voedsel, oliebooring, papiermeule en die kosmetiese bedryf in. Dit is ‘n polimeer wat uit twee komponete: amylose en amylopektien bestaan. Stysel metabolisme, wat die vervaardiging en afbreek van dit insluit, is al baie goed bestudeer. Die ensieme wat ‘n kritiese rol speel is al gevind, maar daar bly nogsteeds ‘n paar vrae wat moet beantwoord word. Is daar nog ensieme wat ‘n rol speel wat nog nie geidentifiseer is nie? Wat is die manier hoe die bekende ensieme met mekaar ‘n interaksie het?

Om die invloed van twee moonlike nuwe stysel metabolisme gene te bestudeer, is T-DNA insersie mutante ondersoek. Hulle word na verwys in die studie as *SP1* (At5g39790) en *CBD1* (At5g01260). cDNAs vir hierdie twee was gemaak vir die vervaardeging van rekombonante proteine. Hierdie rekombinante proteine was dan ondersoek vir moonlike aktiwiteite. ‘n Oormaat stysel was wel gevind in die *cbd1* mutant plante wanner n jodium vlek tegniek gebruik was. Ongelukkig kon hierdie oormaat die bevestig word wanner n kwantitatiewe metode gebruik was nie. Daar was nie ‘n beduidende verskil in stysel wanner die *sp1* mutante plante vergelyk was met die wilde tiepe nie. Daar kon ook geen verbintenis gevind word tussen die kinase area en die fosforilasie van stysel nie. Volgens hierdie data kon daar die n duidelike verbintenis gevind word tussen die twee gene en stysel metabolisme nie.

Om die interaksies tussen bekende stysel metabolisme proteine (BEI, BEII, GWD en ISA2) te bestudeer was chimeriese RNAi konstrukte gebou en toe in aartappels in getransformeer. Slegs die StBEI and StBEII kon geanalisser word en daar was bevind dat die G6P hoeveelheid in beide hoër was. Amilose was in groter hoeveelheide teenwoordig in beide BEII blare en knolle. ‘n Onverwagse obserwasie was gemaak toe die BEI lyne ondersoek was. Daar was gevind dat in die blare en knolle daar ‘n laer hoeveelheid amilose was. Die blare het wel baie laer amilose gehad as die knolle. Die obserwasie moet bevestig word met n ander tegniek en die orige RNAi lyne moet nog bestudeer word.

Om al die fasette van stysel metabolisme te ken is uiters belangrik vir die vervaardiging van gewasse wat groter opbrengste lewer in n kleiner area. Met die wereld bevolking wat al verby 8 biljoen individue gestyg het is dit moontlik al hoe almal voor gesorg kan word in terme van voeding.

Acknowledgements

A whole page is dedicated for the mention of one man.

R.I.P. Hermanus Josias Andreas van der Merwe

The man that made all of this possible. The man that died. The man which last wish was for his grandchildren to have an university education. A great man, one which will live through me to the day I die. I wish he was still here.

Sonder hierdie uitmuntende man sou hierdie werkstuk nie moontlik gewees het nie. Die man wat ongelukkig nie meer met ons is en ons kan eer met sy lewens wysheid, geloof en etiek nie. Sy laste wens was dat sy klein kinders moet universitiet to gaan. n' Man van waarde en respek. Ek streef om n fraksie van sy merkwaardigheid te wees. Deur my gaan ek seker maak dat sy nagelatenis staan tot die dag wat ek my hoof neerle^ en tot die aarde gestel word. Ek het n groot begeerte dat hy nog hier kon wees.

In Afrikaans and English there are not words available to do just to the amount of appreciation that I have for the lady that is named Anthea Bester. All the support and heartfelt “medelye” really kept me going in the harshest of cold Stellenbocsh nights. That ginger is the spice of my life.

The academic staff that had a profound impact on my development was Dr Hills, Dr George, Dr Lloyd and Dr Peters

Dr Gavin Gavin for teaching me how to work hard and to throw myself into it.

Dr Paul Hills must receive special mention that he believed in my passion at the honors interview.

Dr James Lloyd taught me how to use my time wisely. I am thankful for his patients with my writing skills or lack thereof and for starting the project.

Dr Peters thanx biatch.

Prof Jens Koßmann for an ever watchful eye, funding and beer.

I would like to thank the CSIR for funding in my second year of my Masters and the lesson that you can plan your life but that you seldom travel that route.

I would like to also take this opportunity to thank my mother and father which have given up their comfort and health to provide me with an undergraduate degree. The food and logistical support also did not go unnoticed.

I would like to thank two women in the lab that was always a constant during the Masters. Paulianne the mother figure for me in Stellenbosch and Christelle Cronje, a true friend. I enjoyed naming her lightning shoulders. She is a true grammar Nazi par excellence.

Christell and the tissue culture staff did great work looking after my plants :)

Last but not least a person who always enjoyed giving a sarcastic stab, finishing my sentences on purpose just to irritate me. One who struggled with me in the lab to early morning hours. He is the brother I never had. Kyle Wiltard

Table of content

List of figures

List of tables

List of abbreviations

3-PGA	: 3-Phosphoglyceric acid
ADP	: Adenosine 5′-diphosphate
ADP-G	: ADP-glucose
AGPase	: ADP-glucose pyrophosphorylase
AMY	: α-amylase
Arabidopsis thaliana	: Arabidopsis
ATP	: Adenosine triphosphate
BAM	: β-amylase
BCIP/NBT	: 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium
bp	: Base pair
BSA	: Bovine serum albumin
$CaCl_2$	: Calcium chloride
CaMV	: Cauliflower mosaic virus
cDNA	: Complimentary DNA
Cef	: Cefotaxime
cv	: Cultivar
DNA	: Deoxyribonucleic acid
Dp	: Distribution pattern
DPE	: Disproportionating enzyme
DTT	: Threo-1,4-Dimercapto-2,3-butanediol
E.coli	: *Escherichia coli*
EDTA	: Ethylenediaminetetraacetic acid
EtOH	: Ethanol
F6P	: Fructose-6-phosphate
FWD	: Forward
G6P	: Glucose 6-phosphate
GA3	: Gibberellic acid
GBSS	: Granule bound starch synthase
gDNA	: Genomic
GST	: Glutatione S-transferase
GWD	: Glucan water dikinase
HCl	: Hydrochloric acid
HEPES	: 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
HG	: Hellsgate
I_2	: Iodine
IPTG	: Isopropyl β-D-1-thiogalactopyranoside
ISA	: Isoamylase
$K_2S_2O_5$	: Potassium disulfite
Kan	: Kanamycin sulphate
KI	: Potassium iodide
KOH	: Potassium hydroxide
LDR	: Limit dextrinases
LSF	: Like Starch-excess four
MEX	: Maltose excess

$MgCl_2$	: Magnesium chloride
Min	: Minutes
MOPS	: 3-(N-Morpholino)propanesulfonic acid, 4-Morpholinepropanesulfonic acid
MOS	: Malto-oligosaccharides
MS	: Murashige and Skoog
NAA	: Naphthalene acetic acid
NAD	: Nicotinamide adenine dinucleotide
NADH	: Nicotinamide adenine dinucleotide, reduced dipotassium salt
NADPH	: Nicotinamide adenine dinucleotide phosphate, reduced: tetra(cyclohexylammonium) salt
OD	: Optical density
PAGE	: Polyacrylamide gel electrophoresis
PBS	: Phosphate buffered saline
PCR	: Polymerase chain reaction
pGlcT	: Glucose transporter
PK/LDH	: Pyruvate kinase/lactic dehydrogenase
PWD	: Phoshoglucan water dikinase
RE	: Restriction enzymes
REV	: Reverse
RNA	: Ribonucleic acid
S.tuberosum	: Solanum tuberosum
BE	: Starch branching enzyme
SDS	: Sodium dodecyl sulphate
SEX	: Starch excess
SqPCR	: Semi quantitative PCR
SS	: Starch synthase
TB	: Terrific broth
TBS	: Tris buffered saline
TBS-T	: Tris buffered saline-Tween 20
Tris	: Tris (hydroxymethyl) aminomethane
YEP	: Yeast extract peptone

Chapter one

Literature review

1 Starch and its metabolism

1.1 Starch

The main storage carbohydrate in most higher plant species is starch, a glucose polymer composed of two distinct fractions, amylose and amylopectin, which pack together to form semi-crystalline granules (Buléon et al., 1998). Amylose contains mainly α1,4-linkages with relatively few (between 2 and 8) α1,6-branch points. Side chain lengths can vary from 4 to 100 glucose moieties (Takeda et al., 1987; Hizukuri et al., 1981). Amylopectin is more highly branched containing glucose monomers that are α1,4-linked, but also contains many α1,6-branchpoints. The α1,6 linkages form 5-6% of the total bonds in the amylopectin molecule (Buléon et al., 1998; Takeda et al., 1984) and the internal chains have been classified by Peat et al. (1952) as A, B or C (Fig. 1). A chains are located on the outside of inner B chains and are glycosidically linked to the 6 carbon of the glucose moiety. The amylopectin molecule has a single C chain containing the sole reducing terminal glucose residue that carries all the A and B chains. A and B chains link together to form clusters in an ordered structure (Hizukuri,1985, 1986; Hanashiro et al., 1996) and it is this regularity which leads to the crystallinity of the semi-crystalline starch granule. As shown in Fig. 1 the clusters are separated by 9nm amorphous layers that contain mainly amylose (Buléon et al., 1998).

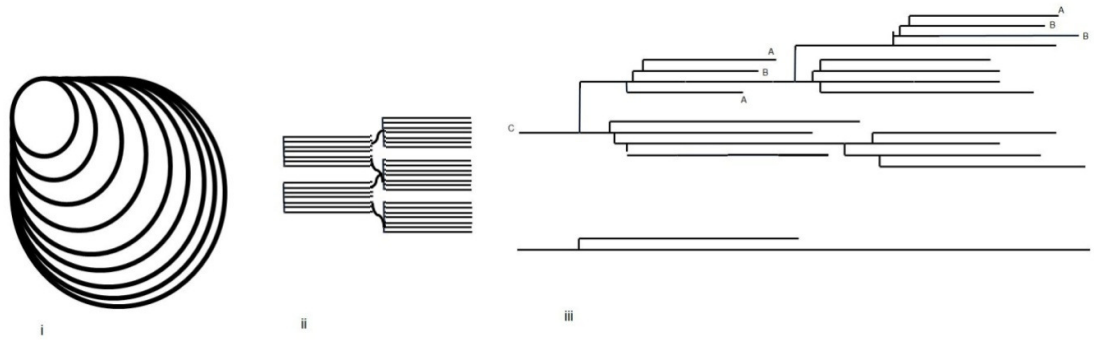


Figure 1. Starch granules in storage organs, such as potato tubers, form growth rings (i). These growth rings are made up of clusters of amylopectin that form helixes and, together with amylose, form amorphous and semi-crystalline layers (ii). A and B chains can be observed and these are linked to a single C terminal chain (iii)

Starch can be found in both photosynthetic and non-photosynthetic tissues and the size and structure of the granules differs immensely between both tissue types and botanical origins (Czaja, 1969; Jobling, 2004). For example, starch granules in Arabidopsis leaves are significantly smaller (0.75-2µm diameter) than those found in potato tubers (5-100µm diameter; Zeeman et al., 2002; Jobling, 2004).

There are many industries where this semi-crystalline glucan polymer is utilized. In the food and oil drilling industries it is used for its ability to alter the viscosity of fluids. The granular texture is important in its use as a filler agent in biodegradable plastics while the smooth texture of small sized starch granules is valuable to the cosmetics industry as it gives an alternative to allergenic magnesium silicate powders (Ellis et al., 1998).

Many enzymes involved in synthesizing the starch polymer have been identified and their individual roles are being elucidated in mutant and transgenic plants. Given the ordered structure of starch granules and the number of enzymes that are involved in manufacturing this biopolymer there is still much research to be performed in order to fully understand the fine control mechanisms affecting its synthesis. In the following sections the enzymes involved in the manufacture and degradation of starch will be reviewed in terms of Arabidopsis leaves and potato tubers as these are the species that this study focuses on.

1.2 Starch synthesis

One of the main differences between starch synthesis in leaves and storage organs is the source of the ADP-glucose that is used as substrate for polymer production as well as the relative contributions of different isoforms of the main polymerizing enzymes. In leaves ADP-glucose is produced from fructose-6-P (Smith, 2012; Streb et al., 2009; Bahaji et al., 2011), a Calvin-Benson cycle metabolite, which is subsequently acted on by phosphoglucose isomerase, phosphoglucomutase and ADP-glucose pyrophosphorylase (extensively reviewed in Stitt and Zeeman, 2012; Zeeman et al., 2010). In potato tubers the ADP-glucose is produced from sucrose that has been transported from leaves via the vascular system (Zrenner et al., 1995; Kühn et al., 1999). Sucrose is converted in the cytosol to glucose 6-phosphate (G6P), which is imported into amyloplasts by a specific G6P transporter (Kammerer et al., 1998) where it is used to produce ADP-glucose by the consecutive actions of phosphoglucomutase and ADP-glucose pyrophosphorylase (Stitt and Zeeman, 2012)

ADP-glucose is a substrate for starch synthase (SS) isoforms which catalyze the first step of polymer formation. In *Arabidopsis* there are five isoforms present in the genome, a granule bound (GBSS) and four soluble SS's (SS1, SS2, SS3, SS4). GBSS is responsible for the formation of amylose (Denyer et al., 1996,1999; Zeeman et al., 2002) while the soluble SS's present seem to have distinct functions in the process of producing glucan chains for amylopectin (Zeeman et al., 2010). Mutant studies in various higher plants led to the general idea that SS1, SS2 and SS3 branch short, medium and long chains respectively but it seems that their functions can also overlap to a degree (Tomlinson et al., 2003; Zeeman et al., 2010; Szydlowski et al., 2011). However, that is too simplistic a view and the process appears to be more intricate. For instance, in potato, the starch synthase isoforms are expressed differentially. SS1 is predominantly found in leaves while the SS2 and SS3 isoforms are more active in the tubers (Kossmann et al., 1999). This would explain the finding that in antisense lines for SS1 the amount of tubers, tuber starch content, amylose content, sucrose content, chain-length distribution and covalently bond phosphate at the C-6 position did not differ significantly from wild-type potatoes (Kossmann et al., 1999). Changes in tuber starch phosphate contents were observed in the SS2 and SS3 antisense lines with a decrease of 50 and increase of 70 % respectively (Abel et al., 1996; Kossmann et al., 1999), but the reason for this is still very poorly understood and requires further investigation. SS1-3 isoforms are clearly involved in amylopectin synthesis; however, SS4 is thought to be responsible in the initiation of starch granules. Arabidopsis *ss4* mutants contain only one starch granule per chloroplast (Roldan et al., 2007) and, when *AtSS4* is overexpressed in Arabidopsis and *S.tuberosum* there is an increase in starch accumulation (Ga´mez-Arjona et al., 2011). Szydlowski et al. (2009) discovered that there might be a functional overlap by

SS3 and SS4 as a *ss3/ss4* double mutant produced almost no starch indicating that SS3 is involved both in synthesizing amylopectin and is also partly responsible for initiating starch granule synthesis.

The glucan polymer produced by the different starch synthases is branched by branching enzymes (BE) to form amylopectin (Fig. 2). In all genomes sequenced so far, multiple BE isoforms have been identified. For example, in Arabidopsis and rice there are three while only two active ones are found in potatoes (Mizuno et al., 1992; Nakamura et al., 1992; Khoshnoodi et al., 1996; Larsson et al., 1996; Larsson et al., 1998; Dumez et al., 2006). The different isoforms can be divided into two classes known as class A (class II) or class B (class I) differentiated by their protein sequence similarity (Burton et al., 1995). Maize BEII, pea BEI, rice BEIII, Arabidopsis BEII, BEIII and potato BEII belong to Class A. Maize BEI, pea BEII, rice BEI , Arabidopsis BEI and potato BEI falls into the class B (Burton et al., 1995; Safford et al., 1998; Larson et al,. 1998; Dumez et al., 2006). In this thesis I will refer to the isoforms as I and II.

BEs are α-1,4-glucan: α-1,4-glucan-6-glycosyltransferases. α1,4 bonds are cleaved by BEs and transferred to the C6 positions of another glucan chain to form a branch point (Borovsky et al., 1979). The two classes of BE have different biochemical actions; the maize and potato class II isoforms transfer shorter chains and have a lower activity on amylose compared with class I enzymes (Guan and Preiss, 1993; Ryberg et al., 2001; Zeeman et al., 2010). Presently there is a trend to utilise genetic alterations to produce plants containing starch with desirable properties (Zeeman et al., 2010). In many plants it has been shown that reductions in BE activity lead to a decrease in amylopectin synthesis with a concomitant increase in the proportion of amylose in starch (Bhattacharyya et al., 1990; Mizuno et al., 1993; Hedman and Boyer, 1982, 1983). Intriguingly, when potato *St*BEI was silenced it was found that there were no significant change in the amylose content of tuber starch, only an increase in phosphate content and the gelatinization temperature (Safford et al., 1998). However, when *St*BEII was silenced an increase in apparent amylose was observed alongside a significant alteration in amylopectin structure. The amylopectin from these transgenic lines contain fewer short chains, leading to an increase in average chain length when compared with wild type (Jobling et al., 1999). When *StBEI* and *StBEII* genes were silenced simultaneously it was found that there was an increase in both amylose and phosphate contents with the amylose increasing to 70% of the starch and a six-fold increase in phosphate (Schwall et al., 2000). These high amylose potatoes were subjected to field trials to evaluate their commercial use and a threefold reduction in starch contents of the transgenic lines was found alongside an increase in glucose and fructose. It was concluded

that the severe starch yield reduction did not make the transgenic potatoes commercially viable (Hofvander et al., 2004).

Debranching is also important in the process of amylopectin production. There are two types of debranching enzymes in plants, namely isoamylases (ISA) and limit dextrinases (LDA) which can be divided by gene sequence and substrate specificities. ISA1 and ISA2 are involved in the synthesis of mature amylopectin while ISA3 is involved in degrading starch (Wattebled et al., 2005). Loss of either ISA1 or ISA2 leads to loss of the other protein, most probably because they form a heterodimer and the monomeric forms are unstable. Elimination or repression of either subunit leads to a reduction in starch and the accumulation of phytoglycogen (Bustos et al., 2004; Wattebled et al., 2005), a polyglucan that is soluble and far more highly branched than amylopectin. This has been observed in a number of mutants including ones in maize, *Arabidopsis* and *Chlamydomonas reinhardtii* (James et al., 1995; Mouille et al., 1996; Zeeman et al., 1998; Kubo et al., 1999; Dauvillee et al., 2000, 2001a, 2001b; Burton et al., 2002). It is proposed that the heterodimeric isoamylase complex is part of an amylopectin trimming process that forms the mature amylopectin molecule (Wattebled et al., 2005). Interestingly in potato, where the expression of either *Isa1* or *Isa2* was reduced it was found the tubers accumulated a large number of very small starch granules, indicating an effect on granule initiation, but only small amounts of phytoglycogen. The increase in starch granule numbers was, however, not associated with a change in amylopectin structure of the transgenic potato lines (Bustos et al., 2004).

The starch that was produced by the above mentioned enzymes during the light period can be either stored or degraded to provide energy in times photosynthesis does not take place. The next section describes the proteins involved in starch catabolism.

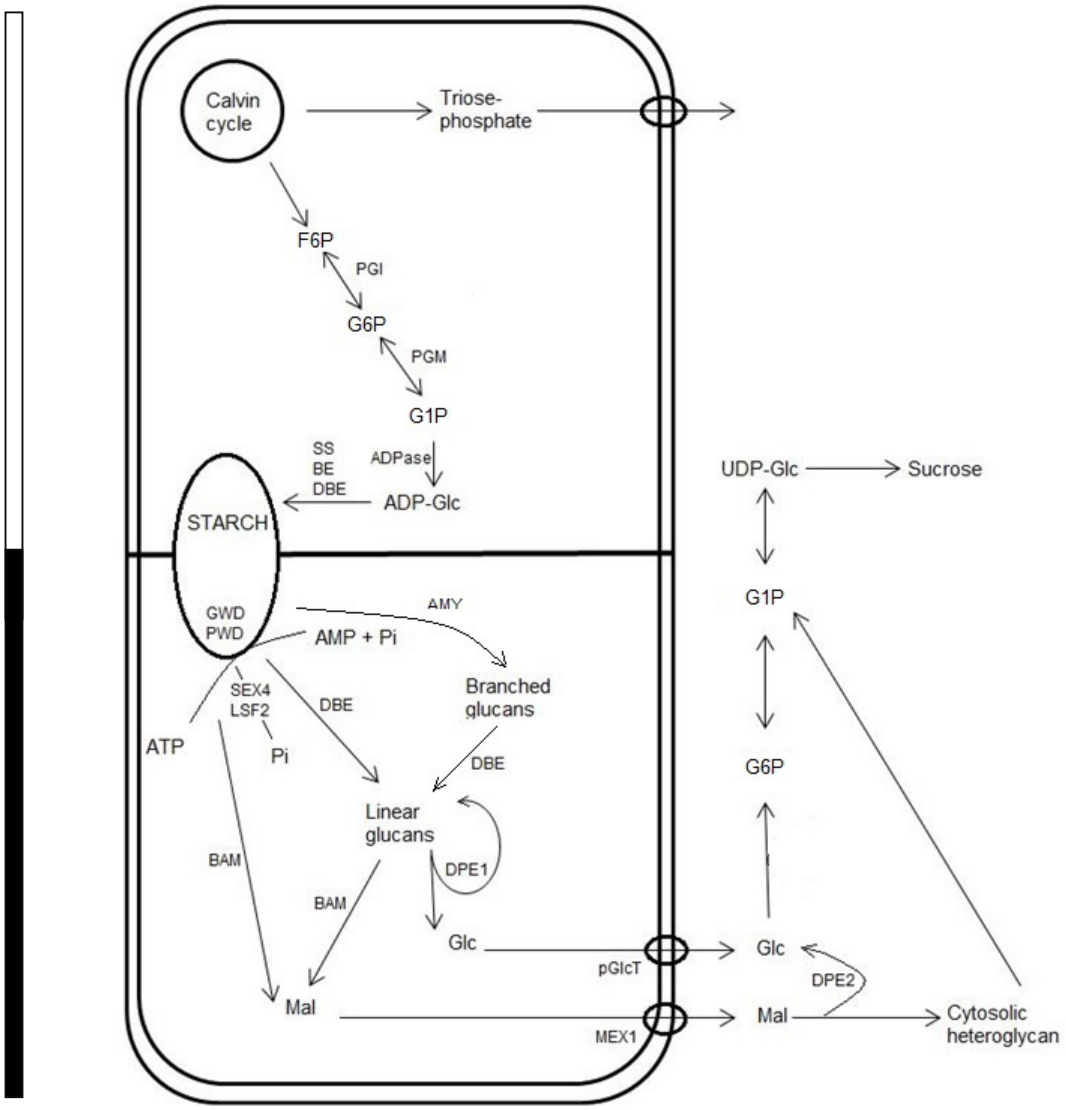


Figure 2. General pathway of starch metabolism in Arabidopsis leaves. Starch manufactured during the day from Calvin cycle intermediates is mobilized at night by a series of enzymes to maltose and glucose. These are transported into the cytosol before being further metabolized to produce sucrose that is exported to sink tissues. Light and dark period reactions are indicated with the bar next to the graph. Unfilled=light, filled=dark. F6P=fructose 6 phosphate, G6P=glucose 6 phosphate, G1P=glucose 1 phosphate, UDP-Glc=UDP glucose, ADP-Glc=ADP glucose, Glc=glucose, Mal=maltose, PGI=phosphoglucoisomerase, PGM=phosphoglucomutase, SS=starch synthases, BE=branching enzymes, DBE=debranching enzymes, GWD=glucan water dikinase, PWD=phosphoglucan water dikinase, SEX4=starch excess 4 , LSF2=Like Starch excess four2, DPE1=disproportionating enzyme1, BAM=β-amylase, AMY=α-amylase, pGlcT=glucose transporter, MEX1=maltose transporter, DPE2=disproportionating enzyme2.

1.3 Starch Degradation

Much has been learned in the past fifteen years about enzymes involved in starch degradation. This has mainly involved an examination of Arabidopsis leaves due to the ease of isolating mutants impaired in starch degradation and which accumulate starch. The lesions present in such *starch excess* (*sex*) mutants have been identified and this has led to the production of a model of how the granule is degraded in Arabidopsis leaves. I will outline what is known about this below.

The first step appears to be that the semi-crystalline, insoluble starch granule is disrupted by the phosphorylating action of glucan water dikinase (GWD) and phosphoglucan water dikinase (PWD; Fig. 2; Lorberth et al., 1998; Yu et al., 2001; Ritte et al., 2002, 2006; Baunsgaard et al., 2005; Edner et al., 2007; Hejazi et al., 2009). It would seem that this is a conserved mechanism because is also reported that GWD homologs are found in many species from unicellular algae to monocots (Worden et al., 2009; Ral et al., 2013). These enzymes phosphorylate glucose moieties within amylopectin at the C6 and the C3 positions respectively (Ritte et al., 2002, 2006). It is thought that this disrupts the double helical structure of the A and B chains within amylopectin allowing access to hydrolytic enzymes. When GWD was silenced or mutated in species like potato, Arabidopsis, tomato and rice there was a large reduction in the phosphate content of the starch and a repression of starch degradation in leaves and tubers of the potatoes (Lorberth et al., 1998; Yu et al., 2001; Nashilevitz et al., 2009; Hirose et al., 2013).

GWD has been demonstrated to have a preference for glucan substrates that contain α-1,6 branch points (Ritte et al., 2002) and, therefore, amylopectin is a better substrate for it than amylose. It was also found that it had a preferential action on specific chain lengths. *In vitro* experiments showed that GWD activity on amylopectin with an average chain-length distribution of 29.5 DP was 20 fold higher than when it is incubated with amylopectin with an average chain-length distribution of 24.9 DP (Mikkelsen et al., 2004). Although GWD can phosphorylate both C6 and C3 positions of the glucan substrate it has a much higher preference for the C6 position (Ritte et al., 2006). A second dikinase is also involved in the phosphorylation process of starch which is known as the phosphoglucan water dikinase (PWD) and which acts on substrates previously phosphorylated by the GWD. (Baunsgaard et al., 2005; Ritte et al., 2006; Hejazi et al., 2009). *In vivo* investigations (Kotting et al., 2005; Ritte et al., 2006; Hejazi et al., 2009) demonstrated that PWD solely phosphorylates C3 positions of the glucan substrate.

Following disruption of the granule by GWD and PWD, the starch becomes degraded by a number of enzymes including endo- and exoamylases, for example α-amylase3 (AMY3), β-amylase3 (BAM3) and debranching enzymes such as isoamylase 3 (ISA3; reviewed in Zeeman et al., 2007, 2010). The *in vitro* action of GWD stimulates activity of specific starch degradative enzymes, while the activities of those enzymes also stimulate GWD (Edner et al., 2007). AMY3 on its own does not seem to be necessary for normal starch turnover in Arabidopsis leaves, however in plants lacking other starch degradative proteins it plays a role (Yu et al., 2005). Two other α-amylases found in Arabidopsis do not contain plastidial transit peptides and mutants of them do not display a starch excess phenotype so it is doubtful that they play a role in degradation (Yu et al., 2005; Kötting et al., 2010). After the surface disruption, β-amylases (BAMs) and isoamylases (ISA) can liberate linear glucans, such as maltose and longer malto-oligosaccharides from the granule surface (Fig. 2). Catalytically active BAM's can act on α1.4-linkages but not where there are branch points at α1.6-linkages in the glucan and these are removed by debranching enzymes, primarily ISA3 (Baba and Kainuma, 1987; Scheidig et al., 2002; Delatte et al.,2006). In Arabidopsis there are nine BAM's, of which four (BAM1, BAM2, BAM3 and BAM4) are localized in the chloroplast. BAM1 and BAM3 have been shown to be catalytic isoforms directly involved in starch degradation (Fulton et al., 2008) while, although BAM4 is non-catalytic, a *bam4* mutant demonstrates reduced starch degradation. Starch levels in *bam2* mutant leaves show no deviation from wild type Arabidopsis plants (Li et al., 2009; Francisco et al., 2010). Interestingly, two of the extra-plastidial BAM's (BAM7 and BAM8) have been demonstrated to act as transcription factors (Reinhold et al., 2011).

The action of these catalytic enzymes produce phosphorylated soluble malto-oligosaccharides where the phosphate needs to be removed before being further degraded (Takeda and Hizukuri, 1981; Edner et al., 2007). This glucan phosphatase activity is catalyzed in *Arabidopsis* by two proteins, Starch EXcess 4 (SEX4) and Like Starch-excess Four2 (LSF2; Fig. 2). SEX4 has the capacity to remove phosphates at both the C6 and C3 positions while LSF2 was shown to be active on phosphate bound at C3 (Kötting et al., 2009; Santelia et al., 2011). Both *sex4* and *lsf2* mutants accumulate phosphorylated malto-oligosaccharides (MOS) and the starch in their leaves is more highly phosphorylated. Like Starch-excess Four1 (LSF1), which is similar to SEX4, also affects starch turnover, although the reason for this is not clear as *lsf1* mutants do not accumulate phosphorylated glucans and there is no measurable reduction in phosphoglucan phosphatase activity (Comparot-Moss et al., 2010).

The dephosphorylated MOS released from starch granules can be acted on by BAMs (discussed above) or a plastidial disproportionating enzyme known as DPE1 (Critchley et al., 2001). This catalyzes a glucanotransferase reaction using MOS with a degree of polymerization equal or greater than three. DPE1 manipulates the sizes of the MOS pool within the chloroplast, but will also produce maltose and glucose which can be exported into the cytoplasm by specific transport proteins, MEX1 and pGlcT, driving the reaction to completion (Niittyla et al., 2004; Cho et al., 2011). Once in the cytoplasm maltose is acted upon by a second disproportionating enzyme (DPE2) leading to the production of glucose (Chia et al., 2004). Starch degradation proceeds in Arabidopsis leaves, therefore, via two distinct pathways. One produces glucose within the chloroplast while the second is extra-plastidial and involves the catabolism of maltose in the cytoplasm. The maltose catabolic route is the one through which the majority flux from starch degradation flows, as shown by the strong repression of starch degradation in *dpe2* and *mex1* mutants compared with *dpe1* and *pglct-1* mutants (Fig. 2; Critchley et al., 2001; Chia et al., 2004; Niittyla et al., 2004; Cho et al., 2011.

The previous sections have outlined the roles of many enzymes in starch turnover in terms of their catalytic activities. Although these have become well characterized in many different species, less is known about coordination of their activities for maximizing plant productivity. The next section will consider how these enzymes are controlled and the importance of this in terms of plant growth.

1.4 Regulation of starch synthesis and degradation

The control of starch metabolism is extremely important for plants. Although great strides have been made in elucidating the enzymes involved in starch synthesis and degradation, knowledge about the mechanisms of how these enzymes are regulated is limited. In the past few years we have begun to understand that this regulation is multi-faceted involving control by redox potential, allosteric regulation, post translational modification, glucan phosphate content, circadian rhythm and the physical and functional interactions between enzymes. (Kötting et al., 2010).

It has become evident that changes in starch turnover can be detrimental for growth. Starch exhaustion in *Arabidopsis* before the next light period was found to lead to alterations in gene expression and an inhibition of growth (Graf et al., 2010; Pantin et al., 2011). The biological clock would appear to have a major influence on the rate of starch degradation as observed when plants were placed in irregular light regimes. It was found that when plants are subjected to either an early or late dark period the rate of starch degradation is altered (either increased or decreased) so that starch becomes almost eliminated at the expected dawn (Lu et al., 2005). This indicates that plants can sense the amount of starch present and adjust the rate of degradation to maintain optimal amounts at dawn. The mechanism by which it is achieved is still not completely understood. It appears that expression of genes encoding known starch degradative enzymes are controlled on the transcriptional level as many of them follow a pattern of up and down regulation over the course of a light-dark cycle (Smith et al., 2004). Transcripts rise late in the light period while their abundances are lower at the end of the dark period (Smith et al., 2004; Lu et al., 2005; Gibon et al.,2006). Remarkably it was found that these shifts in gene expression levels do not have such a big influence on enzyme activities and it is thought to only change turnover mid to long term (Gibon et al., 2006). The control of starch degradation rate in the short term is, therefore, most likely under post-translational regulation.

The first committed step in starch metabolism is the control of the substrate for starch synthesis, ADP-glucose, by AGPase which is known to be partly controlled by post translational modification. One of the first control mechanisms identified for AGPase was the use of allosteric co-factors where it is inhibited by Pi and ADP and activated by 3-PGA (Ghosh and Preiss, 1966). This allosteric activation is also dependent on the redox status of AGPase where the small subunits of the heterotetrameric protein are stabilised though a cysteine disulphide bridge under oxidising conditions. Reduction of AGPase is thought to be dependent on the action of thioredoxin-f and NADPH-thioredoxin C. Initial work by Ballicora et al. (2000) demonstrated that recombinant potato AGPase was reduced and activated by

thioredoxin f from spinach, while it was later shown *in planta* that NADP-thioredoxin reductase C stimulated the activation of AGPase (Michalska et al., 2009). Tiessen et al. (2002) found that AGPase activity was increased when potato tuber slices were incubated with sucrose due to redox activation. The exact mechanism linking sucrose to AGPase activity is still not fully understood. Experiments using isolated chloroplasts by Kolbe et al. (2005) indicated that the link is a trehalose-6-phosphate (Tre6P) mediated mechanism but recent data from Martins et al. (2013) indicates that this is unlikely to be the case *in vivo*. Their data indicates that Tre6P is rather involved in influencing starch breakdown. Other enzymes in starch metabolism have also been found to be sensitive to the redox status. For example the starch dikinase and phosphatase, GWD and SEX4, appear to be activated when reduced (Mikkelsen et al., 2005; Sokolov et al., 2006) as is BAM1 (Sparla et al., 2006). The influence of redox on starch degradation would seem counterproductive because it's generally seen that the plastid is more reduced during photosynthetic periods (Kötting et al., 2010). More studies are, therefore, required to fully understand this mechanism and the interplay of the above mentioned factors.

As discussed above (Section 1.3) reversible glucan phosphorylation in Arabidopsis leaves by GWD, PWD, SEX4 and LSF2 is an important step in the control of starch degradation. Protein phosphorylation has also been linked to the activation of various enzymes (see Hunter et al., 1995; Kennelly et al., 2002 and references within those reviews). Recently Kötting et al. (2010) interrogated the *PhosPhAt* database (http://phosphat.mpimp-golm.mpg.de) and found in *Arabidopsis* 12 proteins involved in starch metabolism which are predicted to be phosphoproteins. A further *in silico* analysis revealed that in Arabidopsis there are genes encoding 45 protein kinases and 21 protein phosphatases predicted to be able to enter the plastids. Subsequent analysis utilizing GFP reporter gene constructs, however, showed that only a low percentage of these were actually transported into the chloroplast. (Lohrig et al., 2009; Schliebner et al., 2008). Taken together these data indicate that control of starch metabolism via protein phosphorylation is possible; however, this has yet to be demonstrated.

There might also be a level of control of starch metabolism based on quaternary structure where proteins physically interact with one another. Several complexes of starch metabolic enzymes have been discovered in *Zea mays* and *Triticum aestivum*. These were mostly formed by SS and BE isoforms, but some also contained PPDK, AGPase and α-glucan phosphorylase (Hennen-Bierwagen et al., 2008,2009; Liu et al., 2009; Tetlow et al., 2004; Tetlow et al., 2008; Table 2). To further complicate matters, some of the complexes described only assemble when the proteins have been phosphorylated and the protein kinase(s) responsible for this is another area which needs to be investigated (Tetlow et al.,

2004; Liu et al., 2009). The role(s) of these complexes in starch metabolism as a whole are still not understood.

Although there are tantalising hints at control of starch metabolism based of post-translational modifications (such as redox and protein phosphorylation), there are only few examples of an effect being observed on recombinant protein *in vitro* being shown to be important *in vivo*. There are, therefore, still a lot of unknown aspects in the starch metabolism regulation that need to be studied. Much of the forthcoming research in starch metabolism is likely to focus on protein interactions and the post-translational modification of these proteins.

In this study the possible interaction between different starch metabolic (BEs, ISA2 and GWD) proteins were investigated, with focus on the phosphate that is present in the cyclical synthesis and degradation of starch. Attempts were also made to link two relatively unstudied proteins to starch metabolism.

Table 1. A list of proteins involved in starch metabolism and and potential regulatory mechanisms derived from a mixture of *in vivo, in vitro* and *in silico* studies. (Kötting et al., 2010)

Starch metabolism proteins and possible regulatory mechanisms			
Enzymes affected by redox			
Enzyme	Gene	Species	Reference
ADP-glucose pyrophosphorylase	*AGPB*	*Solanum tuberosum*	Fu et al., 1998
Glucan, water dikinase	*GWD*	*Solanum tuberosum*	Mikkelsen et al., 2005
Phosphoglucan phosphatase	*SEX4*	*Arabidopsis thaliana*	Sparla et al., 2006
Proteins that might be regulated by phosphorylation, in vivo			
Phosphoglucoisomerase	*PGI1*	*Arabidopsis thaliana*	Heazlewood et al., 2008
Phosphoglucomutase	*PGM1*	*Arabidopsis thaliana*	Heazlewood et al., 2008
Phosphoglucomutase		*Pisum sativum*	Salvucci et al., 1990
AGPase (big subunit)	*APL1*	*Arabidopsis thaliana*	Heazlewood et al., 2008
AGPase (small subunit)	*APS1*	*Arabidopsis thaliana*	Lohrig et al., 2009
Starch synthase II		*Triticum aestivum*	Tetlow et al., 2008
Starch synthase III	*STS3, SSIII*	*Arabidopsis thaliana*	Heazlewood et al., 2008
Starch branching enzyme I		*Triticum aestivum*	Tetlow et al., 2004
Starch branching enzyme IIb		*Zea mays*	Grimaud et al., 2008
Starch branching enzyme II and IIb		*Triticum aestivum*	Tetlow et al., 2004: Tetlow et al., 2008
Granule-bound starch synthase		*Zea mays*	Grimaud et al., 2008
Glucan, water dikinase 1	*GWD1, SEX1*	*Arabidopsis thaliana*	Heazlewood et al., 2008
Glucan, water dikinase 2	*GWD2*	*Arabidopsis thaliana*	Heazlewood et al., 2008
Transglucosidase	DPE2	*Arabidopsis thaliana*	Heazlewood et al., 2008
α-Amylase 3	AMY3	*Arabidopsis thaliana*	Heazlewood et al., 2008
β-Amylase 1	BAM1	*Arabidopsis thaliana*	Heazlewood et al., 2008
β-Amylase 3	BAM3	*Arabidopsis thaliana*	Lohrig et al., 2009
Limit dextranase	LDA1	*Arabidopsis thaliana*	Lohrig et al., 2009
Glucose transporter	GLT1	*Arabidopsis thaliana*	Heazlewood et al., 2008
Maltose transporter	MEX1	*Arabidopsis thaliana*	Heazlewood et al., 2008

Table 2. Proteins demonstrated to form complexes in cereal endosperm. Adapted from Kotting et al. (2010)

Proteins in complex	Species	Reference
SSIIa,SSIII, BEIIa,BEIIb,PPDK,AGPase	*Zea mays*	Hennen-Bierwagen et al., 2008,2009
SSIIa, BEIIa, BEIIb, α-glucan phosphorylase	*Zea mays*	Hennen-Bierwagen et al., 2008
SSI, SSIIa, BEI, BEIIa, α-glucan phosphorylase	*Zea mays*	Liu et al., 2009
BEI, BEIIb, α-glucan phosphorylase	*Triticum aestivum*	Tetlow et al., 2004
SSI, SSIIa, BEIIa or BEIIb	*Triticum aestivum*	Tetlow et al., 2008

References

Abel GJW, Springer F, Willmitzer L, Kossmann J (1996) Cloning and functional analysis of a cDNA encoding a novel 139 kDa starch synthase from potato (Solanum tuberosum L). Plant Journal **10:** 981-991

Baba T, Kainuma K (1987) Partial Hydrolysis of Sweet-Potato Starch with Beta-Amylase. Agric Biol Chem **51:** 1365-1371

Bahaji A, Li J, Ovecka M, Ezquer I, Munoz FJ, Baroja-Fernandez E, Romero JM, Almagro G, Montero M, Hidalgo M, Sesma MT, Pozueta-Romero J (2011) Arabidopsis thaliana Mutants Lacking ADP-Glucose Pyrophosphorylase Accumulate Starch and Wild-type ADP-Glucose Content: Further Evidence for the Occurrence of Important Sources, other than ADP-Glucose Pyrophosphorylase, of ADP-Glucose Linked to Leaf Starch Biosynthesis. Plant and Cell Physiology **52:** 1162-1176

Ballicora MA, Frueauf JB, Fu YB, Schurmann P, Preiss J (2000) Activation of the potato tuber ADP-glucose pyrophosphorylase by thioredoxin. J Biol Chem **275:** 1315-1320

Balmer Y, Vensel WH, Cai N, Manieri W, Schurmann P, Hurkman WJ, Buchanan BB (2006) A complete ferredoxin/thioredoxin system regulates fundamental processes in amyloplasts. Proc Natl Acad Sci U S A **103:** 2988-2993

Bhattacharyya MK, Smith AM, Ellis THN, Hedley C, Martin C (1990) The Wrinkled-Seed Character of Pea Described by Mendel is Caused by a Transposon-Like Insertion in a Gene Encoding Starch-Branching Enzyme. Cell **60:** 115-122

Borovsky D, Smith EE, Whelan WJ, French D, Kikumoto S (1979) Mechanism of Q-Enzyme Action and its Influence on the Structure of Amylopectin. Arch Biochem Biophys **198:** 627-631

Buléon A, Colonna P, Planchot V, Ball S (1998) Starch granules: structure and biosynthesis. Int J Biol Macromol **23:** 85-112

Burton RA, Bewley JD, Smith AM, Bhattacharyya MK, Tatge H, Ring S, Bull V, Hamilton WDO, Martin C (1995) Starch Branching Enzymes Belonging to Distinct Enzyme Families are Differentially Expressed during Pea Embryo Development. Plant Journal **7:** 3-15

Burton RA, Jenner H, Carrangis L, Fahy B, Fincher GB, Hylton C, Laurie DA, Parker M, Waite D, van Wegen S, Verhoeven T, Denyer K (2002) Starch granule initiation and growth are altered in barley mutants that lack isoamylase activity. Plant Journal **31:** 97-112

Bustos R, Fahy B, Hylton CM, Seale R, Nebane NM, Edwards A, Martin C, Smith AM (2004) Starch granule initiation is controlled by a heteromultimeric isoamylase in potato tubers. Proc Natl Acad Sci U S A **101:** 2215-2220

Chia T, Thorneycroft D, Chapple A, Messerli G, Chen J, Zeeman SC, Smith SM, Smith AM (2004) A cytosolic glucosyltransferase is required for conversion of starch to sucrose in Arabidopsis leaves at night. Plant Journal **37:** 853-863

Cho M, Lim H, Shin DH, Jeon J, Bhoo SH, Park Y, Hahn T (2011) Role of the plastidic glucose translocator in the export of starch degradation products from the chloroplasts in Arabidopsis thaliana. New Phytol **190:** 101-112

Comparot-Moss S, Koetting O, Stettler M, Edner C, Graf A, Weise SE, Streb S, Lue W, MacLean D, Mahlow S, Ritte G, Steup M, Chen J, Zeeman SC, Smith AM (2010) A Putative Phosphatase, LSF1, Is Required for Normal Starch Turnover in Arabidopsis Leaves. Plant Physiol **152:** 685-697

Critchley JH, Zeeman SC, Takaha T, Smith AM, Smith SM (2001) A critical role for disproportionating enzyme in starch breakdown is revealed by a knock-out mutation in Arabidopsis. Plant Journal **26:** 89-100

Dauvillee D, Colleoni C, Mouille G, Buléon A, Gallant DJ, Bouchet B, Morell MK, d'Hulst C, Myers AM, Ball SG (2001) Two loci control phytoglycogen production in the monocellular green alga Chlamydomonas reinhardtii. Plant Physiol **125:** 1710-1722

Czaja AT (1969) Die Mikroskopie der Stärkekörner. In: Handbuch der Stärke in Einzeldarstellungen Vol. 6, Verlag Paul Parey, Berlin, Deutsland

Dauvillee D, Colleoni C, Mouille G, Morell MK, d'Hulst C, Wattebled F, Lienard L, Delvalle D, Ral JP, Myers AM, Ball SG (2001) Biochemical characterization of wild-type and mutant isoamylases of Chlamydomonas reinhardtii supports a function of the multimeric enzyme organization in amylopectin maturation. Plant Physiol **125:** 1723-1731

Dauvillee D, Mestre V, Colleoni C, Slomianny MC, Mouille G, Delrue B, d'Hulst C, Bliard C, Nuzillard JM, Ball S (2000) The debranching enzyme complex missing in glycogen accumulating mutants of Chlamydomonas reinhardtii displays an isoamylase-type specificity. Plant Science **157:** 145-156

Delatte T, Umhang M, Trevisan M, Eicke S, Thorneycroft D, Smith SM, Zeeman SC (2006) Evidence for distinct mechanisms of starch granule breakdown in plants. J Biol Chem **281:** 12050-12059

Denyer K, Clarke B, Hylton C, Tatge H, Smith AM (1996) The elongation of amylose and amylopectin chains in isolated starch granules. Plant Journal **10:** 1135-1143

Denyer K, Waite D, Edwards A, Martin C, Smith AM (1999) Interaction with amylopectin influences the ability of granule-bound starch synthase I to elongate malto-oligosaccharides. Biochem J **342:** 647-653

Dumez S, Wattebled F, Dauvillee D, Delvalle D, Planchot V, Ball SG, D'Hulst C (2006) Mutants of Arabidopsis lacking starch branching enzyme II substitute plastidial starch synthesis by cytoplasmic maltose accumulation. Plant Cell **18:** 2694-2709

Edner C, Li J, Albrecht T, Mahlow S, Hejazi M, Hussain H, Kaplan F, Guy C, Smith SM, Steup M, Ritte G (2007) Glucan, water dikinase activity stimulates breakdown of starch granules by plastidial beta-amylases. Plant Physiol **145:** 17-28

Edwards A, Marshall J, Sidebottom C, Visser RGF, Smith AM, Martin C (1995) Biochemical and Molecular Characterization of a Novel Starch Synthase from Potato-Tubers. Plant Journal **8:** 283-294

Ellis RP, Cochrane MP, Dale MFB, Duffus CM, Lynn A, Morrison IM, Prentice RDM, Swanston JS, Tiller SA (1998) Starch production and industrial use. J Sci Food Agric **77:** 289-311

Francisco P, Li J, Smith SM (2010) The gene encoding the catalytically inactive beta-amylase BAM4 involved in starch breakdown in Arabidopsis leaves is expressed preferentially in vascular tissues in source and sink organs. J Plant Physiol **167:** 890-895

Fu YB, Ballicora MA, Leykam JF, Preiss J (1998) Mechanism of reductive activation of potato tuber ADP-glucose pyrophosphorylase. J Biol Chem **273:** 25045-25052

Fulton DC, Stettler M, Mettler T, Vaughan CK, Li J, Francisco P, Gil D, Reinhold H, Eicke S, Messerli G, Dorken G, Halliday K, Smith AM, Smith SM, Zeeman SC (2008) beta-AMYLASE4, a noncatalytic protein required for starch breakdown, acts upstream of three active beta-amylases in Arabidopsis chloroplasts. Plant Cell **20:** 1040-1058

Ga'mez-Arjona FM, Li J, Raynaud S, Baroja-Fernandez E, Munoz FJ, Ovecka M, Ragel P, Bahaji A, Pozueta-Romero J, Merida A (2011) Enhancing the expression of starch synthase class IV results in increased levels of both transitory and long-term storage starch. Plant Biotechnology Journal **9:** 1049-1060

Ghosh HP, Preiss J (1966) Adenosine diphosphate glucose pyrophosphorylase. A regulatory enzyme in the biosynthesis of starch in spinach leaf chloroplasts. The Journal of biological chemistry **241:** 4491-504

Gibon Y, Usadel B, Blaesing OE, Kamlage B, Hoehne M, Trethewey R, Stitt M (2006) Integration of metabolite with transcript and enzyme activity profiling during diurnal cycles in Arabidopsis rosettes. Genome Biol **7:** R76

Graf A, Schlereth A, Stitt M, Smith AM (2010) Circadian control of carbohydrate availability for growth in Arabidopsis plants at night. Proc Natl Acad Sci U S A **107:** 9458-9463

Grimaud F, Rogniaux H, James MG, Myers AM, Planchot V (2008) Proteome and phosphoproteome analysis of starch granule-associated proteins from normal maize and mutants affected in starch biosynthesis. J Exp Bot **59:** 3395-3406

Guan HP, Preiss J (1993) Differentiation of the Properties of the Branching Isozymes from Maize (Zea-Mays). Plant Physiol **102:** 1269-1273

Hanashiro I, Abe J, Hizukuri S (1996) A periodic distribution of the chain length of amylopectin as revealed by high-performance anion-exchange chromatography. Carbohydr Res **283:** 151-159

Heazlewood JL, Durek P, Hummel J, Selbig J, Weckwerth W, Walther D, Schulze WX (2008) PhosPhAt: a database of phosphorylation sites in Arabidopsis thaliana and a plant-specific phosphorylation site predictor. Nucleic Acids Res **36:** D1015-D1021

Hedman KD, Boyer CD (1983) Allelic Studies of the Amylose-Extender Locus of Zea-Mays-L - Levels of the Starch Branching Enzymes. Biochem Genet **21:** 1217-1222

Hedman KD, Boyer CD (1982) Gene Dosage at the Amylose-Extender Locus of Maize - Effects on the Levels of Starch Branching Enzymes. Biochem Genet **20:** 483-492

Hejazi M, Fettke J, Paris O, Steup M (2009) The Two Plastidial Starch-Related Dikinases Sequentially Phosphorylate Glucosyl Residues at the Surface of Both the A- and B-Type Allomorphs of Crystallized Maltodextrins But the Mode of Action Differs. Plant Physiol **150:** 962-976

Hennen-Bierwagen TA, Lin Q, Grimaud F, Planchot V, Keeling PL, James MG, Myers AM (2009) Proteins from Multiple Metabolic Pathways Associate with Starch Biosynthetic Enzymes in High Molecular Weight Complexes: A Model for Regulation of Carbon Allocation in Maize Amyloplasts. Plant Physiol **149:** 1541-1559

Hennen-Bierwagen TA, Liu F, Marsh RS, Kim S, Gan Q, Tetlow IJ, Emes MJ, James MG, Myers AM (2008) Starch biosynthetic enzymes from developing maize endosperm associate in multisubunit complexes. Plant Physiol **146:** 1892-1908

Hirose T, Aoki N, Harada Y, Okamura M, Hashida Y, Ohsugi R, Akio M, Hirochika H, Terao T (2013) Disruption of a rice gene for alpha-glucan water dikinase, OsGWD1, leads to hyperaccumulation of starch in leaves but exhibits limited effects on growth. Frontiers in plant science **4:** 147-147

Hizukuri S (1986) Polymodal Distribution of the Chain Lengths of Amylopectins, and its Significance. Carbohydr Res **147:** 342-347

Hizukuri S (1985) Relationship between the Distribution of the Chain-Length of Amylopectin and the Crystalline-Structure of Starch Granules. Carbohydr Res **141:** 295-306

Hizukuri S, Takeda Y, Yasuda M, Suzuki A (1981) Multi-Branched Nature of Amylose and the Action of Debranching Enzymes. Carbohydr Res **94:** 205-213

Hofvander P, Andersson M, Larsson CT, Larsson H (2004) Field performance and starch characteristics of high-amylose potatoes obtained by antisense gene targeting of two branching enzymes. Plant Biotechnology Journal **2:** 311-320

Hunter T (1995) Protein-Kinases and Phosphatases - the Yin and Yang of Protein-Phosphorylation and Signaling. Cell **80:** 225-236

James MG, Robertson DS, Myers AM (1995) Characterization of the Maize Gene Sugary1, a Determinant of Starch Composition in Kernels. Plant Cell **7:** 417-429

Jobling S (2004) Improving starch for food and industrial applications. Curr Opin Plant Biol **7:** 210-218

Jobling SA, Schwall GP, Westcott RJ, Sidebottom CM, Debet M, Gidley MJ, Jeffcoat R, Safford R (1999) A minor form of starch branching enzyme in potato (Solanum tuberosum L.) tubers has a major effect on starch structure: cloning and characterisation of multiple forms of SBE A. Plant Journal **18:** 163-171

Kammerer B, Fischer K, Hilpert B, Schubert S, Gutensohn M, Weber A, Flugge UI (1998) Molecular characterization of a carbon transporter in plastids from heterotrophic tissues: The glucose 6-phosphate phosphate antiporter. Plant Cell **10:** 105-117

Kennelly PJ (2002) Protein kinases and protein phosphatases in prokaryotes: a genomic perspective. FEMS Microbiol Lett **206:** 1-8

Khoshnoodi J, Blennow A, Ek B, Rask L, Larsson H (1996) The multiple forms of starch-branching enzyme I in Solanum tuberosum. European Journal of Biochemistry **242:** 148-155

Kolbe A, Tiessen A, Schluepmann H, Paul M, Ulrich S, Geigenberger P (2005) Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase. Proc Natl Acad Sci U S A **102:** 11118-11123

Kossmann J, Abel GJW, Springer F, Lloyd JR, Willmitzer L (1999) Cloning and functional analysis of a cDNA encoding a starch synthase from potato (Solanum tuberosum L,) that is predominantly expressed in leaf tissue. Planta **208:** 503-511

Kötting O, Kossmann J, Zeeman SC, Lloyd JR (2010) Regulation of starch metabolism: the age of enlightenment? Curr Opin Plant Biol **13:** 321-329

Kötting O, Santelia D, Edner C, Eicke S, Marthaler T, Gentry MS, Comparot-Moss S, Chen J, Smith AM, Steup M, Ritte G, Zeeman SC (2009) STARCH-EXCESS4 Is a Laforin-Like Phosphoglucan Phosphatase Required for Starch Degradation in Arabidopsis thaliana. Plant Cell **21:** 334-346

Kötting O, Pusch K, Tiessen A, Geigenberger P, Steup M, Ritte G (2005) Identification of a novel enzyme required for starch metabolism in Arabidopsis leaves. The phosphoglucan, water dikinase. Plant Physiol **137:** 242-252

Kubo A, Fujita N, Harada K, Matsuda T, Satoh H, Nakamura Y (1999) The starch-debranching enzymes isoamylase and pullulanase are both involved in amylopectin biosynthesis in rice endosperm. Plant Physiol **121:** 399-409

Kühn C, Barker L, Burkle L, Frommer WB (1999) Update on sucrose transport in higher plants. J Exp Bot **50:** 935-953

Larsson CT, Hofvander P, Khoshnoodi J, Ek B, Rask L, Larsson H (1996) Three isoforms of starch synthase and two isoforms of branching enzyme are present in potato tuber starch. Plant Science **117:** 9-16

Larsson CT, Khoshnoodi J, Ek B, Rask L, Larsson H (1998) Molecular cloning and characterization of starch-branching enzyme II from potato. Plant Mol Biol **37:** 505-511

Li J, Francisco P, Zhou W, Edner C, Steup M, Ritte G, Bond CS, Smith SM (2009) Catalytically-inactive beta-amylase BAM4 required for starch breakdown in Arabidopsis leaves is a starch-binding-protein. Arch Biochem Biophys **489:** 92-98

Liu F, Makhmoudova A, Lee EA, Wait R, Emes MJ, Tetlow IJ (2009) The amylose extender mutant of maize conditions novel protein-protein interactions between starch biosynthetic enzymes in amyloplasts. J Exp Bot **60:** 4423-4440

Lohrig K, Mueller B, Davydova J, Leister D, Wolters DA (2009) Phosphorylation site mapping of soluble proteins: bioinformatical filtering reveals potential plastidic phosphoproteins in Arabidopsis thaliana. Planta **229:** 1123-1134

Lorberth R, Ritte G, Willmitzer L, Kossmann J (1998) Inhibition of a starch-granule-bound protein leads to modified starch and repression of cold sweetening. Nat Biotechnol **16:** 473-477

Lu Y, Gehan JP, Sharkey TD (2005) Daylength and circadian effects on starch degradation and maltose metabolism. Plant Physiol **138:** 2280-2291

Marshall J, Sidebottom C, Debet M, Martin C, Smith AM, Edwards A (1996) Identification of the major starch synthase in the soluble fraction of potato tubers. Plant Cell **8:** 1121-1135

Michalska J, Zauber H, Buchanan BB, Cejudo FJ, Geigenberger P (2009) NTRC links built-in thioredoxin to light and sucrose in regulating starch synthesis in chloroplasts and amyloplasts. Proc Natl Acad Sci U S A **106:** 9908-9913

Mikkelsen R, Baunsgaard L, Blennow A (2004) Functional characterization of alpha-glucan,water dikinase, the starch phosphorylating enzyme. Biochem J **377:** 525-532

Mikkelsen R, Mutenda KE, Mant A, Schurmann P, Blennow A (2005) alpha-Glucan, water dikinase (GWD): A plastidic enzyme with redox-regulated and coordinated catalytic activity and binding affinity. Proc Natl Acad Sci U S A **102:** 1785-1790

Mizuno K, Kawasaki T, Shimada H, Satoh H, Kobayashi E, Okumura S, Arai Y, Baba T (1993) Alteration of the Structural-Properties of Starch Components by the Lack of an Isoform of Starch Branching Enzyme in Rice Seeds. J Biol Chem **268:** 19084-19091

Mizuno K, Kimura K, Arai Y, Kawasaki T, Shimada H, Baba T (1992) Starch Branching Enzymes from Immature Rice Seeds. J Biochem **112:** 643-651

Mouille G, Maddelein ML, Libessart N, Talaga P, Decq A, Delrue B, Ball S (1996) Preamylopectin processing: A mandatory step for starch biosynthesis in plants. Plant Cell **8:** 1353-1366

Nakamura Y, Yamanouchi H (1992) Nucleotide-Sequence of a Cdna-Encoding Starch-Branching Enzyme, Or Q-Enzyme-I, from Rice Endosperm. Plant Physiol **99:** 1265-1266

Nashilevitz S, Melamed-Bessudo C, Aharoni A, Kossmann J, Wolf S, Levy AA (2009) The legwd mutant uncovers the role of starch phosphorylation in pollen development and germination in tomato. Plant Journal **57:** 1-13

Niittyla T, Messerli G, Trevisan M, Chen J, Smith AM, Zeeman SC (2004) A previously unknown maltose transporter essential for starch degradation in leaves. Science **303:** 87-89

Pantin F, Simonneau T, Rolland G, Dauzat M, Muller B (2011) Control of Leaf Expansion: A Developmental Switch from Metabolics to Hydraulics. Plant Physiol **156:** 803-815

Peat S, Whelan WJ, Hinson KA (1952) Synthetic action of almond emulsin. Nature **170:** 1056-7

Ral J, Bowerman AF, Li Z, Sirault X, Furbank R, Pritchard JR, Bloemsma M, Cavanagh CR, Howitt CA, Morell MK (2013) Down-regulation of glucan, water-dikinase activity in wheat endosperm increases vegetative biomass and yield (vol 10, pg 871, 2012). Plant Biotechnology Journal **11:** 390-391

Reinhold H, Soyk S, Simkova K, Hostettler C, Marafino J, Mainiero S, Vaughan CK, Monroe JD, Zeeman SC (2011) beta-Amylase-Like Proteins Function as Transcription Factors in Arabidopsis, Controlling Shoot Growth and Development. Plant Cell **23:** 1391-1403

Ritte G, Lloyd JR, Eckermann N, Rottmann A, Kossmann J, Steup M (2002) The starch-related R1 protein is an alpha-glucan, water dikinase. Proc Natl Acad Sci U S A **99:** 7166-7171

Ritte G, Heydenreich M, Mahlow S, Haebel S, Koetting O, Steup M (2006) Phosphorylation of C6- and C3-positions of glucosyl residues in starch is catalysed by distinct dikinases. FEBS Lett **580:** 4872-4876

Roldan I, Wattebled F, Lucas MM, Delvalle D, Planchot V, Jimenez S, Perez R, Ball S, D'Hulst C, Merida A (2007) The phenotype of soluble starch synthase IV defective mutants of Arabidopsis thaliana suggests a novel function of elongation enzymes in the control of starch granule formation. Plant Journal **49:** 492-504

Rydberg U, Andersson L, Andersson R, Aman P, Larsson H (2001) Comparison of starch branching enzyme I and II from potato. European Journal of Biochemistry **268:** 6140-6145

Safford R, Jobling SA, Sidebottom CM, Westcott RJ, Cooke D, Tober KJ, Strongitharm BH, Russell AL, Gidley MJ (1998) Consequences of antisense RNA inhibition of starch branching enzyme activity on properties of potato starch. Carbohydr Polym **35:** 155-168

Salvucci ME, Drake RR, Broadbent KP, Haley BE, Hanson KR, Mchale NA (1990) Identification of the 64-Kilodalton Chloroplast Stromal Phosphoprotein as Phosphoglucomutase. Plant Physiol **93:** 105-109

Santelia D, Kötting O, Seung D, Schubert M, Thalmann M, Bischof S, Meekins DA, Lutz A, Patron N, Gentry MS, Allain FH-, Zeeman SC (2011) The Phosphoglucan Phosphatase Like Sex Four2 Dephosphorylates Starch at the C3-Position in Arabidopsis. Plant Cell **23:** 4096-4111

Scheidig A, Frohlich A, Schulze S, Lloyd JR, Kossmann J (2002) Downregulation of a chloroplast-targeted beta-amylase leads to a starch-excess phenotype in leaves. Plant Journal **30:** 581-591

Schliebner I, Pribil M, Zuehlke J, Dietzmann A, Leister D (2008) A survey of chloroplast protein kinases and phosphatases in Arabidopsis thaliana. Curr Genomics **9:** 184-190

Schwall GP, Safford R, Westcott RJ, Jeffcoat R, Tayal A, Shi YC, Gidley MJ, Jobling SA (2000) Production of very-high-amylose potato starch by inhibition of SBE A and B. Nat Biotechnol **18:** 551-554

Smith AM (2012) Starch in the Arabidopsis plant. Starch-Starke **64:** 421-434

Smith SM, Fulton DC, Chia T, Thorneycroft D, Chapple A, Dunstan H, Hylton C, Zeeman SC, Smith AM (2004) Diurnal changes in the transcriptome encoding enzymes of starch metabolism provide evidence for both transcriptional and posttranscriptional regulation of starch metabolism in arabidopsis leaves. Plant Physiol **136:** 2687-2699

Sokolov LN, Dominguez-Solis JR, Allary AL, Buchanan BB, Luan S (2006) A redox-regulated chloroplast protein phosphatase binds to starch diurnally and functions in its accumulation. Proc Natl Acad Sci U S A **103:** 9732-9737

Sparla F, Costa A, Lo Schiavo F, Pupillo P, Trost P (2006) Redox regulation of a novel plastid-targeted beta-amylase of Arabidopsis. Plant Physiol **141:** 840-850

Stitt M, Zeeman SC (2012) Starch turnover: pathways, regulation and role in growth. Curr Opin Plant Biol **15:** 282-292

Streb S, Egli B, Eicke S, Zeeman SC (2009) The Debate on the Pathway of Starch Synthesis: A Closer Look at Low-Starch Mutants Lacking Plastidial Phosphoglucomutase Supports the Chloroplast-Localized Pathway. Plant Physiol **151:** 1769-1772

Szydlowski N, Ragel P, Hennen-Bierwagen TA, Planchot V, Myers AM, Merida A, d'Hulst C, Wattebled F (2011) Integrated functions among multiple starch synthases determine both amylopectin chain length and branch linkage location in Arabidopsis leaf starch. J Exp Bot **62:** 4547-4559

Szydlowski N, Ragel P, Raynaud S, Mercedes Lucas M, Roldan I, Montero M, Jose Munoz F, Ovecka M, Bahaji A, Planchot V, Pozueta-Romero J, D'Hulst C, Merida A

(2009) Starch Granule Initiation in Arabidopsis Requires the Presence of Either Class IV or Class III Starch Synthases. Plant Cell **21:** 2443-2457

Takeda Y, Hizukuri S, Juliano BO (1987) Structures of Rice Amylopectins with Low and High Affinities for Iodine. Carbohydr Res **168:** 79-88

Takeda Y, Shirasaka K, Hizukuri S (1984) Examination of the Purity and Structure of Amylose by Gel-Permeation Chromatography. Carbohydr Res **132:** 83-92

Tetlow IJ, Wait R, Lu ZX, Akkasaeng R, Bowsher CG, Esposito S, Kosar-Hashemi B, Morell MK, Emes MJ (2004) Protein phosphorylation in amyloplasts regulates starch branching enzyme activity and protein-protein interactions. Plant Cell **16:** 694-708

Tetlow IJ, Beisel KG, Cameron S, Makhmoudova A, Liu F, Bresolin NS, Wait R, Morell MK, Emes MJ (2008) Analysis of protein complexes in wheat amyloplasts reveals functional interactions among starch biosynthetic enzymes. Plant Physiol **146:** 1878-1891

Tiessen A, Hendriks JHM, Stitt M, Branscheid A, Gibon Y, Farre EM, Geigenberger P (2002) Starch synthesis in potato tubers is regulated by post-translational redox modification of ADP-glucose pyrophosphorylase: A novel regulatory mechanism linking starch synthesis to the sucrose supply. Plant Cell **14:** 2191-2213

Tomlinson K, Denyer K (2003) Starch synthesis in cereal grains. Advances in Botanical Research, Vol 40 **40:** 1-61

Wattebled F, Dong Y, Dumez S, Delvalle D, Planchot R, Berbezy P, Vyas D, Colonna P, Chatterjee M, Ball S, D'Hulst C (2005) Mutants of Arabidopsis lacking a chloroplastic isoamylase accumulate phytoglycogen and an abnormal form of amylopectin. Plant Physiol **138:** 184-195

Worden AZ, Panaud, Piegu (2009) Green evolution and dynamic adaptations revealed by genomes of the marine picoeukaryotes Micromonas (vol 324, pg 268, 2009). Science **325:** 147-147

Yu TS, Kofler H, Hausler RE, Hille D, Flugge UI, Zeeman SC, Smith AM, Kossmann J, Lloyd J, Ritte G, Steup M, Lue WL, Chen JC, Weber A (2001) The Arabidopsis sex1 mutant is defective in the R1 protein, a general regulator of starch degradation in plants, and not in the chloroplast hexose transporter. Plant Cell **13:** 1907-1918

Yu TS, Zeeman SC, Thorneycroft D, Fulton DC, Dunstan H, Lue WL, Hegemann B, Tung SY, Umemoto T, Chapple A, Tsai DL, Wang SM, Smith AM, Chen J, Smith SM (2005) alpha-Amylase is not required for breakdown of transitory starch in Arabidopsis leaves. J Biol Chem **280:** 9773-9779

SC, Smith SM, Smith AM (2002) The priming of amylose synthesis in Arabidopsis leaves. Plant Physiol **128:** 1069-1076

Zeeman SC, Tiessen A, Pilling E, Kato KL, Donald AM, Smith AM (2002) Starch synthesis in arabidopsis. Granule synthesis, composition, and structure. Plant Physiol **129:** 516-529

Zeeman SC, Umemoto T, Lue WL, Au-Yeung P, Martin C, Smith AM, Chen J (1998) A mutant of Arabidopsis lacking a chloroplastic isoamylase accumulates both starch and phytoglycogen. Plant Cell **10:** 1699-1711

Zeeman SC, Delatte T, Messerli G, Umhang M, Stettler M, Mettler T, Streb S, Reinhold H, Kötting O (2007) Starch breakdown: recent discoveries suggest distinct pathways and novel mechanisms. Functional Plant Biology **34:** 465-473

Zeeman SC, Kossmann J, Smith AM (2010) Starch: Its Metabolism, Evolution, and Biotechnological Modification in Plants. Annual Review of Plant Biology, Vol 61 **61:** 209-234

Zrenner R, Salanoubat M, Willmitzer L, Sonnewald U (1995) Evidence of the Crucial Role of Sucrose Synthase for Sink Strength using Transgenic Potato Plants (Solanum-Tuberosum L). Plant Journal **7:** 97-107

Chapter two

Production of transgenic plants to examine potential functional interactions between starch metabolic proteins.

2.1 Introduction

Due to the complexity of starch metabolism the regulation of how the granule is manufactured has not yet been fully been unravelled. For example, it has been shown that simultaneous repression of both SS2 and SS3 affected synthesis of the granule far more drastically than could be explained simply by the reduction in total SS activity (Edwards et al., 1999; Lloyd et al., 1999). One theory to account for this is that the enzymes act in complexes and that repression of one enzyme disrupts the concerted action of this complex. Another possibility is that there is functional interaction, where each isoform produces a substrate within the granule that the other isoform utilizes. Although several potato lines have been produced where more than one starch metabolic enzyme has been repressed (Edwards et al., 1999; Lloyd et al., 1999; Schwall et al., 2000; Fulton et al., 2002; Jobling et al., 2002), there are still many combinations that have not been examined even though there is circumstantial evidence for functional interactions between specific enzymes. For example, repression of some SS isoforms as well as BE isoforms leads to starch with altered phosphate (Abel et al., 1996; Safford et al., 1998; Jobling et al., 1999; Kossmann et al., 1999) indicating that they may interact with the GWD.

The aim of this part of the study is to produce transgenic plants that will help to investigate the nature of any interaction between starch branching enzymes and the GWD in manufacturing starch in potato tubers. In addition, as isoamylase isoforms make up part of the same alpha-amylase supergroup of proteins as BE, interaction between the ISA1/ISA2 complex and GWD will also be examined. To this end it was intended that transgenic plants lacking each of these enzymes individually, as well as in combination with each other, would be produced in order to study the starch that they manufacture. The starch that is produced by these available lines would be analysed for amylose and G6P contents.

2.2 Materials and Methods

2.2.1 Chemicals used

All chemicals used in the studies were of analytical grade. They were ordered from Roche Biochemicals (Mannheim, Germany), Sigma-Aldrich (St. Louis, MO, USA), Qiagen (Dusseldorf, Germany), Promega (Madison, WI, USA) and Megazyme (Co. Wicklow, Ireland). Primers were designed using Primer3Plus (Untergasser and Nijveen, 2007) and were purchased from Integrated DNA Technologies (Coralville, IA, USA) or Inqaba Biotech (Pretoria, South Africa)

2.2.1 Plant material and growth conditions

Wild type *Solanum tuberosum* (cv. Désirée) plants were grown on MS (Murashige and Skoog, 1962) media (4.32g/L MS basal salts , 2% (w/v) sucrose, 0.8% (w/v) agar) in 1 L tissue culture pots with a 16h/8h day night cycle. The plants were grown for two months prior to sub culturing or transfer to soil. Two month old plantlets were potted in a soil mixture (2 parts potting soil, 1 part sand and 1 part vermiculite) and grown in ambient greenhouse conditions.

2.2.2 Plasmid construction

S.tuberosum RNA was extracted using the Qiagen RNAeasy Plant Mini Kit and stored at -80^{o}C until further use. cDNA was produced using Fermentas cDNA first strand kit and was used as template in PCR. Fragments of between 299 and 301 bp for the *GWD, BE1, BE2, ISA2* genes were amplified using primers shown in Table 3. The fragments were purified by using the Fermentas gel-purification kit and ligated into pGem-t-easy (Fermentas).

Gene fragments were restricted out of the pGem-t-easy vector using enzymes shown in Table 3 and ligated individually, or in combination with one another, in pBK-CMV (Agilent). The gene fragments in pBK-CMV were subsequently PCR amplified using primers that contained *att*B1 and *att*B2 recombination sites. These fragments were recombined into the pHellsgate 2 (Helliwell et al., 2002) vector using BP clonase (Invitrogen).

Table 3. PCR primers used for the amplification of gene fragments for the RNAi constructs. Incorporated *Att* and restriction enzyme sites are bold. Annealing sites are underlined. RE= Restriction Enzyme; Na = Not applicable

Target	Primer sequences	RE sites	Product size (bp)
GWD FWD	5'-AT**GGATCC**TGGTGCTTCCATACAGGACA-3'	*BamHI*	300
GWD REV	5'-AT**GAGCTC**TTCAGGTGCTTTTCCACCTT-3'	*SacI*	
BEI FWD	5'-AT**CTGCAG**CAGCTCTGAGCCACGTGTTAA-3'	*PstI*	300
BEI REV	5'-AT**GAATTC**TGGCCAATATCAAAGCCATT-3'	*EcoRI*	
BEII FWD	5'-AT**CTCGAG**GAGGATTGGAGAGTGGGTGA-3'	*XhoI*	299
BEII REV	5'-ATT**CTAGA**GGGAAATCAAACTCAGG-3'	*XbaI*	
ISA2 FWD	5'-AT**CTGCAG**TCTCACGCAGTGCAAGAAGT-3'	*PstI*	301
ISA2 REV	5'-AT**GAATTC**TAGCCAGGTTCCAGGCATAG-3'	*EcoRI*	
T7	5'-GGG**GACAAGTTTGTACAAAAAAGC**A GGCTGTAATACGACTCACTATAGGGC-3'	Na	500-1100
T3	5'-GGG**GACCACTTTGTACAAGAAAGC**TGG GTAATTAACCTCACTAAAGGG-3'	Na	500-1100

2.2.3 Agrobacterium mediated transformation and plant generation

The *A. tumefaciens* strain C5610 was transformed with the silencing constructs using the freeze thaw method (Höfgen and Willmitzer, 1988). Bacterial cells were grown on YEP (5g/L NaCl, 10g/L Peptone, 10g/L Yeast extract, 1.5% (w/v) agar) media containing standard working concentrations of spectinomycin, carbenicillin and rifampicin. Transformed *A.tumefaciens* were grown in YEP broth to an OD_{600} between 1.0 and 1.2. The cells were centrifuged at 3000xg for 10 minutes and pelleted cells were re-suspended into MS30 (4.43g/L MS, 3% (w/v) sucrose pH 5.8) to an OD_{600} of 0.8 and placed on ice.

Leaf explants from wild type (WT) potatoes were cut into 1cm^2 squares and incubated for 15 minutes on a shaker (35rpm) in 20ml MS30 containing 100µL of the *A. tumefaciens* solution. After 15 minutes incubation the explants were sealed and placed in the dark under growth room conditions at 24^oC and 16hour/8hour day night cycle for 2 days. They were then washed with sterile H_2O containing 250mg/L Cefotaxime (Cef).

After two days the washed explants were placed onto MGC (4.43g/L MS, 16g/L glucose, 5mg/L Naphthalene acetic acid (NAA), 0.1mg/L 6-Benzylamino-purine (BAP), 250mg/L Cef, 50mg/L Kanamycin sulphate (Kan)). One week later the leaf squares were placed onto MGS (4.43g/L MS,16g/L glucose, 2mg/L zeatin riboside, 20µg/L NAA, 20µg/L Gibberellic acid (GA_3), 300mg/L Cef, 50mg/L Kan, 2.21g/L gelrite) and subsequently sub cultured every two weeks until shoots started to form. These were placed onto rooting medium (4.43g/L MS,

20g/L sucrose, 250mg/L Cef, 50mg/L Kan, 2,21g/L gelrite) until they formed true leaves and were then placed onto new rooting medium.

2.2.4 Protein extraction

Approximately 40mg leaf material was homogenised in 200 µL protein extraction buffer (50mM Tris-HCL pH 7.5, 0.1% Triton x100, 5mM DTT, 0.005% v/v β mercaptoethanol, 2mM EDTA). The homogenate was centrifuged at 18 000xg and 4°C for 20 minutes, the supernatant was removed and protein concentrations determined using the Biorad Protein Assay Dye Reagent and bovine serum albumin as standard.

2.2.5 Protein analysis

Branching enzyme I activity was investigated via in-gel activity (Kossmann, 1992) 30µg of protein was loaded onto an 8% continuous native PAGE gel containing 0.1% potato amylopectin (Fluka BioChemika, Steinheim). A native buffer system was used. The proteins were separated under native conditions at 4°C for 3 hours at 80 volts and gels were incubated overnight at room temperature in 50mM HEPES-KOH pH 7.0, 1mM DTT, 1mM $MgCl_2$, 1mM $CaCl_2$ and 10% v/v glycerol. BEI activity was revealed by staining with Lugols solution (13.1mM I_2, 39.6mM KI).

ISA activity was analysed with the in-gel technique of Bustos et al., 2004. 30µg protein was loaded per well in a 6% continuous native polyacrylamide gel containing 0.2% β-limit dextrin (Megazyme). The separation took place in 4°C for 3 hours at 80 volts and the gel was incubated at 37 °C in activity buffer (100mM MES-KOH pH 6.0, 5mM DTT, 5% (v/v) Ethanediol) for two hours. Activity was revealed by staining the gel with Lugol's solution.

The presence of the GWD protein was investigated using immunoblots. 40µg of denatured protein was separated by 6% SDS-PAGE and transferred to a nitrocellulose membrane using a semi-dry blotting system. The membrane was blocked in 30mL of Tris buffered saline (TBS) containing 3.3% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Tween 20 for 2 hours. The solution was removed and primary antibody (Lorberth et al., 1998) diluted 1:500 in TBS-T was added and incubated overnight at 4°C. The membrane was washed for 5 minutes using TBS-T and this step was repeated three times. The membrane was incubated in Anti-Rabbit IgG Alkaline Phosphatase secondary antibody (1:7500) in TBS-T for 2 hours, washed 3 times with TBS-T, after which the presence of protein was visualised by incubating it in BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) solution (Sigma*FAST*™, Sigma).

2.2.6 SqPCR

Primers (Table 4) for the semi-quantitative PCRs were designed for *StActin2* and *StBEII* using the Primer3 tool. cDNA was produced from 500µg total RNA for each line using the ThermoScientific Maxima H Minus First strand cDNA Synthesis kit. All PCRs were performed using MyTaq^{TM} Red Mix (Bioline) according to the manufacturer's instructions. The cycle where the *StACT2* and *StBEII* primers reached linearity was identified by performing a PCR using Wt cDNA as template and stopping the reactions with the addition of 5µL of 0.5M EDTA at 0, 5, 7, 9, 11, 15, 17, 20, 22, 25, 27 cycles. The PCR products were separated on a 0.8% agarose gel where it could be visualised in which cycle linearity was reached. The amplification of the *StActin2* and *StBEII* fragments for the sqPCR was performed at the optimised temperature and for 27 cycles.

Table 4. PCR primers used for the amplification of gene fragments for the sqPCRs

Primer name	Primer sequence	Product size
SqACT2 FWD	5'-TGGTTACTCGTTCACCACCT-3'	350
SqACT2 REV	5'-ATCAGCAATACCAGGGAACA-3'	
SqBEII FWD	5'-CAAGTATGAGGGTGGTTTGG-3'	300
SqBEII REV	5'-AGTTGATCCAAGCAGGAATG-3'	

2.2.7 Starch granule purification

2.2.7.1 Tuber starch extraction

Potato tubers were homogenised in extraction buffer (50mM Tris-HCl pH 7.5, 1mM EDTA and 10mg/L K-metabisulphite). Insoluble material was allowed to sediment for 2 hours and the supernatant removed. The starch containing pellet was resuspended in 15mL extraction buffer and filtered through two layers of Miracloth (Calbiochem). The filtrate was centrifuged for 10 minutes at 2000xg and this process was repeated twice. 10ml of acetone at -20^{o}C was added to the starch pellet, vortexed and then centrifuged at 2000xg. The wash step was performed three times before the purified starch was allowed to air dry.

2.2.7.2 Leaf starch extraction

Leaf starch was purified by a modified protocol based on those of Tomlinson et al. (1997) and Zeeman et al. (1998). Two extraction buffers were used; buffer A (30mM MOPS-KOH ph7.3, 5mM EDTA, 5mM DTT and 10mg/L $K_2S_2O_5$) and buffer B (30mM MOPS-KOH ph7.3, 5mM EDTA, 5mM DTT, 10mg/L $K_2S_2O_5$ and 0.5% SDS). The amount 2 ml of buffer A and approximately fifteen 1mm diameter glass beads were added to a sterile 10ml tube. Frozen ground leaf material of approximately 2g was added the tube which was vortexed for 2 min.

The homogenate was filtered through a 100µm filter and the filtrate centrifuged at 5000xg for 10 minutes after which the supernatant was discarded. The pellet was resuspended in buffer A and centrifuged at 5000xg for 10 minutes, a step that was repeated a further three times. The same process was repeated three times using buffer B. 1mL of acetone at -20 ^{o}C was added to the pellet which was allowed to sediment, after which the acetone was removed. The acetone wash step was repeated six times before the pellet was air-dried.

2.2.8 Analysis of starch components

Apparent amylose content was determined as a percentage of total starch through a spectrophotometric assay (Hovenkamp-Hermelink et al., 1988). 500µl of 45% perchloric acid was added to 20mg starch. This was incubated for 5 minutes and 8ml of H_2O was added. The samples were vortexed and allowed to sediment for 10 minutes. 50µl was combined with 450µL H_2O and 500µl half strength Lugols solution (6.55mM I_2, 19.8mM KI) before the absorbance was determined at 618nm and 550nm. The apparent amylose content was estimated using the following formula: P= (3.5-5.1*R)/(10.4*R-19.9) where P is the apparent amylose fraction and R is the Absorption at 618nm/Absorption at 550nm.

The glucose-6-phosphate content of the starch was determined by enzymatic assay (Nielsen et al., 1994). 65mg of starch was digested by adding 500µl of 0.7M HCl and this was incubated at 95^{o}C for 4 hours. The sample was neutralized by addition of 500µl 0.7M KOH. 30 µl of the neutralized solution was added 230µl assay buffer (0.2M Tris-HCl ph 7.5, 2mM NAD, 10mM $MgCl_2$, and 2mM EDTA). The reaction was started by the addition of 10.6 U/ml glucose-6-phosphate dehydrogenase and monitored at 340nm. The glucose 6-phosphate content was derived from the increase in absorbance.

3.2.7 Potato leaf staining

Harvested potato leaves were incubated in 10ml 80% EtOH for three hours. After the incubation excess EtOH was decanted and 5ml Lugols solution (13.1mM I_2, 39.6mM KI) was added for 5 minutes. After the iodine staining leaves was washed six times with H_2O and the image recorded.

2.3 Results and Discussion

2.3.1 Construction of RNAi silencing vectors

To investigate potential interactions between starch metabolic proteins a range of chimeric RNAi silencing constructs were produced in the plant transformation vector pHellsgate2 (Helliwell et al., 2002). The basis behind the vector construction is shown in Fig. 3 and all the constructs built in this study to reduce the expression of *BEI, BEII, GWD* and *ISA2* genes are listed in Table 5.

Table 5. Vectors for RNAi silencing

Vector backbone	Vectors for silencing one gene	Vectors for silencing two genes	Vectors for silencing three genes
pHellsgate2	pHG::BEI	pHG::BEI::GWD	pHG::BEI::BEII::GWD
	pHG::BEII	pHG::BEII::GWD	
	pHG::GWD	pHG::BEI::BEII	
	pHG::ISA2	pHG::ISA2::GWD	

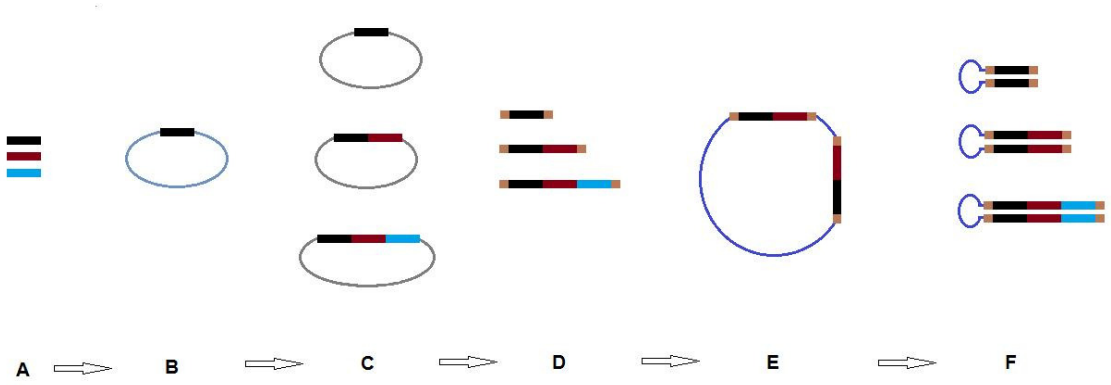


Figure 3. A general schematic of the strategy used in the construction of the RNAi silencing vectors. A: PCR amplification of the inserts of *GWD, BEI* and *BEII*. B: Ligation of amplified vectors into the pGem-T-Easy vector system. C: cloning of the desired fragments into pBK-CMV to form the chimeric combinations. D: Addition of AttB recombination sites with PCR for the recombination reaction. E: Recombination of the fragments into the pHellsgate 2 vector. F: hairpin structures that form in plants after successful transformation.

The RNAi vectors were transformed into *A.tumefaciens* strain C5610 and the presence of the silencing inserts was confirmed by a mixture of restriction digests and colony PCR (Supplementary data).

2.3.2 *A.tumefaciens* mediated transformation and plantlet regeneration

Wild type Désirée potatoes were transformed with *A.tumefaciens* containing the pHellsgate vectors. When plantlets reached the age of 3 months they were planted in a soil mixture and left to grow in ambient conditions in the greenhouse for 2-3 months. Leaf material was then collected for screening to examine gene silencing.

2.3.3 Screening of transgenic *S.tuberosum*

Lines repressed for BEI or ISA2 activity were screened using activity gels (Fig. 4) while semi-quantitative PCRs were used to show a reduction in *BEII* gene expression (Fig.5) and immunoblots were utilized to showing the down regulation of the GWD proteins (Fig. 6).

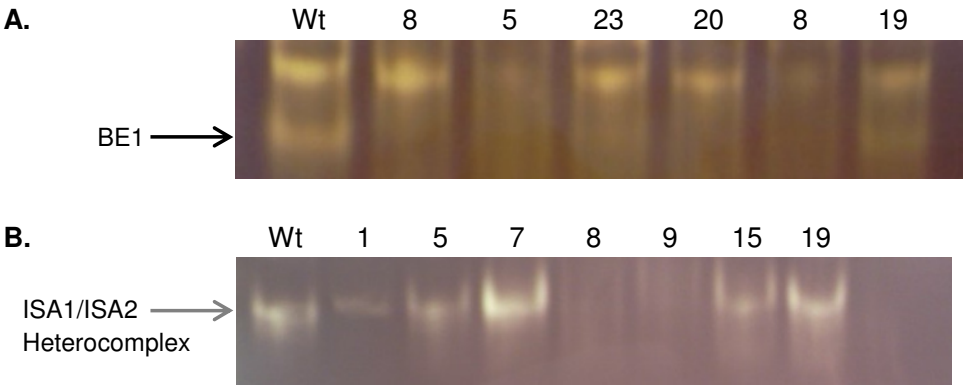


Figure 4. Native gels showing BEI (A) and ISA2 (B) activities in transgenic RNAi potato lines. The arrow denotes the activities of the enzymes. Wt=wild type positive control.

A BEI activity band can be seen in Fig. 4A, which was lacking in a number of lines. For plants where BEI alone was targeted 3 lines were found with good repression, for those where both GWD and BEI were targeted 7 lines were identified with reduced BEI and where both BEI and BEII were targeted 9 repressed lines were found (Supplementary data). The debranching enzyme activities were investigated in lines repressed for ISA2 using native gels containing β-limit dextrin as substrate. Three lines (1, 5 and 15) show a moderate decrease in activity while in lines 8 and 9 the activity was almost eliminated (Fig. 4B).

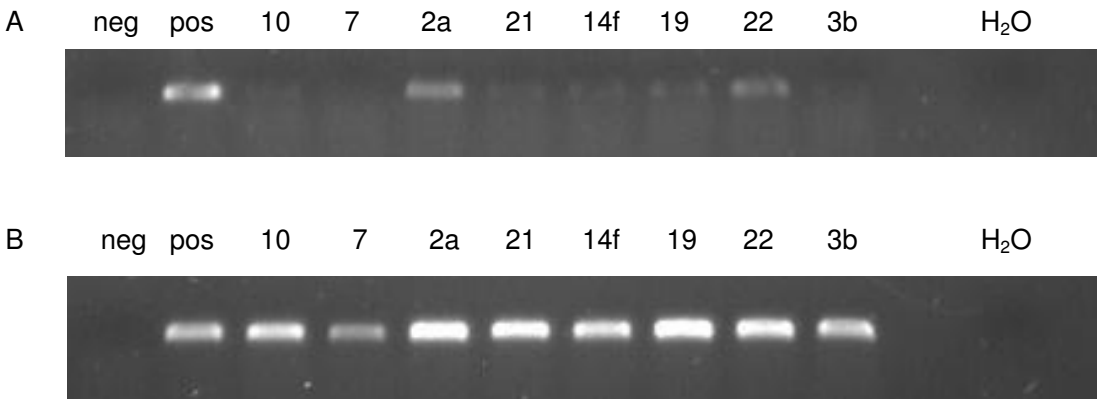


Figure 5. SqPCR to examine expression of *StBEII*. A, amplicons for *StBEII* and B, for *ACTIN2* . Neg = Wt potato RNA, pos= Wt potato cDNA.

SqPCR revealed that for *St*BEII lines 3b, 7 and 10 were nearly completely repressed for *StBEII* expression, while lines 21, 14f and 19 showed a moderate reduction.

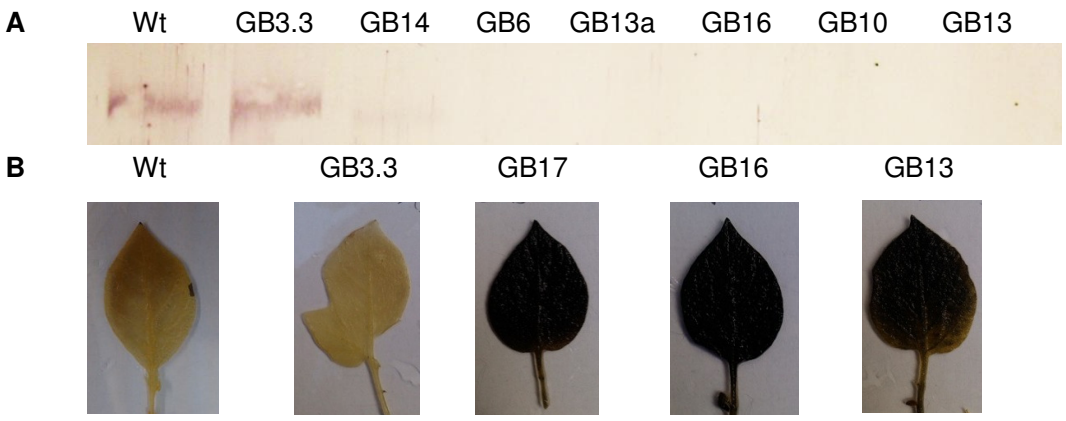


Figure 6. GWD immunoblots and demonstration of a starch excess phenotype in potato leaves of lines lacking GWD. 40µg protein were separated by SDS-PAGE before blotting onto membranes. A, Lines for GWD-BEI (GB). where Wt is protein extract from wild type potato leaf. B, Potato leaves of GWD-BEI lines stained with Lugols solution after three days of darkness. Immunoblots for lines GWD and GWD-ISA2 can be found in the supplementary data.

Immunoblots identified several lines lacking GWD. For the GWD alone five lines were identified with reduced GWD protein, while in the GWD-BEI transformation ten lines contained reduced GWD. Leaves from three of those lines were darkened and stained with iodine to see if they demonstrated a starch excess phenotype known to occur in GWD deficient potato leaves (Lorberth et al., 1998) and it was shown that several of them did (Fig. 6B). None of the GWD-ISA2 lines showed a reduction in GWD.

2.3.4 Analysis of starch from leaves and tubers of primary transformants

In a preliminary assessment of the screened BEI and BEII primary transformants, the amylose and G6P contents were measured in starch from tubers and amylose content for the leaves. Due to the fact that only primary transformants were available intra plant variation alone was assessed.

Starch from wild type tubers had an amylose content of approximately 21% when measured with the I_2-KI method, consistent with a previous study (Hovenkamp-Hermelink et al., 1988). Although previous work by Safford et al. (1998), who also repressed BEI activity, concluded that this led to no significant alteration in amylose contents my work indicates a slight decrease in all of the primary transformants (Fig. 7A). In agreement with Jobling et al. (1999), lines where BEII expression was repressed led to increased concentrations of amylose in starch of 23.2% to 31% (Fig. 7A). Intriguingly, this increase is not as large an reported by Jobling et al. (1999) possibly due to differences in the method used to analyse amylose contents, or because the repression of BEII transcription is less effective in the current study. The data in this study for glucose 6-phosphate contents in tuber starch is almost identical to that of Safford et al. (1998) and Jobling et al. (1999) with increases in all lines where *BE* expression was repressed (Fig. 7C).

To date there is no explanation to account for the increase in covalently bound phosphate content in starch when BEs are silenced. In potato it was found that amylopectin from lines deficient in BEII had longer average chain lengths (Jobling et al., 1999) and it is known that the majority of starch phosphate is located on the amylopectin fraction of starch (Buléon et al., 1998; Blennow et al., 1998). It could be argued that, as the GWD has a preference for longer chains (Mikkelsen et al., 2004, 2005), the increased average chain lengths would lead to more substrates for GWD and the increased phosphate. This theory does not help to explain the increased phosphate in starch from BEI deficient potatoes as these present no difference in chain length (Safford et al., 1998). One possible explanation for this is that when BEI (but not BE2) branches a linear chain it acts close to a phosphate moiety and removes that phosphate moiety in the process. When its activity is removed the amount of phosphate would, therefore, increase as BE2 would be the prevalent activity. BE isoforms have been shown to be able to utilize phosphorylated glucans as substrates (Viksø-Nielsen et al., 1998), but no reports are present about whether they remove phosphate during the branching process. This theory would, therefore, have to be tested using recombinant protein

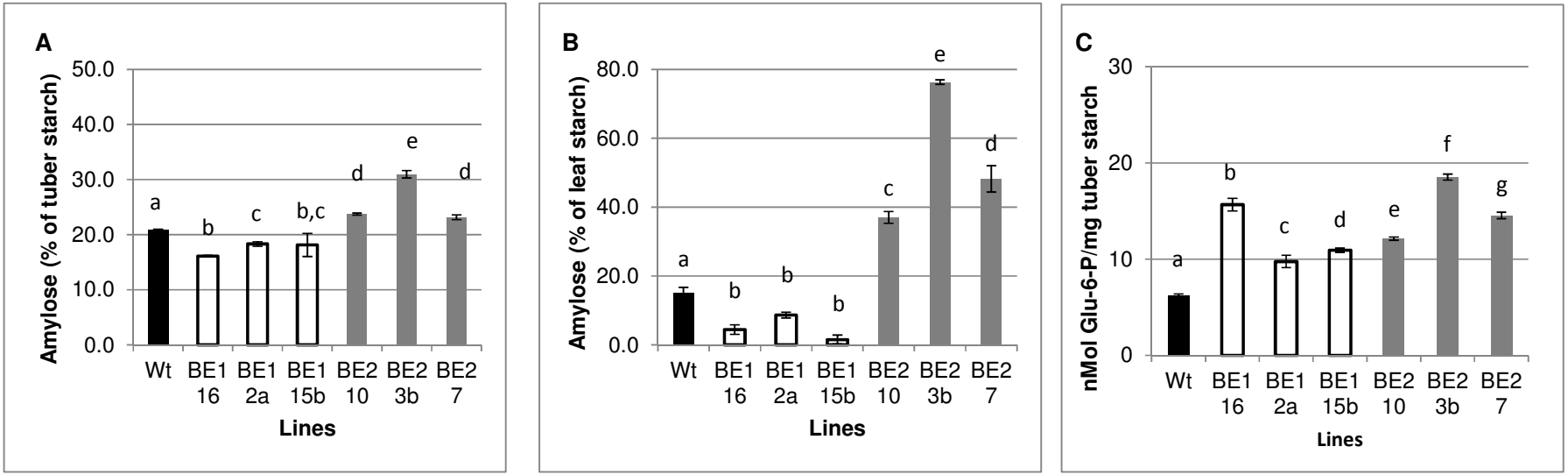


Figure 7. Proportion of tuber (A) and leaf (B) starch that is amylose in lines repressed in either *StBEI* (BEI) or *StBEII* (BEII). (C),The amounts of glucose-6-phosphate present in starch isolated from tubers of transgenic potato lines repressed in either BEI or BEII. Analysis of variance with the *ad hoc* Fisher's (least significant difference) LSD test was performed and letters indicate lines which are similar/different from each other at the 5% level.

When leaf starch samples from the *St*BEI lines were analysed it was found that, while the control lines had an amylose content similar to that previously reported (Hovenkamp-Hermelink et al., 1988) starch from the *St*BEI lines contained very low amylose. This finding should be investigated further by confirming the result using an alternative method for determining amylose, such as through selective precipitation of amylopectin using concanavalin a. If the result is confirmed one explanation could be that potato BEII produces chains that are better substrates for soluble SS1 (the main SS in leaves, but not tubers). Therefore, when *St*BEI is silenced in leaves the activity of SS1 might be be favoured over GBSS. Dumez et al. (2006) observed that starch in *be1* Arabidopsis mutants contained 6% more amylose when the mutation was in the Col-0 background while but no difference when in the Wassilewskija background so a decrease in amylose in lines lacking BEI has not been observed previously. The amylose content in leaf starch from the *St*BEII lines, however, showed an increase over that found in the Wt (Fig. 7B). This was even higher than that found in tuber starch (Fig. 7A) and greater than that found in any BE mutant in Arabidopsis (Dumez et al., 2006). Interestingly the *St*BEII protein is present in much higher quantity in leaves that in tubers (Larsson et al., 1998) which may explain why there was a greater effect in leaves when it was repressed.

Unfortunately due to tissue culture contamination only the BEI and BEII lines' starch could be biochemically analysed for amylose content and G6P content. The other lines are currently being produced and will be used in a large scale experiment in the future.

2.4 Conclusions

In this chapter I have described the production of chimeric RNAi silencing constructs designed to reduce protein levels of BEI, BEII, GWD and ISA2 in potato plants. This was done in order to examine functional interactions between these proteins in starch synthesis.

RNAi silencing vectors was built and successfully transformed into potato explants, lines with reduced activity were produced for BEI, BEII, BEI-BEII, GWD, and GWD-BEI. Starch form the BEI and BEII lines were biochemically analysed for amylose content and G6P content. Samples showed an increase in G6P and, in the StBE2 lines, an increase in amylose. Both of these observations are similar to previously studies (Safford et al., 1998; Jobling et al., 1999). The amylose content in starch from StBE1 repressed leaves seemed to be eliminated, however, further investigation and conformation of this is required. A number of other lines still need ot be generated and analysed. Once this has been done it will lead to insight into the interactions between these enzymes in making starch.

References

Abel GJW, Springer F, Willmitzer L, Kossmann J (1996) Cloning and functional analysis of a cDNA encoding a novel 139 kDa starch synthase from potato (Solanum tuberosum L). Plant Journal **10:** 981-991

Blennow A, Bay-Smidt AM, Olsen CE, Moller BL (1998) Analysis of starch-bound glucose 3-phosphate and glucose 6-phosphate using controlled acid treatment combined with high-performance anion-exchange chromatography. Journal of Chromatography a **829:** 385-391

Borovsky D, Smith EE, Whelan WJ, French D, Kikumoto S (1979) Mechanism of Q-Enzyme Action and its Influence on the Structure of Amylopectin. Arch Biochem Biophys **198:** 627-631

Buléon A, Colonna P, Planchot V, Ball S (1998) Starch granules: structure and biosynthesis. Int J Biol Macromol **23:** 85-112

Bustos R, Fahy B, Hylton CM, Seale R, Nebane NM, Edwards A, Martin C, Smith AM (2004) Starch granule initiation is controlled by a heteromultimeric isoamylase in potato tubers. Proc Natl Acad Sci U S A **101:** 2215-2220

Dumez S, Wattebled F, Dauvillee D, Delvalle D, Planchot V, Ball SG, D'Hulst C (2006) Mutants of Arabidopsis lacking starch branching enzyme II substitute plastidial starch synthesis by cytoplasmic maltose accumulation. Plant Cell **18:** 2694-2709

Edwards A, Fulton DC, Hylton CM, Jobling SA, Gidley M, Rossner U, Martin C, Smith AM (1999) A combined reduction in activity of starch synthases II and III of potato has novel effects on the starch of tubers. Plant Journal **17:** 251-261

Fulton DC, Edwards A, Pilling E, Robinson HL, Fahy B, Seale R, Kato L, Donald AM, Geigenberger P, Martin C, Smith AM (2002) Role of granule-bound starch synthase in determination of amylopectin structure and starch granule morphology in potato. J Biol Chem **277:** 10834-10841

Helliwell CA, Wesley SV, Wielopolska AJ, Waterhouse PM (2002) High-throughput vectors for efficient gene silencing in plants. Functional Plant Biology **29:** 1217-1225

Hofgen R, Willmitzer L (1988) Storage of Competent Cells for Agrobacterium Transformation. Nucleic Acids Res **16:** 9877-9877

Hovenkamphermelink JHM, Devries JN, Adamse P, Jacobsen E, Witholt B, Feenstra WJ (1988) Rapid Estimation of the Amylose Amylopectin Ratio in Small Amounts of Tuber and Leaf Tissue of the Potato. Potato Res **31:** 241-246

Jobling SA, Schwall GP, Westcott RJ, Sidebottom CM, Debet M, Gidley MJ, Jeffcoat R, Safford R (1999) A minor form of starch branching enzyme in potato (Solanum tuberosum L.) tubers has a major effect on starch structure: cloning and characterisation of multiple forms of SBE A. Plant Journal **18:** 163-171

Jobling SA, Westcott RJ, Tayal A, Jeffcoat R, Schwall GP (2002) Production of a freeze-thaw-stable potato starch by antisense inhibition of three starch synthase genes. Nat Biotechnol **20:** 295-299

Kossmann J, Abel GJW, Springer F, Lloyd JR, Willmitzer L (1999) Cloning and functional analysis of a cDNA encoding a starch synthase from potato (Solanum tuberosum L,) that is predominantly expressed in leaf tissue. Planta **208:** 503-511

Larsson CT, Khoshnoodi J, Ek B, Rask L, Larsson H (1998) Molecular cloning and characterization of starch-branching enzyme II from potato. Plant Mol Biol **37:** 505-511

Liu F, Makhmoudova A, Lee EA, Wait R, Emes MJ, Tetlow IJ (2009) The amylose extender mutant of maize conditions novel protein-protein interactions between starch biosynthetic enzymes in amyloplasts. J Exp Bot **60:** 4423-4440

Lloyd JR, Landschutze V, Kossmann J (1999) Simultaneous antisense inhibition of two starch-synthase isoforms in potato tubers leads to accumulation of grossly modified amylopectin. Biochem J **338:** 515-521

Lorberth R, Ritte G, Willmitzer L, Kossmann J (1998) Inhibition of a starch-granule-bound protein leads to modified starch and repression of cold sweetening. Nat Biotechnol **16:** 473-477

Mikkelsen R, Baunsgaard L, Blennow A (2004) Functional characterization of alpha-glucan,water dikinase, the starch phosphorylating enzyme. Biochem J **377:** 525-532

Mikkelsen R, Mutenda KE, Mant A, Schurmann P, Blennow A (2005) alpha-Glucan, water dikinase (GWD): A plastidic enzyme with redox-regulated and coordinated catalytic activity and binding affinity. Proc Natl Acad Sci U S A **102:** 1785-1790

Morell MK, Blennow A, KosarHashemi B, Samuel MS (1997) Differential expression and properties of starch branching enzyme isoforms in developing wheat endosperm. Plant Physiol **113:** 201-208

Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol Plantarum **15:** 473-497

Nielsen TH, Rung JH, Villadsen D (2004) Fructose-2,6-bisphosphate: a traffic signal in plant metabolism. Trends Plant Sci **9:** 556-563

Nielsen T, Wischmann B, Enevoldsen K, Moller B (1994) Starch Phosphorylation in Potato-Tubers Proceeds Concurrently with De-Novo Biosynthesis of Starch. Plant Physiol **105:** 111-117

Rydberg U, Andersson L, Andersson R, Aman P, Larsson H (2001) Comparison of starch branching enzyme I and II from potato. European Journal of Biochemistry **268:** 6140-6145

Safford R, Jobling SA, Sidebottom CM, Westcott RJ, Cooke D, Tober KJ, Strongitharm BH, Russell AL, Gidley MJ (1998) Consequences of antisense RNA inhibition of starch branching enzyme activity on properties of potato starch. Carbohydr Polym **35:** 155-168

Schwall GP, Safford R, Westcott RJ, Jeffcoat R, Tayal A, Shi YC, Gidley MJ, Jobling SA (2000) Production of very-high-amylose potato starch by inhibition of SBE A and B. Nat Biotechnol **18:** 551-554

Tomlinson KL, Lloyd JR, Smith AM (1997) Importance of isoforms of starch-branching enzyme in determining the structure of starch in pea leaves. Plant Journal **11:** 31-43

Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JAM (2007) Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res **35:** W71-W74

Viksø-Nielsen A, Blennow A, Nielsen TH, Moller BL (1998) Phosphorylated alpha(1 -> 4)glucans as substrate for potato starch-branching enzyme I. Plant Physiol **117:** 869-875

Zeeman SC, Umemoto T, Lue WL, Au-Yeung P, Martin C, Smith AM, Chen J (1998) A mutant of Arabidopsis lacking a chloroplastic isoamylase accumulates both starch and phytoglycogen. Plant Cell **10:** 1699-1711

Chapter Three

Investigation of genes putatively involved in starch metabolism

3.1 Introduction

A large number of enzymes involved in starch metabolism have been identified, however, it is possible that there are other activities which remain to be identified. For example, it isn't clear how plants sense leaf starch concentrations allowing them to adjust their rates of synthesis and degradation, or how granule size is controlled. It is likely that these aspects are controlled by unknown proteins.

One method of identifying genes involved in starch metabolism has been screening mutant collections to identify plants that demonstrate differential staining with Lugols solution (for example see Caspar et al., 1991). Since the publication of the Arabidopsis genome (Arabidopsis Genome Initiative, 2000) it has become possible to identify genes as being putatively involved in specific metabolic pathways using *in silico* analysis. For example, by co-expression analysis or through identification of genes which encode proteins with specific activity domains.

In order to find remaining undiscovered proteins involved in starch metabolism two candidate genes (At5g39790 and At5g01260) were identified based on the presence of a starch binding domain within the proteins they encode. At5g39790 was previously identified as a scaffold protein (SP1; Lohmeier-Vogel et al., 2008) due to its ability to bind to starch granules and the presence of a coiled-coiled domain, normally involved in protein protein interactions Lohmeier-Vogel et al. (2008). They concluded that this protein is necessary for other starch metabolic proteins to associate with the starch granule. However, the protein also contains an AMP-activated kinase domain and no investigation was performed on to examine if it acts as a kinase. For example as either a starch or protein kinase. Although Arabidopsis *sex1* mutants contain very little measurable G6P in starch (Yu et al., 2001), it is not impossible that very small amounts of phosphate could be incorporated by another protein. The possibility of it acting as a protein kinase comes from studies that indicate that starch metabolic proteins can be phosphorylated (For examples see Kötting et al., 2010). In addition, Tetlow et al. (2004) reported that protein complexes form in wheat as a result of protein phosphorylation.

The second locus, At5g01260 provisionally designated *CBD1* (carbohydrate binding domain 1), encodes a protein containing a carbohydrate binding domain which is found in other starch metabolic proteins such as DPE2, GWD and PWD (Baunsgaard et al., 2005; Steichen et al., 2008; Christiansen et al., 2009).To examine if these two proteins are involved in starch metabolism Arabidopsis mutants lacking them will be studied and the cDNA's used to produce recombinant proteins for activity determinations.

3.2 Materials and Methods

3.2.1 Arabidopsis seed sterilization

Arabidopsis seeds were incubated for 8 minutes in sterilization buffer (0.25% (v/v) NaClO, 90% (v/v) EtOH, 5% H_2O and 0.01% Tween 20) after which they were washed three times for 5 minutes in sterile H_2O.

3.2.2 Plant material and growth conditions

Arabidopsis seeds were vernalised at 4°C for 3 days. They were germinated on ½ MS media containing 3% (w/v) sucrose. After the formation of four true leaves, the plants were transferred to peat discs (Jiffy Products International AS). All the plants were grown (growth room) in a 10/14 hour light/dark cycle where the temperature ranged from 22°C in the light to 15°C in the dark. Plants were sub irrigated every 2 days and received phosphogen plant food (Bayer Garden) (1.4g/L) at 7 and 21 days post germination.

3.2.3 Plasmid construction

For recombinant protein expression cDNAs encoded at the At5g01260 and At5g39790 loci were amplified from Colombia-0 Arabidopsis cDNA using PCR. The following sets of forward (FWD) and reverse (REV) primers were used: CBD1-FWD 5'-CTGCAGAAGATGATTGGCATTGGA-3', CBD1-REV 5'-GAGGCTCTTTTGCCGATGTA and SP-FWD 5'-TCATGGGATGTGTACCCAGA-3', SP-REV 5'-CAAATGACTCCTGCCACCTA-3'. PCR products were separated by agarose gel electrophoresis and purified using a commercially available kit (Fermentas). The cDNAs were ligated in the *Eco*RV restriction site of the pBluescript-sk (pSK) vector to make pSK::SP and pSK::CBD1. The SP and CBD1 fragments were restricted from the vectors using *Eco*RI, XhoI and PstI, XhoI respectively. These were ligated into the same restriction sites of pET41 (A and C respectively) in frame with the GST tag.

3.2.4 Bacterial transformation

Heatshock competent *E. coli* were manufactured and transformed according to the method of Sambrook et al. (1989).

3.2.5 E.coli screening

Two *E. coli* strains which produce high amounts of linear glucans, a phosphoglucomutase (PGM) mutant (CGSG# 8761) and KV832::pACAG (Kiel et al., 1987; Kossmann et al., 1999) were transformed by the heat shock method with the pBluescript-sk (pSK), pSK::SP and pSK::CBD1 vectors. These transformants were then streaked onto LB agar plates containing either 1% (v/v) maltose (PGM) or 1% (w/v) glucose (KV832) with the appropriate antibiotics. Plates were incubated overnight at 37°C before colonies were stained with iodine vapour to see if the respective proteins had an influence on the linear glucans that were formed

3.2.6 Arabidopsis mutant genotyping

Arabidopsis tDNA insertion lines for At5g01260 and At5g39790 were ordered from the European Arabidopsis Stock Centre. The lines were SALK_024994.55.50.x (SP1-1), SALK_090838.49.15.x (SP1-2), SALK_129717.55.00.x (SP1-3), SALK_025022.56.00.x (SP1-4), SALK_124278.41.45.x (CBD1-1), SAIL_1152_B07 (CBD1-2) and SALK_109816.49.05.x (CBD1-3).

Genomic DNA (gDNA) was extracted by homogenizing one fresh leaf in 400uL gDNA extraction buffer (100mM Tris-HCL pH 8.0, 2% CTAB, 20mM EDTA, 1.4M NaCl and 1% PVP) and incubating for 20 minutes at 65°C and 400µL chloroform was added. The mixture was vortexed for 5 seconds and centrifuged at 18600xg for 5 minutes. 200µL of the upper phase was removed and placed into a new microcentrifuge tube and 200µL propan-2-ol was added. Precipitated gDNA was isolated by centrifugation at 18600xg for 20 min. Following removal of the supernatant, the pellet was washed twice with 70% (v/v) ethanol after which the supernatant was removed and the pellet air dried. PCRs were performed to identify plants that contained an insertion in the gene using gDNA as a template and the primers shown in table 4.

3.2.7 Iodine staining of Arabidopsis leaves

Harvested Arabidopsis cauline leaves were incubated at 80°C in 1ml 80% EtOH for three hours. The EtOH was periodically replaced with 80% to hasten the de-staining process. After the incubation the liquid was decanted and 1ml Lugols solution (13.1mM I_2, 39.6mM KI) was added for 5 minutes. After the iodine staining leaves was washed six times with H_2O after which the Wt and Mt leaves were photographed.

3.2.8 Quantification of Arabidopsis leaf starch

Leaf discs were taken using a size 3 cork borer, placed in a microcentrifuge tube, and frozen in liquid nitrogen. The samples were incubated in 80% (v/v) ethanol for one hour at 80°C, the liquid removed and the sample was washed with 80% (v/v) ethanol. Once the ethanol was removed, 400µl 0.2M KOH was added and heated to 95°C for one hour after which it was cooled and neutralised with 70µl of a 1M acetic acid solution. Samples were stored at 4°C until assayed

30µl of the neutralised solution was digested in digestion buffer (30µl 50mM NaAC-AcOH pH5.6, 10U/ml amyloglucosidase) for 2 hours at 37°C. 250µl of assay buffer (20mM Tris-HCL pH 6.9, 5mM $MgCl_2$, 1mM ATP, 1mM NAD) was added and the assay started by addition of 1µl/ml glucose-6-phosphate dehydrogenase. The reaction was followed at 340nm and the amounts of starch are calculated based on the increase in absorbance.

3.2.9 DNA sequencing

All cDNA fragments were sequenced using a commercial service.

Table 6. Primer sets used to genotype of the T-DNA insertion mutants. The internal primer and the expected size of wild type (Wt) and mutant (Mt) fragment amplicons are indicated.

Screening primers and expected product sizes				
At line	Primer name	Primer sequence	Internal primer	Product size
SP1-1	R1	5'-TGGTTCTGGGGTTTATCAGTG-3'	LBb1.3	Wt 1178 bp
	L1	5'-TTCCAATTTGATACAATGCCG-3'		Mt 606-906 bp
SP1-2	R2	5'-TTTTGAAAAAGTGACGGTTGC-3'	LBb1.3	Wt 1205 bp
	L2	5'-GATTTCACAGATTCACGACGC-3'		Mt 556-856 bp
SP1-3	R3	5'-TTCCAATTTGATACAATGCCG-3'	LBb1.3	Wt 1178 bp
	L3	5'-TGGTTCTGGGGTTTATCAGTG-3'		Mt 508-808 bp
SP1-4	R4	5'-TGGTTCTGGGGTTTATCAGTG-3'	LBb1.3	Wt 1178 bp
	L4	5'-TTCCAATTTGATACAATGCCG-3'		Mt 606-906 bp
CBD1-3	R5	5'-TGTTATTTGGTTGATGCAAAATG-3'	LBb1.3	Wt 1181 bp
	L5	5'-GCGATAAATTCCAGGAGGAAG-3'		Mt 511-811 bp
CBD1-2	R6	5'-TGTTATTTGGTTGATGCAAAATG-3'	LB1	Wt 1181 bp
	L6	5'-GCGATAAATTCCAGGAGGAAG-3'		Mt 514-814 bp
CBD1-1	R7	5'-GTTTCCTCCACACGACTTGTC-3'	LBb1.3	Wt 1115 bp
	L7	5'-ACATTGCCATCAGACCAGTTC-3'		Mt 448-748 bp

3.2.10 Protein expression and purification

E.coli (BL21 codon plus RIPL, Agilent Technologies Company) was used for expression of recombinant protein. Cells containing vectors for protein production were transferred to 200mL Terrific Broth (TB) and grown at room temperature with shaking to an optical density of 0.4-0.6 prior to isopropyl β-D-1-thiogalactopyranoside (IPTG) being added at a final concentration of 1mM. The induced bacteria were then grown in the same conditions overnight.

E.coli cells were recovered by centrifugation at 5000xg for 10 min. The pellet was weighed and resuspended in the specified amount off B-PER solution containing DNaseI, Lysozyme (Thermo Scientific) and Sigma FAST (Sigma) protease inhibitor cocktail. The protein extract was centrifuged at 18600xg and the supernatant recovered.

1ml Protino GST/4B purification columns (Macherey-Nagel) were used to isolate the fusion protein. 4.5mL of protein extract was combined with 0.5mL 10x phosphate buffered saline (PBS) solution. The column was equilibrated with 10 volumes of 1x PBS at a flow rate of 1ml/min. The protein solution was loaded onto the column at a flow rate of 0.2ml/min. The column was washed with 20 volumes of PBS at a flow rate of 1ml/min and bound proteins were eluted from the column in fifteen 1ml fractions using reduced glutathione elution buffer (50mM Tris-HCL pH 8.0, 10mM reduced glutathione).

3.2.11 SDS PAGE

Samples were denatured in Laemmli buffer (375mM Tris-HCL pH6.8, 9% (v/v) SDS, 50% (v/v) Glycerol, 0.03% (w/v) Bromophenol blue) at 95℃ for 5 min and separated by 10% SDS-PAGE at room temperature. After electrophoresis the proteins was fixed and stained by incubating in 40ml staining solution (0.1% (w/v) Coomassie brilliant blue, 20% (v/v) HPLC grade methanol, 10% (v/v) acetic acid) overnight. The stained gels were repeatedly washed with destaining solution (50% (v/v) methanol, 10% (v/v) acetic acid) until the protein bands could be visualized.

3.2.12 Kinase activity

Two methods were used to investigate the possible polyglucan kinase activity of the recombinant protein. The first examined production of ADP using pyruvate kinase/lactate dehydrogenase in a continuous assay. The second determined *in vitro* modification of starch from corn.

For the enzyme coupled kinase assay 10µL of purified protein (1.5µg/µL) was added to assay buffer (50mM HEPES-KOH pH7.0, 5mM $MgCl_2$, 0.15mM NADH, 5mM PEP, 1µL/ml PK/LDH enzyme mix and 1mg/ml solubilised potato starch). The assay was started by adding 1mM ATP and was run for 7 hours till completion.

For the *in vitro* assay 65mg of waxy corn starch was incubated overnight in 1.5ml of 50mM HEPES-KOH pH7.0, 5mM $MgCl_2$, 0.1mM AMP and 1mM ATP plus 120µl of purified protein (1.5µg/µL). The starch was washed three times with 80% ethanol followed by centrifugation at 2000xg for 10 minutes after which the ethanol was removed. A final acetone wash step was performed and the pellet was left to air-dry. Recovered starch was incubated in 500µL of 0.7M HCl for 4 hours at 95^{o}C before being neutralized by addition of 500µL 0.7M KOH. 30uL of the sample was added to 230µL assay buffer (200mM Tris-HCL pH7.5, 2mM NAD, 10mM $MgCl_2$, 2mM EDTA). The assay was started by adding 1µl/ml glucose-6-phosphate dehydrogenase and the reaction was followed at 340nm.G6P contents were calculated based on the increase in absorbance.

3.3 Results and discussion

As mentioned in section 3.1 SP1 and CBD1 were identified through *in silico* analysis as genes that might play a role in starch metabolism. To study their functions T-DNA Arabidopsis insertion mutants lacking the respective proteins were obtained. In addition recombinant versions of the two proteins were produced to try and identify an activity for them.

3.3.2 Insertion mutant analysis

3.3.2.1 Genotyping

Four lines for SP were obtained SALK_024994.55.50.x (SP1-1), SALK_090838.49.15.x (SP1-2), SALK_129717.55.00.x (SP1-3), SALK_025022.56.00.x. (SP1-4) and 3 lines for CBD1 SALK_124278.41.45.x (CBD1-1), SAIL_1152_B07 (CBD1-2) and SALK_109816.49.05.x (CBD1-3)

Arabidopsis plants for the seven mutant lines were genotyped to identify individuals containing an insert. 24 idivituals each for SP1-1 and CBD1-3 were genotyped and found to be homozygotic wild types (data not shown). These two lines were, therefore, assumed to be only Wt and discarded. 24 plants of each of the SP1-2, SP1-4, SP1-3, CBD1-1 and CBD1-2 were successfully genotyped and for each line homozygotic wild-type and mutant individuals were found (Fig. 8).

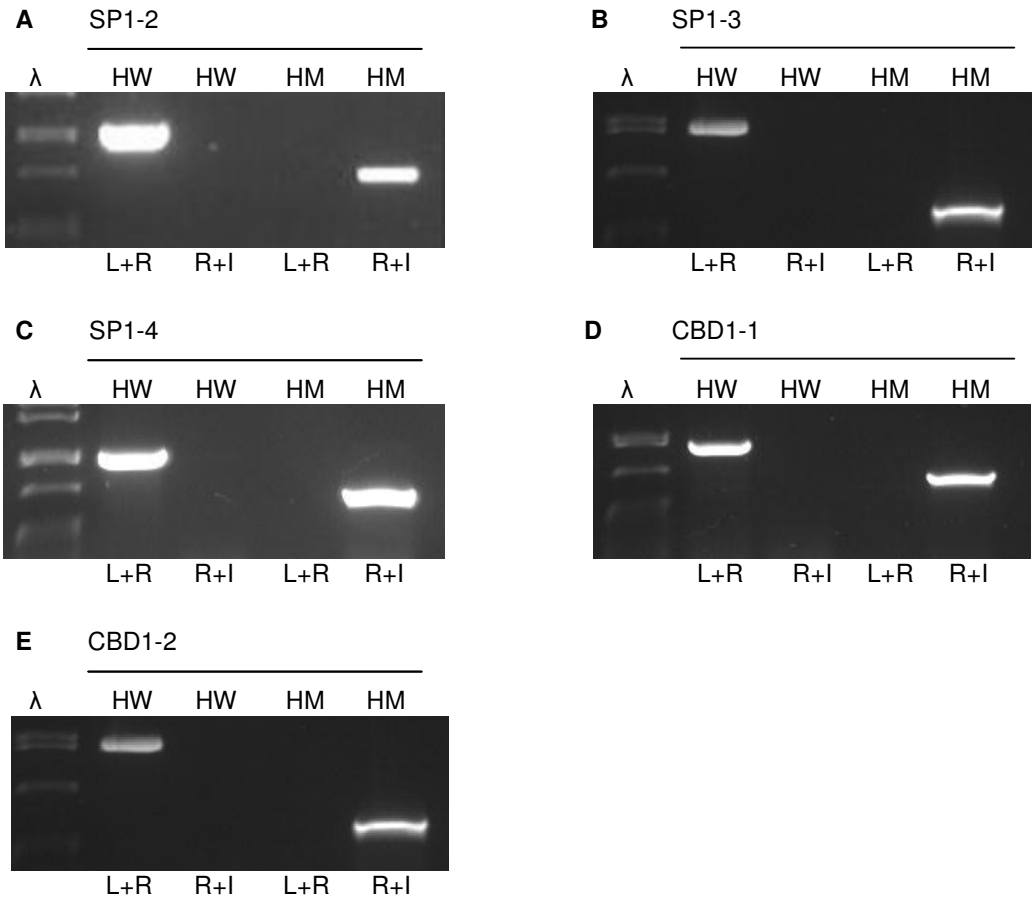


Figure 8. Genotyping of Arabidopsis mutants for CBD1 and SP. A-E, Homozygotic Wt and homozygotic Mt for SP1-2, SP1-3, SP1-4, CBD1-1 and CBD1-2 respectively. Wt and mutant alleles was genotyped using LP and RP primers (Table 6) λ= Lambda DNA digested with PstI. HW=homozygotic wild type. HM =homozygotic mutant. L=left border primer. R=right border primer. I=either LBb1.3 or LB1. See Table 6 for details.

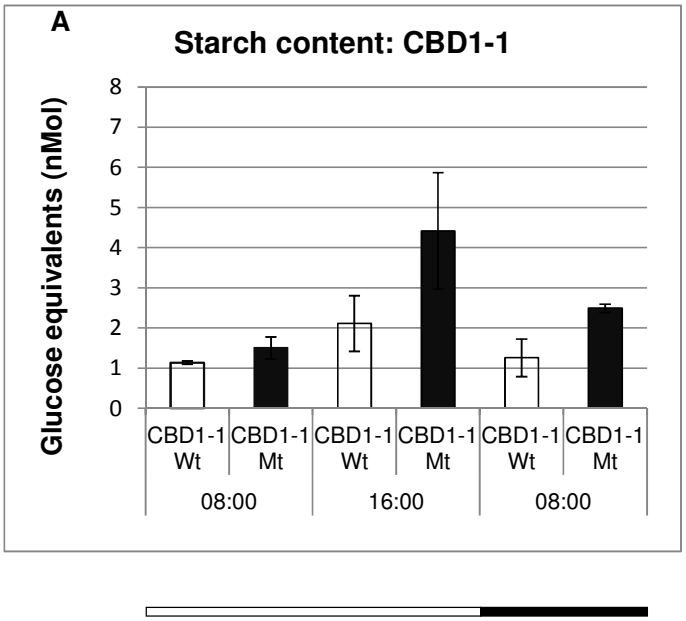
3.3.3 Starch analysis of *Arabidopsis* insertion mutants

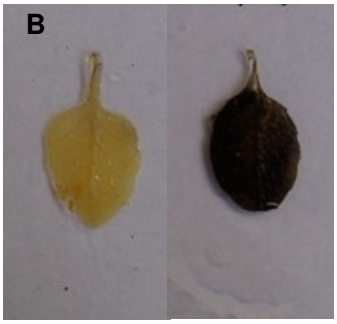
3.3.3.1 Iodine staining and quantitative starch enzymatic assays

Leaves from the homozygotic lines were subjected to iodine staining at the end of the dark period. Both CBD1-2 and CBD1-1 developed a starch excess phenotype when grown in ambient temperature and lighting conditions of the glasshouse during October (Fig. 9B&D). These observations are currently being confirmed on a larger sample size and more controlled lighting conditions. SP1-2 was also subjected to iodine when harvested in the middle of the day, and this indicated that starch contents in this were unaltered in most parts of the leaf (Fig. 10D).

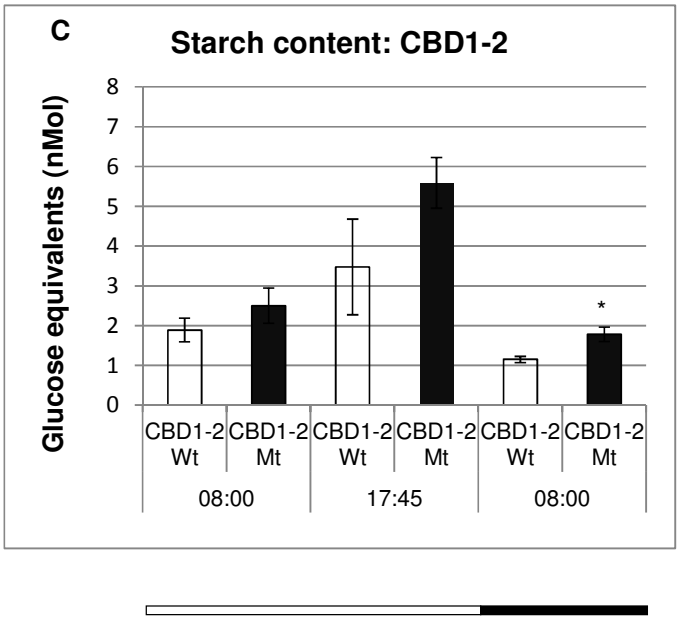
Starch was then quantified over a day/night cycle. In both mutants *cbd* lines at one time point the end of the dark period there was a slight increase in starch amounts. In CBD1-1 this was close to being significant ($p=0.08$) and in CBD1-2 with the increase was significant ($p=0.03$) (Fig. 9A,C). This experiment needs to be repeated under more controlled conditions and on a larger scale .

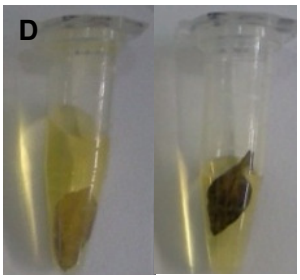
The starch contents in SP1-2, SP1-4 and SP1-3 lines were unchanged, indicating that if the SP protein is involved in starch metabolism, this is not important for yield (Fig. 10A-C).





CBD1-1 Wt CBD1-1 Mt





CBD1-2 Wt CBD1-2 Mt

Figure 9. A and B, Quantitative leaf starch measurements for CBD1-1 and CBD1-2. C and D, Iodine staining of CBD1-2, CBD1-1 wild type and mutant (07:45 am). A and B, 10 hour light and 14 hour dark regime was followed. Light and dark periods are indicated as bars underneath. See through = light period, Black = Dark period. * denotes differences from the Wt control at the 5% level (Two tailed student *t*-test)

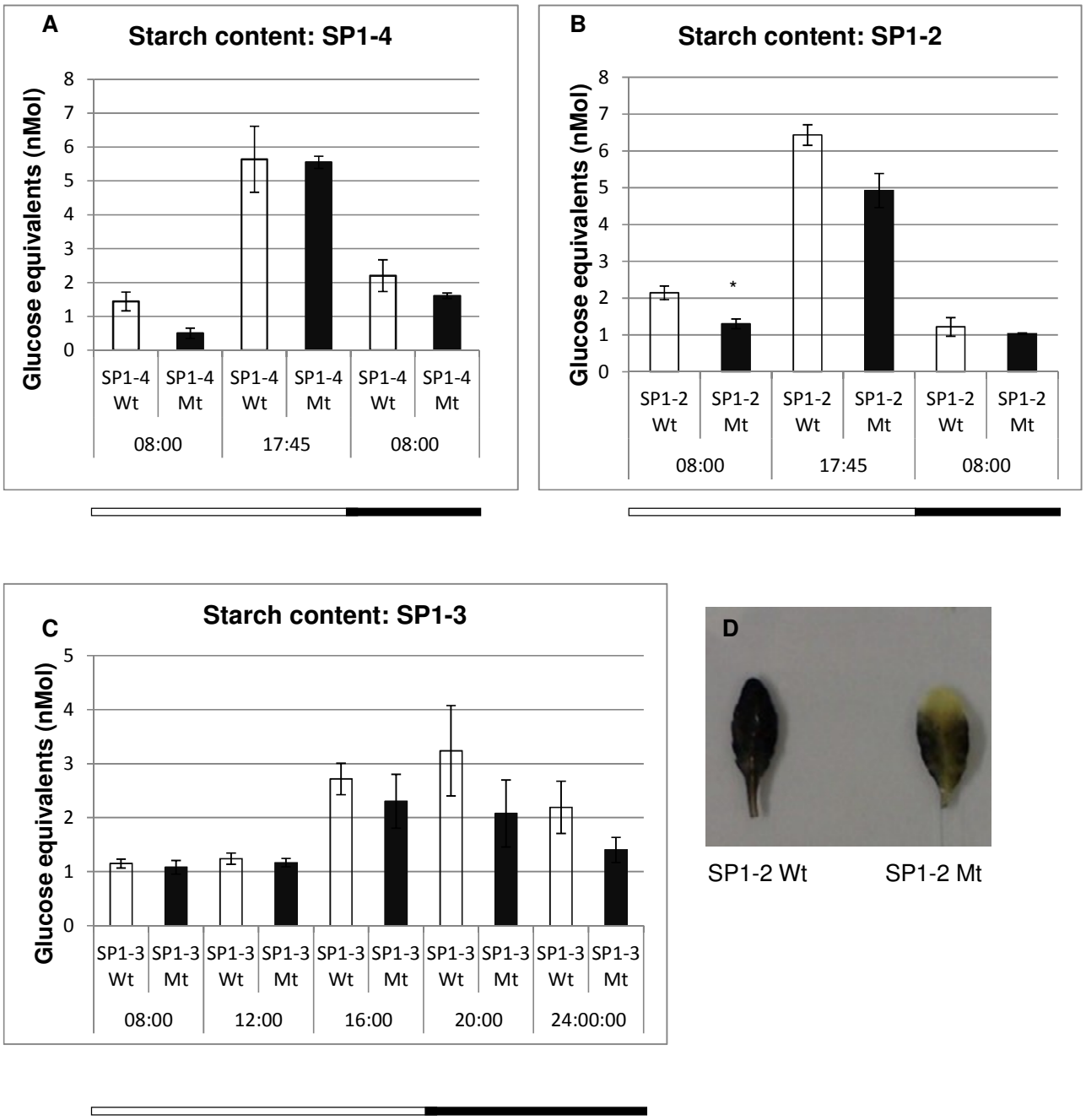


Figure 10. A-C, Quantitative leaf starch content for SP1-4, SP1-3 and SP1-2. D, Iodine staining of SP 838 wild type and mutant (11:45 am). A 10 hour light and 14 hour dark regime was followed. Light and dark periods are indicated as bars underneath. See through = light period, Black = Dark period. * denotes differences from the Wt control at the 5% level (Two tailed student *t*-test)

3.3.1 Recombinant protein

3.3.1.1 *E.coli* screening

cDNAs were amplified by PCR in order to produce recombinant SP1 and CBD1 proteins and ligated into pBluescript SK in sense orientation with respect to the lac promoter. Sequencing demonstrated no errors had been introduced by the PCR.

To investigate the possible effect these two proteins might have on α1,4 glucans, they were transformed into two different *E.coli* strains which produce large amounts of linear glucans. These were KV832::pACAG (Kiel et al., 1987; Kossmann et al., 1999) and a phosphoglucomutase (PGM) mutant. The KV832::pACAG strain lacks the glycogen branching enzyme gene GlgB and carries the *glgC16* ADP-glucose pyrophosphorylase allele on a pACYC184 derived plasmid. The glycogen synthase present in this strain will produce the linear glucans from ADP-glucose synthesised by the allosterically unregulated glgC16 (Creuzat-Sigal et al., 1972). *pgm* mutants, on the other hand, produce linear glucans from maltose by the action of amylomaltase and are unable to degrade them as the glucose 1-phosphate, produced by maltodextrin phosphorylase, cannot be further metabolized to glucose 6-phosphate due to the lack of PGM.

Expression of neither gene influenced accumulation of glucans in KV832::pACAG as determined by iodine staining (Fig 11, B&D). When the pSK::SP vector was present in the PGM mutant, however, it appeared that the cells accumulate lower amounts of glucans (Fig 11, A). This indicates either that the SP protein reduces the amounts of linear glucans accumulating, or acts by helping catabolism of the glucans manufactured by amylomaltase.

pgm$^-$ KV832::pACAG

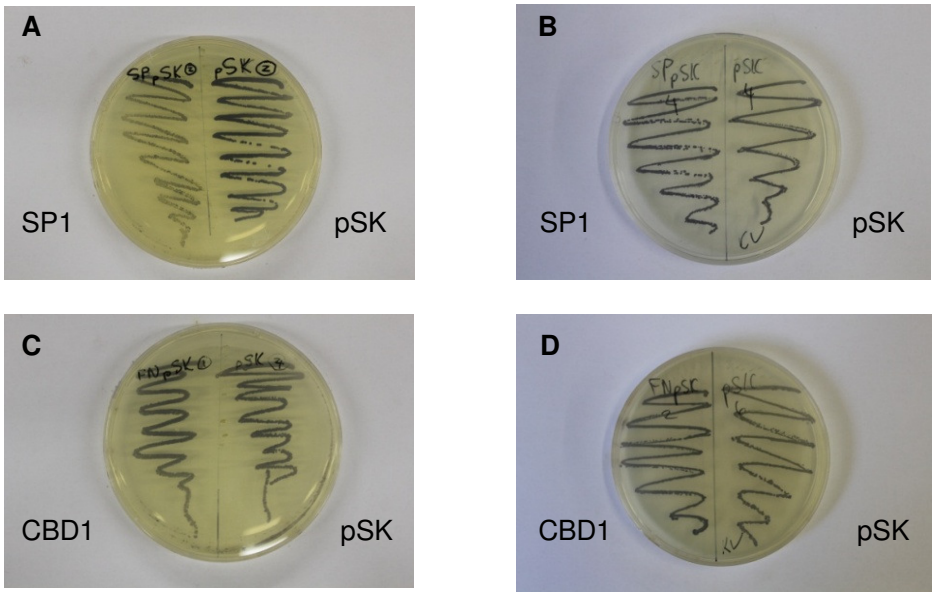


Figure 11. Iodine staining of *E.coli* strains that produce linear glucans and which are expressing *SP1* or *CB1* genes.(A,C) *pgm* mutant, (B,D) KV832::pACAG. . A, pSK::SP1 (left) and pSK::Empty (right). B, pSK::SP1 (left) and pSK::Empty (right) C, pSK::CBD1 (left) pSK::Empty (right) D, pSK::CBD1 (left) and pSK::Empty (right).

3.3.1.2 Recombinant protein activity

To test for possible enzymatic activity of the CBD1 and SP1 proteins on amylopectin containing native gels under non-denaturing conditions the pSK plasmids containing the cDNAs were transformed into the *E. coli* BL-21 strain. Crude extracts were separated by native page and, after overnight incubation, the gels were stained with iodine, however, no unique activity bands corresponding to SP1 and CBD1 were found.

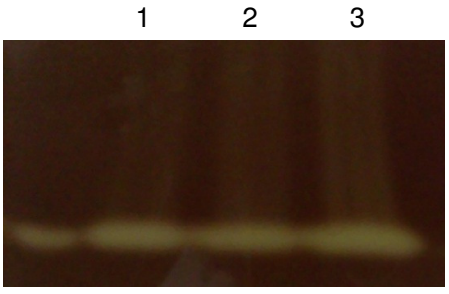


Figure 12. Crude protein extracts from BL21 codonplus RIPL containing pSK (Lane1) pSK::SP1 (Lane 2) or pSK::CBD1 (Lane 3) were separated by PAGE in gels containing no SDS, but 0.1% (w/v) amylopectin. After overnight incubation gels were stained with iodine to visualise any activity bands.

Because of the lack of additional activity bands when crude extracts were separated on native gels (Fig. 12) the cDNAs encoding the proteins were ligated into pET41 in frame with a GST tag and recombinant proteins purified by affinity purification.

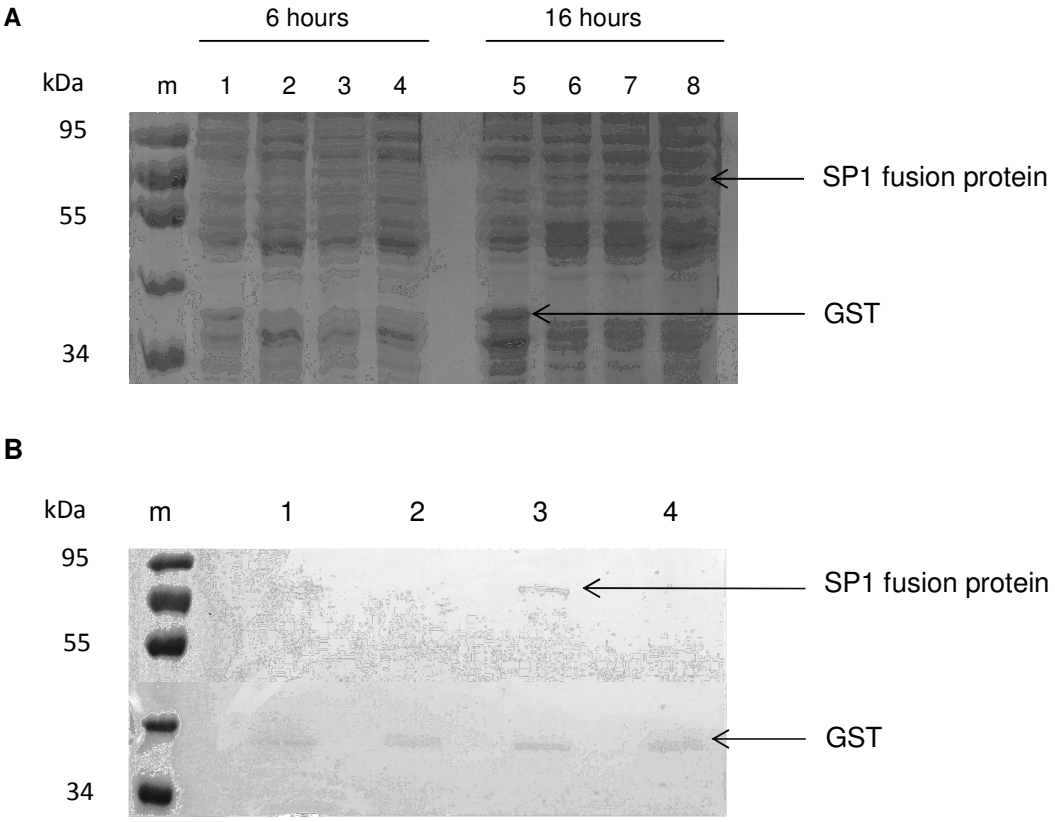


Figure 13. A, Coomassie brilliant blue stained 10% SDS-PAGE gel showing expression optimisation for recombinant SP1 with IPTG induction. Concentrations of 0.1mM, 0.5mM and 1mM. Lanes 1-4: 6 hours after induction. Lanes 5-8: 16 hours after induction. Lanes 1 & 5, pET41. Lanes 2 & 6 pET41::SP1 induced with 0.1mM IPTG. Lanes 3 & 7 pET41::SP1 induced with 0.5mM IPTG. Lanes 4 & 8 pET41::SP1 induced with 1mM IPTG. B, Coomassie brilliant blue stained 8% gel showing protein purification for SP1 and the GST tag protein complex. 1&3, SP1 protein in fraction 4 and 5 respectively which was used in the kinase activity assays. 2&4, empty vector which expressed the GST-tag complex (36 kDa) and was used as the vector control in the kinase activity assays. m= PageRuler^{TM} Prestained protein ladder (Invitrogen).

Expression was optimised based on addition of different concentrations of IPTG (Fig. 13). For SP1, the recombinant protein was purified on a GST column and the fusion protein found in the eluant was of the expected 70 kDa size. Purified protein was then examined for kinase activity by measuring production of ADP using pyruvate kinase and lactate dehydrogenase in a continuous assay. Small amounts of AMP were included in the reaction buffer as SP1 shows significant similarity to an AMP dependant kinase which acts as a

glycogen sensor in mammals (Grahame, 2011). Although ADP production was observed, it was not due to the fusion protein as this was also found in extracts from *E. coli* carrying the empty pET41 vector where GST alone was purified (Fig. 14). To exclude the possibility of adenylate kinase contamination AMP was excluded from the reaction mix, but activity was still seen (Fig. 14). The observed activity is most likely due to a DNAk protein that has been discovered to bind non-specifically to GST columns (Rial and Ceccarelli, 2002) and is known to demonstrate ATPase activity (Zylicz et al., 1983). It was decided that this assay is not suited to examine potential glucan kinase activity.

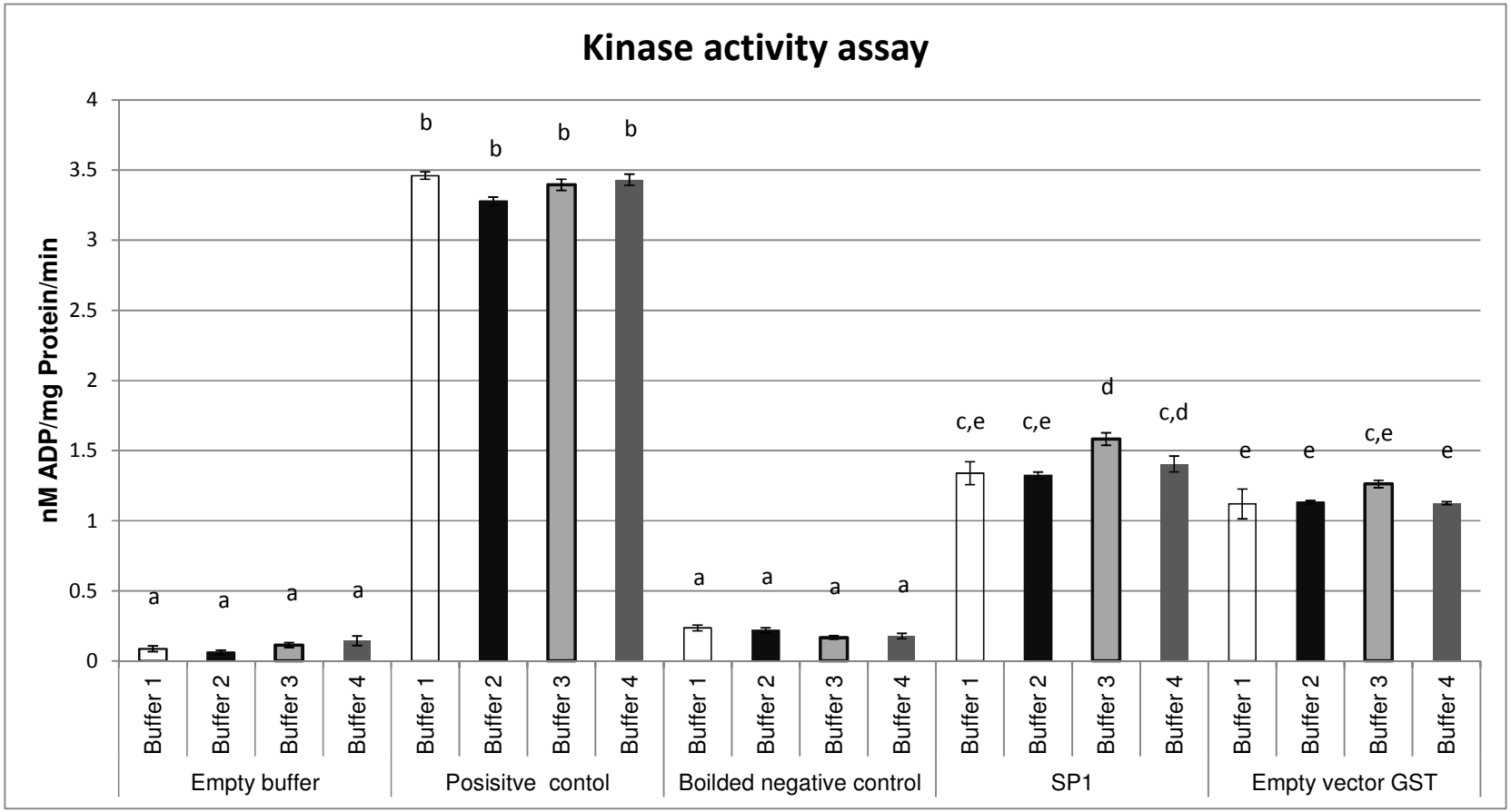


Figure 14. Kinase activity assays in with different substrates. Assay buffer1 contained 1mM ATP, starch and 0.1mM AMP. Buffer 2 contained 1mM ATP, 0.1mM AMP but no starch. Buffer 3 contained 1mM ATP and buffer 4 contained 1mM ATP plus starch. The activities from the different controls, buffers and proteins were grouped together using ANOVA with a Bonferroni *ad hoc* adjustment. . a-e denotes groups that did not show a statistically significant difference between one another at (0.09%).

Due to this problem another method was attempted. Recombinant protein was incubated with corn starch and an activity buffer containing 50mM HEPES-KOH pH7.0, 5mM $MgCl_2$, 0.1mM AMP and 1mM ATP. No significant increase in starch phosphate was detected and, therefore, it seems unlikely that the SP1 acts as a starch kinase. As SP1 is known to bind to starch granules (Lohmeier-Vogel et al., 2008), contains a kinase domain and is postulated to be involved in protein protein interactions I think in future that a role for it in in protein phosphorylation should be investigated.

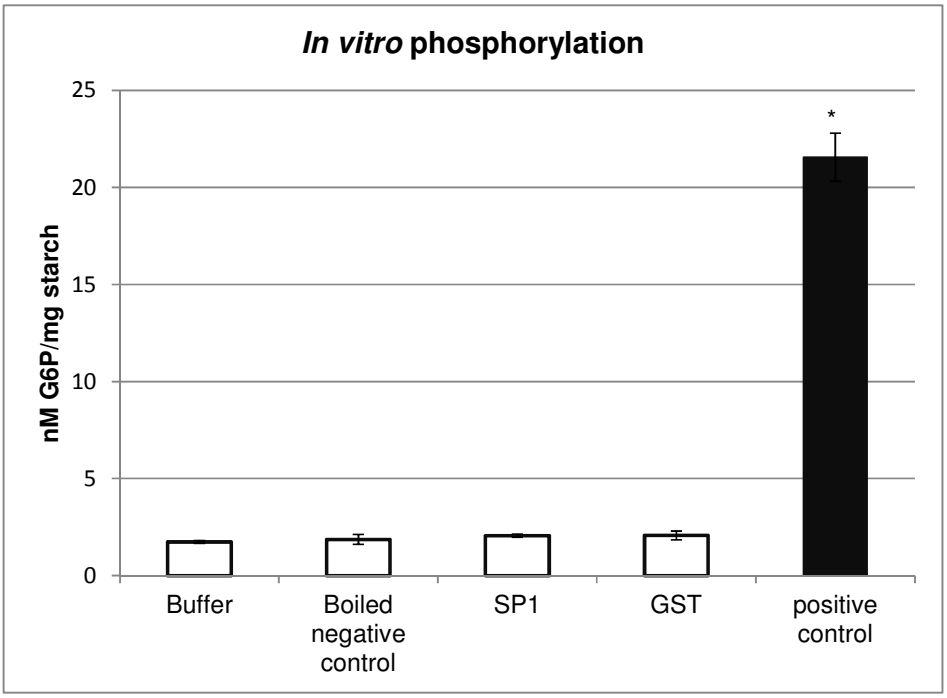


Figure 16. *In vitro* kinase activity assay. This assay investigated if SP1 added a phosphate at the C6 position of starch. Two negative controls were used, buffer without the recombinant protein and SP1 that was boiled. The positive control was starch from potatoes where activity of BEII was reduced. Its known to be rich in covalently bound G6P. * denotes differences from the negative control at the 5% level (Two tailed student *t*-test).

3.4 Conclusions

This chapter is divided into two parts which attempt to elucidate if SP1 and CBD1 play a role in starch metabolism. The first is the study of Arabidopsis T-DNA insertion mutants and the second investigates the activity of recombinant proteins of manufactured by the two cDNAs in *E.coli.*

It was found that CBD1 might play a role in starch degradation. Two Arabidopsis mutant lines appeared to contain more starch when leaves were exposed to iodine at the end of the dark period. However, quantitative analysis of starch was inconclusive. It is possible that environmental differences between the experiments (in one the plants were grown in the glasshouse and in the other in a growth room) would help to explain the results. As such the experiment should be repeated under the conditions where a starch excess phenotype was noted. A clear answer still cannot be given to what extent CBD1 is involved en starch degradation. The recombinant protein also did not show any activity on amylopectin native gels. My data does not show any clear role for SP1 in starch metabolism. Only one mutant line (SP1-2) showed any difference in starch amounts. On the other hand the expression of SP1 in a *E. coli pgm* mutant grown on maltose indicated that it affected the manufacture of linear glucan chains. This might indicate that it acts more on amylose than amylopectin and it would be interesting to include linear glucans in the activity assays that I attempted instead of amylopectin.

References

Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature **408:** 796-815

Baunsgaard L, Lutken H, Mikkelsen R, Glaring MA, Pham TT, Blennow A (2005) A novel isoform of glucan, water dikinase phosphorylates pre-phosphorylated alpha-glucans and is involved in starch degradation in Arabidopsis. Plant Journal **41:** 595-605

Caspar T, Lin TP, Kakefuda G, Benbow L, Preiss J, Somerville C (1991) Mutants of Arabidopsis with Altered Regulation of Starch Degradation. Plant Physiol **95:** 1181-1188

Christiansen C, Abou Hachem M, Glaring MA, Vikso-Nielsen A, Sigurskjold BW, Svensson B, Blennow A (2009) A CBM20 low-affinity starch-binding domain from glucan, water dikinase. FEBS Lett **583:** 1159-1163

Creuzat-Sigal N, Latil-Damotte M, Cattaneo J, Puig J (1972) Genetic analysis and biochemical characterization of mutants impairing glycogen metabolism in Escherichia coli K-12. In: Biochemistry of the glycoside linkage. Academic Press, New York, USA

Hardie DG (2011) AMP-activated protein kinase-an energy sensor that regulates all aspects of cell function. Genes Dev **25:** 1895-1908

Kiel JAKW, Vossen JPMJ, Venema G (1987) A General-Method for the Construction of Escherichia-Coli Mutants by Homologous Recombination and Plasmid Segregation. Molecular & General Genetics **207:** 294-301

Kötting O, Kossmann J, Zeeman SC, Lloyd JR (2010) Regulation of starch metabolism: the age of enlightenment? Curr Opin Plant Biol **13:** 321-329

Kossmann J, Abel GJW, Springer F, Lloyd JR, Willmitzer L (1999) Cloning and functional analysis of a cDNA encoding a starch synthase from potato (Solanum tuberosum L,) that is predominantly expressed in leaf tissue. Planta **208:** 503-511

Lohmeier-Vogel EM, Kerk D, Nimick M, Wrobel S, Vickerman L, Muench DG, Moorhead GBG (2008) Arabidopsis At5g39790 encodes a chloroplast-localized, carbohydrate-binding, coiled-coil domain-containing putative scaffold protein. Bmc Plant Biology **8:** 120

Rial DV, Ceccarelli EA (2002) Removal of DnaK contamination during fusion protein purifications. Protein Expr Purif **25:** 503-507

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, New York, USA

Steichen JM, Petty RV, Sharkey TD (2008) Domain characterization of a 4-alpha-glucanotransferase essential for maltose metabolism in photosynthetic leaves. J Biol Chem **283:** 20797-20804

Tetlow IJ, Wait R, Lu ZX, Akkasaeng R, Bowsher CG, Esposito S, Kosar-Hashemi B, Morell MK, Emes MJ (2004) Protein phosphorylation in amyloplasts regulates starch branching enzyme activity and protein-protein interactions. Plant Cell **16:** 694-708

Yu TS, Kofler H, Hausler RE, Hille D, Flugge UI, Zeeman SC, Smith AM, Kossmann J, Lloyd J, Ritte G, Steup M, Lue WL, Chen JC, Weber A (2001) The Arabidopsis sex1 mutant is defective in the R1 protein, a general regulator of starch degradation in plants, and not in the chloroplast hexose transporter. Plant Cell **13:** 1907-1918

Zylicz M, Lebowitz JH, Mcmacken R, Georgopoulos C (1983) The Dnak Protein of Escherichia-Coli Possesses an Atpase and Autophosphorylating Activity and is Essential in an Invitro Dna-Replication System. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences **80:** 6431-6435

Chapter four

General conclusions

This project attempted to study two aspects of starch metabolism: the interactions of different starch metabolic protein by repressing their activities in transgenic potatoes and the linking of two relatively unstudied genes and their protein products to starch metabolism in Arabidopsis leaves.

The first part of the study involved branching enzymes (BEI & BEII), debranching enzymes (ISA2) and a glucan kinase (GWD). RNAi vectors was produced and these vectors were transformed into potato and transgenic lines lacking BEI, BEII, GWD, ISA2, BEI-BEII, GWD-BEI and GWD-ISA2 were isolated. The RNAi lines for BEI and BEII generally followed the same phenotypically changes seen previously (Safford et al., 1998; Jobling et al., 1999), with an increase in G6P is observed in both sets of lines (Fig. 7C). The amylose content of starch from BEII tubers was also increased (Fig. 7A), but not to the same extent as previously published (Jobling et al., 1999). Intriguingly it was observed that in some of the BEI lines amylose in the leaf starch was almost eliminated (Fig. 7B). It is known that the amylose content of leaf starch is generally less than storage organ starch (Hovenkamp-Hermelink et al., 1988) but not to this extent. This result should be verified utilising another method and, if shown to be true, examined further. A more complete picture about the interactions between the starch metabolic proteins (BEs, GWD and ISA2) will only be gained once all RNAi chimeric lines can be analysed under the same growing conditions. Two lines lacking GWD-BEII and GWD-BEI-BEII still need to be produced and screened. Once that is done it will be possible to examine if/how they interact in manufacturing starch.

The second part of the thesis involved the study of two genes and the possible activity recombinant proteins expressed in *E. coli* have. Seven Arabidopsis mutant lines were obtained, four for SP1 and three for CBD1. These lines were genotyped and three homozygotic mutants for SP1 (SP1-4, SP1-2 and SP1-3) and two for CDB1 (CDB177 and CBD1-1) were identified. Both the homozycotic mutants for CBD1 demonstrated a starch excess phenotype when harvested in the morning. It was decided in the subsequent generation to quantitatively determine starch contents. An increase in starch was observed in both mutant lines but not to such a large extent as would have been expected based on the iodine staining. That CBD1 protein may play a role in starch degradation but, maybe only under specific environmental conditions or developmental stages. Expression of CBD1 is predicted to be up regulated in older plants. CBD1 protein expressed in *E.coli* did not show any additional activity on amylopectin containing zymograms and without a more exact idea of the putative function it is difficult to design a more targeted assay to assess its activity.

The Arabidopsis SP1-2 and SP1-4 mutant lines did not present a clear change in starch content. The analysis of recombinant proteins indicates that the kinase domain present in SP1 might not be a starch kinase but it cannot be excluded that necessary co-factors may not have been present. If that is the case, it is possible that this domain confers protein kinase activity to SP1. As SP1 also contains a coiled-coil (Lohmeier-Vogel et al., 2008) in addition to the kinase domain, it could perhaps be involved in phosphorylating an enzyme involved in starch metabolism which contains a similar coiled-coil domain, such as GBSS1, SS1, SS2, SS3, SS4, AMY3 and GWD1 (Lohmeier-Vogel et al., 2008; Kötting et al., 2010).

Taken together the finer mechanism of starch phosphorylation could not be elucidated in the current experiment, but this will be better understood once all the transgenic lines are produced and analysed. With regards to CBD1 and SP1 further analysis on the mutant lines is required to see if they are involved in starch metabolism.

References

Hovenkamphermelink JHM, Devries JN, Adamse P, Jacobsen E, Witholt B, Feenstra WJ (1988) Rapid Estimation of the Amylose Amylopectin Ratio in Small Amounts of Tuber and Leaf Tissue of the Potato. Potato Res **31:** 241-246

Jobling SA, Schwall GP, Westcott RJ, Sidebottom CM, Debet M, Gidley MJ, Jeffcoat R, Safford R (1999) A minor form of starch branching enzyme in potato (Solanum tuberosum L.) tubers has a major effect on starch structure: cloning and characterisation of multiple forms of SBE A. Plant Journal **18:** 163-171

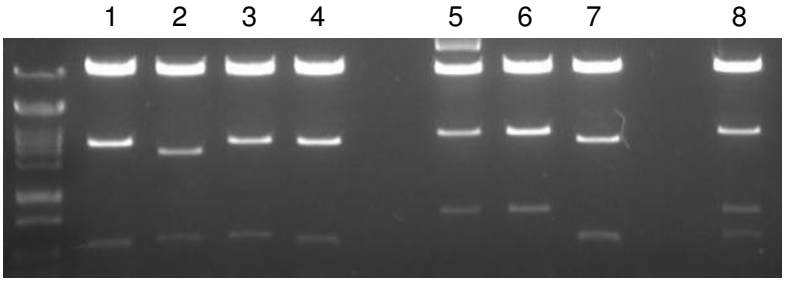
Kotting O, Kossmann J, Zeeman SC, Lloyd JR (2010) Regulation of starch metabolism: the age of enlightenment? Curr Opin Plant Biol **13:** 321-329

Lohmeier-Vogel EM, Kerk D, Nimick M, Wrobel S, Vickerman L, Muench DG, Moorhead GBG (2008) Arabidopsis At5g39790 encodes a chloroplast-localized, carbohydrate-binding, coiled-coil domain-containing putative scaffold protein. Bmc Plant Biology **8:** 120

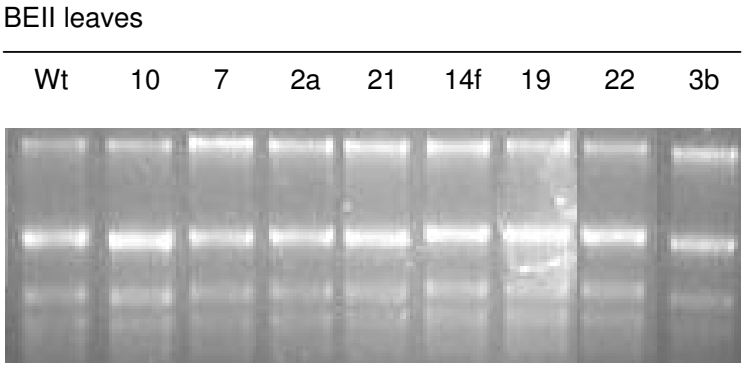
Safford R, Jobling SA, Sidebottom CM, Westcott RJ, Cooke D, Tober KJ, Strongitharm BH, Russell AL, Gidley MJ (1998) Consequences of antisense RNA inhibition of starch branching enzyme activity on properties of potato starch. Carbohydr Polym **35:** 155-168

Supplementary data

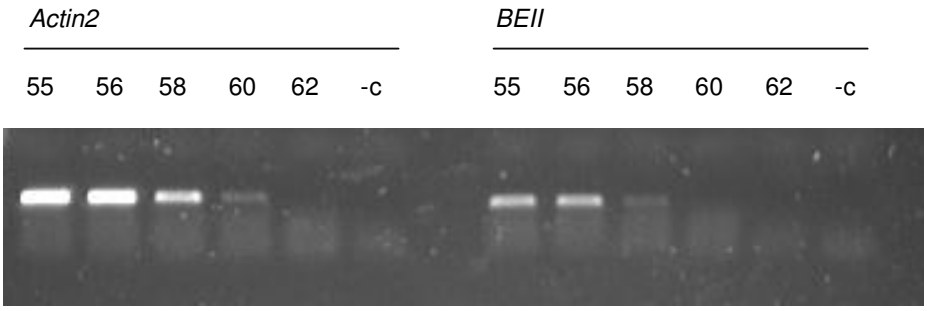
Chapter two supplementary figures



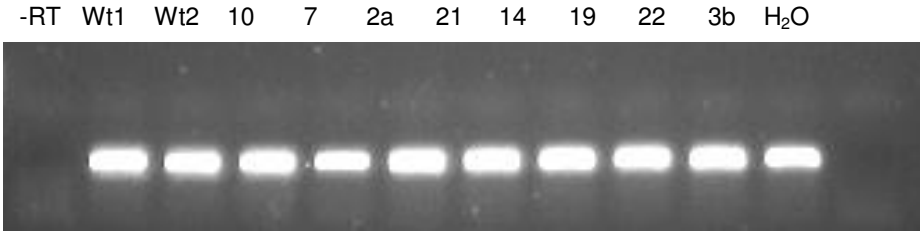
Supplementary figure 1. XhoI RE digest of the HG RNAi vectors to confirm the inserts entered correctly. The same vectors were also digested with XbaI (Data not shown). 1, pHG-GWD. 2, pHG-BE1. 3, pHG-BE2. 4, pHG-ISA2. 5, pHG-GWD-BE1. 6, pHG-GWD-ISA2. 7, pHG-BE1-BE2. 8, pHG-GWD-BE1-BE2.



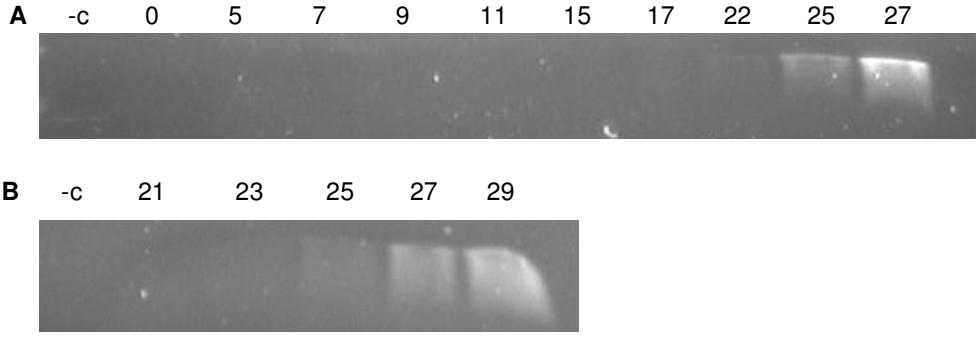
Supplementary figure 2. Intact total RNA that was extracted from RNAi lines which is down regulated in BEII and run under denaturing conditions on a 1.2% agarose gel. Wt=wild type Désirée leaf material.



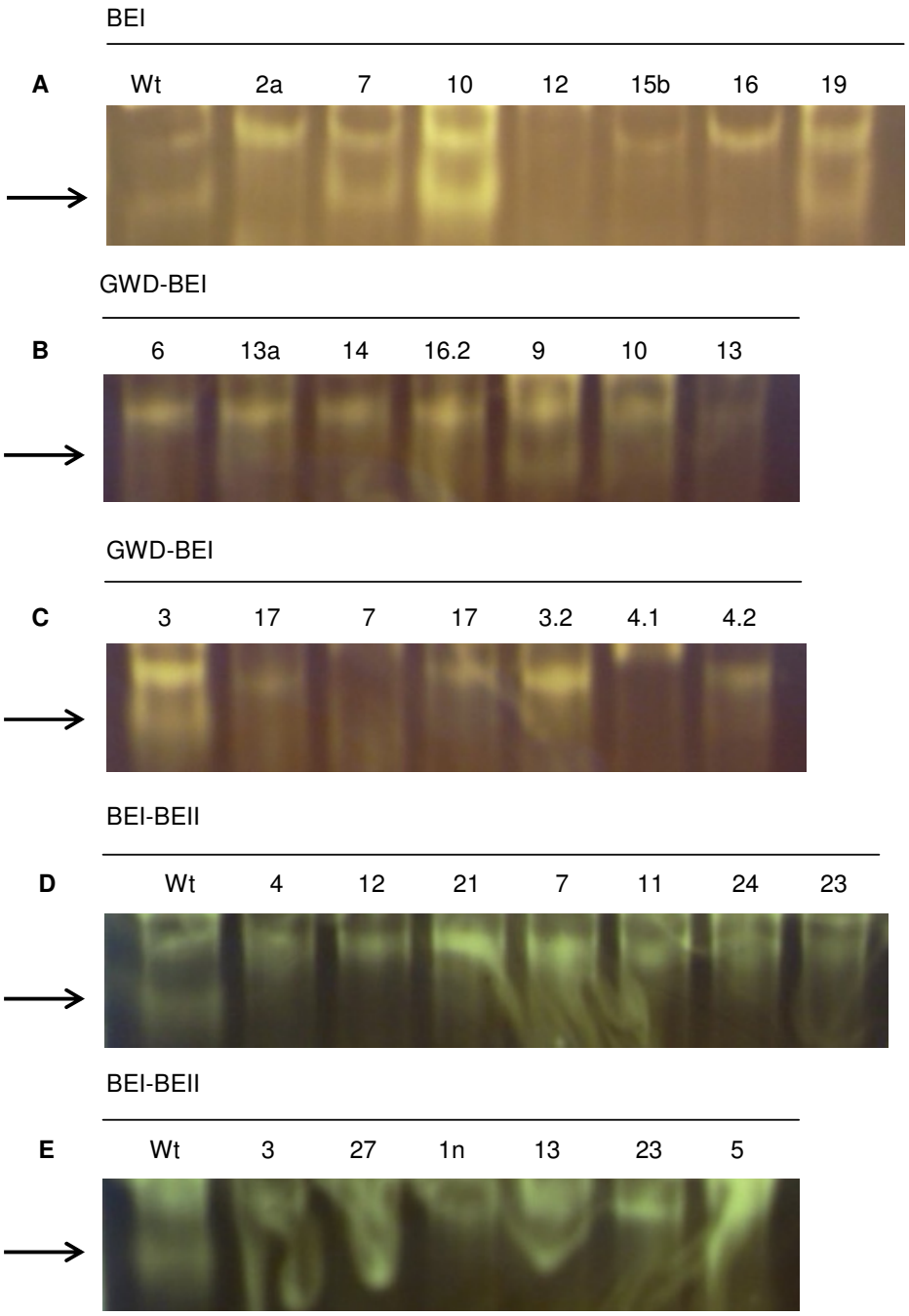
Supplementary figure 3. Primer annealing temperature optimization was done for the *Actin2* and *BEII* primers. A temperature range between 55 and 62°C were investigated and all subsequent PCR reaction was done at 56°C. –c= H_2O



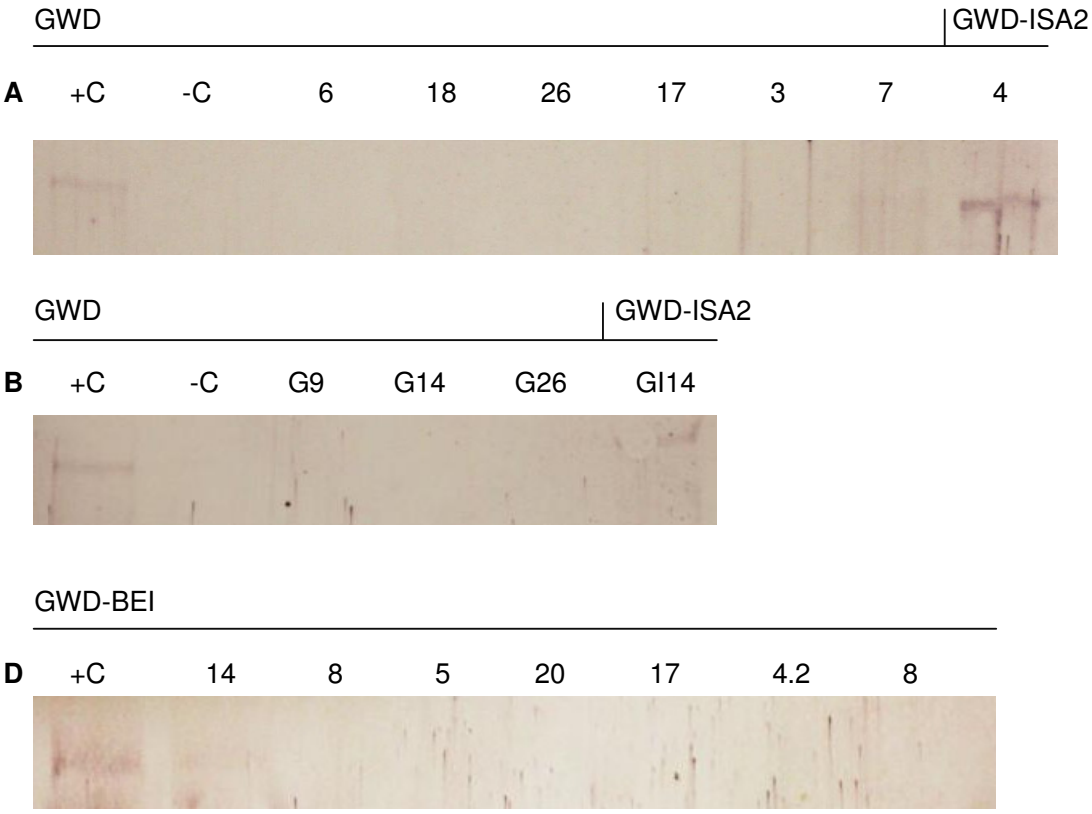
Supplementary figure 4. The integrity and quality of the cDNA was investigated with a PCR where the *Actin2* primer set was used. –RT= no reverse trancriptase control for gDNA contamination determination. Wt1= wild type potato cDNA reaction1, Wt2= wild type potato cDNA reaction 2.



Supplementary figure 5. PCR reactions were done to ascertain where the *Actin2* and *BEII* primers reach linearity. A, PCRs were done on Wt potato cDNA with the *Actin2* primer pair. Reactions were stopped by adding 5µl of 0.5M EDTA. B, PCRs were done on Wt potato cDNA with the *BEII* primer pair. Reactions were stopped by adding 5µl of 0.5M EDTA. –c= H_20

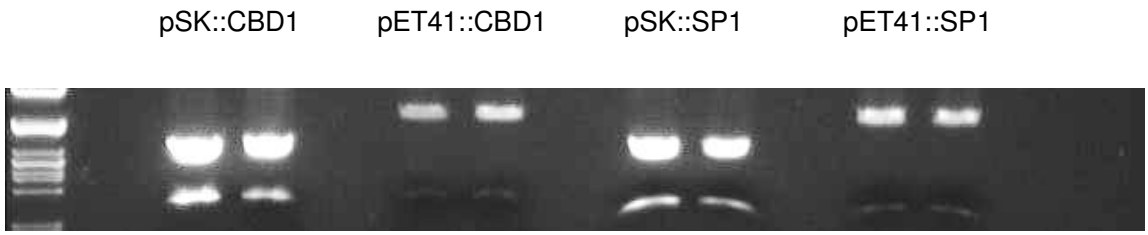


Supplementary figure 6. Native gels showing BEI activity on amylopectin. A, lines transformed with the HG::BEI. B&C, Lines transformed with the HG::GWD::BEI. D&E, lines transformed with the HG::BEI::BEII construct.



Supplementary figure 7, Immunoblots to indicate a reduction in the presence of GWD. A&B, GWD and GWD-ISA2 lines. D, GWD-BE1 lines. +C= Wt potato

Chapter three supplementary figures



Supplementary figure 8, RE digestion to investigate if the CBD1 and SP1 was successfully ligated into the pSK and pET41 vectors. CBD1 double enzyme digestion was performed with PstI and XhoI while SP1 RE digestions were performed with EcoRI and XhoI.