Analysis of interactions between glucan, water dikinase and either isoamylase or starch branching enzymes in determination of starch structure

By

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Declaration

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Abstract

Starch is the main form in which carbon is stored in plants and it is used in a wide array of applications, from its role as a source of calories in human diets, to livestock feed and as an industrial feedstock. It is composed of two types of glucan polymer, amylose which is composed mainly of α-1,4 linked chains and highly branched amylopectin which contains α1,4 chains which are connected together with α1,6 branchpoints. Synthesis of the starch polymer involves co-ordinated activities of starch synthases, starch branching enzymes (SBE) and debranching enzymes (DBE). In addition, the enzyme glucan, water dikinase (GWD) can introduce phosphate into amylopectin. The critical roles these enzymes play during starch metabolism have been investigated by knockout or silencing of genes encoding them in some plants, mainly *Arabidopsis thaliana*. Interactions between some of these enzymes have also been demonstrated, but there are still many questions about how these interactions occur and how they influence starch metabolism.

In this study two sets of experiments were performed. The first involved analyses of potato (*Solanum tuberosum* L.) plants where the expression of *SBEI*, *SBEII* and/or *Glucan, Water Dikinase 1* (*GWD1*) were repressed using RNAi technology. Individual or joint repression of the two *SBE* isoforms resulted in a significant increase in starch phosphate, whereas repression of *GWD1* led to synthesis of low phosphate starch. Starch phosphate decreased in SBEI/GWD1 and SBEII/GWD1 lines but not to the levels found in lines where only *GWD1* was repressed. The apparent amylose content increased in starch from SBEI/GWD1 lines was greater than either the SBEI or GWD1 lines. These alterations in starch composition influenced its granule morphology, swelling power and freeze-thaw stability. Silencing of *GWD1* reduced starch degradation in cold-stored tubers, but this was not the case in lines repressed in either starch branching enzyme.

The second set of experiments examined polyglucan metabolism in potato lines repressed in isoamylase 2 (*ISA2*) and/or *GWD1*. Transgenic potato lines were produced by RNAi gene silencing and tuber starch metabolism analysed. The water-soluble glucan content in ISA2 and ISA2/GWD1 lines tuber was higher than GWD1 lines. Analysis of starch structure indicated that there was an increase in the apparent amylose content of starches from GWD1 lines whereas it was unchanged ISA2/GWD1 lines. There was a reduction in the starch phosphate in both GWD1 and ISA2/GWD1 lines, and this reduction was greatest in the GWD1 lines. The ISA2 lines contained a proliferation of small starch granules, but this was not observed in ISA2/GWD1 lines which indicates that ISA2 and GWD1 interact during granule initiation. The effect of both enzymes on cold-induced sweetening (CIS) was also investigated. As expected, CIS was reduced in lines where *GWD1* was silenced. Interestingly CIS was also inhibited in the ISA2 lines indicating that the pathway of starch degradation may differ between cold-stored potato tubers and Arabidopsis leaves.

Opsomming

Stysel word hoofsaaklik as koolstof in plante geberg en dit word in 'n wye verskeidenheid toepassings gebruik wat wissel van ‘n rol as bron van kalorieë in menslike dieet, tot veevoer en as 'n industriële voerstof. Dit bestaan uit twee tipes glukaanpolimere naamlik, lineêre amilose wat hoofsaaklik uit α-1,4-gekoppelde-kettings saamgestel is en hoogs vertakte amilopektien wat α-1,4-kettings bevat wat met α-1,6-takpunte verbind is. Styselsintese van die styselpolimeer en degradasie behels gekoördineerde aktiwiteite van verskeie ensieme soos styselsintase, styselvertakkingsensieme (SBE) en onttakkende ensieme (DBE). Daarbenewens kan die ensiem, glukaan waterdikinase (GWD) fosfaat in amilopektien invoeg. Die kritieke rolle wat hierdie ensieme tydens styselmetabolisme speel is ondersoek deur gene wat hulle kodeer in sommige plante, hoofsaaklik *Arabidopsis thaliana*, uit te skakel of stil te maak. Interaksies tussen sommige van hierdie ensieme is reeds gedemonstreer, maar daar is steeds baie vrae oor hoe hierdie interaksies plaasvind en hoe hulle styselmetabolisme beïnvloed.

In hierdie studie is twee stelle eksperimente uitgevoer. Die eerste het ontledings van aartappel (*Solanum tuberosum* L.) plante behels waar die uitdrukking van *SBEI, SBEII* en/of *glukaan, water dikinase 1* (*GWD1*) onderdruk is deur gebruik te maak van RNAi tegnologie. Individuele of gesamentlike onderdrukking van die twee SBE isovorme het gelei tot 'n beduidende toename in styselfosfaat, terwyl onderdrukking van *GWD1* gelei het tot sintese van lae fosfaatstysel. Styselfosfaat het in SBEI/GWD1- en SBEII/GWD1-lyne afgeneem, maar nie tot die vlakke wat gevind is in lyne waar slegs *GWD1* onderdruk is nie. Die oënskynlike amilose-inhoud het verhoog in stysel vanaf SBEI/GWD1-lyne tot hoër vlakke as in óf die SBEI- of GWD1-lyne. Hierdie veranderinge in styselsamestelling het die korrelmorfologie, swelkrag en vries-ontdooi-stabiliteit beïnvloed. Onderdrukking van *GWD1* uitdrukking het styselafbraak in koudgebergte knolle verminder, maar dit was nie die geval in lyne waar enige van die styselvertakkingsensieme onderdruk is nie.

Die tweede stel eksperimente het poliglukaanmetabolisme ondersoek in aartappellyne waar *isoamilase 2 (ISA2*) en/of *GWD1* geen-uitdrukking onderdruk is. Transgeniese aartappellyne is geproduseer deur RNAi-geenuitkikker tegnologie en knolstyselmetabolisme is ontleed. Die wateroplosbare glukaan inhoud in ISA2 en ISA2/GWD1 lyne se knolle was hoër as in GWD1 lyne. Analise van styselstruktuur het aangedui dat daar 'n toename in die oënskynlike amilose-inhoud van stysels vanaf GWD1-lyne was, terwyl dit onveranderde gebly het in ISA2/GWD1-lyne. Daar was 'n verlaging in die styselfosfaat in beide GWD1 en ISA2/GWD1 lyne, en hierdie verlaging was die grootste in die GWD1 lyne. Die ISA2-lyne het 'n proliferasie van klein styselkorrels bevat, maar dit is nie in ISA2/GWD1-lyne waargeneem nie, wat aandui dat ISA2 en GWD1 interaksie het tydens korrelinisiasie. Die effek van beide ensieme op koue-geïnduseerde versoeting (CIS) is ook ondersoek. Soos verwag is CIS verminder in lyne waar GWD1 stilgemaak is. Interessant genoeg is CIS ook in die ISA2-lyne geïnhibeer, wat aandui dat die pad van styselafbraak kan verskil tussen koudgestoorde aartappelknolle en Arabidopsis-blare.

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Yes, I finally made it!

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are both part of my success story and I thank you so much for bearing with my absence, I promise to me more available from now. Thank you and I love you all very much.

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Abbreviations

ADP	Adenosine diphosphate
ADPGlc	ADP-glucose
AMP	Adenosine monophosphate
AMY	α-amylase
$AgNO_3$	Silver nitrate
AGPase	ADP-glucose pyrophosphorylase
AIM	Agrobacterium infection media
ATP	Adenosine 5'-triphosphate;
BAM	β-amylase
BCIP/NBT	5-Bromo-4-Chloro-3-Indolyl Phosphate p-Toluidine/ Nitro-Blue Tetrazolium Chloride
bp	Base pairs
BSA	Bovine serum albumin
C-3	Carbon-3
C-6	Carbon-6
CBM	Carbohydrate binding module
cDNA	Complementary deoxyribonucleic acid
CIM	Callus induction medium
CIS	Cold-induced sweetening
cm	centimeter;
CPM	Clonal propagation medium
DBE	Debranching enzyme
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
DPE1	Disproportionating enzyme 1
DPE2	Disproportionating enzyme 2
DSP	Dual specificity phosphatase
EC	Enzyme commission

DTT	Dithiotreitol
EDTA	Ethylenediaminetetraacetic acid
EF-1α	Elongation factor-1 alpha
ETOH	Ethanol
F6P	Fructose 6-phosphate
FW	Fresh Weight
G1P	Glucose 1-phosphate
GA_3	Gibberellic acid
GPT	Glucose phosphate translocator
g	Gram
GBSS	Granule-bound starch synthase
GH	Glycoside hydrolase
GWD	Glucan, water dikinase
HCl	Hydrochloric acid
h	Hours
I_2	Iodine
ISA	Isoamylase
KCL	Potassium chloride
KI	Potassium iodide
KOH	Potassium hydroxide
kDa	Kilodalton
KH_2PO_4	Potassium dihydrogen phosphate
KOH	Potassium hydroxide
L	Litre
LB	lysogeny broth
LDA	Limit Dextrinase
LSF1	Like Sex Four–1
LSF2	Like Sex Four–2
M	Molar
MES	2-N-morpholino ethane sulfonic acid

MEX	Maltose excess
µl	microliter
mg	Milligram
$MgCl_2$	Magnesium cloride
µg	Microgram
Ml	Microlitre
Mmol	Micromole
Min	Minutes
ml	Millilitre
MOPS	4-Morpholinepropanesulfonic acid
MOS	Malto-oligosaccharides
mM	Millimolar
MFP	MAR BINDING FILAMENT LIKE PROTEIN
MRC	MYOSIN-RESEMBLING CHLOROPLAST PROTEIN
MS	Murashige and Skoog
M_w	Molecular weight
Ng	Nanogram
NAA	Naphthalene acetic acid
NaAC	Sodium acetate
NAD	Nicotinamide adenine dinucleotide
NaCl	Sodium chloride
Na_2HPO_4	Disodium hydrogen phosphate
NCBI	National Centre for Biotechnology Information
NH_2	Ammonia
Nm	Newton meter
NMR	Nuclear magnetic resonance
NTT	Nucleotide translocator
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline

PBS-T	Phosphate-buffered saline-Tween 20
PCR	Polymerase chain reaction
PDS	Phytoene desaturase
PGI	phosphoglucoisomerase
pGlcT	Plastidial glucose transporter
3-PGA	3-phosphoglycerate
PGM	Phosphoglucomutase
pH	measure of the acidity or basicity
PHS	Plastidial phosphorylase
P_i	Inorganic phosphate
PP_i	Inorganic pyrophosphate
PTST	Protein Targeting to Starch
PUL	Pullulanase
PWD	Phosphoglucan, water dikinase
RE	Restriction enzyme
RIM	Root induction medium
RDS	Rapidly digestible starch
RNA	Ribonucleic acid;
RNAi	Ribonucleic acid interference
Rpm	Revolutions per minutes
RS	Resistant starch
SBE	Starch branching enzyme
SDS	Slowly digestible starch
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscope
SEX4	Starch excess-4
SIM	Shoot induction medium
sqRT-PCR	Semi-quantitative reverse transcriptase PCR
SS	Starch synthase
SuSy	Sucrose synthase

Tris-HCl	Tris(Hydroxymethyl)-aminomethane
U	Units
UDP	Uridine diphosphate
UGPase	UDP-glucose pyrophosphorylase
(v/v)	(Volume/volume)
V	Volts
(w/v)	(Weight/volume)

Chapter 1: Literature review

1.1 Origin and history of the cultivated potato

The cultivated potato (*Solanum tuberosum* L.) is classified within the asterid clade alongside other eudicot plants, which make up 25% of angiosperms. It is predicted to have diverged from grape about 89 million years ago, an event which is thought to correspond to the break between rosids and asterids (Xu et al., 2011). As a member of the family Solanaceae it is closely related to many economically important crops such as tomato, tobacco, eggplant, petunia and pepper. Diversification of potato's wild species has been traced to Mexico and South America, where it has been used as a food source for more than 10 000 years (Louderback & Pavlik, 2017). It was domesticated about 8 000 years ago and its cultivation was pioneered in the Andes mountains of Peru and Bolivia. Spanish and British explorers introduced it to Europe in the late 1500s (Thornton and Sieczka, 1980; Louderback & Pavlik, 2017) and it arrived much later in Africa at the end of 17th century with Christian missionaries. Although it was initially fed to livestock in Europe, the famines experienced during the Napoleonic wars led to its adoption as human food and its high vitamin C content may have been pivotal in preventing scurvy among its early consumers (Buckenhüskes, 2005). Potatoes are currently cultivated in over 158 countries worldwide (FAOSTAT, 2020) and the most recent edition of the world catalogue of potato varieties published by the International Potato Center in 2010, reported 4500 cultivated potato varieties (www.cipotato.org). These have become adapted to growth in different ecological zones and have been selected for utilization either for consumption or for industrial purposes.

1.2 Taxonomy and features of cultivated potato

Potato belongs to the *Solanum* genus, which is characterized by plants where the basic chromosome number is 12. Presently a total of 111 species of potato have been identified and

these are subdivided into 4 cultivated and 107 wild species (Spooner et al., 2014). Cultivated potato species consist of *Solanum ajanhuiri* a diploid (2n=24); *Solanum curtilobum* a pentaploid (5n=60); *Solanum juzepczukii* a triploid (3n=36) and *Solanum tuberosum* which is grouped into two cultivars. The Andigenum group includes upland Andean genotypes and can be diploids, triploids or tetraploids (4n=48). The second group - Chilotanum - is tetraploid and represents the Chilean lowland landraces (Spooner et al., 2007) and is the most widely cultivated potato (Brown, 1993). There is relatively low genetic variation in the North American and European potato gene pools due to the founder effect caused by the widespread use of this group in breeding programs (Haan & Rodriguez, 2016).

Solanum tuberosum is a dicotyledonous and annual herbaceous plant, also referred to as perennial because of its tuber production (www.cipotato.org). The life cycle of potato is made up of several developmental stages including stolon formation, tuberization, tuber filling, dormancy and tuber sprouting (Ewing & Struik, 1992). Some cultivars bear green fruits (berries) which contain about 300 seeds (Centro Interncional de la Papa (CIP) 1984). Potato tuberization is initiated at the tip of the underground stolon leading to increased metabolic activity and occurrence of two major biochemical changes; formation of storage protein and starch accumulation (Appeldoorn et al., 1997). Upregulation of genes associated with starch biosynthesis and storage proteins coincide with transition of the stolon to tuber (Xu et al., 2011). The common method used in commercial cultivation of potato is by vegetative propagation, which involves planting tubers or tuber sections. Potato can also be propagated through *in vitro* tissue culture because of its ease of regeneration, but somaclonal variation may occur when callus is included in the culture technique (Bordallo et al., 2004).

The beginning of 19th century marks the start of potato breeding, however, progress in potato improvement using conventional breeding methods have been slow due to several factors. Firstly cultivated tetraploid potato has a very narrow genetic basis unlike other major crop

species (Davies, 2002; Xu et al., 2011). Secondly being a highly heterozygous, difficulties in segregation and purifying targeted genes into a single line is a major challenge (Xu et al., 2011; Wang et al., 2014). Other limiting factors for potato breeding through conventional methods are self-incompatibility, acute inbreeding depression due to fixation of unfavourable alleles and susceptibility of tubers to several devastating pest and pathogens. This slows down generation of sufficient quality seed tubers for multilocation field selection (Xu et al., 2011; Stokstad, 2019; Bonierbale et al., 2020). Although introgression of genes from wild relatives of potato which confer disease resistance into cultivated potato have been successful on few occasions (Ross and Hunnius, 1986; Sinden and Sanford, 1981; Bradshaw and Ramsay, 2005; Bradshaw, 2009; Finkers-Tomczak et al., 2011), unwanted side effects from hybridisation events imply that between 12-15 years is required for cultivar development (Davies, 2002; Bonierbale et al., 2020). Using backcrossing to eliminate these unwanted effects is not an easy option for potato, therefore, improvement of existing potato cultivars through genetic engineering methods, has received positive attention in recent times. It is important to note that genetic engineering methods may not render the conventional breeding of potato obsolete, rather it can serve a complementary purpose in modifying the genome of potato for commercial and environmental benefit.

1.3 Importance of potato

Potato was the fourth most important food crop based on its global production in 2020, which was reported to be 359 million tons with only rice (756 million tons), wheat (761 million tons) and maize (1.16 billion tons) yielding more (FOASTAT, 2020). In terms of human consumption, it ranks as the third most important crop as only 14% of maize production is used as food. It is consumed by more than one billion people worldwide and harvested from around 16 million hectares of land in over 158 countries with an average yield of 20 tons ha^{-1} (FOASTAT, 2020). Potato cultivation can be categorised into consumption (by both humans and animals), starch production (as a renewable polymer and energy source in industry) and

the formation of seed potatoes (to allow planting of disease-free tubers). Harvesting of tubers for consumption is the most commercially important aspect as they can act as a universal staple food which contributes significantly to long-term human health (Kruger, 1997).

Approximately 80% of the dry weight of mature tuber is made up of starch (Kruger, 1997) and, therefore, the majority of calories derived from potato consumption is contributed by this component. Compared with most cereals, potato yields more protein in their tubers for the same growing area. Because potatoes contain all essential amino acids in relatively high amounts their tubers have a high biological value (a measure of the proportion of protein absorbed from a food and retained for use) of between 90-100% when compared with popular legumes and cereals such as soybean (84%) and rice (83%) (Buckenhüskes, 2005). It also contains essential vitamins (Augustin, 1975), minerals (White & Broadley, 2005) and important phytochemicals such as phenolics, flavonoids and carotenoids. Many of these phytochemicals have antioxidant properties making it a healthy diet for people that are prone to age-related chronic ailments such as certain cancers, cardiovascular diseases, and diabetes (Ezekiel et al., 2013).

In addition to its importance as a food source, starch from potatoes is gaining traction as a renewable and pollution-free industrial feedstock. It is useful in specific industries, like noodle production, because it forms a particularly viscous gel when heated in water (Lu et al., 2012a). Potato starch is unique amongst industrial starch sources because of its high levels of covalently bound phosphate (Slattery et al., 2000), which gives it an electrostatic charge allowing it to bind to cellulose during paper manufacture. Research on potato has many advantages: it is easily transformable and therefore amenable to genetic manipulation, and it can be rapidly propagated both in tissue culture and through cuttings (Jackson, 1999). This makes it a plant with many advantages for the study of starch metabolism especially with a focus on how it can be altered to improve it for industrial use.

1.4 Importance of starch

Starch is a principal storage carbohydrate in most vascular plants, which plays important roles during the life cycle of the plant, particularly as a metabolite involved in influencing growth (Sulpice et al., 2009). In leaves it is synthesized and degraded diurnally in chloroplasts, serving as a nocturnal energy resource where its glucose monomers become converted to sucrose to maintain energy supply for biological processes (Bahaji et al., 2014). Leaf starch is frequently referred to as 'transitory starch' because of its role as short-term carbohydrate reservoir. Under most conditions Arabidopsis mutant unable to synthesis or degrade starch at night have reduced growth rates (Smith & Stitt, 2007). Accumulation of starch occurs in amyloplasts of non-photosynthetic plant organs such as seeds (for example from wheat, rice, maize, sorghum), tubers (potato) and roots (cassava). In these organs it is often referred to as storage starch. Storage starch in potato maintains the energy demand of the dormant tuber and powers growth of new shoots after dormancy is broken (Streb et al., 2012). Starch is furthermore emerging as a key molecule mediating plant responses to abiotic stresses, such as water deficit, high salinity, or extreme temperatures. Under these challenging environmental conditions, plants generally mobilize it to provide energy and carbon at times when photosynthesis may be limited. The sugars and other derived metabolites released support plant growth under stress, and function as osmo-protectants to mitigate the negative effect of the stress (Krasensky & Jonak, 2012).

In addition to the vital role that starch plays in plant growth, it is the most important carbohydrate used for food and feed purposes and represents the primary source of calories in the human diet. Based on the rate and extent of digestibility, most starches contain fractions that are digested rapidly (rapidly digestible starch (RDS)), digested slowly (slowly digestible starch (SDS)) or are resistant to digestion (resistant starch (RS)) (Huang et al., 2018). Intake of RDS-rich foods results in speedy increase in blood glucose and insulin levels which can lead to health problems, such as type II diabetes and cardiovascular diseases (Lehmann & Robin,

2007; Miao et al., 2015). Unlike RDS, digestion of SDS is slow in the small intestine, resulting in slow release of glucose into the blood stream (Zhang & Hamaker, 2009; Miao et al., 2015). Although RS escapes digestion in the small intestine, it may be fermented in the colon (Englyst et al., 1999). The health benefit of SDS and RS relates to postprandial glucose metabolism (Harbis et al., 2004, Ells et al., 2005), prevention of meal-associated hyperglycaemia leading to type II diabetes (Axelsen et al., 1999), and enhanced mental performance (Benton et al., 2003; Benton & Nabb, 2003). Moreover, fermentation of RS in the colon as dietary fibre helps to ensure gut health (Lockyer & Nugent, 2017). Resistant starch also contributes as a prebiotic helping to stimulate microorganisms that inhibit fat accumulation and bile stone formation (Ashwar et al., 2016).

Generally, potato starch is high in glycaemic index (GI) value than other foods (Kaur & Singh 2016). Glycaemic index is a measure of degree and rapidity of elevation of blood glucose levels after consumption of a particular food (Gagné, 2008). The consumption of potato by diabetic patients, therefore may be deleterious (Holt et al., 1995). Considerable differences occur in the GI values among potato cultivars. For example, a study on eight British potato cultivars divided them into high and medium GI cultivars, while low GI values were also reported by Ramdath et al. (2014) for purple potato varieties. Other factors which can alter the GI values of potato includes, maturity of potato tubers, cooking method, nature of the starch (especially the amount of amylopectin) extent of starch modification and cooling after cooking (Soh & Brand-Miller, 1999; Xavier & Sunyer, 2002; Fernandes et al., 2005; Henry et al., 2005; Singh et al., 2010). The likelihood of reducing GI of potato starch remains a topic for further research.

The demand for starch from non-food industries continues to increase (Bertoft, 2017). It was estimated that in 2015 about 85 million tons were extracted and used in industrial applications and the annual growth rate for starch production for this purpose is about 4% (http://www.starch.dk/isi/stat/rawmaterial.asp). Most starch used in industry is obtained from

maize seeds, though the demand for potato starch is increasing because of its high phosphate content (Blennow et al., 2000a), which helps it form stable, clear, gelatinous pastes that have elevated swelling power (Santelia & Zeeman, 2011). The diversity of both composition and physical parameters of starches from different botanical sources gives rise to their diverse physico-chemical properties and applications. This is true for both non-food sectors (such as sizing agents in textile and paper industry, adhesive gums, and biodegradable materials) and in food industries (particularly in bakery, thickening, confectionary and emulsification) (Slattery et al., 2000; Burrell, 2003; Mooney, 2009). Extracted starch often needs to be modified using costly and waste-generating chemicals, or by physical or enzymatic treatments. These enhance the required functional properties of the starches making them more amenable to industrial use. Several studies have shown that the structure and physico-chemical properties of starch can be affected by altering the activities of enzymes involved in starch metabolism through use of biotechnological tools (Lorberth et al., 1998; Lloyd et al., 1999; Schwall et al., 2000; Xu et al., 2017a; Xu et al., 2017b). This can make them usable by industries without prior modification and provides a route for the production of starches that are more environmentally friendly.

1.5 Starch structure

Starch is a polyglucan which is stored as granules of varying shapes and sizes dependant on the botanical source (Gallant et al., 1992). For example, starch granules of potato are oval or spherical, while those from maize are spherical or polygonal and from wheat are discoid and spherical. The diameter of starch granules ranges between less than 1 µm to 100 µm, and those of cereal seeds are generally smaller compared with starches isolated from cassava and potato (Vamadevan & Bertoft, 2015). Starch grains from most plants exhibit a unimodal size distribution, but starch from the endosperm of wheat, barley and rye exhibit a bimodal granule size distribution containing A (less than 10 µm diameter) and B (greater than 10 µm diameter) granules (He et al., 2012).

Starch granules are composed of two distinct polyglucans named amylose and amylopectin. The minority of the starch granule is amylose (normally about 30%) with the remainder being amylopectin (Wang et al., 2018). Amylose is a predominantly linear molecule (Figure 1) composed mainly of α-1,4-linked D-glucose monomers with occasional α-1,6-linked branchpoints, and it appears to make no contribution to the starch granule's structural organization (Koroteeva et al., 2007; Kozlov et al., 2007). Its average degree of polymerization (DP) ranges between 900 and 3300 with an average chain length of 270-525 glucosyl units (Vamadevan & Bertoft, 2015) and molecular weight of ~10^5-10^6 kDa. Amylopectin on the other hand is a highly branched molecule consisting of α1,4-linked D-glucose units with many (approximately 5% of glucose moieties) α-1,6-linked branchpoints. It consists of alternating crystalline (composed of glucan chain double helices formed from adjacent chains within a cluster) and amorphous lamellae (the branch-point zone linking two clusters), which are repeated at intervals of 9 nm along the molecule's axis (Jenkins et al., 1993) and make up part of the granule's matrix (Figure 1). The regularity of its structure means that amylopectin is responsible for the crystallinity observed in the starch granule. The average molecular weight (M_w) of amylopectin among various starch sources (for example from storage organs of maize, wheat, barley, tapioca, and potato) ranges between 10^7-10^8 kDa and contains an average DP between 4800 and 15,900 (Vamadevan & Bertoft, 2015).

Due to the high degree of α-1,6-linked branchpoints in amylopectin the average chain length is lower than in amylose, ranging between 18-27 glucosyl units (Hanashiro et al., 1996; Ao & Jane, 2007). The chains are mainly composed of two types: short chains consisting of 6 to 36 and long chains with more than 36 glucosyl units (Hanashiro et al., 1996; Jane et al., 1999; Bertoft et al., 2008; Gomand et al., 2010). Its chains are further classified based on (α-1,6)-linkages. Chains that do not carry other chains via (α-1,6)- linkages are referred to as A-chains, while those that carry other chains are known as B-chains (Peat et al., 1952). There is one C-

chain per amylopectin molecule that carries other chains, but it differs from B-chains as it contains the sole reducing group (Peat et al., 1952). All A-chains are external, meaning that they are wholly positioned outside the outermost branches, whereas all B-chains are either part of external chain or form the amylopectin's internal unit chain profile (Vamadevan & Bertoft, 2015).

Varying types of crystallinity has been detected in starch granules, depending on the arrangement of double helices that form between C chains, and these are usually termed A and B allomorphs (Figure 1). The A-type is commonly found in cereal starches, B-type crystalline allomorph is found in several starches from roots, rhizomes, and tubers and accommodates more water molecules in its lattice than A-type, while legume starches often contain a mixture of the two allomorphs and are referred to as C-type (Imberty & Perez, 1988; Buléon et al., 1998). The constitutive chains of amylopectin are longer in B-allomorphs than in A-allomorphs (Hizukuri, 1986). Branching points of B-allomorphs cluster in the amorphous areas while those in the A-allomorph are dispersed into amorphous and crystalline areas (Jane et al., 1997).

Apart from amylopectin and amylose, varying amounts of minor components such as phosphate monoester groups, proteins, and lipids (including phospholipids) are also present in starch granules. Although these minor components are present in low amounts, they can have a large influence on the physicochemical properties of starch (Muhrbeck & Eliasson, 1991). For example, the physical properties which makes potato starch useful in some industries are caused by an electrostatic charge conferred by covalently attached phosphate monoester groups. The extent of starch phosphorylation varies between plant organs and botanical sources. Phosphorylation of glucose residues is approximately 0.1% of the total in leaf starch and up to 0.5% in potato tubers. The phosphate content of potato starch is second only to *Curcuma zedoaria* (which contains about 1.5%), while starch phosphate in cereal endosperm is less than 0.01% (Tabata et al., 1975; Blennow et al., 1998; Blennow et al., 2000b; Kozlov et

al., 2007). These starch phosphate esters are linked to glucose monomers at either C-6 or C-3 positions (Hizukuri et al., 1970; Lim & Seib, 1993; Bay-Smidt et al., 1994; Blennow et al., 2000a; Kozlov et al., 2007), with the ratio of the distribution at C-6 and C-3 ranging from between 3:2 (Bay-Smidt et al., 1994) to 10:1 (Blennow et al., 1998).

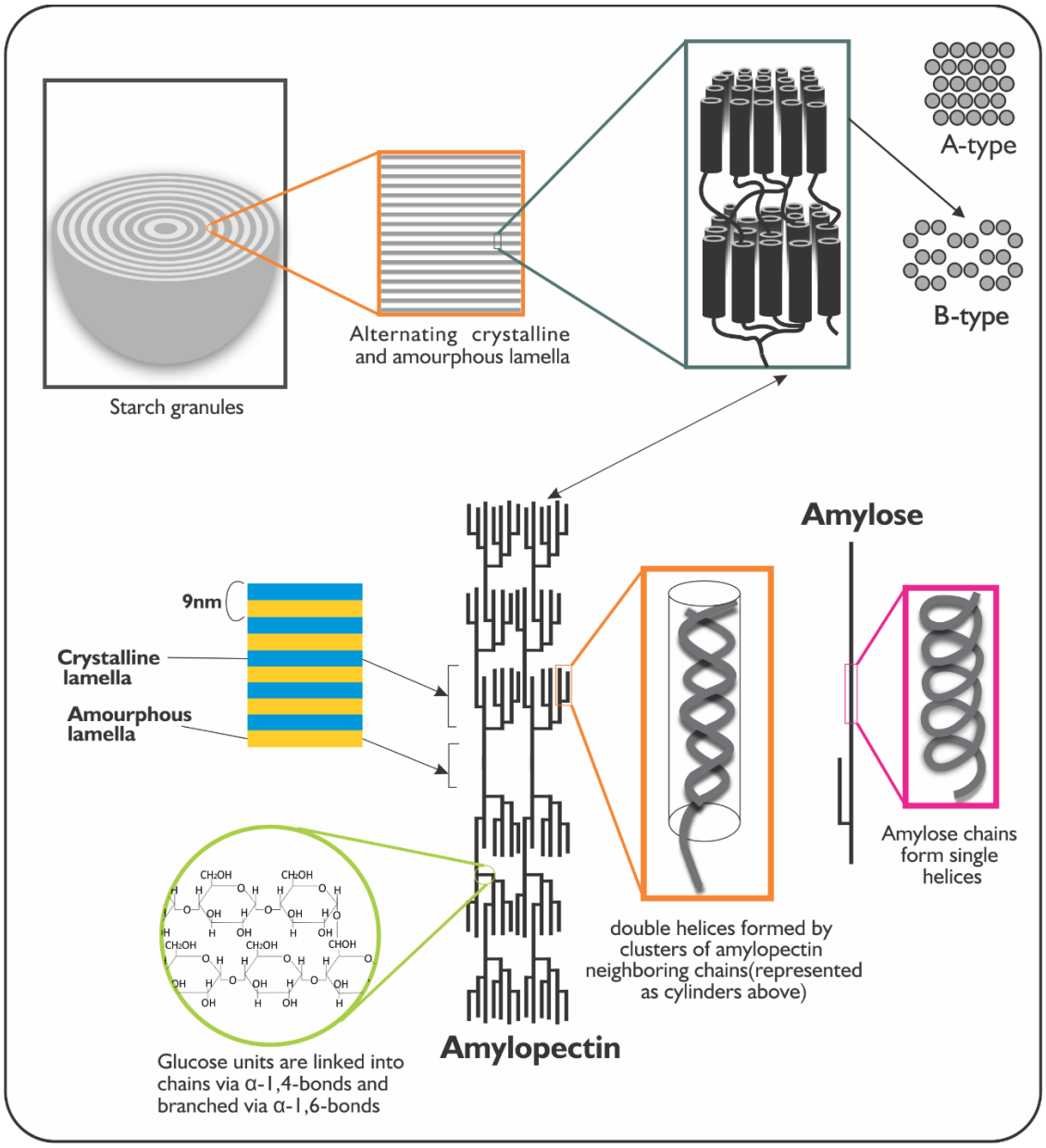

Figure 1: Structural composition of starch granules. (A) Growth rings within potato tuber starch following enzymatic digestion (B) plane view sketch of amylopectin structure showing allomorph types. (C) schematic representation of amylopectin and amylose structure. The chains in the amylose molecule are made up of about 1000 or more glucosyl residues in length. The short chains within the clusters of the amylopectin molecule are characteristically 12–20 glucosyl residues in length. Image was re-drawn from (Zeeman et al., 2010).

In native starches the phosphate residues are almost exclusively located in amylopectin (Samec, 1914) and are preferentially found on longer chains (Takeda and Hizukuri, 1982; Blennow et al., 1998), mostly sited more than nine glucosyl units away from the amylopectin branch points. Various studies conducted on potato starch have shown that the concentration of phosphate monoesters is greatest at the core (hilum) of the granule (Jane & Shen, 1993; Blennow et al., 2005a) and phosphate content is higher in smaller granules than larger ones (Jane & Shen, 1993; Nielsen et al., 1994), but the extent of starch phosphorylation is not contingent on tuber size (Nielsen et al., 1994). The enzyme which catalyses starch phosphorylation was identified by Lorberth et al. (1998) and will be discussed in more detail below. Various techniques have been deployed to manipulate this enzyme which have increased our understanding on the key roles of phosphate groups in determining starch structure. For instance, starch phosphorylation may play a significant role in ensuring normal packing of starch granules because disruption of granule matrix in potato starches containing very low phosphate content was observed by (Xu et al., 2017a). They proposed that phosphate esters may operate as identifiers to differentiate amylose chains from amylopectin chains and serve to inhibit erroneous interactions between segments of these two macromolecules.

1.6 Starch biosynthesis

The complex structure of the starch molecule described above indicates that its synthesis will rely on a coordinated process requiring several enzyme classes. It is believed that these enzymes carry out their functions simultaneously and their activities are interdependent. Most of these enzyme classes are composed of multiple isoforms that play specific roles in polymer synthesis (Nakamura et al., 1995; Patron, et al., 2002; Seung et al., 2015), allowing starch granule initiation, the arrangement of varying chain length in amylopectin (Tetlow & Emes, 2011), phosphorylation of glucosyl residues (Ritte et al., 2002; Baunsgaard et al., 2005; Kötting

et al., 2005; Lütken et al., 2010; Lloyd & Kötting, 2016), and regulation of granule morphology (Szydlowski et al., 2009; Crumpton-Taylor et al., 2013).

Even though the steps involved in starch biosynthesis are largely similar in all plants, the granules produced still exhibit structural diversity and variable numbers, and the reasons for this are unclear (Sonnewald & Kossmann, 2013; Lloyd & Kötting, 2016). Most work on starch metabolism has occurred in the model plant *Arabidopsis thaliana*, with significant work having also occurred in potato, cereals and the green alga, *Chlamydomonas reinhardtii*. I will concentrate on outlining the generally accepted pathways of synthesis and degradation identified in Arabidopsis and compare them to other species where appropriate. As my project examines starch metabolism in potato, I will also concentrate on knowledge about that plant.

1.6.1 ADP-glucose biosynthesis

The substrate for amylose and amylopectin biosynthesis in higher plants is the sugar nucleotide adenosine diphosphate glucose (ADP-glucose) which is synthesised from a reaction utilising glucose 1-phosphate (G1P) and adenosine triphosphate (ATP) (Zeeman et al., 2010). The supply of substrate for ADP-glucose synthesis depends on whether the pathway is present in photosynthetic or non-photosynthetic tissue. In leaves (Figure 2) the G1P is supplied directly from the Calvin-Benson-cycle (Bahaji et al., 2014) and in *A. thaliana* leaves up to 50 percent of photoassimilates are used for starch synthesis (Stitt & Zeeman, 2012). In potato tubers the G1P comes from sucrose that is imported from leaves and is cleaved into UDP-glucose and fructose by sucrose synthase (SuSy) in the cytosol (Zrenner et al., 1995). The UDP-glucose is used to synthesize G1P by UDP-glucose pyrophosphorylase (UGPase), followed by glucose 6-phosphate (G6P) formation by a cytosolic phosphoglucomutase (PGM) isoform (Figure 3). A glucose 6-phosphate/phosphate translocator (GPT) facilitates entry of G6P into the amyloplast (Zhang et al., 2008a; Kunz et al., 2010; Jonik et al., 2012) where plastidial PGM converts G6P into G1P (Tauberger et al., 2000). Synthesis of ADP-glucose alongside liberation of pyrophosphate

(PP_i) in both tissues is catalysed by ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27). Although the AGPase reaction is reversible the PP_i becomes quickly hydrolysed to inorganic phosphate by a plastidial alkaline inorganic pyrophosphatase (Gross & Ap Rees, 1986; George et al. 2010) making it irreversible *in vivo*.

In dicotyledonous plants AGPase is localised in the plastid where it is allosterically activated by 3-phosphoglycerate and inhibited by P_i, both of which contribute to the regulation of the rate of starch synthesis. It is a heterotetrameric enzyme consists of two small and two large subunits (Preiss, 1991; Smith-White & Preiss, 1992; Ballicora et al., 2003). While the small subunits contain a catalytic site, the large subunits are generally thought to have a regulatory function and modulate the small subunits sensitivity to allosteric effectors (Ballicora et al., 1998). A study by Hwang et al., (2007), however, demonstrated that although the large subunits lack catalytic activity a specific amino acid within their N-terminal region is essential for net catalysis of the heterotetrameric enzyme.

In potato tubers the large subunit modulates the affinity of the small subunit for the allosteric regulators 3PGA and P_i (Iglesias et al., 1993; Ballicora et al., 1995). Small subunit encoding genes are often expressed constitutively, whereas the expression of large subunit encoding genes may be distributed across different tissues (Crevillén et al., 2003, 2005), indicating that different large subunits might modulate the AGPase to possess allosteric properties specific for the needs of different tissues. Redox regulation of AGPase activity occurs through the reduction of an intermolecular disulphide bridge that forms between cysteine residues of the small subunits (Fu et al., 1998; Tiessen et al., 2002; Hädrich et al., 2012). The combination of these two regulatory mechanisms enables starch synthesis only when enough substrates are present.

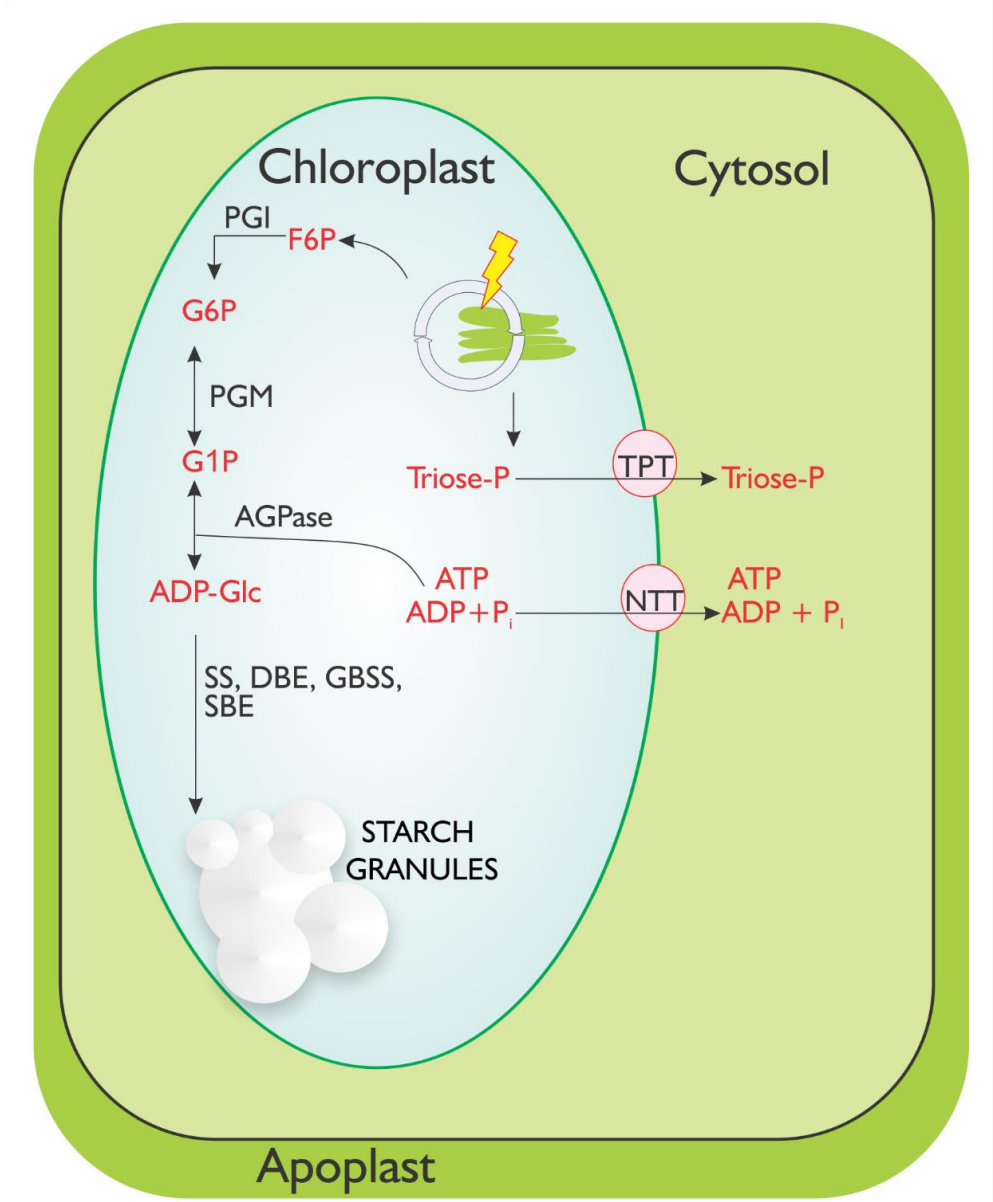

Figure 2: Pathway of starch synthesis in potato leaves as proposed by Van Harsselaar et al. (2017). Leaf photoassimilates in the form of fructose-6-phosphate (F6P), are produced in the Calvin-Benson-Cycle. F6P in two subsequent reactions which are catalysed by phosphoglucoisomerase (PGI) and phosphoglucomutase (PGM) respectively, are converted to glucose 1-phosphate (G1P). G1P is used as substrate in a reaction catalysed by ADP-glucose pyrophosphorylase (AGPase) to produce ADP-Glucose which is used to form the starch polymer by a combination of granule bound starch synthase (GBSS); starch synthase isoforms (SS); starch branching enzyme (SBE); debranching enzyme (DBE).

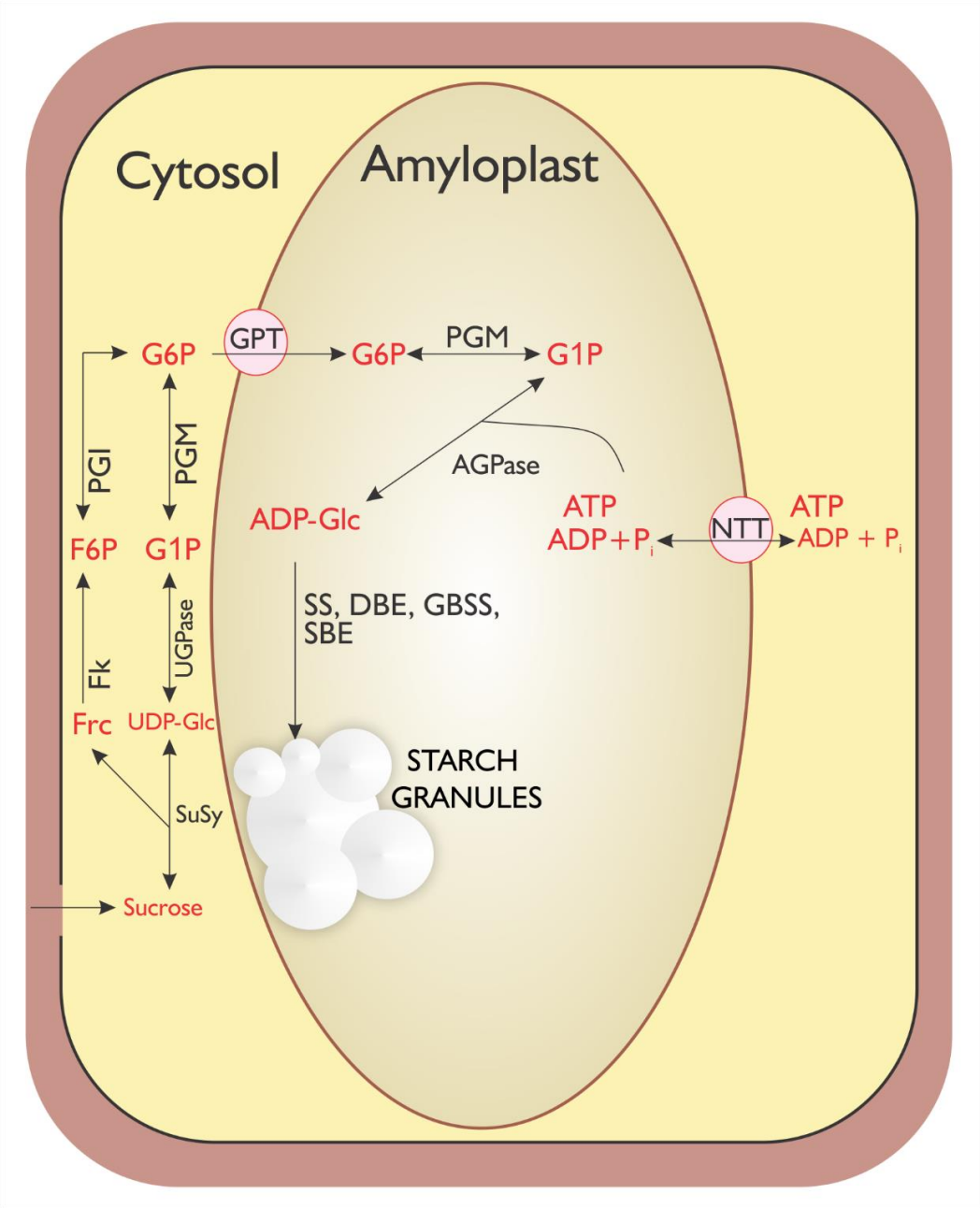

Figure 3: Pathway of starch synthesis in storage organs. Sucrose synthase (SuSy) cleaves sucrose into UDP-glucose and fructose. UDP-glucose is used to form glucose 1-phosphate (G1P) by UDP-glucose pyrophosphorylase (UGPase). The G1P formed is then converted to glucose-6-phosphate and imported into the amyloplast. Glucose-6-phosphate then reconverted to into G1P in the amyloplast by the catalytic activity of plastidal PGM, the G1P can now serve as the substrate for the synthesis of starch. Nucleotide translocator; (NTT), fructokinase; (Fk), triose-phosphate; (TP), inorganic pyrophosphate; (PP_i), inorganic phosphate; (P_i).

1.6.2 Elongation of the glucan chain

The glucosyl moieties of ADP-glucose are transferred and elongated successively to the non-reducing end of existing glucosyl chains (Figure 4) to form linear α1,4 polyglucan chains (Martin & Smith, 1995; Smith et al., 1997). The class of enzyme involved in this reaction is starch synthase ((SS); EC 2.4.1.21) and they can be further subdivided into multiple isoforms. Synthesis of amylose is catalysed by granule bound starch synthase (GBSS), which is exclusively found bound to starch granules (Zeeman et al., 2010). In the stroma many soluble SSs are found, but the number depends greatly on the plant species. Their roles have been best characterised in Arabidopsis leaves where three of these SS isoforms (SSI; SSII and SSIII) are involved in amylopectin synthesis (Delvallé et al., 2005; Zhang et al., 2008b). Two other isoforms SSIV (Roldán et al., 2007; Crumpton-Taylor et al., 2013) and SSV (Abt et al., 2020) are non-catalytic and combine with SSIII in starch granule initiation. A species-specific isoform of SS called SS6 has been identified in potato (Helle et al., 2018), but its function in starch biosynthesis is yet to be characterised.

Mutations that eliminate GBSS production are often referred to as *waxy*. Such mutants synthesis starch where amylose is decreased or eliminated and have been reported in many species including rice (Wang et al., 1995), maize (Sprague et al., 1943), wheat (Nakamura et al., 1995), barley (Patron et al., 2002), potato (Jacobsen et al., 1989) and Arabidopsis (Seung et al., 2015, 2020a). Suppression of GBSS activity in transgenic cassava (Raemakers et al., 2005; Koehorst-van Putten et al., 2012) and potato (Visser et al., 1991; Kuipers et al., 1994; Tatge et al., 1999) similarly induces a drastic reduction in the amount of amylose. It is thought that amylose synthesis occurs inside the granular matrix rather than at the surface of the granule (Denyer et al., 1993; Mu-Forster et al., 1996; Tatge et al., 1999; Grimaud et al., 2008), which is why GBSS becomes entrapped in the granule.

Each soluble SS isoform appears to play distinct role in amylopectin biosynthesis as demonstrated by the mutations which eliminate them. SSI is responsible for elongating chains up to DP7 as amylopectin in Arabidopsis *ssi* mutants contains lower numbers of chains between DP7 to 12 and increased numbers between from DP14 to 23 (Delvallé et al., 2005; Pfister et al., 2014). Characterization of starch from plants with SSII deficiency in Arabidopsis mutants (Zhang et al., 2008b; Szydlowski et al., 2011; Pfister et al., 2014) and potato transgenics (Lloyd et al. 1999) shows amylopectin containing increased chains with a DP lower than 15 and decrease in abundance of chains between DP15 and 80. This indicates that SSII elongates chains of between DP14-30. Arabidopsis *ssiii* mutant lines had very long chains estimated to be greater than DP60 (Zhang et al., 2005), whereas transgenic potato tubers repressed in SSIII activity (Lloyd, et al., 1999) had fewer long chains of between 36 to 50 DP. Studies on other SSIII mutants in maize (Inouchi et al., 1987; Wang et al., 1993), rice (Fujita et al., 2007) endosperm are more similar to the observation in the transgenic potato, which may indicate a difference in the role of this enzyme in amylopectin synthesis in leaf and storage tissues. Overall these data suggest that SSI and SSII tend to produce short A- and B glucan chains while longer chains are attributed to SSIII (Tetlow & Emes, 2011).

Interestingly Arabidopsis *ssii* and *ssiii* double mutants and potato transgenic lines lacking SSII and SSIII activities demonstrated alterations in amylopectin structure that cannot be explained simply by the combined reductions in their activities (Edwards et al., 1999; Lloyd et al., 1999; Zhang et al., 2008b). It was suggested that the two isoforms produce structures that other isoforms rely on for activity, and so simultaneous repression of the two isoforms disrupts normal synthesis of amylopectin. This indicates that starch synthesising enzymes rely on each other's activities to help synthesise the complex structure within the starch granule.

1.6.3 The roles of branching enzymes in amylopectin synthesis

The formation of α-(1,6)-linkages is catalysed by starch branching enzyme (SBE; EC 2.4.1.18). This non-reversible reaction involves cleavage of α-(1,4)-linkage within a chain and formation of α-(1,6)-linkage between the reducing end of the severed glucan chain and a C-6 linked oxygen of a flanking chain (Figure 4) resulting in the creation of additional substrates for SSs (Pfister & Zeeman, 2016). Multiple isoforms of SBE from various botanical sources are broadly classified into A-family and B-family based on the relatedness of their amino acid sequences (Burton et al., 1995) and enzymatic properties (Sawada et al., 2009). For example pea SBEI, maize SBEII and potato SBEII belong to the A-family while rice SBEI, maize SBEI, pea SBEII and potato SBEI belong to the B-family (Vos-Scheperkeuter et al., 1989; Koßmann et al., 1991; Burton et al., 1995; Larsson et al., 1996; Safford et al., 1998; Jobling et al., 1999). In Arabidopsis two isoforms of SBE (SBEII and SBEIII) belong to the B-family (Fisher et al., 1996), but this plant also contains a third isoform (SBEI) which has been placed in a third clade alongside SBEIII from rice, maize and poplar (Han et al., 2007; Yan et al., 2009).

Expression patterns vary between *SBE* genes depending on developmental and environmental factors. In Arabidopsis *SBEI* expression occurs in most tissues but was particularly high in inflorescences and flowers suggesting that the expression is triggered during embryogenesis (Wang et al., 2010). On the other hand, transcript of *SBEII* increases when illuminated and decreases in the dark, but *SBEIII* expression does not seem to be affected by either light or dark (Khoshnoodi et al., 1998). Transcripts of both *SBEII* and *SBEIII* accumulated when plants were incubated in combinations of light and exogenously supplied glucose, fructose, or sucrose. Similarly *SBEI* expression was induced in potato leaves incubated with sucrose (Koßmann et al., 1991). This implies that the expression of the two genes in Arabidopsis as well as *SBEI* in potato are triggered by carbohydrate signals. Additionally the expression of *SBEI* in potato was

abundant in the tuber whereas expression of *SBEII* is greatest in the leaves indicating that *SBE* genes expression is organ dependent (Jobling et al., 1999).

There is good evidence that the SBE isoforms play distinct roles in starch synthesis. The branching pattern of amylopectin is known to influence starch structure and function and so knowledge about the distribution of branches by SBE isoforms is essential to help determine the relationship between amylopectin structure and physical properties. Studies conducted on various plants have revealed members of the B-family have a propensity to transfer longer chains than those within the A-family (Mizuno et al., 1993; Guan et al., 1997; Nozaki et al., 2001; Rydberg et al., 2001; Nakamura et al., 2010). In potato SBEI (member of the B-family) was shown to be generally more active towards long linear substrate (such as amylose) whereas SBEII (member of the A-family) typically prefer branched substrates (such as amylopectin) (Rydberg et al., 2001).

Mutation in either *SBEII* or SBE*III* in Arabidopsis did not lead to marked alteration in the chain length distribution of amylopectin whereas mutations of both SBE*II* and SBE*III* caused elimination of starch accumulation coupled with the presence of high amounts of maltose which is formed during starch degradation (Dumez et al., 2006). It was hypothesised that in these plants α1,4 glucans are synthesised by starch synthases, but the lack of branching enzyme activity means that starch cannot form, and they become rapidly degraded to maltose.

In potato repression of SBEI activity leads to no increase in amylose and minor alterations in starch structure (Safford et al., 1998; Tuncel et al., 2019; Zhao et al., 2021), but decreases in SBEII activity leads to an increase of amylose from 30 to 40% and an increase in small starch granules (Jobling et al., 1999). Transgenic repression of activities of both SBEI and SBEII leads to large increases in amylose in potato (Hofvander et al., 2004; Blennow et al., 2005b;

Andersson et al., 2006) and in plants where both *StSBEI* and *StSBEII* are mutated only amylose accumulates (Tuncel et al., 2019; Zhao et al., 2021).

The elimination of starch synthesis in Arabidopsis *sbeii* and *sbeiii* double mutants (Dumez et al., 2006) points to a function of SBE in the formation of semi-crystalline structures within starch granules and indicates that functional redundancy exists between *SBEII* and *SBEIII* in leaves of this plant. In potato at least one allele of the SBEII isoform is enough for the formation of normal starch structure because significant alteration in the amylose fraction was only noticed when indels were created in all alleles of both *sbei* and s*beii* genes (Zhao et al., 2021).

Reduction in SBE activity also leads to phenotypes unrelated to the alterations in amylopectin branching. In potato for example, starch phosphate becomes increased when either *SBEI* or *SBEII* is repressed and this is greater in lines where both isoforms are repressed than when either is repressed alone (Safford et al., 1998; Jobling et al., 1999; Schwall et al., 2000). The mechanism leading to this increase is still poorly understood.

1.6.4 The roles of debranching enzymes in amylopectin synthesis

Formation of the cluster structure of amylopectin is dependent on the activity of another class of hydrolytic enzymes called debranching enzymes (DBEs). In plants DBEs exist as either isoamylase ((ISA); EC 3.2.1.68) or limit dextrinase ((LDA); EC 3.2.1.142 also known as pullulanase (PUL)) types (Manners, 1971; Doehlert & Knutson, 1991). Both can hydrolyse α-1-6 linkages directly, but they differ in their activities toward specific glucan polymers. The genome of Arabidopsis encodes three isoamylase (ISA1-ISA3) type polypeptides and one limit dextrinase. While ISAs have little effect on degrading pullulan *in vitro*, they can hydrolyze branched linkages in amylopectin and glycogen. Interestingly, although both ISA1 and ISA3 are catalytically active, ISA2 is not (Hussain et al., 2003).

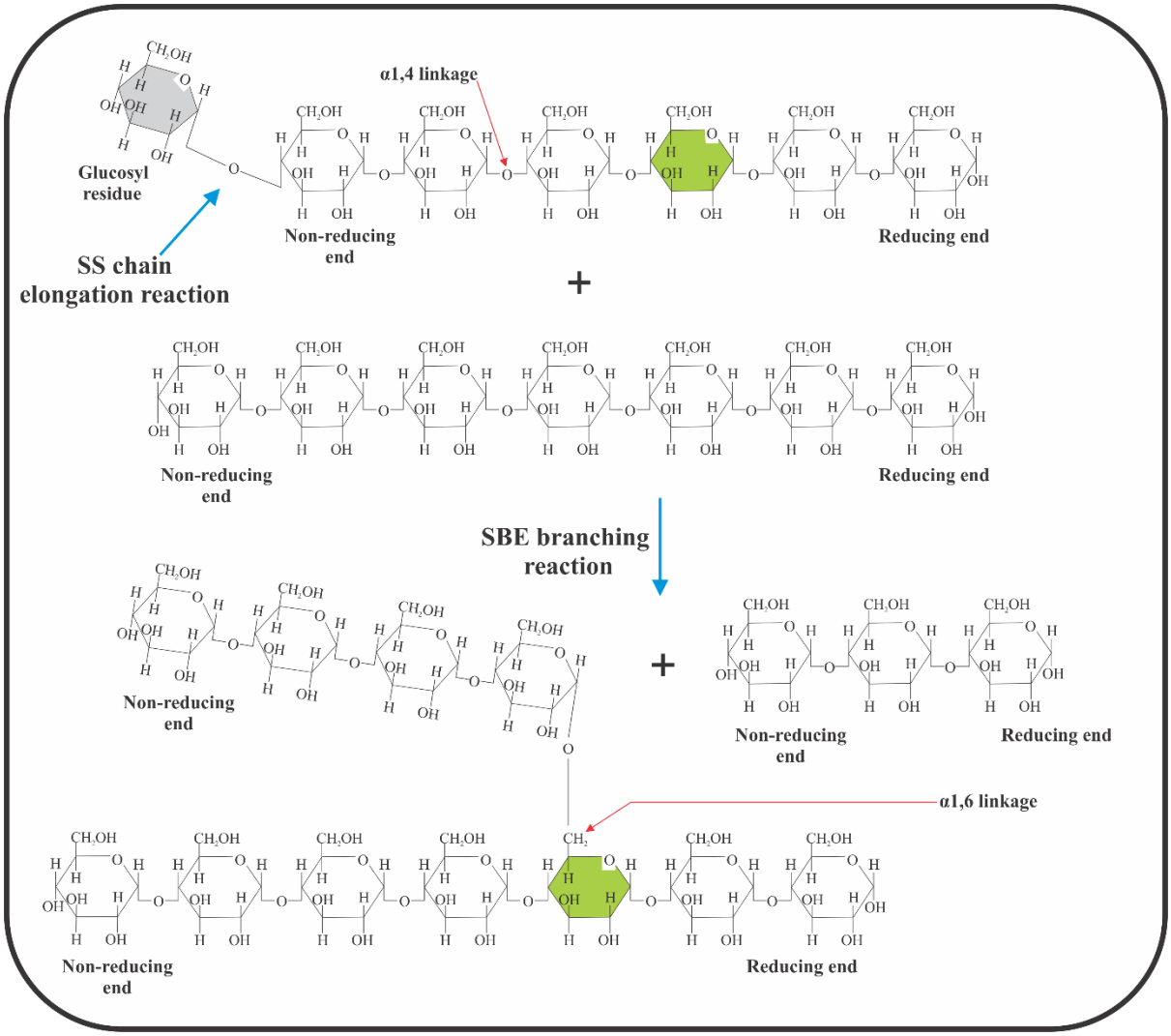

Figure 4: Development of starch polymers by addition of glucosyl residue of ADP-glucose to the non-reducing end of chain through an α1,4 linkage and reaction is catalysed by Starch synthase (SS). Starch-branching enzyme (SBE) cleaves portions of chains from the non-reducing end and join them to the side of contiguous or the same chain by α1,6 linkage. The glucosyl residue used to elongate the developing chain is shaded gray and the residue that accept cleaved chains for the synthesis of the α1,6 glucosidic (branch) linkage is shaded green.

On the other hand LDA can cleave the α-1,6-linkages of polyglucans in pullulan, but seldomly in amylopectin and glycogen (Nakamura et al., 1996; Zeeman et al., 2010).

Mutant and transgenic plants lacking either ISA1 or ISA2 activity have been shown to lead to accumulation of both starch and a structurally related polyglucan called phytoglycogen (Pan & Nelson, 1984; Matuo et al., 1987; Zeeman et al., 1998; Bustos et al., 2004; Delatte et al., 2005;

Wattebled et al., 2005; Ferreira et al., 2017). Like amylopectin this contains short α1,4 glucan chains linked by α1,6 branchpoints, but is more highly branched. The current model for phytoglycogen synthesis assumes that the molecule is formed by the combined actions of SS and SBE which then become trimmed to amylopectin by ISA1 and ISA2 (Ball et al., 1996; Dauvillée et al., 2001; Myers et al., 2000). This process is thought to be universal in plants as such mutants have been found to have the same effect in both green algae (Mouille et al., 1996) and a Cyanobacterium (Cenci et al., 2013), and it was shown also to be essential for the formation of starch like molecules when starch synthesis was recreated in yeast (Pfister et al., 2016). Isoamylase 3 appears uninvolved in this process but is likely involved in starch degradation (Dinges et al., 2003; Hussain et al., 2003; Delatte et al., 2006) that will be discussed in a later section. Limit dextrinase may also be involved in phytoglycogen formation as amounts of that polyglucan increased in maize endosperm when limit dextrinase was mutated alongside isoamylase (Dinges et al., 2003).

In Arabidopsis and potato it has been demonstrated that ISA1 and ISA2 function as a heterocomplex (Bustos et al., 2004; Delatte et al., 2005) and neither of the two polypeptides is stable without the other isoform (Bustos et al., 2004; Delatte et al., 2005; Streb et al., 2008; Wattebled et al., 2005, 2008). However in rice and maize the situation is somewhat different as ISA1 not only forms heteromultimeric complex with ISA 2, but it also forms homomeric complex with itself (Utsumi & Nakamura, 2006; Kubo et al., 2010). This depends on tissue and plant species. Rice leaves only contains the ISA1 and ISA2 heteromultimeric complex, while endosperm contains both (Utsumi et al., 2011). On the other hand, maize leaves and endosperm contain both type of complexes (Kubo et al. 2010; Lin et al. 2013). Mutation analysis in rice has shown that the ISA1 homocomplex is sufficient to remove excess branchpoints in endosperm tissue as *osisa2* mutants have little effect on starch amount or structure. In maize on the other hand, mutations in *ISA2* do not affect starch in endosperm

(Kubo et al., 2010) but leads to some alterations in starch content and granule morphology in leaves (Lin et al., 2013). Similar to Arabidopsis, mutations in *ISA1* lead to phytoglycogen accumulation in maize and rice endosperm (Kubo et al., 2010; Takahashi et al., 2019).

1.6.5 Structural relationship between starch branching enzyme and debranching enzyme

Analysis of the primary sequence of starch branching enzyme and debranching enzymes shows that they belong to α-amylase enzyme superfamily, termed glycoside hydrolase family 13 (GH13) (Tetlow & Emes, 2014). Member of this superfamily act on α-glucoside linkages of starch but they catalyse a range of reactions including hydrolysis, condensation, transglycosylation and cyclization (Ali et al., 2006). All enzymes belonging to GH13 superfamily share highly symmetrical $(\beta/\alpha)_8$ barrel catalytic domain (Svensson, 1994) including members which lack catalytic activity (Fort et al., 2007). They are further subdivided into 42 subfamilies, based on their specificity and diversity of their ancillary domains (Plaza-Vinuesa et al., 2019). Within the GH13 superfamily isoamylases and starch branching enzymes share a similar domain architecture (Tetlow and Emes, 2014). Both enzyme types are composed of three domains (Figure 5); a NH_2-terminal domain, a catalytic domain and a C-terminal domain (MacGregor et al., 2001; Abad et al., 2002).

1.7 Starch phosphorylation

The phosphate within amylopectin is incorporated by glucan, water dikinases (GWD). Three isoforms of this enzyme have been identified in Arabidopsis namely, GWD1 (or SEX1; E.C. 2.7.9.4), GWD2 and GWD3 (also known as phosphoglucan, water dikinase (PWD); E.C.2.7.9.5). Genes encoding these enzymes are present in many plants including red algae (Mdodana et al. 2019) although their functions have not been assessed outside off angiosperms and in one bryophyte.

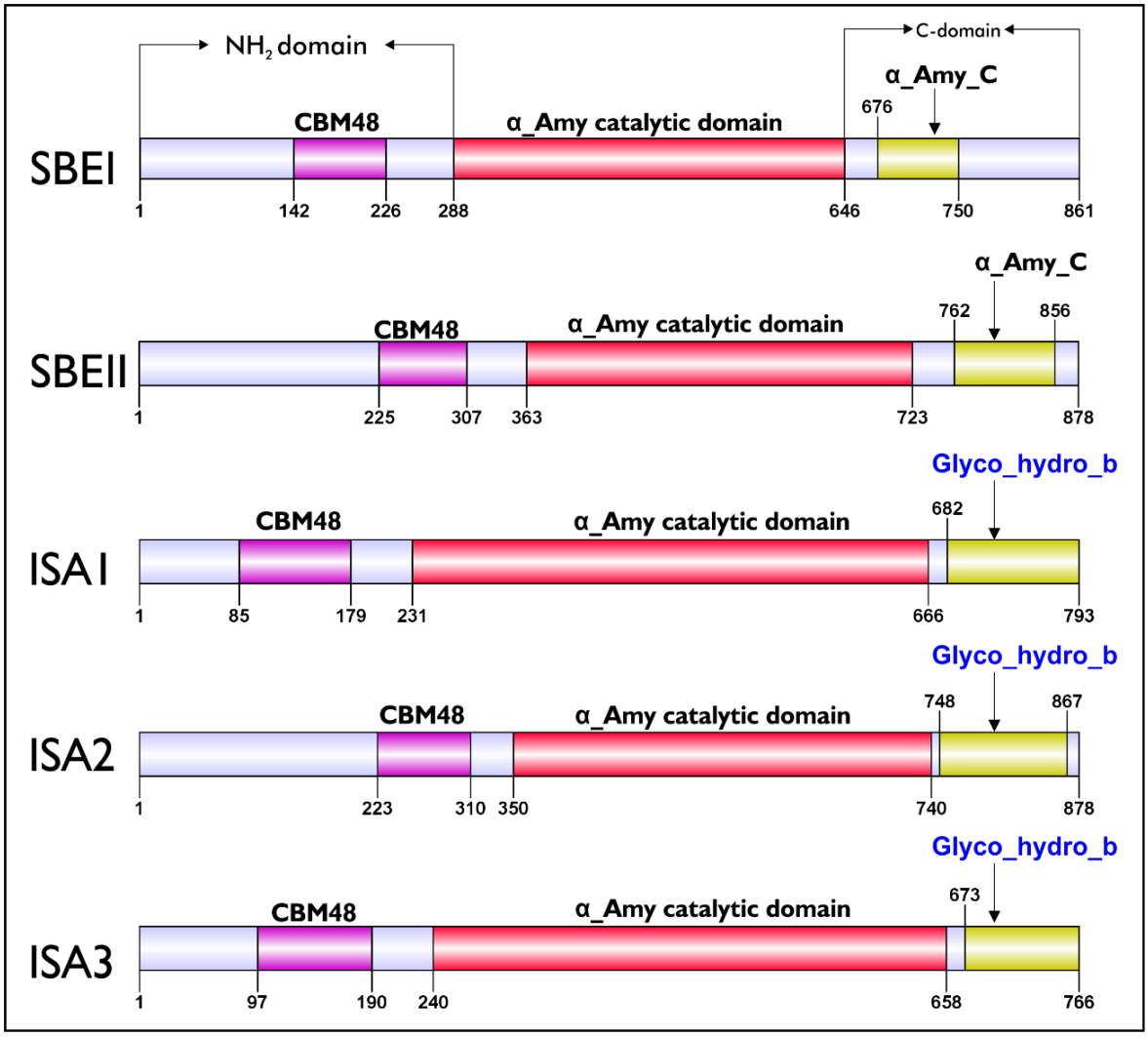

Figure 5: Illustration of structural architecture between starch branching enzyme (SBE) and isoamylase (ISA) isoforms of potato. Compositions and distributions of domain structures are marked and annotated in distinct colours. CBM, carbohydrate binding module family 48. The text in blue is uncharacterized domain but found in InterPro database to be homologous to other protein in GH superfamily. Structural architecture was reproduced with DOG (Ren et al., 2009), according to the domain classification by InterProScan (www.ebi.ac.uk). The number indicate the amino acid position of the polypeptide of respective enzyme.

In angiosperms GWD1 and GWD3 localise to the plastid and incorporate phosphate into the amylopectin fraction of starch (Baunsgaard et al., 2005; Kötting et al., 2005; Mikkelsen et al., 2006). It is unclear what the role of GWD2 is as it is cytosolic and so cannot be involved in starch metabolism, but Arabidopsis mutants lacking GWD2 were affected in growth and seed development (Pirone et al. 2017).

The biochemical mechanism of starch phosphorylation by GWD was described by Ritte et al. (2002; 2006) and involves the transfer of β-phosphate of ATP to either the C-6 or C-3 position of glucosyl residues within amylopectin, with the α-phosphate being transferred to water. Glucan, water dikinase 1 and GWD3 phosphorylate the C-6 and C-3 positions on the glucan chain respectively (Ritte et al., 2002; Baunsgaard et al., 2005; Kötting et al., 2005; Lloyd & Kötting, 2016) and phosphorylation of C-6 position by GWD1 is essential for the catalytic activity of GWD3 (Baunsgaard et al., 2005; Kötting et al., 2005). Glucan water dikinase 1 exhibits reversible binding to starch granules at the beginning of the night (Ritte et al., 2000) and partitioning of GWD between its soluble and granule-bound forms has been shown to be redox regulated (Mikkelsen et al., 2005). Interestingly plants lacking GWD1 or GWD3 isoforms are affected in starch degradation, and it has been suggested that starch phosphorylation initiates transitory starch degradation (Mikkelsen et al., 2005).

1.8 Starch degradation

Research into the catabolism of starch has made rapid progress over the past two decades (Smith et al., 2005; Fettke et al., 2009, Smith and Zeeman, 2020) due to its relevance in several important processes. For example, it has been shown that leaf starch catabolism is important in determining plant yield as mutants unable to degrade starch efficiently are often smaller that wild type. In Arabidopsis this is thought to lead to decreased soluble sugars at night leading to a starvation response that inhibits gibberellic acid synthesis (Stitt & Zeeman, 2012; Paparelli et al., 2013). Starch degradation is also involved in several important industrial processes. One example is a specific issue in potato which occurs when tubers are stored at temperatures below 6ºC to elongate sprout dormancy (Wu et al., 2011). Such cold-stored tubers degrade starch and accumulate reducing sugars (Schippers, 1975; Ewing et al., 1981), a phenomenon called cold induced sweetening (CIS) first described by Müller-Thurgau (1882). These reducing sugar react with free amino acids during frying (Shallenberger et al., 1959) to produce an

unacceptable dark coloured products during potato chips or French fries' production and accumulation of the toxin acrylamide (Kumar et al., 2004).

As was stated above evidence suggests that starch degradation in leaves is initiated by reversible phosphorylation of glucan at the starch granule surface by GWD isoforms (Yu et al., 2001; Ritte et al., 2004; Edner et al., 2007). Starch phosphorylation is thought to diminish crystallinity within amylopectin by disrupting the packing of its double helices and allowing access to hydrolytic enzymes (Edner et al., 2007; Hejazi et al., 2008, 2009; Blennow & Engelsen, 2010). β-amylases, ISA3 and AMY3 are the main exo-acting enzymes that release maltose, maltotriose and phosphorylated malto-oligosaccharides (MOS) from the starch granule (Niittylä et al., 2004; Zeeman et al., 2010; Thalmann et al., 2019). The removal of phosphate groups from these is necessary for the efficient digestion of starch molecules, because β-amylases are unable to act on a glucan chain carrying a phosphate group adjacent to the nonreducing end (Takeda & Hizukuri, 1982; Kötting et al., 2005) and this is achieved by two glucan phosphatases known as SEX4 and Like-Sex Four 2 (LSF2) (Gentry et al., 2007; Kötting et al., 2009; Hejazi et al., 2010; Santelia et al., 2011).

The importance of SEX4 in starch degradation is shown by accumulation of phospho-oligosaccharides and an impairment of starch degradation in Arabidopsis *sex4* mutants (Kötting et al., 2009). *lsf2* mutants are unaltered compared with the wild type in terms of starch degradation, but *sex4/lsf2* double mutants show a more severe phenotype in this regard than the *sex4* single mutant (Santelia et al., 2011), indicating some redundancy between the two proteins. Repression of *SEX4* or *LSF2* in transgenic potatoes affected starch degradation in leaves indicating that LSF2 plays a bigger role in leaf starch degradation in potato than in Arabidopsis, however, suppression of neither SEX4 nor LSF2 affected soluble sugar accumulation from starch degradation in cold-stored tubers (Samodien et al., 2018).

Unphosphorylated MOS can be mobilised to maltose by β-amylases and ISA3. They are also metabolized in the plastid by disproportionating enzyme 1 (DPE1) to form glucose and maltose (Critchley et al., 2001), which are transported to the cytosol by separate transporters (Figure 6). Knockout mutants lacking the glucose transporter (pGlcT) did not show any visible phenotype but *mex1* mutants lacking the maltose exporter accumulate a high level of both starch and maltose in leaves and show reduced growth (Niittylä et al., 2004). Arabidopsis *pglct-1/mex1* double mutants show greater growth retardation than *mex1* single mutants (Cho et al., 2011) demonstrating that the two plastidial sugar transporters contribute jointly to starch mobilization and are that this process is important for plant growth.

In the cytosol, maltose is converted to glucose through a transglucosylation reaction catalyzed by disproportionating enzyme 2 (DPE2) which releases one of the glucosyl residues as free glucose and transfers the other to a heteroglycan (Figure 6). Arabidopsis *dpe2* mutants and potato transgenics lacking DPE2 accumulate large amounts of both maltose and starch (Chia et al., 2004; Lloyd et al., 2004; Lu et al., 2006), while transgenic potato lines with repression of both DPE1 and DPE2 activities accumulated large amounts of starch, maltose and longer chain malto-oligosaccharides (Lütken et al., 2010). Those plants also demonstrate chlorotic leaves and growth retardation similar to the Arabidopsis *mex1/pGlcT* double mutant (Cho et al., 2011). The above description involves degradation by only hydrolytic enzymes, but a plastidial starch phosphorylases also exists in plants. Degradation by this enzyme seems unimportant in *A. thaliana* except under abiotic stress conditions (Niittylä et al., 2004), although recent evidence indicates a key role for this enzyme in starch degradation in CAM plant leaves (Ceusters et al., 2021).

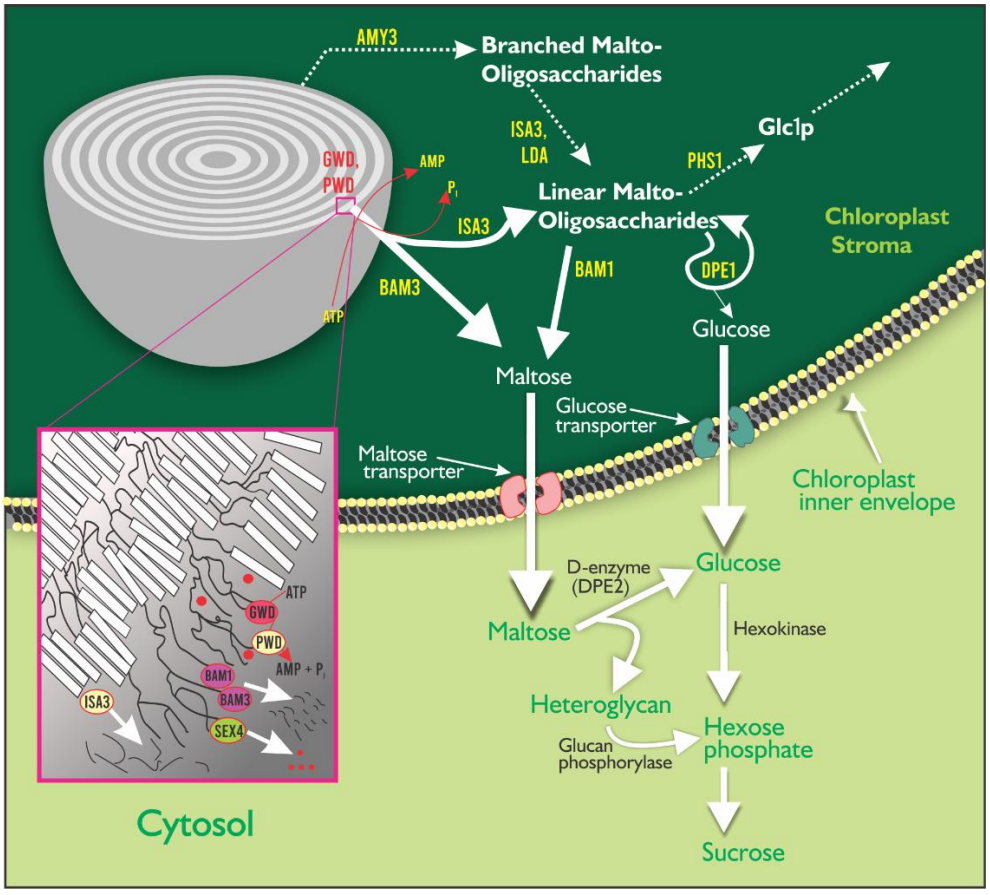

Figure 6: The pathway of starch degradation in transitory starch of Arabidopsis leaves at night. Degradation is initiated by phosphorylation of amylopectin in a reaction catalysed by glucan, water dikinase GWD. Dephosphorylation of glucan chain and phosphorylated MOS is catalysed by SEX4 and LSF2. Dephosphorylated amylopectin on the granule surface is debranched mainly by isoamylase 3 (ISA3), resulting in the formation of linear glucans which are subsequently metabolised by β-amylase (BAM1 & BAM3) to release maltose as the main product and malto-oligosaccharides (maltotriose) as minor product. Branched glucan may also be release from the granule surface by α-amylase and debranched by ISA3 and limited dextrinase (LDA). Dashed arrows indicated minor pathways in Arabidopsis because it is unimportant in starch degradation.

Inset is a model illustrating the role of phosphorylation in starch by GWD and PWD in disordering the packing of amylopectin double helices (white boxes). Phosphate (*red dots*) is concurrently released by Starch Excess 4 (SEX4) to permit full degradation. This permits the access by ISA3, BAM1 and BAM3 which releases maltose and maltotriose (*black lines*) by ISA3, BAM1 and BAM3. Adapted from Lloyd and Kötting (2016); Zeeman et al., 2010. GWD, glucan, water dikinase; PWD, phosphoglucan, water dikinase; BAM, beta-amylase; AMY, alpha-amylase; ISA3, Isoamylase; LDA, Limit dextrinase; SEX4, starch excess 4; LSF, Like starch-excess Four; DPE, disproportionating enzyme; PHS1, plastidial phosphorylase; MEX, maltose transporter; Glucose transporter.

1.9 Other proteins involved in starch metabolism

Most studies on starch biosynthesis have focused on enzymes that help synthesise or degrade the starch polymer, however recent research has identified proteins which affect starch metabolism without demonstrating catalytic activity; for example, proteins belonging to a class known as Protein Targeting To Starch ((PTST); Peng et al., 2014; Seung et al., 2015; Lloyd, 2020; Seung, 2020b). All PTST proteins contain coiled coil domains that are believed to be involved in protein-protein interactions (Peng et al., 2014; Seung et al., 2015) *A. thaliana* contains three *PTST* genes (*PTST1*, *PTST2* and *PTST3*) and evidence is emerging as to proteins that each interacts with. PTST1's interaction appears to be with GBSS as this protein is eliminated for the starch granule and amylose synthesis is reduced in *ptst1* mutants of *A. thaliana*, casava and rice seeds (Seung et al., 2015; Bull et al., 2018; Zhong et al., 2019; Wang et al., 2020); although a barley's *ptst1* mutant contained no endosperm starch (Zhong et al., 2019). Arabidopsis *ptst2* and *ptst3* mutants contain larger but fewer starch granules in their chloroplasts (Seung et al., 2015, 2017) and are thought to be involved in starch granule initiation. PTST2 has been shown to interact with SSIV (Seung et al., 2017; Vandromme et al., 2019), MYOSIN-RESEMBLING CHLOROPLAST PROTEIN (MRC) and MAR BINDING FILAMENT LIKE PROTEIN1 (MFP1) (Seung et al., 2018). The rice ortholog to *PTST2* (known as *FLOURY ENDOSPERM 6* (*FLO6*)) on the other hand interacts with ISA1 (Peng et al., 2014).

1.10 How do starch metabolising enzymes interact?

Enzymes often do not act alone but interact with others involved in the same metabolic pathway. Such associations may involve physical interactions where enzymes form a complex, or a functional interaction where the activity of one enzyme influences the activity of another. Above I discussed a physical interaction observed between ISA1 and ISA2 in many plants and a functional interaction where GWD3 is dependent on GWD1 phosphorylating starch for its

activity. Examples of functional interactions where SSII and SSIII rely on each other's activities to synthesise amylopectin (Edwards et al., 1999; Lloyd et al., 1999), but which do not necessarily interact physically were also discussed above. More complex physical interactions have also been demonstrated in wheat and maize where complexes involving SSIa, SSIII, SBEIIa, and SBEIIb have been identified (Tetlow et al., 2008; Hennen-Bierwagen et al., 2008, 2009; Liu et al., 2009).

Based on the observation that repression of *SBEI* and/or *SBEII* leads to increased starch phosphate (Jobling et al., 1999; Safford et al., 1998; Schwall et al., 2000). I hypothesize that a functional interaction may exist between SBEI and/or SBEII and GWD. As isoamylases demonstrate some similarity to SBE's, it may show a similar interaction with GWD. To examine this, I decided to repress genes encoding glucan, water dikinase (*GWD1*) and either of starch branching enzymes I or II or the *ISA2* isoform in potato.

1.11 Aims and objectives

The aim of Chapter 2 in this dissertation is to assess if GWD1, SBEI and SBEII functionally interact with each other in the synthesis of starch.

1. This will be achieved by manufacturing transgenic potato in which *GWD1*, *SBEI* or *SBEII* are repressed singularly as well as transgenic lines in which the following genes combinations were repressed (*SBEI/SBEII*), (*GWD1*/*SBEI*), (*GWD1/SBEII*), and (*GWD1/SBEI/SBEII*) through RNA interference (RNAi) silencing.

2. Repression of the genes will be examined by semi-quantitative reverse transcription PCR and immunoblotting.

3. Tubers of respective transgenic potato lines will be analysed for determination of starch amounts, alteration in structure and physicochemical properties and inhibition of CIS.

The aim of Chapter 3 is to assess functional interaction between GWD1 and ISA2 enzymes during starch synthesis.

1. This will also be achieved by manufacturing transgenic potato lines in which *GWD1*, and/or *ISA2* are repressed using RNAi silencing.

2. Repression of the gene/s will be confirmed by semi-quantitative reverse transcription PCR, activity gels and immunoblotting.

3. Tubers of respective transgenic potato lines will be analysed for starch amounts also analysis of storage starches will be conducted to check for any structural alteration. Accumulation of water-soluble glucan in the *ISA2* repressed lines will also be examined and cold-stored tubers will be analysed for CIS inhibition.

CHAPTER 2: Interaction of starch branching enzyme isoforms and glucan water dikinase in storage starch biosynthesis of potato

2.1 Introduction

Starch is the most common storage carbohydrate in plants. It is as a major source of calories and is also utilized as an industrial feedstock. Two major polyglucans - amylose and amylopectin – are found in starch granules and amylopectin contains small amounts of phosphate monoesters (Hizukuri et al., 1970). Amylose is a largely linear α(1-4) glucan molecule whereas amylopectin contains many α(1-6) branchpoints.

Starch is synthesised within plastids by the activities of several enzymes. The first step of polymer formation involves starch synthases (SS) which use ADP-glucose to synthesise linear glucans (Kossmann et al., 1999; Lloyd et al., 1999; Pfister & Zeeman, 2016). These are branched by starch branching enzymes (SBE) to form amylopectin (Safford et al., 1998; Jobling et al., 1999; Blennow et al., 2005b; Zhao et al., 2021) and phosphate is introduced into amylopectin by two dikinases, glucan water dikinase (GWD) 1 and 3 (Lorberth et al., 1998; Yu et al., 2001; Baunsgaard et al., 2005; Ritte, et al., 2002; Ritte et al., 2006). In angiosperms most starch biosynthetic enzyme classes contain several isoforms and their roles have been elucidated in several plants. There is still lack of knowledge, however, about many aspects of their involvement in starch biosynthesis. For example, evidence exists that enzymes can bind together in complexes and suppression of one of the enzymes of that complex alters the concerted actions of the other enzymes present (Ahmed et al., 2015; Hennen-Bierwagen et al., 2008, 2009). Functional interactions have also been observed between some of the enzymes in starch biosynthesis, where one isoform is thought to produce a substrate which the other isoform utilizes during starch granule formation (Edwards et al., 1999; Lin et al., 2012; Lloyd et al., 1999).

Experiments where amounts of GWD1 or the two main SBE isoforms were reduced in potato were performed more than 20 years ago. In the case of GWD1 antisense inhibition of the gene encoding it led to decreased starch phosphate (Lorberth et al. 1998), while in the case of repression of both SBE isoforms increased amylose and starch phosphate was observed (Jobling et al., 1999; Safford et al., 1998; Schwall et al., 2000). More recently both *SBE* isoforms have been mutated using genome editing techniques which lead to a more severe phenotype with only amylose present when both isoforms were mutated (Zhao et al., 2021). The increased starch phosphate observed in the SBE antisense plants may indicate an interaction between those enzymes and GWD1. This could be caused by functional interaction with reduced SBE activity leading to altered glucan substrate that GWD1 acts on more efficiently. Interestingly Mikkelsen et al (2004) showed that potato GWD1 phosphorylates longer chains better than shorter chains and repression of SBE activity led to increase average chain length (Jobling et al., 1999; Schwall et al., 2000). It has also been suggested that the prevalence of covalently bound phosphate in the amylopectin fraction indicates a functional link between phosphate incorporation and starch branching in potato tuber starch synthesis (Viksø-Nielsen et al. 1998). This implies that reductions in starch phosphate would affect starch branching through reducing the ability of SBE's to branch polyglucans. As there are two SBE isoforms in potatoes, I decided to examine how repression of both SBE and GWD1 isoforms would affect starch in potato tubers.

2.2 Materials and methods

2.2.1 Plant material and growth conditions

Explant material was sourced from micro-propagated *Solanum tuberosum* (cv. Désirée) plants. Plants were grown on sterile media (4.32 g L^{-1} MS (Murashige & Skoog, 1962), 20 g L^{-1} sucrose, 0.5 g L^{-1} casein, 2.23 g L^{-1} Gelrite). $AgNO_3$ at 1.7 mg L^{-1} was added to the media 20

minutes after autoclaving. Plants in tissue culture were grown at 25 °C under 16 h/8 h cycle with fluorescent light.

2.2.2 Plasmid construction

RNAi constructs designed to repress transcription of combinations of *GWD1*, *SBEI* and/or *SBEII* had been produced previously in pHellsgate2 (Helliwell et al., 2002) by Claassens, (2013). One exception was a construct able to repress *SBEII* and *GWD1* simultaneously. This was constructed by amplifying fragments of between 299 and 301 bp for *SBEII* (NM_001288538.1) and *GWD1* (NM_001288123.1) by PCR using leaf and tuber tissues of *S. tuberosum* (cv. Désirée) cDNA as template. The primers used are shown in Table 1. Amplicons were purified using Promega PCR clean-up kits and ligated into pGEM-T Easy (Fermentas). Individual gene fragments were restricted out of the pGEM-T Easy vectors using enzymes shown in Table 1 and chimeric constructs containing more than one fragment were created in pBK-CMV (Agilent) by ligation of individual *BEII* or *GWD1* sequences into the polylinker of that vector (Supplementary figure 4). The combined genes fragment in pBK-CMV were subsequently amplified using primers (GWD1 FWD2 and SBEII REV) allowing directional cloning into pENTR-D-TOPO and the amplicon was inserted into that vector. LR clonase (Invitrogen) was then used to move the BEII::GWD1 fragment into the pK7GWIWG2(II) RNAi vector (Karimi et al., 2002).

2.2.3 *Agrobacterium* transformation

Agrobacterium tumefaciens strain GV2260 was transformed with the silencing vectors by electroporation. Transformed cells were selected at 28ºC on lysogeny broth ((LB) 10 g L^{-1} peptone, 5 g L^{-1} Yeast extract, 10 g L^{-1} NaCl, 15 g L^{-1} Agar) plates containing 50 µg ml^{-1} kanamycin, 50 µg ml^{-1} rifampicin and 50 µg ml^{-1} spectinomycin for 48 hrs.

2.2.4 Plant transformation

Plant transformation was performed according to the method of Chetty et al. (2015) with slight modifications. Leaf explant material was sliced into either approximately 1 cm^2 squares or

Table 1. PCR primers used for the amplification of gene fragments for the RNAi constructs. Incorporated restriction enzyme sites are bold. Bold and underlined sequence is the incorporated topo adapter

Target	Primer Sequences	Restriction enzyme site	PCR product size (bp)
BEII FWD	5'AT**CTCGAG**GAGGATTGGAGAGTGGGTGA-3'	*XhoI*	299
BEII REV	5'-AT**TCTAGA**GGGAAATCAAACTCAGG-3'	*XbaI*	
GWD1 FWD	5'AT**GGATCC**TGGTGCTTCCATACAGGACA-3'	*BamHI*	300
GWD1 REV	5'-AT**GAGCTC**TTCAGGTGCTTTTCCACCTT-3'	*SacI*	
GWD1 FWD2	5'-**CACC**TGGTGCTTCCATACAGGACA-3'	*BamHI*	300

approximately 5mm long internodes, which were transferred to sterile filter paper placed over ~50 ml of clonal propagation medium (CPM; Table 2) in Petri dishes. These were incubated for 48 hours at 25 °C with a photoperiod of 16/8 h light/dark with fluorescent light. A single colony of *Agrobacterium* were inoculated in 2 ml LB with kanamycin, 50 µg ml^{-1} rifampicin and 50 µg ml^{-1} spectinomycin and cultured with shaking at 28°C. After 48 hours 100 µl of the *Agrobacterium* culture was added to 50 ml of the same medium and incubated overnight with shaking at 28 °C. The cells were collected by centrifugation at 5152 × *g* for 10 min and pellets re-suspended in 10 ml of *Agrobacterium* infection media (AIM; Table 2). *Agrobacterium* culture was adjusted to optical density (OD_{600}) of about 0.6 with AIM. For plant transformation 20 µl of acetosyringone stock (74 mM) was added to 30 ml of AIM-diluted *Agrobacterium* culture. Explants were transferred into a 50 ml tube containing 30ml of the *Agrobacterium* culture followed by incubation with shaking for 20 mins at 50rpm.

Table 2: Composition of *Agrobacterium* infection medium (AIM), Clonal propagation medium (CPM), Callus induction medium CIM), shoot induction medium (SIM), root induction medium (RIM). NAA = naphthalene acetic acid; MS = Murashige and Skoog salts without vitamin; GA_3 = Gibberellic acid; B5 Vitamins [0.2 g, nicotinic acid; 0.2 g, pyridoxine hydrochloride; and 2 g of thiamine hydrochloride dissolved in 200 ml double distilled H_2O (Gamborg et al., 1968)]; MS vitamin (0.1 g thiamine HCl, 0.05 g pyridoxine HCl, 0.05 mg nicotinic acid, and 0.02 g glycine dissolved in 100 ml of ddH_2O (Murashige & Skoog, 1962)

Media Components	AIM	CPM	CIM	SIM	RIM
MS salts (g)	4.3	4.3	4.3	4.3	4.3
B5 vitamins (µl)	0	200	0	0	0
MS vitamin(ml)	1		1	1	1
Sucrose (g)	30	30	20	20	20
Myoinositol (mg)		100	100	100	100
NAA (mg)		0.02	0.2	0.02	
Zeatin riboside (µg)			2.5	2	0
GA_3 (mg)			0.02	0.02	
Gelrite (g)		2.23	2.23	2.23	2.23
Water (L)	1	1	1	1	1

Explants were blotted-dry using filter paper and transferred to Petri dishes containing filter paper placed on CIM. This was incubated for 48 hours in a growth room at 25°C with a photoperiod of 16/8 h light/dark with fluorescent light. Explants were collected after 48 hours of co-cultivation and rinsed for 5 minutes with ddH_20 containing 250 mg L^{-1} of cefotaxime. They were then blotted-dry using filter paper and transferred to fresh CIM containing 500 mg L^{-1} carbenicillin, 100 mg L^{-1} kanamycin, and 250 mg L^{-1} cefotaxime. Explants were transferred to fresh CIM every two weeks until shoot primordia began to emerge. Explants with callus producing shoot primordia were transferred onto shoot induction medium (SIM; Table 2) and after four weeks shoots of about 2 cm in length were transferred to root induction medium

(RIM; Table 2) supplemented with 250 mg L^{-1} carbenicillin, 125 mg L^{-1} cefotaxime, 50 mg L^{-1} kanamycin under similar growth condition. Plants that grew roots inside the RIM were selected as putative transgenic lines.

2.2.5 Cultivation of transgenic plants

About ten putative transgenic potato plants (representing 10 independend transformation events per construct) were initially generated for each construct. To confirm insert of each construct into potato genome PCR was used to analyse all the putative transgenic lines (Supplementary figure 5). Semi quantitative RT-PCR was then used to examine repression of the targeted genes in all the putative transgenic lines. The phenotypic effect of repressing targeted genes on inhibition of starch degradation in leaves was also conducted on the 10 putative transgenic lines after 6 weeks of planting in a greenhouse. Two selected transgenic potato lines per construct were grown for 2 months on sterile media (4.32 g L^{-1} MS (Murashige & Skoog, 1962), 20 g L^{-1} sucrose, 0.5 g L^{-1} casein, 2.23 g L^{-1} Gelrite) in tissue culture pots under 16 h/8 h day and night cycle. Two-month-old transgenic plants were then potted in a soil mixture (1.5 parts potting soil and 1 part sand) and grown in ambient greenhouse conditions in Stellenbosch for 4 months (30th September 2020-31st January 2021), after which tubers were harvested.

2.2.6 RNA isolation and reverse transcription-PCR

Plant tissue was homogenized by grinding the sample into powder with mortar and pestle after the addition of liquid nitrogen and used for RNA isolation using Maxwell 16 LEV plant RNA kits (Promega) and a Maxwell 16 MDx AS3000 (Promega) machine following the manufacturer's instruction. Concentration of total RNA was determined using Nanodrop Lite (Thermo Scientific). One hundred nanograms of RNA was converted to cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) according to manufacturer's instructions.

2.2.7 Semi-quantitative reverse transcriptase-PCR

Primers (Table 3) for semi-quantitative RT-PCR (SqRT-PCR) were designed against potato cDNA sequences for the respective enzymes using Primer3 (Untergasser et al., 2012). To ensure that total RNA is not contaminated with gDNA, primers was designed to amplify larger fragments if gDNA was used as template. The housekeeping gene *EF-1α* (Nicot et al., 2005; Tang et al., 2017; NCBI accession number AJ536671.1) was used to determine the number of cycles used for the SqRT-PCR for expression in the linear range. The cycle where the EF-1α primer reached linearity was identified by performing a PCR using cDNA from wild type plants as template where reactions were stopped with the addition of 0.05mM EDTA at 0, 5, 7, 10, 15, 17, 20, 22, 25 and 27 cycles.

Table 3. PCR primers used in semi-quantitative RT-PCR

Primer name		Primer sequence
SqEF-1α	Forward	5-ACTCCCCGGTGACAATGTTG-3
SqEF-1α	Reverse	5-TGGTCACTTTGGCACCAGTT-3
SqBEI	Forward	5-CCGAGCCCCACGAATCTATG-3
SqBEI	Reverse	5-ACAGCCTGCTATCCCACAAC-3
SqBEII	Forward	5-CCGTTCAAGATGGGGGTGTT-3
SqBEII	Reverse	5-GGTGTTGTTCAGCCCTAGGG-3
SqGWD1	Forward	5-AGGTGGGAGAGGAAGGGAAA-3
SqGWD1	Reverse	5-TGTACTGCAGGACTGGAAGG-3

The PCR products were separated on a 1% (w/v) agarose gel in Tris acetate EDTA buffer stained with 3 µl (lab stock solution 0.4 µg ml^{-1}) of ethidium bromide to visualise where cycle linearity was reached by observation under ultraviolet light. Semi-quantitative RT-PCR was conducted subsequently for all the fragments at 27 cycles using GoTaq DNA polymerase (Promega) in a 50 µl reaction with annealing temperature of 57°C for all primer pairs.

2.2.8 Iodine staining

Potato leaves were covered with aluminium foil before sunset and left for three days after which they were harvested and incubated in 20 ml of 80% (v/v) ethanol for three hours at 80°C. Excess ethanol was decanted after incubation and leaves were washed several times with H_20 before addition of Lugol's solution (13.1 mM iodine, 39.6 mM potassium iodide) and left for 5 minutes. Leaves were then washed several times with water and imaged.

2.2.9 Immunoblots

Core samples of freshly harvested potato tubers were taken using a cork borer and frozen in liquid Nitrogen. Ten milligram of the potato tuber core samples were homogenized in 200 µl of ice-cold protein extraction buffer (100 mM MOPS pH 7.0; 2 mM EDTA; 1 mM DTT; 10% (v/v) ethanediol), centrifuged at 13000x g and the supernatant containing crude soluble protein was transferred to a fresh microcentrifuge tube. Total protein was determined using a commercially available kit (Thermo Scientific) based on the method of Bradford (1976). Twenty micrograms of total protein were denatured by incubating at 95°C for 5 min in 2% (w/v) SDS, 10% (v/v) glycerol, 60 mM Tris-HCL (pH 6.8) prior to separation on 8% (v/v) SDS-PAGE at 100 V for 90 minutes. The gel was blotted onto nitrocellulose using a semi-dry blotting system (BioRad) and the membrane was blocked for 2 hours at room temperature in 20 ml buffer containing phosphate-buffered saline (PBS; 8 g NaCl, 200 mg KCl, 1.44 g Na_2HPO_4, 240 mg KH_2PO_4 combine and dissolve in ddH_20 adjusted to pH 7.4 with HCL), 2% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Tween 20 with gentle shaking. The solution

was removed and primary antibody raised against GWD1 (Lorberth et al., 1998) diluted 1:500 in PBS-T (PBS containing 0.1% (v/v) Tween 20) was added to the membrane and incubated overnight at 4ºC. Primary antibody was decanted and the membrane washed for 10 minutes with PBS-T, with the wash step being repeated three times before the membrane was incubated with anti-rabbit IgG bound to alkaline phosphatase (Sigma) secondary antibody (diluted 1:7500 in PBS-T) for 2 hours. The membrane was then washed a further 3 times with PBS-T, after which the presence of GWD1 protein was visualised by incubating it in BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) solution (Sigma*FAST*, Sigma).

2.2.10 Determination of starch and soluble sugars

2.2.10.1 Sample preparation

To remove soluble sugars approximately 10 mg of tuber material was placed in 2 ml tube with 1 ml of 80% (v/v) ethanol at 80°C for 60 minutes. After centrifugation at 2000 x g for 5 minutes, the supernatant was transferred to another 2 ml tube for sugar determinations while the remaining material was washed a further twice with 80% (v/v) ethanol and the supernatant discarded. To solubilise starch the pellet was incubated at 95°C with 400 µl of 0.2 M KOH for 60 minutes before neutralization by addition of 70 µl of 1 M acetic acid.

2.2.10.2 Starch determinations

One hundred microlitres of the neutralised KOH solution was incubated with 10 U ml^{-1} α-amylase (Sigma), 10 U ml^{-1} amyloglucosidase from *Aspergillus niger* (Megazyme) and 100 µl of 50 mM NaAC pH 5.6 at 37°C for 120 minutes. This was diluted tenfold in water and 10µl combined with 250 µl of assay buffer (10 mM MOPS-KOH pH 6.9, 5 mM $MgCl_2$, 1 mM ATP, 1 mM NAD). Glucose was determined by adding, 1 U ml^{-1} glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and 1 U ml^{-1} hexokinase from yeast (Megazyme) and increase absorbance at 340 nm were monitored. The change in absorbance was used to calculate the amount of hexose equivalents present in the sample.

2.2.10.3 Soluble sugars

Fifty microlitres of the aqueous ethanol supernatant was combined with 250 µl assay buffer (10 mM MOPS-KOH pH 6.9, 5 mM $MgCl_2$, 1 mM ATP, 1 mM NAD). Glucose concentrations were determined by adding 1 U ml^{-1} glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and 1 U ml^{-1} hexokinase from yeast (Megazyme) and following the increase in absorbance at 340 nm. Fructose concentrations were then determined by addition of 1 U ml^{-1} phosphoglucose isomerase from *Saccharomyces cerevisiae* (Megazyme) and monitoring the increase in absorbance at 340 nm. Finally, sucrose concentrations were determined by adding 1 U ml^{-1} invertase (β-fructosidase) from *Candida utilis* (Megazyme) and monitoring the increase in absorbance at 340 nm.

2.2.11 Tuber starch extraction and purification

Starch was isolated from potato tubers according to the method of Edwards et al. (1995). Tuber samples were ground in a mechanical juicer (Braun MP 80) with 10ml extraction buffer consisting of 50 mM Tris-HCl pH 7.0, 1 mM EDTA and 10 mg L^{-1} Na-metabisulphite. Insoluble material was allowed to sediment for 60 minutes at room temperature and the supernatant was removed. The starch containing pellet was re-suspended in extraction buffer and filtered through two layers of Miracloth (Calbiochem Califo., USA), followed by centrifugation of the filtrate at 4°C and 2000 x g for 10 minutes This step was repeated 3 times. The starch pellet was washed three times in 80% (v/v) ethanol and centrifuged at 4°C for 10 minutes. Finally the starch was washed with 25 ml of acetone at -20°C and air dried.

2.2.12 Analysis of starch components

2.2.12.1 Determination of the glucose 6-phosphate content of purified starch

An enzymatic assay (Nielsen et al., 1994) was used to determine the glucose-6-phosphate content of the purified starch. One hundred and twenty-five milligrams of starch were digested in 0.5 ml of 0.7 M HCL and incubated for 4 hours at 95°C before by neutralization by addition

of 0.5 ml of 0.7 M KOH. Thirty microlitres of this was added to 230 µl assay buffer (0.25 M MOPS-KOH pH 7.5, 10 mM $MgCl_2$, 2 mM NAD and 2 mM EDTA) before addition of 10.6 U ml^{-1} glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Megazyme) and the increase in absorption monitored at 340 nm. The glucose 6-phosphate content was calculated based on the increase of OD_{340}.

2.2.12.2 Measurement of amylose contents of purified starch

Apparent amylose content of purified starch was determined by the method of Hovenkamp-Hermelink et al. (1988) where 20 mg of starch was dissolved by addition of 500 µl of a 45% (v/v) perchloric acid solution. Samples were incubated at room temperature for 5 minutes before 8 ml of H_2O was added and the samples vortexed for 2 minutes before being allowed to rest for 10 minutes. Fifty microlitres were combined with 450 µl H_2O and 500 µl 0.5 X Lugols solution. Absorbance was determined at 618 nm and 550 nm and the apparent amylose content was measured 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 optical density at 618 nm/optical density at 550 nm.

2.2.12.3 Starch swelling power determination

Swelling power was determined by the method of (Howard et al., 2014) where 10 mg samples of purified starch were suspended in 1 ml water inside pre-weighed 2 ml tubes. Samples were heated for 20 minutes at 80°C with shaking at 750 rpm, followed by cooling for 5 minutes at room temperature and centrifugation for 5 minutes at 1500 x g. The supernatant was removed, and the weight of the starch gel determined. Swelling power was then calculated as;

(Weight of tube + starch gel) - (weight of empty tube)/ Weight of starch.

2.2.12.4 Freeze-thaw stability determination

Freeze-thaw stability was also determined according to the method of Howard et al. (2014) where 25 mg of purified starch samples were suspended in 1ml of water was heated for 20 minutes at 80°C with shaking at 750 rpm. The starch gel was allowed to cool at room temperature and was frozen at -80°C overnight. The frozen starch gel was then allowed to thaw for 60 minutes and centrifuged for 15 minutes at 8000 x g. The supernatant was removed, and weight recorded before the freeze-thaw cycles was repeated 4 times. Freeze-thaw stability was calculated as the % weight loss of the gelatinised starch after 5 cycles.

2.2.12.5 Scanning electron microscopy

Prior to imaging, the samples were mounted on aluminium stubs with double sided carbon tape. The samples were then coated with a thin (~10 nm thick) layer of carbon, using a Quorum Q150T E Sputter Coater. Samples were loaded in a Zeiss MERLIN Field Emission Scanning Electron Microscope (SEM) at Stellenbosch University's Central Analytical Facility. Beam condition 3 kV and 200 pA IProbe were use.

2.2.12.6 Starch granule size determination

Starch granule particle size determination Granule size distributions were determined according to the International Standardization Method ISO 13320 for particle size analysis. Starch samples were measured in aqueous (water) suspension using laser diffraction, Saturn DigiSizer 5200 v1.12 which utilize a CCD detector for high-resolution particle size distribution analysis. The conditions of sample analysis were as follows; flow rate 12.0 L min^{-1}, circulation time 30 sec, ultrasonic intensity 50 %, ultrasonic time 60 sec.

2.2.13 Data Analysis

One-way analysis of variance (ANOVA) and post hoc Bonferroni–Holm partitioning were carried out in Microsoft Excel 2013 using add-in from Daniel's XL Toolbox (Kraus, 2014).

2.3 Results

2.3.1 Production of transgenic potato plants repressed in *SBEI*, *SBEII* and/or *GWD1*

Silencing of single genes by RNAi has been used routinely in potato and studies where multiple genes were repressed by a single RNAi chimeric construct have also been reported (Miki et al., 2005; Andersson et al., 2006; Carciofi et al., 2012; Ferreira et al., 2017). Using that approach in this study, transgenic potato plants were manufactured where transcription of *SBEI*, *SBEII* and *GWD1* were repressed either individually or in combinations with each other. To investigate whether the RNAi constructs successfully repressed the targeted genes in transgenic plants, expression analysis was performed using RNA isolated from freshly harvested tuber material. Transcript accumulation of all three genes decreased in the transgenic lines where they were targeted, but in all transgenic lines *GWD1* transcript also appeared to be reduced (Figure 1A). Immunoblots show that GWD1 protein accumulation was reduced in all lines in which *GWD1* was silenced and appeared reduced in lines SBEI-2 and SBEII-1 but was unaltered in the others (Figure 1B). Two transgenic lines/construct with good repression of targeted genes were selected and used for further analysis. Immunoblotting analysis showed that the transgenic lines containing a construct designed to repress all three genes had similar GWD1 protein amount to the wild type (Figure 1B) and based on those observations these lines were not analysed further.

Repression of *GWD1* in Arabidopsis and potato leads to decreased starch phosphate (Lorberth et al. 1998; Viksø-Nielsen et al., 2001; Ritte et al. 2002; Mikkelsen et al., 2004; Xu et al., 2017a) whereas this parameter increased when *SBEs* were repressed in potato (Safford et al., 1998; Jobling et al., 1999; Schwall et al., 2000; Blennow et al., 2005b). I decided, therefore, to examine the starch phosphate contents in the transgenic potato tubers by quantifying the amounts of glucose 6-phosphate present in starch (Figure 1C). Within the SBEI and SBEII lines this increased to about 8-10 nmol glucose 6-phosphate µmol^{-1} hexose equivalents, the

SBEI/SBEII lines were even further increased to 18-27 nmol glucose 6-phosphate µmol^{-1} hexose equivalents. The lowest level of phosphorylation of 0.8 nmol glucose 6-phosphate µmol^{-1} hexose equivalents was found in the GWD1 lines. The starch phosphate of SBEI/GWD1 and SBEII/GWD1 lines also decreased to between 2-3.5 nmol glucose 6-phosphate µmol^{-1} hexose equivalents.

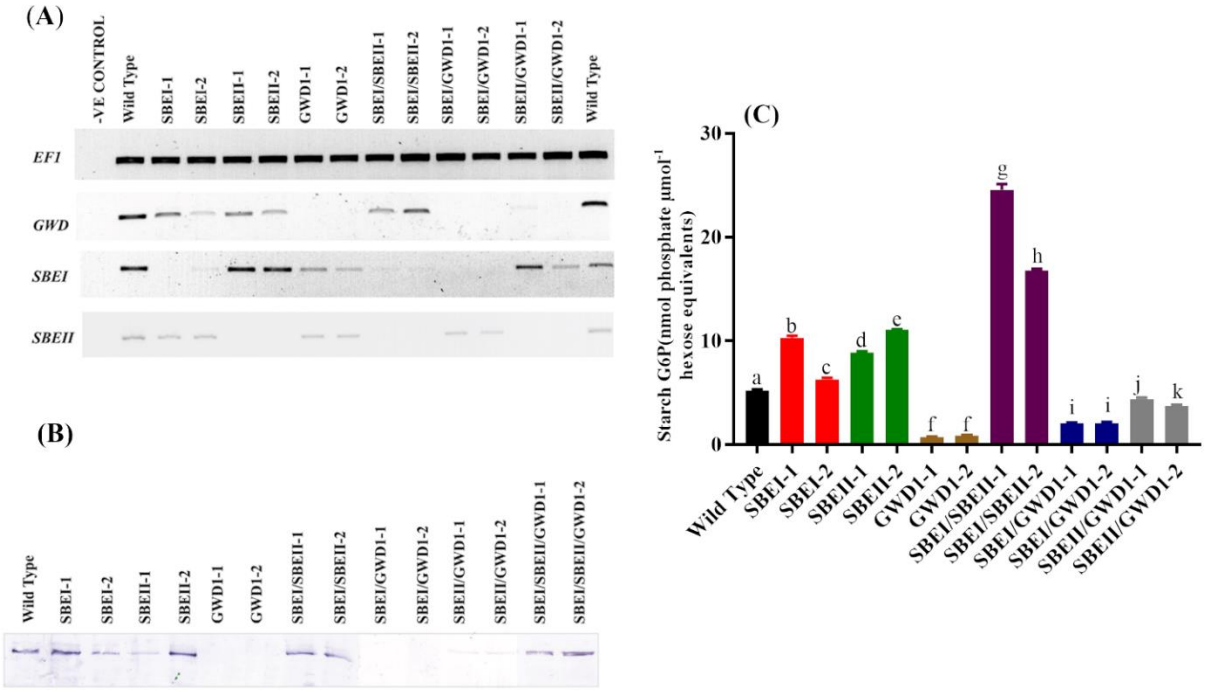

Figure 1 | Repression of *GWD1* and *SBE* genes alters starch phosphate (A) Semi-quantitative RT-PCR of *EF1*, *SBEI*, *SBEII* and *GWD1* expression in transgenic potato tubers (B) GWD1 Protein amounts examined by immunoblotting. Equal amounts (20 µg) of crude protein extracts from freshly harvested tubers were separated by SDS-PAGE and blotted onto nitrocellulose membranes prior to immunoblotting (C) Analysis of glucose 6-phosphate in isolated starches pooled from tubers of at least five plants.

The aerial parts of soil grown plants were phenotypically similar (Figure 2A). Various plant species lacking GWD1 are affected in starch degradation and demonstrate a leaf starch excess phenotype (Caspar et al., 1991; Lorberth et al., 1998; Yu et al., 2001; Nashilevitz et al., 2009; Vriet et al., 2010; Hirose et al., 2013; Zhou et al., 2017). To examine if this occurred in any of the transgenic lines, iodine staining of leaves was used to visualise starch. Source leaves

darkened for 72 hours demonstrated that all lines repressed in *GWD1* (GWD1, SBEI/GWD1 and SBEII/GWD1) still contained significant amounts of starch (Figure 2B) while the other lines did not. Analysis of tuber weights and starch content of tubers immediately after harvest revealed no significant differences between any of the transgenic lines and the wild type (Figure 2C and D). Images of tubers from all the transgenic lines also demonstrated that the tubers were morphologically unaltered in all the transgenic lines (Figure 2E).

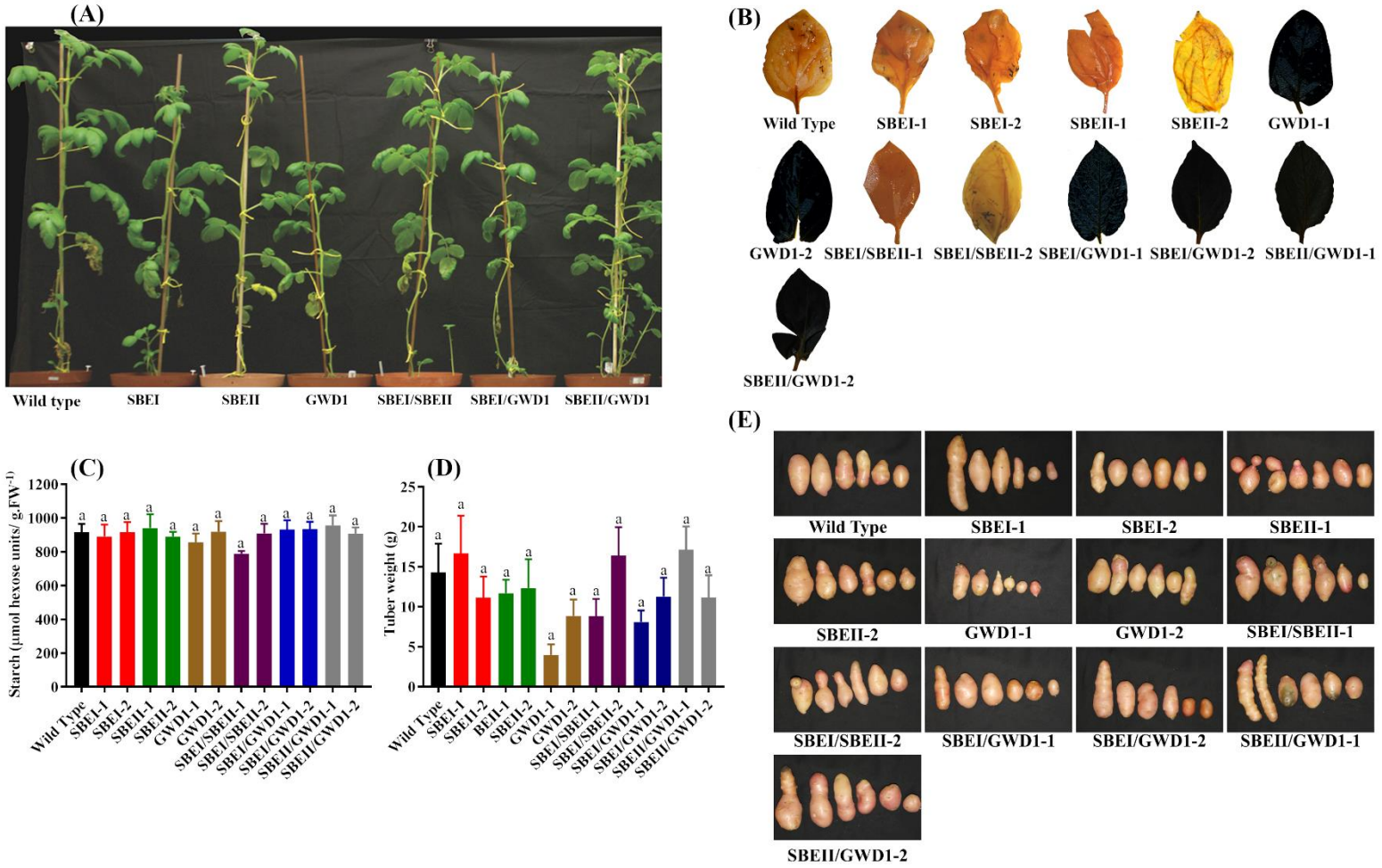

Figure 2 | Analysis of transgenic plants. (A) Plant morphology after 10 weeks of growth in the greenhouse (B) Staining of starch in potato leaves using iodine after incubation in the dark for 3 days (D) Average tuber weight of transgenic plants (C) Starch content after tuber harvest. Data represent the mean ± SEM of at least five plants. Letters represent groups with similar means at the 5% significance level as determined using the Bonferroni–Holm post hoc test following a one-way analysis of variance (E) Tubers from potato line.

2.3.2 Analysis of tuber starch

Alterations in the amylose content of starch from some plant species have been reported in studies where GWD1 or SBE isoforms were suppressed (Mizuno et al., 1993; Jobling et al., 1999; Schwall et al., 2000; Viksø-Nielsen et al., 2001; Sestili et al., 2010). In this study I examined the apparent amylose content of starches isolated from the transgenic lines (Figure 3A) using an iodine binding technique (Hovenkamp-Hermelink et al., 1988). No significant alterations in apparent amylose were observed in the SBEI (20%-21%) or SBEII/GWD1 (20%-22%) lines while one of the two SBEII lines contained starch with a significant increase in amylose (28%). The apparent amylose content of the SBEI/SBEII lines in this study was higher (between 28%-30%) than the wild type and was also increased in both the GWD1 (28%-29.4%) and SBEI/GWD1 (29.5-33.2%) lines with the SBEI/GWD1 lines demonstrating the highest values (29.0% - 33.4%; Figure 3A).

The swelling power of starches from the lines were examined due to the observed changes in their starch bound phosphate and amylose contents. No alterations were observed in starches from the SBEI, SBEII and SBEI/SBEII lines. Starches from all GWD1 and SBEI/GWD1 lines and one of the two SBEII/GWD1 lines had significantly decreased swelling power than all other lines (Figure 3B).

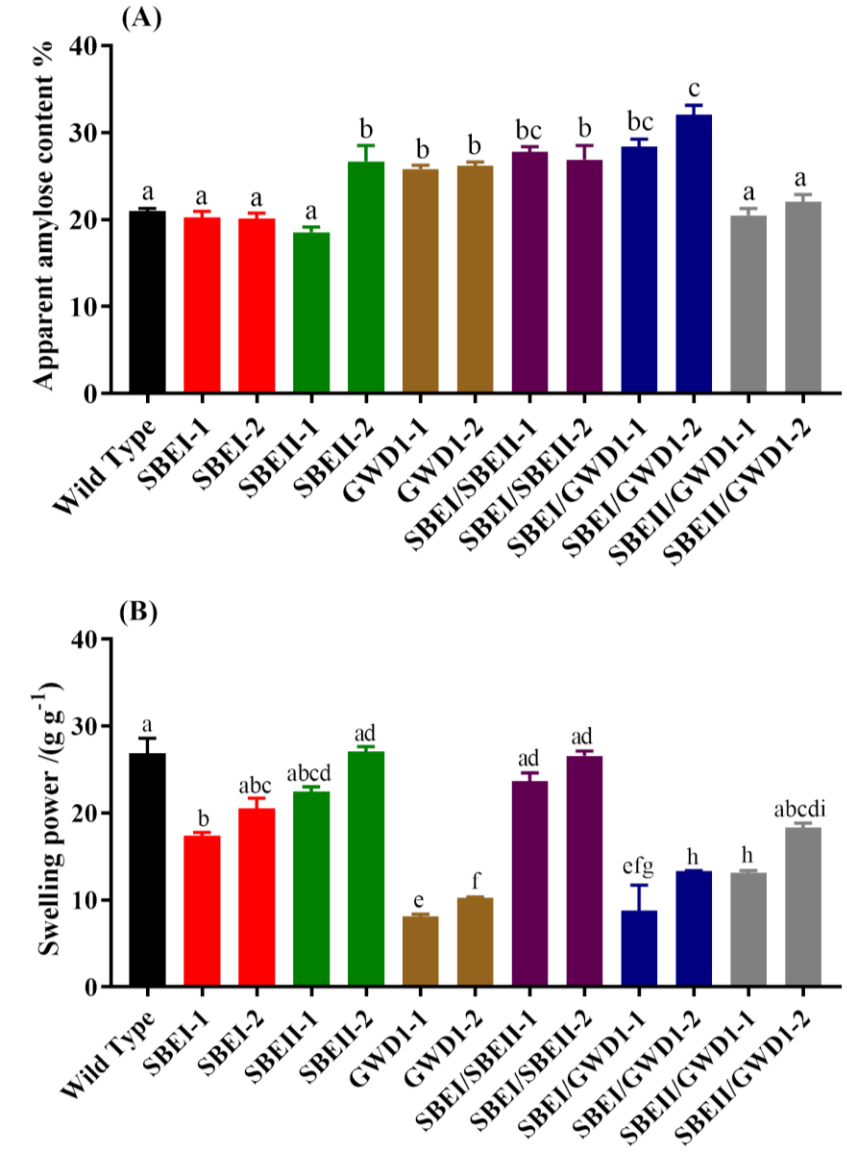

Figure 3 | Analysis of pooled samples of starches isolated from tubers of at least five plants. (A) Apparent amylose (B) Swelling power. The data represent the mean ± SEM of at least four experiments on the pooled starches form each line. Letters represent groups with similar means at the 5% significance level as determined using the Bonferroni–Holm post hoc test following a one-way analysis of variance.

Since freeze-thaw stability of starch has been previously linked to starch phosphate levels (Xu, et al., 2017a), I measured this parameter as percentage weight loss of gelatinized starches after multiple freeze-thaw cycles. Starches from the SBEI lines were similar to the WT, while those from SBEII and SBEI/SBEII lines showed decreased freeze-thaw stability. Starches from the GWD1 and SBEI/GWD1 lines demonstrated better stability in first three cycles (Figure 4).

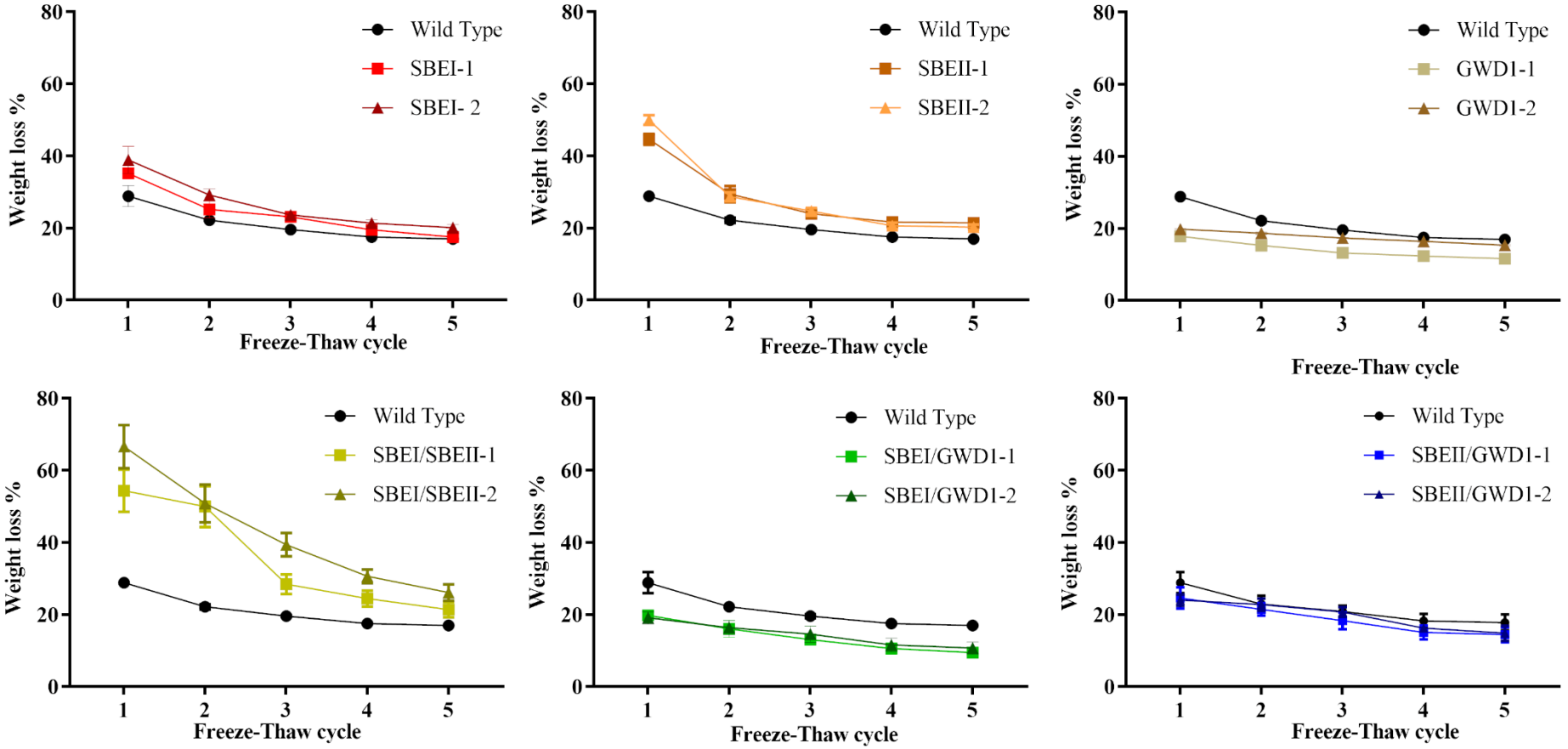

Figure 4 | Freeze-Thaw stability of isolated tuber starches. Measurements are expressed as percentage weight loss per freeze-thaw cycle. The data represent the mean ± SEM of at least four individual samples per line.

Starch granule morphology can impact its physical behaviour (Blennow et al., 2013), such as processability (Jane et al., 1999). I decided to examine granule morphology of various transgenic lines by visualizing them under the scanning electron microscopy (Figure 5A). Granule morphology was unaltered except in two lines. Starches from SBEI/SBEII repressed lines demonstrated heterogeneous granule morphologies, for example some of the granules were ovoid with rough surfaces, some had cracked granules while some of the granules were multi-lobed. Starches from SBEI/GWD1 lines also contained a fraction of granules that appeared lobed (Figure 5A).

Starch granule size can influence its rheological properties, digestibility, crystallinity, swelling and solubility (Lindeboom et al., 2004; Huang et al., 2018; Wu & Zhou, 2018). In this study starches from all the transgenic lines were examined for alterations in granule size. Although starch granules from all the transgenic lines were reduced in size compared to the untransformed wild type, there were no consistent differences between them (Figure 5B).

2.3.3 Repression of *GWD1* inhibits starch degradation and accumulation of soluble sugars in cold-stored tubers

Tubers harvested from the lines were stored at room temperature or 4^{o}C for 8 weeks before starch content and accumulation of glucose, fructose and sucrose were determined. Starch contents were lower in tubers stored at 4^{o}C than those at room temperature (Figure 6). The starch contents of cold-stored tubers from all GWD1 and SBEI/GWD1 lines were significantly higher than the other lines. Glucose and fructose concentration in cold-stored tubers were significantly lower in all the lines repressed in *GWD1* than other transgenic lines where *GWD1* was not repressed. There was no significant different in the amount of sucrose that accumulated in cold store tubers of all the lines.

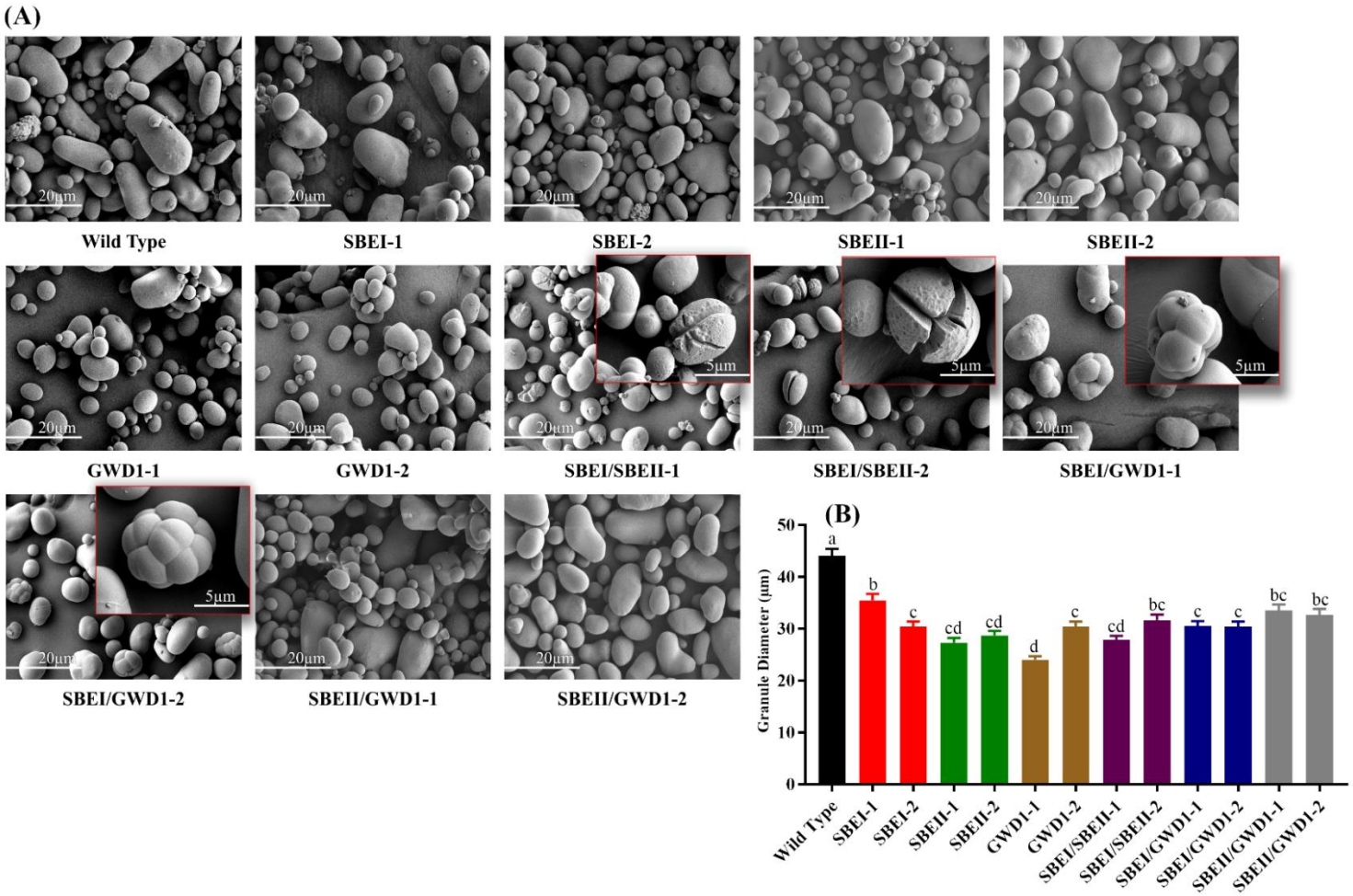

Figure 5 | Analysis of granule size and shape. (A) Granule diameter (B) Scanning electron micrographs of purified tuber starch granules. The data represent the mean ± SEM of at least four individual samples per line. Letters represent groups with similar means at the 5% significance level as determined using the Bonferroni–Holm post hoc test following a one-way analysis of variance.

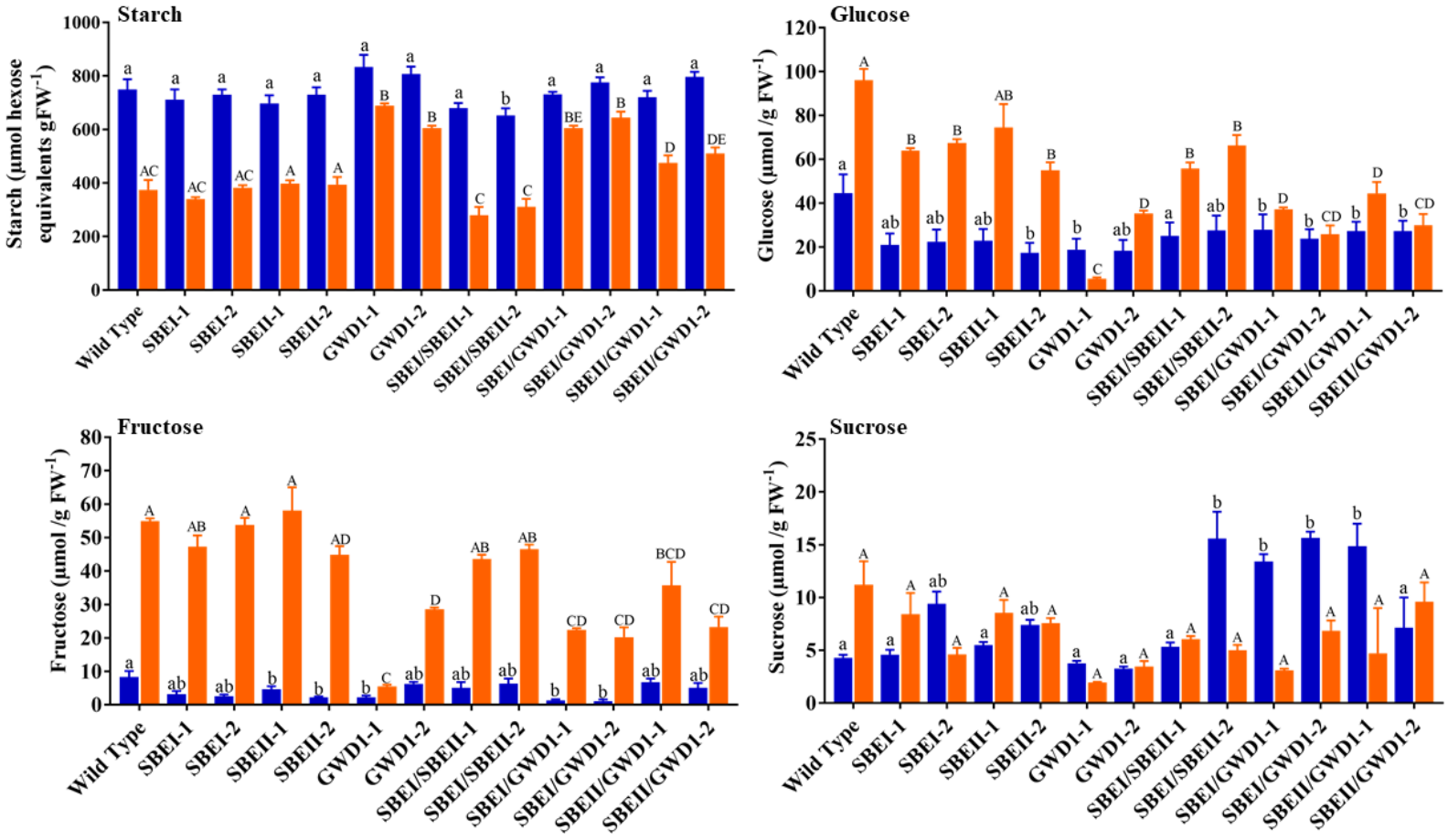

Figure 6 | Starch degradation and accumulation of reducing sugars in tubers stored for 2 months at 4°C (orange) or room temperature (blue). Data represent means ± SEM of measurements from at least five individual tubers of each line. Letters represent groups with similar means at the 5% significance level as determined using the Bonferroni–Holm post hoc test following a one-way analysis of variance.

2.4 Discussion

This study sought to assess possible interactions between SBEs and GWD1 in potato tuber starch synthesis. Branching enzymes play a crucial role in amylopectin biosynthesis by catalysing the formation of α1,6 branch points (Tetlow & Emes, 2014; Pfister & Zeeman, 2016) and starch phosphate is mostly found within this fraction (Hizukuri et al., 1970; Takeda & Hizukuri, 1982). Elevation of C-6 linked phosphate groups have been demonstrated to result from the repression of either of *SBEI* and/or *SBEII* (Safford et al., 1998; Jobling et al., 1999; Schwall et al., 2000), while GWD1 catalyses the incorporation of phosphate to the C-6 position of glucose moieties within starch (Ritte, et al., 2002). Based on this I hypothesised that there may be a potential interaction between SBE and GWD1 and decided to examine this by repressing both *SBE* and *GWD1*. Chimeric RNAi constructs were used to downregulate these genes in transgenic potato plants and semi-quantitative RT-PCR analysis showed that the appropriate genes were repressed in the isolated lines (Figure 1A). Interestingly *GWD1* transcript appeared to be reduced in all transgenic lines (Figure 1A), but this did not appear to alter GWD1 protein amounts (Figure 1B) which were only consistently lowered in lines transformed with a construct designed to repress that *GWD1* expression. A previous study on potato with repressed *GWD1* expression reported that *SBEII*, *SSII* and *SSIII* were also downregulated in tubers (Xu et al., 2017a). Since starch biosynthesis requires concerted activities of several enzymes (Smith & Zeeman, 2020), it is plausible that the expression of some of the genes encoding these enzymes will be co-regulated. The reduction in the transcript level of *GWD1* in all the SBE lines may, therefore, be caused by its pleiotropic regulation caused by SBE repression.

In the current study, alterations in starch phosphate in SBEI, SBEII, SBEI/SBEII and GWD1 lines aligned with previous data (Lorberth et al., 1998; Safford et al., 1998; Jobling et al., 1999; Schwall et al., 2000; Viksø-Nielsen et al., 2001; Blennow et al., 2005b) as starch phosphate

was reduced in GWD1 lines and increased in SBEI, SBEII and SBEI/SBEII lines. The SBEI/GWD1 and SBEII/GWD1 lines have not been examined previously and amounts of glucose 6-phosphate in starches isolated from them were below that of the wild type. They were, however, increased compared with the GWD1 lines, which also contained a small amount of starch phosphate (Figure 1C). As GWD1 is the only enzyme known to incorporate phosphate to the C-6 position of starch *in vivo*, this is an indication that there must be a small amount of that enzyme remaining in all the GWD1 transgenic lines. Similar amounts of GWD1 enzyme appeared to be present in the SBEI/GWD1 and SBEII/GWD1 lines compared with the GWD1 lines and the increased starch phosphate in those lines may be caused by two potential mechanisms. Firstly, it is possible that they contain more GWD1 protein than the GWD1 lines, but that the immunoblots are not sensitive enough to demonstrate this. Secondly, repression of either of the two SBE isoforms might lead to the synthesis of polyglucan that acts as a more suitable substrate for any remaining GWD1. Mikkelsen et al. (2004) demonstrated that GWD1 was more active *in vitro* when longer chains were provided as substrate compared with shorter chains, consistent with an observation made by Blennow et al. (1998) that longer chains are phosphorylated more than the shorter once. Analysis of amylopectin in potatoes repressed in starch branching enzymes has demonstrated that it contained increased numbers of long chains (Safford et al., 1998; Jobling et al., 1999; Schwall et al., 2000) which may act better as a substrate for GWD1. It would be useful to assess average chain length as well as the distribution of phosphate on those chains in all the lines of this study.

Apparent amylose contents were unchanged in the SBEI and SBEII/GWD1 lines, but increased in all the SBEI/ SBEII, GWD1, SBEI/GWD1 lines as well as one of the SBEII lines (Figure 3A) similar to data from previous studies (Safford et al., 1998; Blennow, et al., 2005b; Zhao et al., 2021). The apparent amylose content in the SBEI/SBEII lines however is lower than that reported in similar lines by Schwall et al. (2000) where amylose contents were as high as 75%,

and this most likely represent differential repression of the *SBE* between the studies. Potato lines where all *SBEI* and *SBEII* alleles were mutated led to elimination of amylopectin (Zhao et al., 2021), but removal of all SBE activity in transgenic plants is difficult which helps to explain the presence of some remaining amylopectin in the current and in previous studies ((Figure 3A); Schwall et al., 2000; Blennow, et al., 2005b). Decreased starch phosphate has been shown to positively influence amylose when below a threshold value of approximately 2 nmol phosphate µmol^{-1} hexose equivalents (Viksø-Nielsen et al., 2001), which helps explain why the amylose contents of the two GWD1 lines were significantly higher than the wild type.

These data indicate that low levels of starch phosphate affect branching by SBE's leading to increased apparent amylose, but it is unclear what the mechanism for this is. It could be that low phosphate starch is not a good substrate for SBE isoforms or, potentially that it increases the activity of granule bound starch synthase which synthesises amylose. The difference in amylose between the SBEI/GWD1 and SBEII/GWD1 lines is more difficult to explain and may represent differences between SBE isozymes in branching non-phosphorylated glucans. Differences in substrate preference of potato SBE isoforms have been demonstrated in an *in vitro* experiment (Rydberg et al., 2001). In that study potato SBEI was more active on amylose while SBEII used amylopectin preferentially. When linear dextrins were used as substrate it was shown that SBEI is more efficient in using longer chains as substrate than SBEII. Further assessment will however be needed to examine the activity of the two isoforms to phosphorylated and non-phosphorylated substrates.

The increased amylose observed in some of the lines in this study, may be an overestimate as this was assessed using an iodine binding method, and this methodology can be affected by alterations both in amounts of amylose as well as in amylopectin structure (Takeda and Hizukuri, 1982; Jane et al., 1999). Indeed in a previous study examining *SBEII* repressed potato lines it was demonstrated that although apparent amylose increased (up to 38.5%) when

determined by iodine binding, this was not the case when the same samples were analysed by gel permeation chromatography. The reason for this difference was because repression of *SBEII* leads to increases in the average chain length within amylopectin (Jobling et al., 1999) which are also stained by iodine. A series of other methods to measure amylose, such as size exclusion chromatography and binding to the lectin concanavalin A, will need to be used alongside the iodine binding method to confirm this apparent increase. It does, nevertheless, indicate that starch structure has been altered in the transgenic lines, either in amylose amount or amylopectin structure, or a combination of the two.

As both starch phosphate and apparent amylose contents were altered in the lines, the starches were also examined for alterations in the industrially useful properties of swelling power and freeze/thaw stability. Starch swelling power indicates the ability of starch to hydrate when heated and a survey of starches from potato cultivars with varying phosphate contents demonstrated a positive correlation between these two parameters (Karim et al., 2007). This was corroborated in the starches with low phosphate (all GWD1 and SBEI/GWD1 lines, and one of the two SBEII/GWD1 lines) where swelling power was lower than the wild type (Figure 3B). Interestingly, the link between starch phosphate and swelling power was not observed in the starches with increased phosphate (isolated from SBEI, SBEII and SBEI/SBEII lines) which differs from data of Karim et al. (2007) and Samodien et al. (2018). In that second study, starch phosphatases were repressed in potato and this increased both starch phosphate and swelling power. The lack of change in this parameter in the high phosphate starches of this study indicates, therefore, that starch swelling power is not determined solely by starch phosphate content. This is unsurprising as it has been demonstrated previously that amylose contents and starch fine structure can both also influence swelling power (Tester & Morrison, 1990; Srichuwong et al., 2005; Karim et al., 2007) and amylose and amylopectin structure has

been shown to be altered in GWD1 and SBE repressed lines ((Figure 3A); Jobling et al., 1999; Schwall et al., 2000; Viksø-Nielsen et al., 2001).

Starch granule sizes were reduced in all the transgenic lines (Figure 5A). For starch from lines repressed in both *SBEI* and *SBEII,* this is similar to data reported both in *sbei/sbeii* mutants (Zhao et al., 2021) and antisense *SBEI/SBEII* potato plants (Hofvander, et al., 2004). A previous study reported, however, that granules from GWD1 repressed lines were similar in size to wild type (Viksø-Nielsen et al., 2001) based on observations using a light microscope. That method is unlikely to lead to accurate measurements and so the determination of granule diameter using a particle sizer in this study is more likely to be correct. The reduction in the granule sizes in all the transgenic lines may be due to changes in starch structure affecting granule initiation. It is unlikely that the alteration in the granule size is solely due to changes in starch phosphate content has no consistent correlation between granule phosphate content and size have been observed. For example increased and decreased granule sizes have been observed in potato tuber starches from lines with increased starch phosphate (Xu, et al., 2017b; Samodien et al., 2018). A number of starch synthases, isoamylase and non-catalytic proteins have been shown to be involved in granule initiation (Bustos et al., 2004; Roldán et al., 2007; Szydlowski et al., 2009; Crumpton-Taylor et al., 2013; Seung et al., 2017, 2018; Vandromme et al., 2019) and alterations in the structure of the starch polymer may influence the activities of these polypeptides leading to the observed phenotype.

Starch freeze-thaw stability is a property desired by the food industry, especially for products that are frozen or stored at low temperature. Numerous factors such as low amylose content, fine structure of amylopectin, phosphate ester content and granule size have all been linked with starch freeze thaw stability (Srichuwong et al., 2005, 2012). In the current study low-phosphate starches maintained high stability after three freeze-thaw cycles, whereas starches

with high phosphate were less stable (Figure 4). It is interesting to note that starches from SBEI/GWD1 lines were more stable than those from the WT control as these starches have both decreased phosphate and increased apparent amylose. As increased amylose has been said to be negatively correlated with freeze-thaw stability (Zheng & Sosulski, 1998; Jobling et al., 2002) this indicates that this parameter is more dependent on decreased starch phosphate than amylose. This data also raises the question about whether the freeze-thaw stability reported for starch from plants repressed in *GBSS1/SSII/SSIII* (Jobling et al., 2002) was caused by a reduction in the amylopectin chain length as the authors suggested, or due to the reduction in starch phosphate that they also reported. It would be interesting to study the effect of repressing *GWD1* alongside *GBSS1* in future research to produce low phosphate amylose free starch which might be even more free-thaw stable than the GWD1 lines in this study.

The alteration in granules size of all the transgenic lines (Figure 5A) was only accompanied by change in granule morphology in two lines (SBEI/SBEII and SBEI/GWD1; Figure 5B). The heterogeneous granule morphology observed in SBEI/SBEII lines agrees with data presented by Hofvander et al. (2004) and Blennow et al. (2005b) where *SBEI* and *SBEII* were jointly repressed leading to irregularly shaped starch granules. A fraction of lobed granules observed in the SBEI/GWD1 lines is similar in appearance to compound granules observed in high amylose pea starch from the *rugosus* mutant which is affected in starch branching enzyme (Bhattacharyya et al., 1990; Lloyd et al., 1996). As no visible alteration in granule morphology was observed in GWD1, SBEI or SBEII lines, but were in SBEI/GWD1 and SBEI/SBEII lines, it appears that potato SBEI influences this when repressed alongside G*WD1* or *SBEII*. Previous studies have shown that alterations in starch granule shape may be caused by slight alterations in crystallinity and packing of starch molecules during biosynthesis (Blennow, et al., 2005b; Xu et al., 2017a). This indicates that the change in shape of SBEI/GWD1 or SBEI/SBEII starch granules are most likely caused by structural alterations such as increased amylose and/or

altered starch phosphate, which can affect normal packing of starch molecules (Jenkins & Donald, 1995; Blennow et al., 2003). Since change in granule morphology is observed in SBEI/GWD1 lines and not in individual lines of SBEI or GWD1, it is plausible that both SBEI and GWD1 are involved in synergistic interaction during granule initiation. Hence this supports the hypothesis that SBEI and GWD1 interract during granules initiation.

Starch degradation was reduced both in leaves of lines repressed in GWD1 (Figure 2B) and in potato tubers harvested from those lines when stored under low temperature (Figure 6). Accumulation of reducing sugars (primarily glucose and fructose) in cold-stored potato tubers is known as cold induced sweetening (CIS). During French fry and potato chip production, glucose and fructose in cold-stored tubers react with free amino acids (Shallenberger et al., 1959), making products that are pigmented and which accumulate acrylamide, both of which are unacceptable to consumers (Bhaskar et al., 2010). Genetic studies have shown that CIS is a complex trait (Menéndez et al., 2002; Li et al., 2008), controlled by multiple loci and may include genes which code for numerous enzymes involved in both starch synthesis and degradation (Xiao et al., 2018). For example, QTL analysis of diploid potato population demonstrated that both *GWD1* and *AGPS2* (encoding an ADP-glucose pyrophosphorylase subunit) genes co-localized with different QTLs contributing to CIS (Xiao et al., 2018). Suppression of some enzymes involved in starch degradation such as GWD1 ((Figure 6); Lorberth et al., 1998), α-amylase23, β-amylase1 and β-amylase 9 (Zhang et al., 2014; Hou et al., 2017) have been approaches which have been used to inhibit CIS in cold-stored tubers. The data from this study indicated that neither SBE affected CIS (Figure 6). As SBE's are involved in starch synthesis, this may not be surprising, but mutation in a SBE in maize decreased leaf starch degradation (Yandeau-Nelson et al. 2011). This is presumably because the lack of SBE altered starch structure in such a way that starch degradative enzymes were less able to act upon it. This appears not to be the case in potato tubers.

Given that the starch phosphate content of the SBEI/GWD1 and SBEII/GWD1 lines is greater than the GWD1 lines, it is possible that this may have affected CIS inhibition in cold-stored tuber. Although reducing sugars were significantly higher in both the SBEI/GWD1 and SBEII/GWD1 lines than in GWD1-1 that was not true when compared with GWD1-2. This indicates that the slight increase in the level of starch phosphorylation in the SBEI/GWD1 or SBEII/GWD1 lines did not reverse inhibition of CIS in these lines. The lines produced in this study, therefore, contain both altered starches that may be used for industrial purposes and demonstrate resistance to cold induced sweetening.

2.5 Conclusion

The study was successful in repressing *SBEI, SBEII, GWD1, SBE/SBEII, SBEI/GWD1* and *SBEII/GWD1* in potato which enhances our knowledge of the combined effects of these enzymes during starch biosynthesis. It was demonstrated that SBEI engages in synergistic interaction with GWD1 during starch biosynthesis. This was demonstrated by examination of the effects of repressing *SBE* and *GWD1* which led to increased apparent amylose above either of the *SBE1* or *GWD1* repressed lines. These alterations influenced both swelling power, freeze-thaw stability and morphology of the isolated starches which may render them more suitable for industrial applications.

CHAPTER 3: Interaction of isoamylase and glucan, water dikinase in storage starch biosynthesis of potato

3.1 Introduction

Starch is composed of amylose — a generally linear polymer of glucose units linked by α-1-4-glucosidic bonds — and amylopectin which is a polymer composed principally of α-1-4-glucan chains with varying proportion of α-1-6-glucan branchpoints. Although several models of amylopectin structure have been proposed, the cluster model is the most widely accepted (Bertoft, 2017) which involves classification of chains within amylopectin based on their position within the molecule. The linear chains within amylopectin have been characterised as short [known as A- and B1- chains with a length of <25 residues] and long B2 (25-50 residues) and B3-chains (>50 residues; Hizukuri, 1986). The A-chains are located in the external part of the cluster while the B-chains are situated in both external and internal parts of the cluster and link clusters together (Hizukuri, 1986). Additionally a single C-chain which bears the sole reducing end-group of the molecule carries only the B-chains (Vamadevan & Bertoft, 2015).

One of the most abundant constituents of crops cultivated globally is starch (Jobling, 2004). it is ingested directly as food or feed and a considerable proportion of starch is also channelled into several industrial uses (Marz, 2006). The cluster structure of amylopectin is highly ordered and provides crystallinity within a starch granule. This influences the physicochemical properties that starch has and its potential industrial uses. Understanding its formation is important from a biotechnological perspective as it may be possible to use that knowledge to alter amylopectin structure and change industrial usefulness. The main polymer forming enzymes – starch synthases and starch branching enzymes – catalyse all the activities necessary to synthesis amylopectin, but it is known that the activity of an isoamylase type debranching enzyme (DBE) is also important in this process (Zeeman et al., 1998; Burton et al., 2002;

Bustos et al., 2004; Delatte et al., 2005; Utsumi & Nakamura, 2006; Kubo et al., 2010). This is thought to accelerate crystallization of amylopectin via a process known as trimming where misplaced branches are removed (Streb et al., 2008; Pfister & Zeeman, 2016).

In plants DBEs exist as two types, isoamylase (ISA) and limit dextranase (LDA; also called pullulanase) (Delatte et al., 2005; Streb et al., 2008). The Arabidopsis genome encodes three isoamylase isoforms - ISA1, ISA2 and ISA3 - and one LDA (Delatte et al., 2005). Mutants lacking ISA1 and/or ISA2 activity partly replace starch by a water-soluble glucan (phytoglycogen) in Arabidopsis leaves and in the endosperm of rice, maize and barley (Pan & Nelson, 1984; James et al., 1995; Nakamura et al., 1996; Burton et al., 2002), which indicates their importance in starch metabolism. ISA1 and ISA2 appear to be involved only in starch biosynthesis as *isa1* and/or *isa2* mutants of Arabidopsis are able to degrade starch normally (Zeeman et al., 1998). On the other hand, ISA3 and LDA are thought to be mainly involved in starch degradation, where ISA1 and ISA2 appear not to act (Delatte et al., 2006).

Previous studies in Arabidopsis and potato have shown that ISA2 is catalytically inactive but that it acts together with ISA1 through the formation of heterocomplex (Bustos et al., 2004; Delatte et al., 2005; Wattebled et al., 2005) as when either protein partner is lost in mutant or transgenic plants, the other also becomes eliminated (Hussain et al., 2003; Delatte et al., 2005). This means that mutation or repression of either *isa1* or *isa2* alone leads to the same phenotype as repression of both genes (Bustos et al. 2004; Delatte et al., 2005).

Amylopectin contains covalently bound phosphate bound either to the C-6 or C-3 positions of the glucose monomers and which varies in amount depending on the organ or botanical source (Hizukuri et al.,1970; Bay-Smidt et al.,1994). The C-6 phosphate is added by glucan water dikinase 1 (GWD1; Ritte et al., 2002) and its activity is essential for the addition of phosphate to the C-3 position which is catalysed by GWD3 (also known as phosphoglucan water dikinase)

(Baunsgaard et al., 2005; Kötting et al., 2005; Ritte et al., 2006). The incorporation of phosphate is the first step in starch degradation (Edner et al., 2007; Smith & Zeeman, 2020) and it could be hypothesised that this enzyme is also involved in phytoglycogen degradation. One way to test if this hypothesis is correct is by examining water-soluble glucan in plants where both *ISA2* and *GWD1* are repressed to identify if there is greater amount in plants lacking both enzymes.

Starch degradation occurs in potato tubers when stored at low temperature causing reducing sugars to accumulate (Müller-Thurgau, 1882; Sowokinos, 2001). This process of sugar accumulation in potato tuber under low temperature is called cold-induced sweetening (CIS) and it is important for the potato processing industry as the reducing sugars react with amino acids upon frying leading to discoloration and formation of acrylamide (Sowokinos, 2001; Bhaskar et al., 2010; Wiberley-Bradford et al., 2016). Inhibition of CIS in potato has been reported through repression of *GWD1* (Lorberth et al., 1998), and by manipulation of some genes involved either in starch degradation or sucrose synthesis (Rommens et al., 2006; Chen et al., 2008; Zhang et al., 2013, 2014; Wiberley-Bradford et al., 2016; Hou et al., 2017). ISA1 and ISA2 are thought to be involved primarily starch biosynthesis (Zeeman et al.,1998), unlike ISA3 which has been shown to be involved in leaf starch degradation (Delatte et al., 2006). The role of the ISA1/ISA2 heterocomplex in starch degradation has only been examined in leaves (Zeeman et al., 1998; Pfister & Zeeman, 2016) and so it will be of interest to examine if they are involved in CIS. This study therefore examines potential interaction between ISA2 and GWD1 in potato by repressing the genes individually and in combination with each other to determine if interaction exist between the two enzymes and how this interaction may affect starch metabolism, starch structure and CIS.

3.2 Materials and Methods

3.2.1 Plasmid construction

RNAi constructs designed to repress transcription of combinations of *ISA2* and/or *GWD1* had been produced previously in pHellsgate2 (Helliwell et al., 2002) by Claassens, (2013).

3. 2.2 Plant transformation

Agrobacterium-mediated plant transformation and selection of transgenic lines used for further study was carried out exactly as described in section (2.2.3 and 2.2.4).

3.2.3 Semi-quantitative RT-PCR

Isolated RNA from plant tissue from the potato transgenic lines and methods for Semi-quantitative RT-PCR (sqRT-PCR) is as described in section (2.2.6 & 2.2.7). Using Primer3 (Untergasser et al., 2012), sq-PCR primers were designed against potato cDNA sequences *ISA2* (NM_001287875.1) and *GWD1* (NM_001288123.1) for the respective genes (Table 1) and the housekeeping gene *EF-1α* (AJ536671.1) was used to determine the number of cycles used for the SqRT-PCR for expression in the linear range.

3.2.4 Iodine staining

It was done according to the method described in section (2.2.8).

3.2.5 Immunoblots

It was carried out as described in section (2.2.9).

3.2.6 Enzyme assay

Isoamylase activity was analysed with the in-gel technique of Bustos et al. (2004). Crude soluble protein was extracted from 500mg tuber in 100mM MOPS-KOH 7.0, 1mM DTT, 2mM EDTA and 5% (v/v) ethanediol. Twenty microgram of crude soluble protein was separated on an 8% (v/v) continuous native polyacrylamide gel containing 0.2% (w/v) limit dextrin (Megazyme) and 1.5 M Tris-HCL (pH 8.8). Separation took place at 4ºC for 4 hours at 100

volts and the gel was incubated overnight at 30ºC in 100 mM MOPS-KOH pH 7.0, 5 mM DTT, 5% (v/v) ethanediol. Activity was revealed by staining the gel with Lugol's solution.

Table 1. PCR primers used in semi-quantitative RT-PCR

Primer name		Primer sequence
SqEF-1α	Forward	5-ACTCCCCGGTGACAATGTTG-3
SqEF-1α	Reverse	5-TGGTCACTTTGGCACCAGTT-3
SqISA2	Forward	5-AGGGTGGGTCTGAGATACGT-3
SqISA2	Reverse	5-CATCCTCTGCAGTGTGAGTGA-3
SqGWD1	Forward	5-AGGTGGGAGAGGAAGGGAAA-3
SqGWD1	Reverse	5-TGTACTGCAGGACTGGAAGG-3

3.2.7 Sample preparation and determination of water-soluble glucan, starch and soluble sugars

Extraction of water-soluble glucan was based on the methods by Bustos et al. (2004) and Ferreira et al. (2017) where frozen tubers were homogenized in water at 0ºC in a blender. To 200 mg of the blended tuber, 1 ml of Ice-cold 1.12 M perchloric acid was added and mixed properly. After which 1 ml of suspension was transferred to a fresh tube and subsequently neutralized by neutralization buffer (0.4 M potassium chloride, 2 M potassium hydroxide, 0.4 M MES) followed by centrifugation for 10 minutes at 3000 x g and 4ºC. The supernatant was subsequently transferred into fresh tube and used to determine the water-solube glucan content by measuring the glucose amount released. Glucose was measured according to the method of

Smith & Zeeman (2006); 100 µL of supernatant was mixed with 100 µL of 50 mM NaAC pH 5.6 containing 10 U ml^{-1} amyloglucosidase from *Aspergillus niger* (Megazyme) and 10 U ml^{-1} α-amylase from (Sigma) incubated at 37°C for 2 hours. A 10 times dilution of the tuber solution was added to 250 µl of assay buffer (10 mM MOPS-KOH pH 6.9, 5 mM $MgCl_2$, 1 mM ATP, 1 mM NAD. Glucose was determined by addition of 1 U ml^{-1} hexokinase from yeast and 1 U ml^{-1} glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and following the increase in absorbance at 340 nm. The change in absorbance recorded was used to calculate the amount of hexose equivalents present in the sample. Sample preparation and determination of starch and soluble sugars was carried out exactly as it was described previously in section (2.2.10).

3.2.8 Analysis of starch components and physicochemical properties

Tuber starch was extracted and purified as described in section (2.2.11). Similarly all analysis of starch components (amylose and glucose 6-phosphate contents of purified starch) and pysicochemical properties (swelling power, freeze-thaw stability, granule morphology and size), was carried out as described from section 2.2.12.1 to 2.2.12.6.

3.2.9 Data Analysis

One-way analysis of variance (ANOVA) and post hoc Bonferroni–Holm partitioning were carried out in Microsoft Excel 2013 using add-in from Daniel's XL Toolbox (Kraus, 2014).

3. 3 Results

3.3.1 Production of transgenic potato plants repressed in *ISA2* and/or *GWD1*

Potato plants designed to be repressed in transcription of *ISA2*, *GWD1* or both genes (*ISA2/GWD1*) were produced using RNAi constructs in the potato cultivar Désirée through Agrobacterium-mediated transformation. To examine whether genes targeted by RNAi

constructs were repressed in transgenic potato plants, RNA extracted from tuber material was used in semi-quantitative RT-PCR experiments. Transcript amounts of both *ISA2* and *GWD1* was reduced in multiple plants and two independent transgenic lines for each construct were chosen for further analysis (Figure 1A).

Formation of a hetero-oligomeric complex between ISA1 and ISA2, which is unstable when either protein partner is absent, has been reported in many dicotyledonous plants. In these species, which includes potato, repression of either *ISA1* or *ISA2* results in loss of one activity band on a native gel (Bustos et al., 2004; Delatte et al., 2005; Wattebled et al., 2005). To examine ISA activity in the lines produced for this study, crude protein extracts were made from tuber material and separated on β-limit dextrin containing polyacrylamide gels under native conditions.

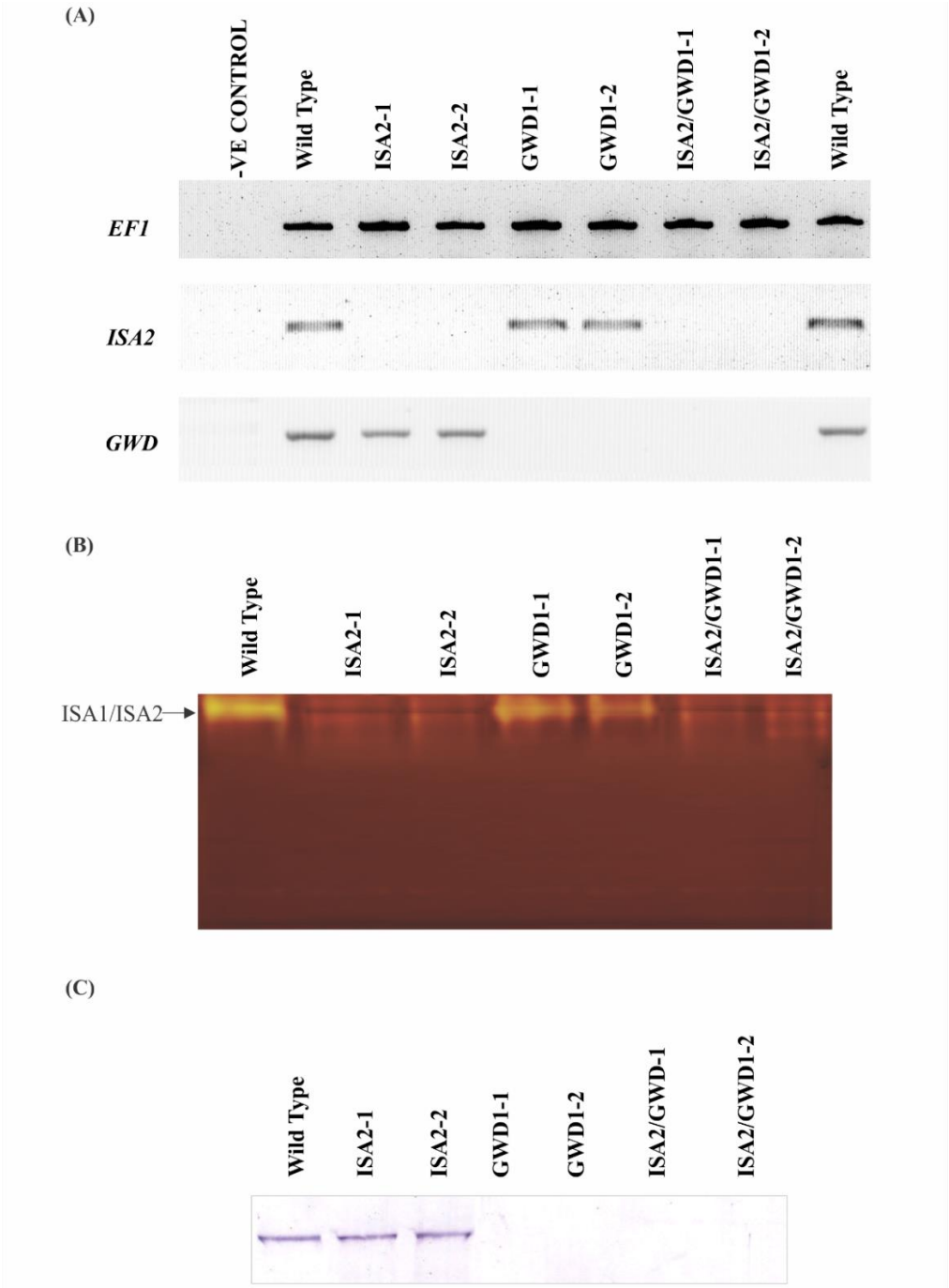

Figure 1 | Analysis of gene expression and protein activity or accumulation in transgenic plants. (A) Semi-quantitative RT-PCR analysis demonstrating *ISA2* and *GWD1* expression in transgenic potato tubers. The *EF1* gene was used as a reference gene. –ve control is a PCR reaction without template. (B) Zymogram analysis of isoamylase activity in the tubers from the various lines. Equal amounts (20 µg) of crude protein extracts were separated under native conditions in polyacrylamide gels containing β-limit dextrin prior to overnight incubation and staining using Lugol's solution. (C) Immunoblot analysis of GWD1 polypeptide. Equal amounts (20 µg) of crude protein extracts from freshly harvested tubers were denatured and then separated by SDS-PAGE and blotted onto nitrocellulose membranes prior to detection using an antibody that recognises GWD1.

There was one main band present in the wild type and GWD1 lines which was greatly reduced in intensity in the ISA2 and ISA2/GWD1 lines (Figure 1B) and presumably corresponds to the ISA1/ISA2 heterocomplex (Bustos et al. 2004). An antibody was used to assess the presence of GWD1 which demonstrated that it was eliminated in all the GWD1 and ISA2/GWD1 lines (Figure 1C).

3.3.2 Silencing does not affect plant growth, tuber morphology and weight but significantly alters tuber number per plants

None of the transgenic plants grown in the soil appeared to be altered in growth (Figure 2A). After three and half months tubers were harvested and imaged (Figure 2B) to examine morphological changes. No visible differences were observed between tubers of the lines. Tuber mass was unaltered in the lines (Figure 2C), however the numbers of tubers produced by GWD1 lines were significantly greater than the wild type but not from the ISA2/GWD1 lines (Figure 2D).

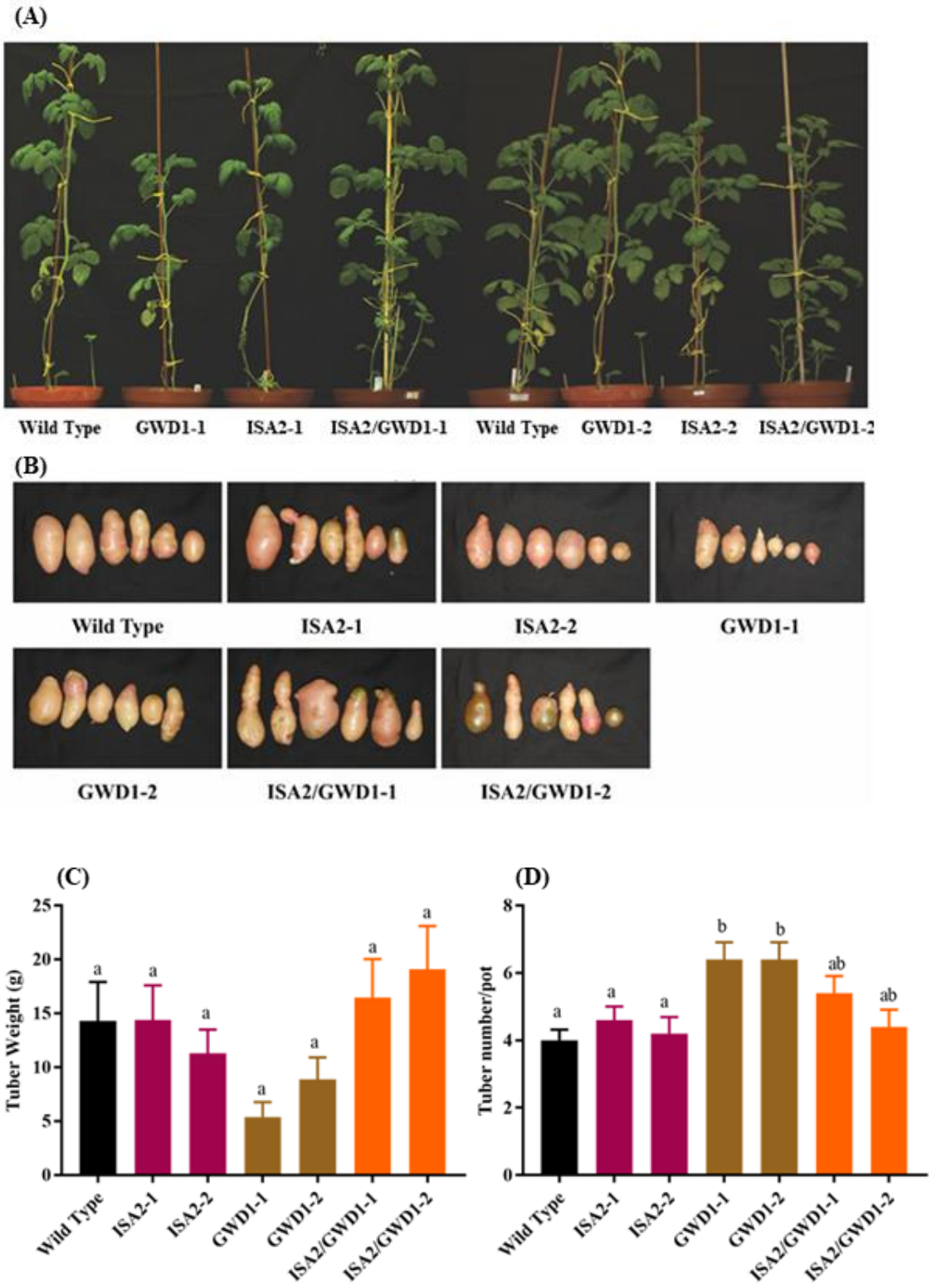

Figure 2 | Plant and tuber morphology and analysis of tubers harvested from transgenic plants: (A) Images of plants after 10 weeks of growth (B) Images of potato tubers (C) Mean weight of tubers (D) Mean tuber number per plant. Data represent the mean ± SEM of measurements from at least five plants . Letters represent groups with similar means.

3.3.3 Repression of *GWD1* results in a starch excess phenotype in leaves

One known phenotype of *GWD1* repression is inhibition of starch degradation leading to accumulation of that polysaccharide in leaves (Lorberth et al., 1998; Yu et al., 2001; Hirose et al., 2013; Zhou et al., 2017). To examine this in the transgenic lines, source leaves were covered with aluminium foil for 72 hours and stained with Lugol's solution. As expected, all transgenic lines in which *GWD1* were silenced demonstrate starch excess phenotype in the source leaves, which was not observed in either the wild type or ISA2 lines (Figure 3).

Figure 3 | Examination of leaf starch degradation. Iodine staining of potato leaves after dark incubation for 3 days. Source leaves were collected from the plants and destained in 80% (v/v) ethanol at 80ºC. Lugol's solution was used to stain the leaves for presence or absence of starch.

3.3.4 Tuber starch content and accumulation of water-soluble glucan

To quantify the effect of repressing *ISA2*, *GWD1* and *ISA2/GWD1* on the major polyglucan pools within potato tubers, water-soluble glucans (WSG) and starch content were determined. Previous studies reported that WSG accumulate in many dicotyledonous plants where *ISA2* was mutated or repressed (Zeeman et al., 1998; Bustos et al., 2004; Delatte et al., 2005; Wattebled et al., 2005, 2008; Ferreira et al., 2017). In the current study, accumulation of WSG

was determined in freshly harvested tubers of all the transgenic lines and it was shown to increase in both ISA2 and ISA2/GWD1 repressed lines, but there were no consistent differences in WSG between the plants from these two lines (Figure 4A). The starch content in freshly harvested tubers did not alter despite increases in WSG in ISA2 and ISA2/GWD1 lines (Figure 4B).

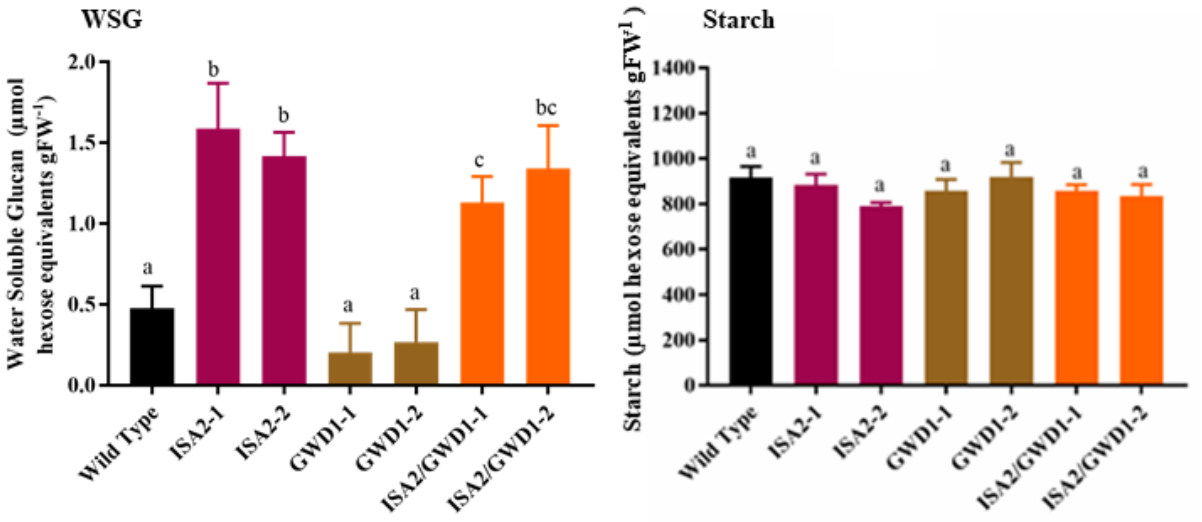

Figure 4 | Glucan contents of tubers from pot grown plants. Data represent means ± SEM of measurements from at least five plants from each line. Letters represent groups with similar means at the 5% significance level as determined using the Bonferroni–Holm post hoc test following a one-way analysis of variance.

3.3.5 Determination of starch phosphate amount and amylose content in tuber

Transgenic potatoes where *GWD1* is silenced synthesise tuber starch with reduced phosphate (Lorberth et al., 1998; Viksø-Nielsen et al., 2001; Blennow et al., 2003). The amounts of glucose 6-phosphate were, therefore, examined in starch isolated from mature tubers in this study and this demonstrated that starch from GWD1 lines were significantly reduced in starch phosphate (Figure 5A). This was also lower in ISA2/GWD1 lines where it was significantly increased compared with the GWD1 lines.

Antisense repression of *GWD1* in potato led to increased amylose when starch phosphate was below 2 nmol glucose 6-phosphate µmol^{-1} hexose equivalent (Viksø-Nielsen et al., 2001). I decided therefore to examine the amylose content in the tuber starches of all the transgenic

lines and this demonstrates that the apparent amylose was significantly higher in the GWD1 lines than all other lines, and that starch from the ISA2 or ISA2/GWD1 was similar to the WT in this regard (Figure 5B).

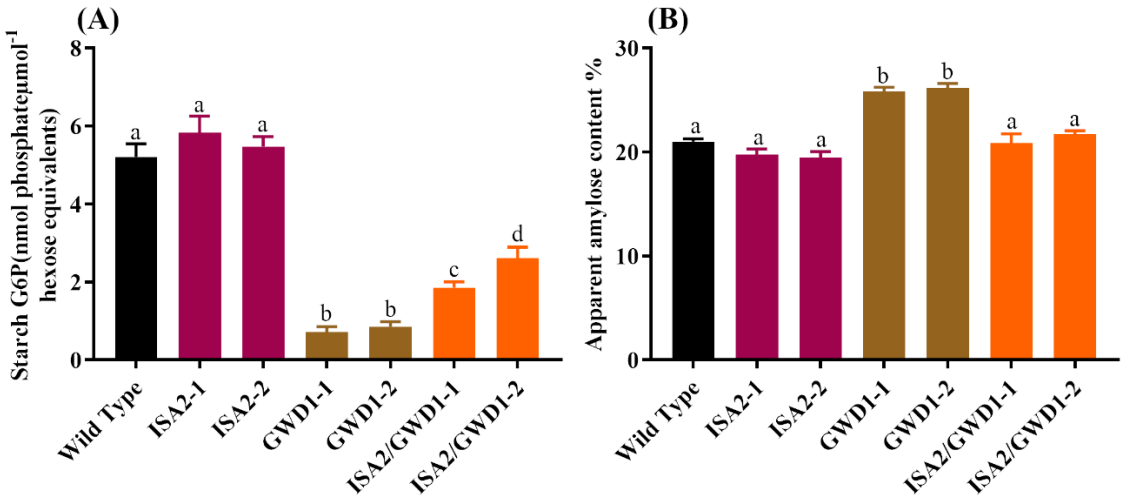

Figure 5 | Analysis of isolated tuber starches. (A) Glucose 6-phosphate content of starch (B) Apparent amylose of starch. The data represent the mean ± SEM of at least five individual tubers of each line. Letters represent groups with similar means at the 5% significance level as determined using the Bonferroni–Holm post hoc test following a one-way analysis of variance.

3.3.6 Starch granule morphology and size

Scanning electron microscopy was used to examine morphology of purified tuber starches. Granules from all the samples were roughly spherical, but the starch from *ISA2* repressed lines contained multiple small granules of less than 0.5 µm in diameter that appear on the surface of larger granules (Figure 6A). These were never observed in starch isolated from the other lines. Diameters of starch granules isolated from the transgenic lines were decreased in all lines compared with the wild-type (Figure 6B), but there were no consistent differences between the transgenic lines.

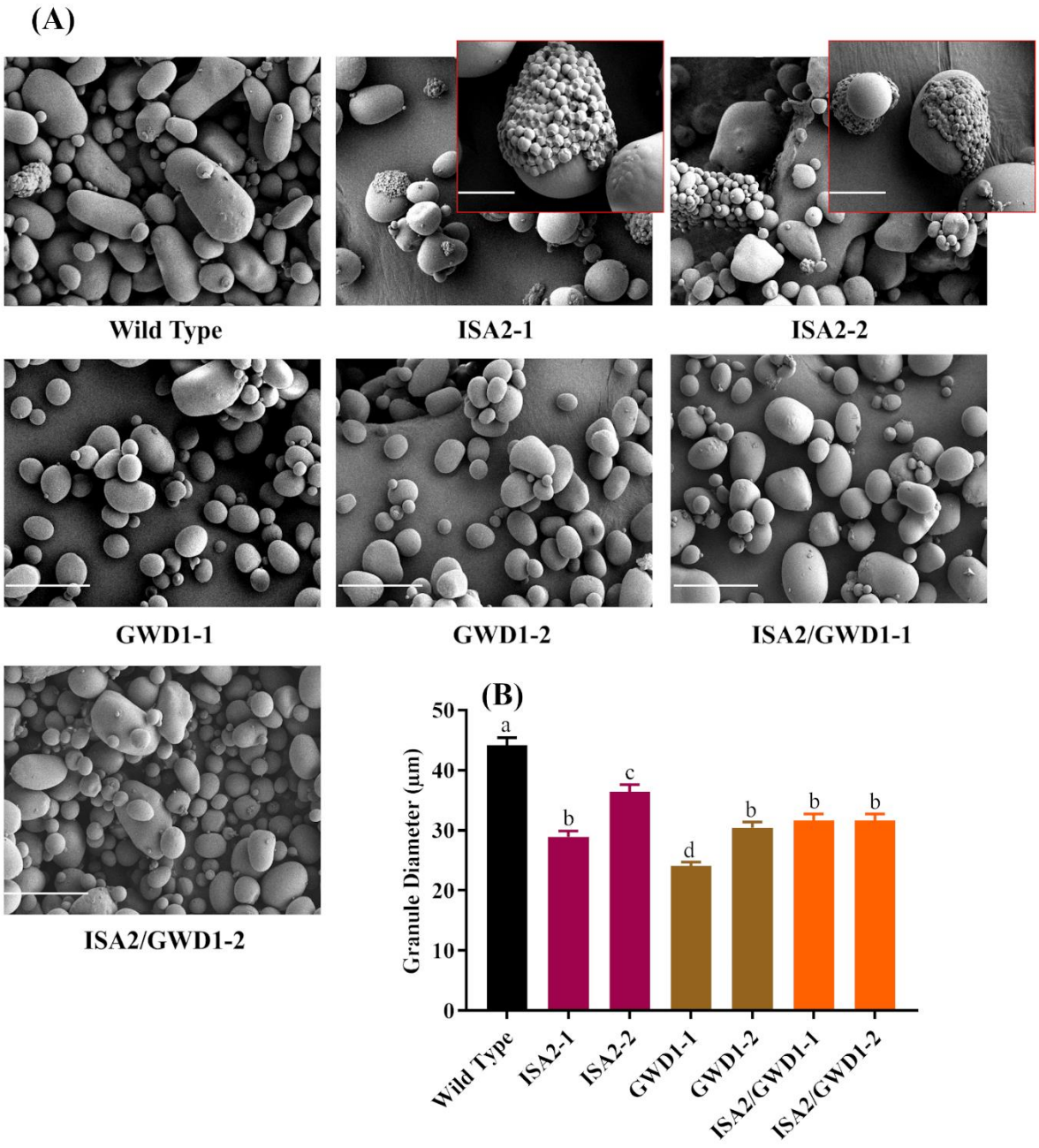

Figure 6 | Starch granule size and morphology. (A) Scanning electron micrographs of purified tuber starch granules. Scale bar represents 20 µm. Inset are images of respective transgenic lines at scale bar 5 µm (B) Mean granule diameter of starch granules determined using a particle counter. The data represent the mean ± SEM of at least five individual tubers of each line. Letters represent groups with similar means at the 5% significance level as determined using the Bonferroni–Holm post hoc test following a one-way analysis of variance.

3.3.7 Starch physical properties

Starch granules are insoluble in cold water, but when heated in water their crystalline structure is disrupted causing them to swell (Vamadevan & Bertoft, 2015; Singh et al., 2016). I decided

to test the swelling power of the starches extracted from all the transgenic lines in this study (Figure 7A). Starches isolated from both GWD1 and ISA2/GWD1 lines had lower swelling power than starch isolated from the WT and ISA2 lines. Swelling power of starch from the ISA2 lines was unchanged in one line and decreased in the other.

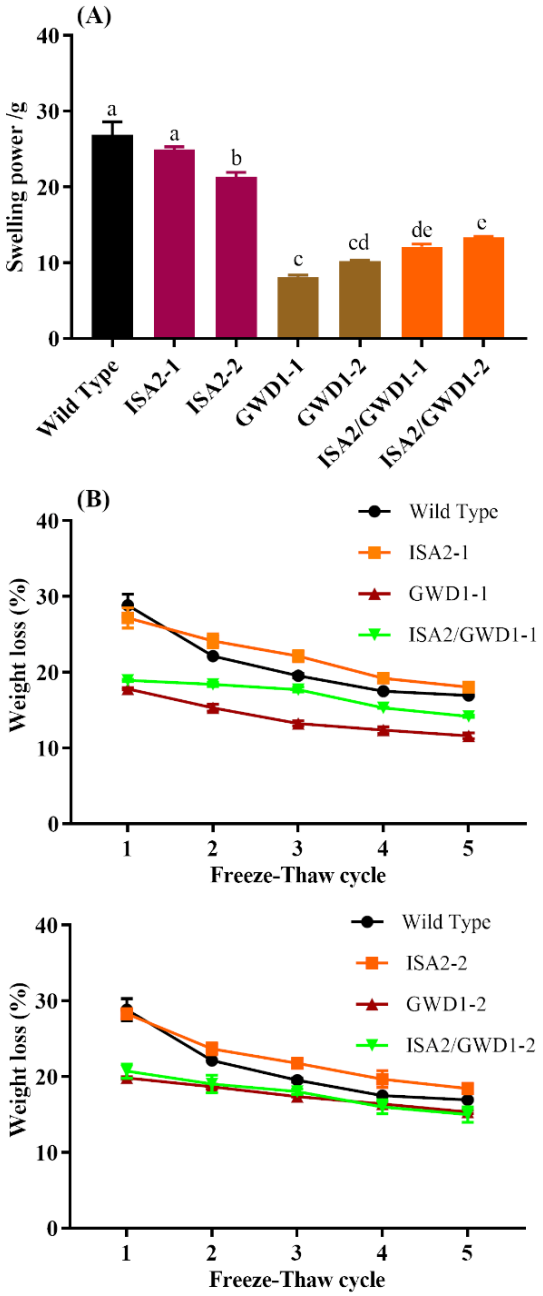

Figure 7 | Physicochemical properties of isolated starches (A) Swelling power (B) Freeze-thaw stability. expressed as percentage weight loss per cycle for 5 cycles. The data represent means ± SEM of at least four measurements. Letters represent groups with similar means at the 5% significance level as determined using the Bonferroni–Holm post hoc test following a one-way analysis of variance.

Improvement of starch freeze-thaw stability is often achieved by chemical treatment but it has also been improved by genetic engineering (Jobling et al., 2002; Vamadevan & Bertoft, 2015). In this study I examined the free-thaw stability of all the transgenic lines and the result indicate that GWD1 and ISA2/GWD1 lines were more stable than others during all cycles (Figure 7B), but the results indicate that ISA2 does not influence freeze-thaw stability of potato starch.

3.3.8 Repression of *ISA2* and *GWD1* affects cold-induced sweetening (CIS) in stored tubers

Transgenic potatoes repressed in *GWD1* have been shown to block CIS in cold-stored potato tubers (Lorberth et al., 1998), but this has not yet been examined in ISA2 lines. I examined CIS in my lines by measuring starch and reducing sugars in tubers stored either at 4°C or room temperature for 8 weeks.

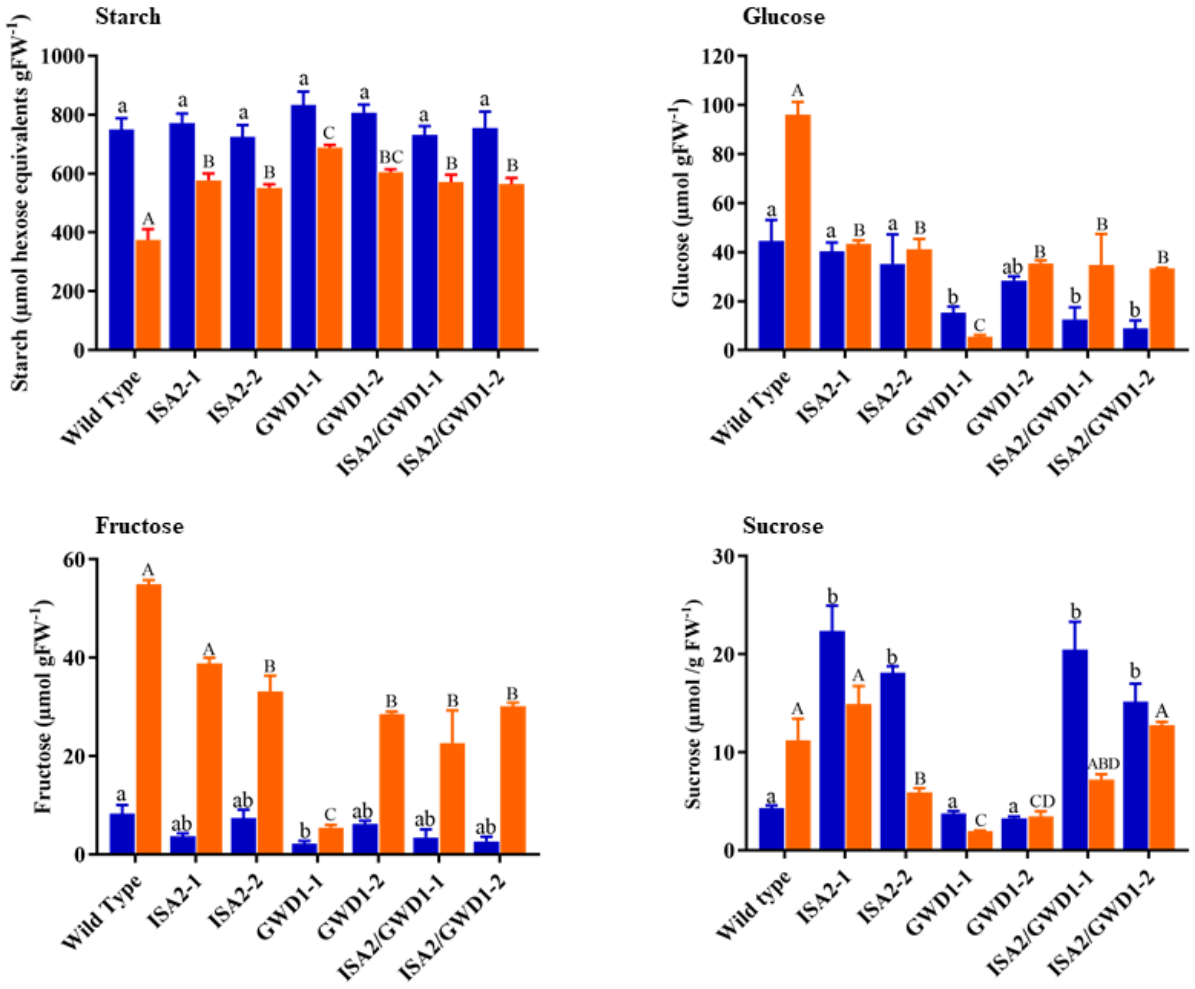

Figure 8 | Starch degradation in tubers. Starch and soluble sugar contents were measured in tubers stored at room temperature (blue bars) or 4ºC (orange bars) for 2 months. Data represent means ± SEM of measurements from at least five individual tubers of each line. Letters represent groups with similar means at the 5% significance level as determined using the Bonferroni–Holm post hoc test following a one-way analysis of variance.

No differences were observed in starch contents of any of the transgenic lines when stored at room temperature (Figure 8), and starch contents were clearly reduced in all cold-stored tubers compared with the room temperature stored tubers. Starch contents in all the transgenic lines were significantly higher in cold-stored tubers than the WT tubers. Decreased starch degradation in cold stored-tubers would be expected to result in greater accumulation of reducing sugars (Wiberley-Bradford et al., 2016) and the data presented here would substantiate this glucose and fructose levels were significantly reduced in most of the transgenic lines except fructose in ISA2-1 (Figure 8), but no consistent difference was observed for sucrose accumulation among the transgenic lines.

3.4 Discussion

In this study I repressed *ISA2* and *GWD1* in potato which have both previously been repressed individually in different studies (Lorberth et al., 1998; Viksø-Nielsen et al., 2001; Bustos et al., 2004). I also repressed *ISA2* and *GWD1* simultaneously to examine interaction between them during starch biosynthesis. None of the potato plants grown in the soil were phenotypically different from each other and the total mass of tubers produced was also unaltered (Figure 2A & 2C). However, there was an increase in the number of tubers produced by GWD1 lines (Figure 2D). Starch levels have been shown to affect tuber numbers and size with plants manufacturing tubers accumulating reduced starch generating many small tubers (Müller-Röber et al., 1992; Tjaden et al., 1998). It is thought that the most likely explanation for this is that the reduced starch increases soluble sugars and these sugars affect downstream, developmental processes. Manipulation of sugar pools in tubers has also been shown to alter

tuber size, with expression of an invertase in the apoplast leading to formation of few, large tubers (Sonnewald et al., 1997). As starch amounts are unaltered in tubers from freshly harvested GWD1 lines (Figure 4B) it seems unlikely that altered tuber sugars will have led to this effect in the current expriments. One explanation is that as repression of GWD1 leads to reduced leaf starch degradation (Figure 3; Lorberth et al. 1998), this will alter export of sugars from leaves to plant stems which may affect stolon formation. However there was no significant increase in tuber number of ISA2/GWD1 lines despite demonstrating reduced leaf starch degradation. It may be that there are differences in sugar export in the ISA2 and ISA2GWD1 lines and this will need to be assessed in more detail.

The starch content in the freshly harvested tubers was unchanged despite increases in the WSG in ISA2 and ISA2/GWD1 lines (Figure 4A & B), consistent with the study by Bustos et al. (2004). In cereal seed endosperm tissue, mutations in *isa1* dramatically decreases starch and leads to accumulation of significant amounts of WSG (James et al., 1995; Nakamura et al., 1996). Similarly Arabidopsis leave from *isa1* or *isa2* mutants accumulate large amounts of WSG accompanied by reduced of starch in mesophyll cells (Zeeman et al., 1998; Delatte et al., 2005), but not in epidermal, sieve element companion and bundle sheath cells which accumulate only starch (Delatte et al. 2005). This demonstrates that the ISA1/ISA2 heterocomplex is not essential for starch synthesis in all cell types and this may help explain why changes in starch contents were not observed in the tubers of ISA2 lines in this study. It is unclear whether the normal starch contents in ISA2 repressed lines (Figure 4; Bustos et al., 2004) is because the ISA1/ISA2 complex does not play as large a role in potato tubers as in Arabidopsis leaves or cereal endosperm, or because there is still some residual activity which is indicated by examination of the activity band in the native gel (Figure 1B). It is unknown whether GWD1 affects WSG formation, and this is the first study to examine this. The data (Figure 4A) indicate that GWD1 does not influence WSG formation as there was no alteration

in WSG amounts in the ISA2/GWD1 plants compared with ISA2. This did not support our initial hypothesis however the question remains whether the WSG is phosphorylated and this will require further investigation.

One effect of repressing *ISA2* in potato has been reported to be the accumulation of numerous small starch granules in the range 0.2–0.5 μm. In a previous study these did not sediment by gravity after being left for 5 hours, and were then collected by centrifugation (Bustos et al., 2004). As these granules are so small, they would not be isolated for starches used for industrial purposes as gravity sedimentation is normally used during the purification process. Because of that I decided to examine starch granules following sedimentation for 60 minutes so that the samples would be similar to those isolated for industrial use. Using these granules it could be shown that there were reductions in starch phosphate content in both GWD1 and ISA2/GWD1 lines, and that the decrease in the ISA2/GWD1 lines was not as great as in GWD1 lines (Figure 5A).

While it is possible that variation in the expression of *GWD1* in these transgenic lines could explain the differences in their starch phosphate, the level of repression of gene expression and protein (Figure 1A, C) appears the same in all the transgenic lines. It is unlikely, therefore, that the increased starch phosphate of ISA2/GWD1 lines is due to increased amount of GWD1 compared with the GWD1 lines. Another possible explanation could be that the suppression of the ISA1/ISA2 complex alters amylopectin structure to make it a better substrate for GWD1. Although antisense repression of *ISA1/ISA2* in potato has been reported not to lead to an alteration of amylopectin structure (Bustos et al., 2004), *isa1* mutants of Arabidopsis and rice accumulate amylopectin with increased numbers of chains below DP10 (Wattebled et al., 2005; Shufen et al., 2019). If this were the case in the starches from the current study this would still not explain the observed phenotype as recombinant GWD1 demonstrated 20-fold more activity when chain lengths increased from 27.8 to 29.5 DP (Mikkelsen et al., 2004). This indicates

either that immunoblot is not sensitive enough to differentiate GWD1 amounts between the GWD1 and ISA2/GWD1 lines, or another mechanism is involved.

According to Viksø-Nielsen et al. (2001) when *GWD1* was repressed in potato, it was reported that the amylose content was increased in tuber starch from some of the transgenic lines when the starch phosphate level was below 2 nmol glucose 6-phosphate μmol^{-1} hexose equivalents. In this study the starch phosphate was below this threshold amount in GWD1 lines but was above it in ISA2/GWD1 lines. It is possible that the increase in apparent amylose observed in starches from GWD1 lines but not in the ISA2/GWD1 lines (Figure 5B), is caused by the starch phosphate in the lines lying either side of this threshold value.

Interestingly starch granules from all transgenic lines were smaller than the controls (Figure 6A). These reductions were not caused simply by reduced tuber starch because this did not decrease in the transgenic lines (Figure 4B), and so must be due to the effect of repressing *ISA2* and/or *GWD1* on granule initiation and size determination. This process is currently poorly understood (Seung & Smith, 2019), but *ISA2* is known to be involved as its repression leads to synthesis of very small granules in potato tubers (Bustos et al. 2004). This may help to explain the reduction in granule size in the ISA2 lines. It has also been proposed that alterations in starch phosphate can affect granule size. Samodien et al. (2018) increased starch phosphate in potato tubers by repressing starch phosphatases and this led to decreased average starch granule diameter, and they proposed that the increased starch phosphate may have directly influenced this. In the current study the starch phosphate contents of various transgenic lines were either reduced or unaltered, yet the average granule size in all the transgenic lines decreased significantly. These data contradict the proposal of Samodien et al. (2018) indicating that any role starch phosphate plays in granule initiation is complex. It is possible that rather than overall starch phosphate being important, it is the distributions in specific parts of a granule which may

influence initiation events by enzymes such as SSIII and SSIV which are known to influence this process (Roldán et al., 2007; Szydlowski et al., 2009).

The alteration in granule size was not accompanied by alterations in granule morphology as all lines produced round granules (Figure 6B), but many small granules were seen attached to the surface of large starch granules from the ISA2 lines. These probably represent the small granule fraction identified by Bustos et al. (2004) which likely co-purified with the larger granules. Interestingly these very small granules were never observed in the ISA2/GWD1 lines indicating that GWD1 influences their formation. As it is unclear how the loss of ISA1 or ISA2 lead to their formation it is difficult to know how GWD1 interferes with that process. It may be that the decreased starch phosphate in the ISA2/GWD1 lines affects their formation, or that reduced GWD1 protein interferes directly with this process.

The alterations observed in starch phosphate and amylose led to an investigation of the starch's physical properties. According to Jobling (2004), starch hydration capacity can be improved by the presence of covalently bond phosphate. Starches from all the transgenic plants with low starch phosphate (GWD1, ISA2/GWD1) had reduced swelling power which indicates decreased water binding capacity. The result also demonstrates that the small increase in amylose in the GWD1 lines is less important in determining the starch swelling power than starch phosphate (Figure 7A) as the amylose content in ISA2/GWD1 lines was unaltered yet it swelling power was similarly low. Starch phosphate has also been negatively correlated with freeze-thaw stability (Xu et al., 2017a). As would be expected, therefore, starches isolated from GWD1 and ISA2/GWD1 lines were more freeze-thaw stable than starches from ISA2 lines or wild type (Figure 7B). In the study by Jobling et al. (2002), starch that is amylose-free and which contains amylopectin with both decreased average chain lengths and low starch phosphate were synthesised in transgenic potatoes. These starches had improved freeze thaw stability which was mainly attributed to a decrease in average chain length within amylopectin.

However, my result provides an alternative explanation for the freeze thaw stability observed in the study, namely that it was caused (at least partly) by the reduced starch phosphate that they observed.

Starch degradation occurs in potato tubers when they are stored at temperatures below 10^{o}C (Hou et al., 2017) and is accompanied by accumulation of reducing sugar. This commercially important process is known as cold-induced sweetening (CIS) and repression of GWD1 can inhibit it (Lorberth et al., 1998). The data in this study demonstrates that the starch content of cold-stored tubers was greater in all the trangenic lines compared with WT (Figure 8), indicating that both GWD1 and ISA2 affect this process. Although ISA2 is catalytically inactive, it is required for the stability of ISA1 (Hussain et al., 2003; Sundberg et al., 2013). This means that their assemblage into heteromultimeric complex does not occur in the absence of ISA2 meaning that the most likely explanation for the repression of CIS in the ISA2 lines is that ISA1 is involved in that process (Bustos et al., 2004). Starch degradation is less well studied during CIS than in leaves at night, but some differences between these processes have been observed. For example, repression of *DPE2*, *SEX4* or *LSF2* affected starch degradation in potato leaves, but not tubers (Lloyd, et al., 2004; Samodien et al., 2018), indicating that the pathways between tuber and leaf starch degradation may differ. The data in the current study indicates a further difference as ISA1/ISA2 heterocomplex is not involved in leaf starch degradation (Delatte et al., 2006).

3.5 Conclusion

This study was successful in repressing *ISA2*, *GWD1* and *ISA2/GWD1* in potato and the analysis of these plants has helped illuminate the roles of these enzymes and how they interact. The effect of repressing *GWD1* alone alters the molecular structure of starch in a different manner than when both *ISA2* and *GWD1* were repressed. This is shown by the differing amylose and starch phosphate contents in the ISA2/GWD1 lines compared with ISA2 and

GWD1 lines. The presence of small granules was a major effect of repressing *ISA2* alone in potato (Bustos et al., 2004), but these granules appeared absent in ISA2/GWD1 lines indicating that GWD1 and ISA2 may interact to initiate their formation. Interestingly, repressing *ISA2* appeared to block CIS which indicates that the pathway of starch degradation in cold-stored tubers may differ from the one established in Arabidopsis leaves.

Chapter 4: General discussion

Starch is a plant product that is a major feedstock utilized by many industries. As reported by the analysts MARKETSandMARKETS, the size of the modified starch market globally in 2020 was worth approximately $13.1 billion and it is expected to rise to about $14.9 billion by 2025 (https://www.marketsandmarkets.com/Market-Reports/modified-starch-market-511.html). Not only is it becoming increasingly important as an inexpensive, renewable and clean energy source, but it also serves as principal major source of calories in the diets of humans and many farm animals (Zeeman et al., 2010; Sonnewald & Kossmann, 2013; Bahaji et al., 2014).

Industrial starch is derived principally from four botanical sources mainly from maize and in much lesser proportion from wheat, cassava, and potato (Zeeman et al., 2010; Avérous & Halley 2014). According to the report by Research and Markets, in 2020 the global potato starch market is approximately 4 million tons per annum (https://www.researchandmarkets.com/report/5330932/potato-starch-market-global-industry-trends) and it is expected to continue to grow throughout the forecasted period (2021-2026). High phosphate in potato starch confers high viscosity on it when heated in water, a unique physicochemical property which makes it valuable in noodle production (Lu et al., 2012b). Modified potato starch called quaternized starch with cationic properties has also been developed (Survase et al., 2016) and it is used to deliver inhibitory RNA therapeutics to treat diseases by targeting genes through small interfering RNA's.

The formation of ordered starch granules is a complex process regulated by the combined activities of enzymes involved in glucan chain elongation, branching, debranching, phosphate substitution, glucan hydrolysis as well as physical packing of newly synthesised α-glucan double helices (Smith, 2001). The molecular constituents of starch granules – amylose, amylopectin and phosphate groups – play critical roles influencing its physico-chemical properties (Parker & Ring, 2001) and they impact on other factors which influences starch

functional properties such as starch granule shape and size (Hoover, 2001; Noda et al., 2002; Kitahara et al., 2007).

Usually the physicochemical properties of native starches need to be modified to enhance their industrial usefulness, which can limit their value (Jobling, 2004). For example, native starches often function inadequately in processed food applications because of their tendency to retrograde, their lack of thermal, pH and shear resistances, and their relatively low solubility (Wu & Zhou, 2018). This makes their modification necessary before industrial use which is normally achieved by means of chemical, physical or enzymatic treatments (Jobling, 2004; Lu et al., 2012b). These are not only cost and labour intensive, but they also impact the ecosystem negatively through pollution. Manipulating metabolism to produce plants which accumulate starch with improved properties for industry may, therefore, be a route to minimize the use of environmental pollutants for starch modification.

The pathway of starch biosynthesis has been demonstrated to be catalysed by the actions of multiple enzymes. The presence of many of these enzymes in starch metabolism has been elucidated in multiple, evolutionarily diverse, plant species from rhodophytes to angiosperms (Sesma & Iglesias, 1998; Coppin et al., 2005; Patron & Keeling, 2005; Zeeman et al., 2010). Their roles have been examined through studies in these plants, but most work has been performed in the model plant *Arabidopsis thaliana* which has led to the description of biochemical pathways of starch synthesis and degradation in photosynthetic tissue. In an industrial context starch has been altered in storage organs of many species through manipulation of, for example, the amylose/amylopectin ratio or starch phosphate amounts. This has provided insight into how starches can be manipulated in planta for improved industrial properties.

So far alteration in the activities of some starch biosynthetic enzymes within several plant species have led to the synthesis of starch altered in its molecular composition and structure. For example low amylose starch has been identified in mutant or transgenic plants where the gene encoding granule bound starch synthase I (*GBSSI*) was inhibited (Weatherwax, 1922; Murata et al., 1965; Shure et al., 1983; Hovenkamp-Hermelink et al., 1987; Kuipers et al., 1991; Ishikawa et al., 1994; Nakamura et al., 1995; Pérez et al., 2019). On the other hand, *SBE* mutant/transgenics led to increased amylose in several plants (Bhattacharyya et al., 1990; Stinard et al., 1993; Jobling et al., 1999; Schwall et al., 2000; Satoh et al., 2003; Hofvander et al., 2004; Blennow et al., 2005b; Andersson et al., 2006; Warthmann et al., 2008; Regina et al., 2006, 2010; Butardo et al., 2011; Carciofi et al., 2012; Sun et al., 2017; Zhao et al., 2021) and many of these starches have distinct physicochemical properties when compared with unmodified starch.

Apart from modified amylose/amylopectin ratios, the level of other starch components like covalently bound phosphate has been altered in plants by genetic engineering. For example, high starch phosphate has been produced in transgenic potato through antisense repression of *SSIII* (Abel et al., 1996), suppression of SBEI and/or SBEII activity (Safford et al., 1998; Jobling et al., 1999; Schwall et al., 2000), repression of starch phosphatases (Samodien et al., 2018), by over-expression of glucan, water dikinase (*StGWD1*) (Xu et al., 2017a), and expression of the human *Laforin* gene (Xu et al., 2017b). On the other hand, decreased starch phosphate has been produced in potato repressed in *GWD1* (Lorberth et al., 1998; Viksø-Nielsen et al., 2001), *SSII* alone (Kossmann et al., 1999), *SSII* and *SSIII* combined (Jobling et al., 2002), or by overexpression of *SBEII* (Brummell et al., 2015).

Although much understanding has been gained on the function of various enzymes involved in starch synthesis in plants, the precise role(s) of each enzyme can remain obscured due to several factors. Firstly, suppression of individual starch biosynthetic enzymes can have pleiotropic

effects on starch metabolism which conceal their roles and those of other enzymes (Safford et al., 1998; Zeeman, et al., 1998; Jobling et al., 1999). Secondly, these enzymes also form complexes with each other, which may have distinct functions during starch synthesis. Some evidence indicates that physical interactions can occur between some starch biosynthetic enzymes in various plant species (Bustos et al., 2004; Tetlow et al., 2004, 2008; Hennen-Bierwagen et al., 2009; Ahmed et al., 2015; Crofts et al., 2015). The functions of these complexes are obscure. They may be present to optimise the rate of starch synthesis, or to help synthesise specific structures within starch granules. This second possibility would imply that some enzymes synthesise specific structures that are then utilised by other members of the complex, with one enzyme affecting the function of the other through substrate supply.

Starch phosphorylation has been demonstrated to occur concurrently with starch biosynthesis (Nielsen et al., 1994) and it has also been shown that starch branching enzymes can utilize phosphorylated glucans as substrate (Viksø-Nielsen et al., 1998). Repression of *SBE* in potato leads to increased starch phosphate (Safford et al., 1998; Jobling et al., 1999; Schwall et al., 2000), indicating an interaction between SBE and GWD1. I examined this by making SBEI/GWD1 and SBEII/GWD1 repressed lines (Chapter 2). Since SBEs and isoamylases are part of the same α-amylase superfamily of proteins, transgenic lines repressed in *ISA2* and/or *GWD1* were produced (Chapter 3) to examine possible interaction between the ISA1/ISA2 heterocomplex (Bustos et al., 2004; Delatte et al., 2005; Wattebled et al., 2005) and GWD1. Even though SBE and the ISA1/ISA2 heterocomplex catalyse opposite activities it would be interesting to examine if their similarities in structure meant that they also interacted with GWD1.

The technique used for simultaneous silencing of genes in this study have been used effectively for this purpose in various plant species (Miki et al., 2005; Andersson et al., 2006; Carciofi et al., 2012; Ferreira et al., 2017). It would have been intriguing to examine the effects of

repressing *SBEI, SBEII* and *GWD1* on starch metabolism, but the RNAi construct designed to do this did not lead to successful repression as gene expression and/or enzyme activity were not eliminated in the SBEI/SBEII/GWD1 lines. More recently studies have used genome editing techniques to generate mutations affecting starch metabolic enzymes (Sun et al., 2017; Bull et al., 2018; Shufen et al., 2019; Tuncel et al., 2019; Wang et al., 2019; Zhao, et al., 2021), but at the time of initiating this research genome editing had not been established in the laboratory where I was based. This type of work would be easier using knockout mutants as there would be no possibility of residual enzyme activity being present. This was clearly present in my lines, for example demonstrated by the presence of residual starch phosphate in lines where *GWD1* transcript accumulation was repressed (Chapter 2: Figures 1B & 1C). Future efforts should concentrate on establishing genome editing to examine the roles of these enzymes further.

4.1.1 Effects on starch biochemistry

In the current study, the interactive effect of supressing SBE and GWD1 or ISA2 and GWD1 was examined to gain more insight into their interactions which otherwise may not be revealed by supressing one of them. In previous studies on Arabidopsis *isa1, isa2* and *isa1/isa2* mutants accumulated little starch in leaves and large amount of a soluble polyglucan – phytoglycogen – a glucan which lacks the capacity to form semi-crystalline granules in some cell type (Zeeman et al., 1998; Kubo et al., 1999; Delatte et al., 2005). In this study low amounts of water-soluble polyglucan were found in tubers of ISA2 and ISA2/GWD1 lines alongside unaltered amounts of starch (Chapter 3: Figure 4), similar to results presented in other studies on potato (Bustos et al., 2004; Ferreira et al. 2017). During starch biosynthesis the ISA1/ISA2 heterocomplex (Bustos et al., 2004; Delatte et al., 2005) is thought to accelerate amylopectin crystallization by removing misplaced branches in a process known as trimming (Delatte et al., 2005; Streb et

al., 2008). As water soluble glucan accumulated to the same extent in ISA2 and ISA2/GWD1, GWD1 appears not to interfere with trimming of misplaced branches by ISA1/ISA2.

Repression of genes encoding enzymes involved in starch biosynthesis and degradation have been shown to result in the alteration of granule size and/or morphology (for example: Edwards et al., 1995; Edwards et al., 1999; Blennow et al., 2005b; Samodien et al., 2018) and this was also demonstrated in this dissertation (Chapter 2: Figure 3C & 5; Chapter 3: Figure 6A). Alteration in starch molecular constituents - such as amylose and phosphate contents - were observed in starches from some of the transgenic lines produced in this study, but reduction in starch granule size (Chapter 2: Figure 3C; Chapter 3: Figure 6A) was observed in all the transgenic lines despite starch content not changing (Chapter 2: Figure 2D; Chapter 3: Figure 4). In contrast to the understanding of the enzymes involved in the starch biosynthetic pathway, the mechanism and regulation of starch granule initiation, variation in size and morphology remains poorly understood (Seung & Smith, 2019). The above-mentioned change in granule size (Chapter 2: Figure 3C; Chapter 3: Figure 6A) in all the transgenic plants suggest that granule initiation and size determination was affected by repressing *SBEs*, *ISA2*, *GWD1* genes. It may also be that the expression or activity of other starch biosynthetic enzymes were influenced in the various transgenic plants. The reduction in the granule size may therefore not be directly caused by alteration in GWD1, SBE or ISA2 directly, but to pleiotropic effects affecting other genes or proteins. According to recent studies on granule initiation in various plant organs, several proteins have been identified to be involved in this process (Seung & Smith, 2019). For example mutation in *ss1*, *ss3* and *ss4* have been shown to influence starch granule initiation in various plant species (Roldán et al., 2007; Szydlowski et al., 2009; Guo et al., 2017). The involvement of isoamylase in starch granule initiation was demonstrated by an *isa1* mutation in barley (Burton et al., 2002) or repression of *ISA1* and *ISA2* in transgenic potato (Chapter 3: Figure 6B; Bustos et al., 2004) which led to proliferation of small granules in their

respective storage organs. A protein called PROTEIN TARGETING TO STARCH 2 (PTST2) also plays a role in the initiation of granule formation in Arabidopsis leaves and endosperm from rice and barley (Peng et al., 2014; Seung et al., 2017; Saito et al., 2018). Other proteins implicated in this process are PTST3, MAR-BINDING FILAMENT PROTEIN and MYOSIN-RESEMBLING CHLOROPLAST PROTEIN (Seung et al., 2018; Vandromme et al., 2019). It is possible, therefore, that repression of targeted gene/s in this study could have influenced the protein concentration or altered the preferred substrate of at least one of these proteins leading to reduction in granule size. Further investigation will be required to confirm this.

Due to alteration in starch granule components from some transgenic plants in this study, I decided to examine their granule morphology by imaging using SEM. Lines repressed in *SBEI* and *GWD1* had multilobed granules (Chapter 2: Figure 5), similar to granules from the *rugosus* pea mutant (Boyer, 1981; Lloyd et al., 1996) – a mutation which causes the loss of the SBEI isoform (Bhattacharyya et al., 1990). This is accompanied by an increase in the amylose ratio from 35% to 65% and altered molecular size of the amylopectin (Colonna & Mercier, 1984). As decrease in starch phosphate was accompanied by increase in apparent amylose in SBEI/GWD1 (Chapter 2: Figure 1C & 3A), this indicates that polyglucan structure is altered and this may have led to the observed granule morphology, but clearly a more detailed analysis is needed to examine this.

The pathway of starch degradation may differ between plant organs and distinct pathways may exist within the same plant. This process is best understood in leaves and it involves reversible phosphorylation of amylopectin by GWD1 and GWD3 at the surface of the starch granules (Edner et al., 2007; Zeeman et al., 2010). This solubilizes the granule surface making it accessible for hydrolytic enzymes such as isoforms of β-amylase (BAM1 and BAM3) and isoamylase (ISA3) (Kaplan & Guy, 2005; Delatte et al., 2006; Fulton et al., 2008; Smith &

Zeeman, 2020) to further degrade starch. The phosphate groups added by GWD isoforms must be removed from malto-oligosaccharides released by the hydrolytic enzymes by the phosphatases SEX4 and LSF2 (Kötting et al., 2009; Santelia et al., 2011) before amylolytic degradation can proceed. This results in the formation of maltose and malto-oligosaccharide which are acted on by disproportionating enzyme 1 to form maltose and glucose (Critchley et al., 2001). These sugars are transported into the cytosol by separate glucose and maltose transporters (Cho et al., 2011; Niittylä et al., 2004) where maltose is converted to glucose by disproportionating enzyme 2 (Chia et al., 2004; Lloyd et al., 2004).

Starch degradation has been demonstrated to be inhibited in the leaves and tubers of transgenic potato repressed in *GWD1* (Chapter 2: Figure 6; Chapter 3: Figure 8; Lorberth et al., 1998), additionally this study revealed that ISA2 but not SBE may also be involved in starch degradation of cold-stored tuber, indicating that some enzymes primarily involved in starch synthesis may influence the process of starch degradation as well. It is possible that the ISA1/ISA2 complex is necessary for synthesis of suitable substrate for starch degrading enzymes like β-amylase to act upon and the activities of these enzymes may have been further inhibited by low storage temperature leading to impaired starch degradation as observed in the cold store tubers.

4.1.2 Effects on starch structure

Altered activities of starch biosynthetic enzymes influences the content and molecular structure of starch polymers such as the ratio of amylose to amylopectin and the branching of amylopectin (Satoh et al., 2003; Vandeputte & Delcour, 2004). Repression of *SBEI* may be expected to increase amylose as it is involved in converting linear chains to amylopectin but *SBEI* repressed lines did not show increased amylose both in this study (Chapter 2: Figure 3A) and in previous studies (Safford et al., 1998; Zhao, et al., 2021). Increased amylose was however observed in SBEII, GWD1 and SBEI/SBEII lines (Chapter 2: Figure 3A) similar to

previous studies in potato (Hofvander et al., 2004; Jobling et al., 1999; Schwall et al., 2000; Viksø-Nielsen et al., 2001; Zhao et al., 2021). Interestingly increased amylose was observed when both *SBEI* and *GWD1* were repressed that was greater than when either *SBEI* or *GWD1* were repressed alone (Chapter 2: Figure 3A). Many factors could have contributed to this. Firstly, it may be due to pleiotropic effect of repressing *SBE* on the expression of other starch synthesis genes. This has been reported in barley and durum wheat where *SBE* was silenced by RNAi leading to upregulation of *GBSSI* during endosperm development accompanied by a marked increase in amylose (Sestili et al., 2010; Carciofi et al., 2012). Secondly, suppression of SBE could have altered amylopectin structures, which may have altered the activities of other enzymes such as SS isoforms through alteration of their polyglucan substrates. Finally, the decreased starch bound phosphate in this line (Chapter 2: Figure 1C) may have affected the activity of SBEII. Although it is known that potato SBE's can utilise phosphorylated glucans as substrates (Viksø-Nielsen et al., 1998), the influence of such phosphorylated glucan on SBE activity has not been assessed. It is, therefore, possible that low phosphate starch may act as a worse substrate for potato SBEII and it is unable to compensate for decreased SBEI activity in the SBEI/GWD1 lines. This would also help explain the increased amylose in *GWD1* repressed lines (Chapter 2: Figure 3A; Viksø-Nielsen et al., 2001; Xu, et al., 2017a) as reduced SBEII activity alone is known to increase amylose in potato (Jobling et al., 1999)

Increased amylose within starch from the GWD1 and SBEI/GWD1 lines may lead to a health benefit as high-amylose starch has been reported to promote better colonic function (Bird et al., 2000). Such starches resist digestion in the small intestine and are fermented in the large intestine where they are used by gut bacteria to synthesise short chain fatty acids which contribute to optimal colon health (Topping & Clifton, 2001; Topping et al., 2003). Indeed high-amylose starch has also been demonstrated to have positive effect on reducing the blood glucose response in rats having type II diabetes (Zhu et al., 2012).

The physicochemical properties of starches from different botanical sources or genetically modified plants have been reported to vary based on altered phosphate content (Lorberth et al., 1998; Blennow et al., 2000b; Zaidul et al., 2007; Xu, et al., 2017a; Xu, et al., 2017b). Increased starch phosphate of SBEI/GWD1 lines when compared with GWD1 lines (Chapter 2: Figure 1) may have occurred due to increased numbers of longer glucan chain in amylopectin acting as a better substrate for a residual GWD1 (Mikkelsen et al., 2004). As mentioned above, the amylose content of starch contributes to its digestion within the gastrointestinal tract, however an *in vitro* experiment has reported a negative correlation between starch digestibility and its phosphate (Wickramasinghe et al., 2009). This indicates that the high starch phosphate content may be beneficial to human health by reducing post prandial blood glucose spikes which can lead to the development of type II diabetes (Zhu et al., 2012; Ting & Yu, 2016). It will be interesting to compare digestibility of starches from SBEI/GWD1 and ISA2/GWD1 lines with GWD1 lines both *in vitro* and *in vivo* to gain more understanding about this.

4.1.3 Industrial properties

Alterations in starch phosphate, apparent amylose, granule size and morphology observed in the starches led to investigation of their physical properties. The results indicate that starch phosphate content is a crucial factor in determining swelling power. This is because reduced starch phosphate led to low swelling power in the GWD1, SBEI/GWD1 and ISA2/GWD1 lines (Chapter 2: Figure 3B; Chapter 3: Figure 7A). However in a study by Wickramasinghe et al. (2009) increased amylose was proposed to be responsible for reducing the swelling power of high phosphate starch isolated from antisense *SBE* transgenic potatoes, and low phosphate starch from antisense *GWD1* potato. Also in another study on swelling power in potatoes, increased branching within amylopectin by overexpression of *SBEII* was identified as the main determinant as increased swelling power was observed despite reduction in starch phosphate (Brummell et al., 2015). This indicates that swelling power is dependent on a number of factors,

including starch phosphate and amylopectin chain length distribution, and that these will need to be considered together when designing genetic manipulations to influence this parameter.

Potato starch has high swelling power when compared with cereal starch, but its industrial application is limited because its swollen granules are unusually large in volume, resulting not only in high viscosity but also in less smooth texture (Hermansson & Svegmark, 1996). The fragility of the swollen potato starch granules (Craig et al., 1989) further exposes them to dispersion during continuous heating and shearing which leads to weak-bodied, stringy and cohesive pastes (Acquarone & Rao, 2003). Crosslinking by chemical modification has been used to stabilise internal starch structures by adding chemical bonds randomly within a granule (Acquarone & Rao, 2003) which has been successfully utilized in reducing the swelling power of potato starch (Kaur et al., 2006). The lower swelling power of starches isolated from GWD1 repressed lines (Chapter 2: Figure 3B; Chapter 3: Figure 7A) suggest greater stability during continuous heating, and these starches may be used in food applications such as alkaline noodles production (Lii & Chang, 1991; Jun et al., 1998) with little or no modification.

The low phosphate starches with improved freeze-thaw stability produced in the current study (Chapter 2: Figure 4; Chapter 3: Figure 7B) might also be utilized in various food applications. For example, better freeze-thaw stability in combination with superior paste clarity of potato when compared with corn or wheat starches (Craig et al., 1989), will make it a preferred starch in food application such as fruit pie fillings (Zheng et al., 1998). In addition, they can also benefit the French fry industry by reducing phosphate pollution. Currently at least 17 litres of wastewater are generated per kilogram of potato tuber (Hung et al., 2004) and this can contain up to 40 mg L^{-1} of phosphate, half of which is derived from the potato starch (Rommens et al., 2006). Utilizing starch with low phosphate would minimize the cost associated with reducing phosphate in wastewater prior to environmental release. Furthermore, starches from antisense *GWD1* repressed lines have been reported to demonstrate reduced viscosity due to decreased

phosphate and low molecular weight of amylopectin (Lorberth et al., 1998; Viksø-Nielsen et al., 2001), and this structural composition may be suitable for seed coating due to an increase ability to form filmogenic solutions (Bello-Perez & Agama-Acevedo, 2017). A full assessment of the physical properties of starches produced in this study using, for example, a rapid viscoanalyser would help to define their potential industrial uses.

Potato tuber starch degradation was discussed above in the context of the roles of ISA2 and GWD1 in cold induced sweetening (CIS). This is an industrially important process where tubers accumulate reducing sugars when stored at temperature below 10^{o}C (Chapter 2: Figure 6; Chapter 3: Figure 8; Smith et al., 2005; Wiberley-Bradford et al., 2016; Hou et al., 2017). Cold induced sweetening has been inhibited successfully through various biotechnological solutions. These include suppressing some of the genes involved in starch degradation as well as sucrose synthesis and degradation such as repression of vacuolar acid invertase (Bhaskar et al., 2010; Clasen et al., 2016), sucrose phosphate phosphatase (Chen et al., 2008), α and β amylases (Hou et al., 2017), as well as overexpression of α-amylase inhibitor (Zhang et al., 2014). Inhibiting CIS by repressing *GWD1* in potato however may be advantageous over both the solutions listed and the repression observed in the ISA2 lines in this study (Chapter 3: Figure 8), because it leads to lines with altered starch structure conferring novel physico-chemical properties in addition to repressed CIS.

4.1.4 Summary

Starch from the transgenic lines indicate that a functional interaction exists between SBEI and GWD1 during starch biosynthesis which facilitates normal granule formation. This was shown as lines repressed in both *SBEI* and *GWD1* synthesised starch with altered granule shapes, while lines repressed only in *SBEI* or *GWD1* did not (Chapter 2: Figure 5). The interaction between SBEI and GWD1 also led to increased apparent amylose as shown by the amylose in the

SBEI/GWD1 lines compared with the GWD1 or SBEI lines (Chapter 2: Figure 3A). This has implications for the functionality of the starch such as pasting, gelling and digestive properties. The project also provided data which suggest that some level of interaction may exist between ISA2 and GWD1 in granule initiation. This was shown as small granules adhering to the surface of large granules isolated from ISA2 lines were not present in starch from ISA2/GWD1 lines (Chapter 3: Figure 5). Starches isolated from some of the transgenic lines exhibited freeze-thaw stability which may make them useful in industrial food applications such as in potato processing, frozen foods and noodles production.

4.1.5 Impact of COVID-19 on this project

Unfortunately, I couldn't ascertain possible interaction between both SBE isoforms and GWD1 because production of transgenic plants repressed in all the three genes was unsuccessful and this will need be examined in future research. The main reason for this was the negative impact that COVID-19 had on the project. During an initial hard lockdown between April - July 2020 many plants died in tissue culture, including putative SBEI/SBEII/GWD1 lines. Since then, it was only been possible to work for 2 weeks out of every 4 to maintain social distancing within the laboratory. Because of this, it was decided to concentrate on analysing the novel lines produced during the study (SBEI/GWD1, SBEII/GWD1 and ISA2/GWD1) alongside the lines where *SBEI*, *SBEII*, *GWD1* or *ISA2* genes were repressed individually. This analysis has led to novel data examining the effects of repressing *GWD1* alongside *SBEI*, *SBEII* or *ISA2* which will be useful in understanding how starch is synthesised and will hopefully help in the rational design of altered starches for industry.

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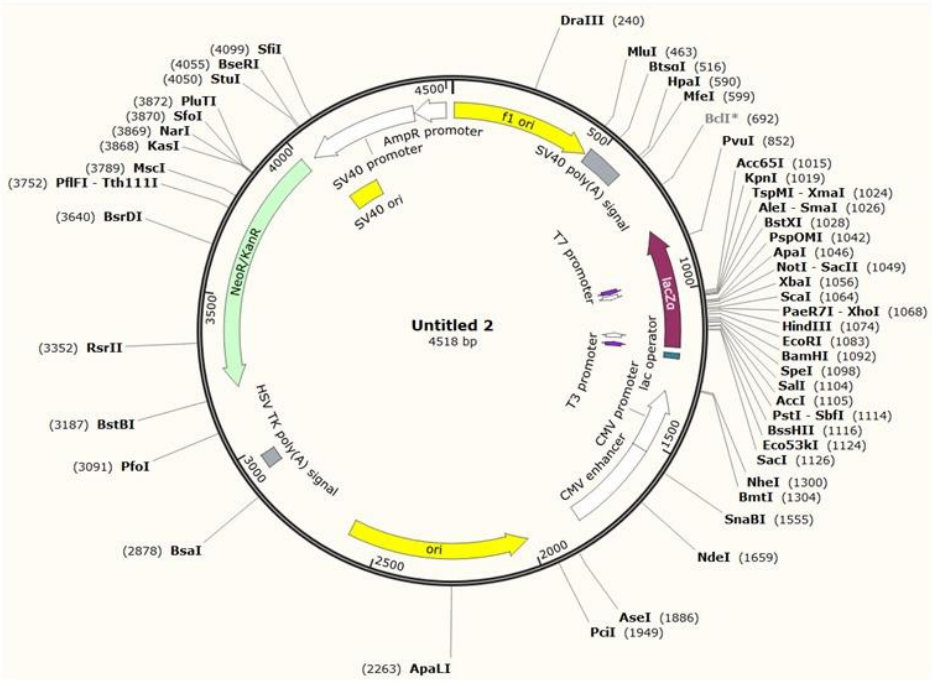
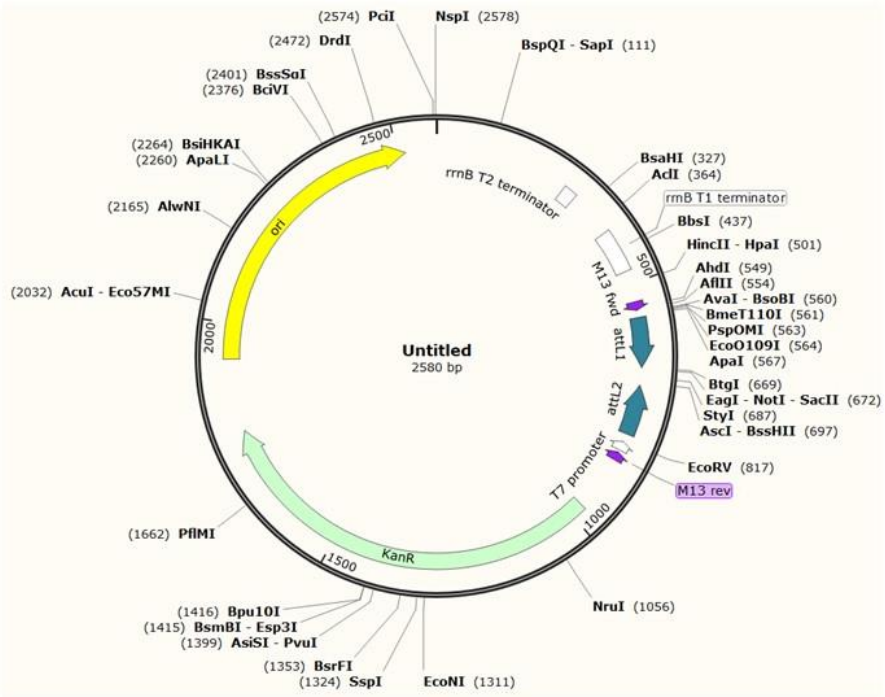
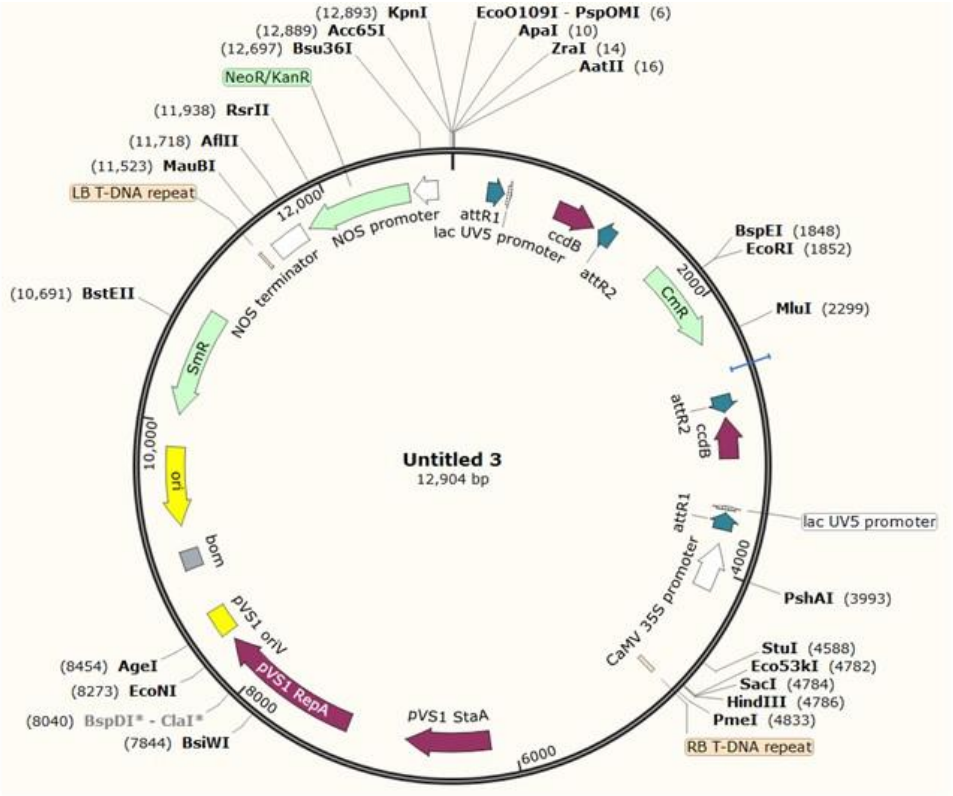
Appendix

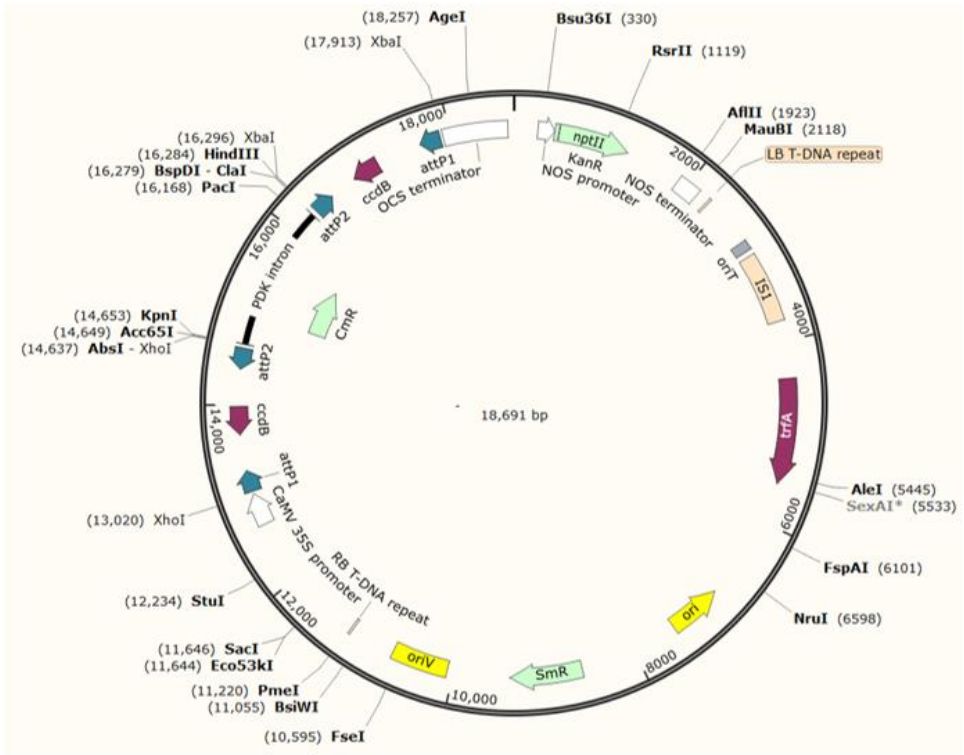


Supplementary Figure 1 | Plasmid map of donor vector (pBK-CMV)

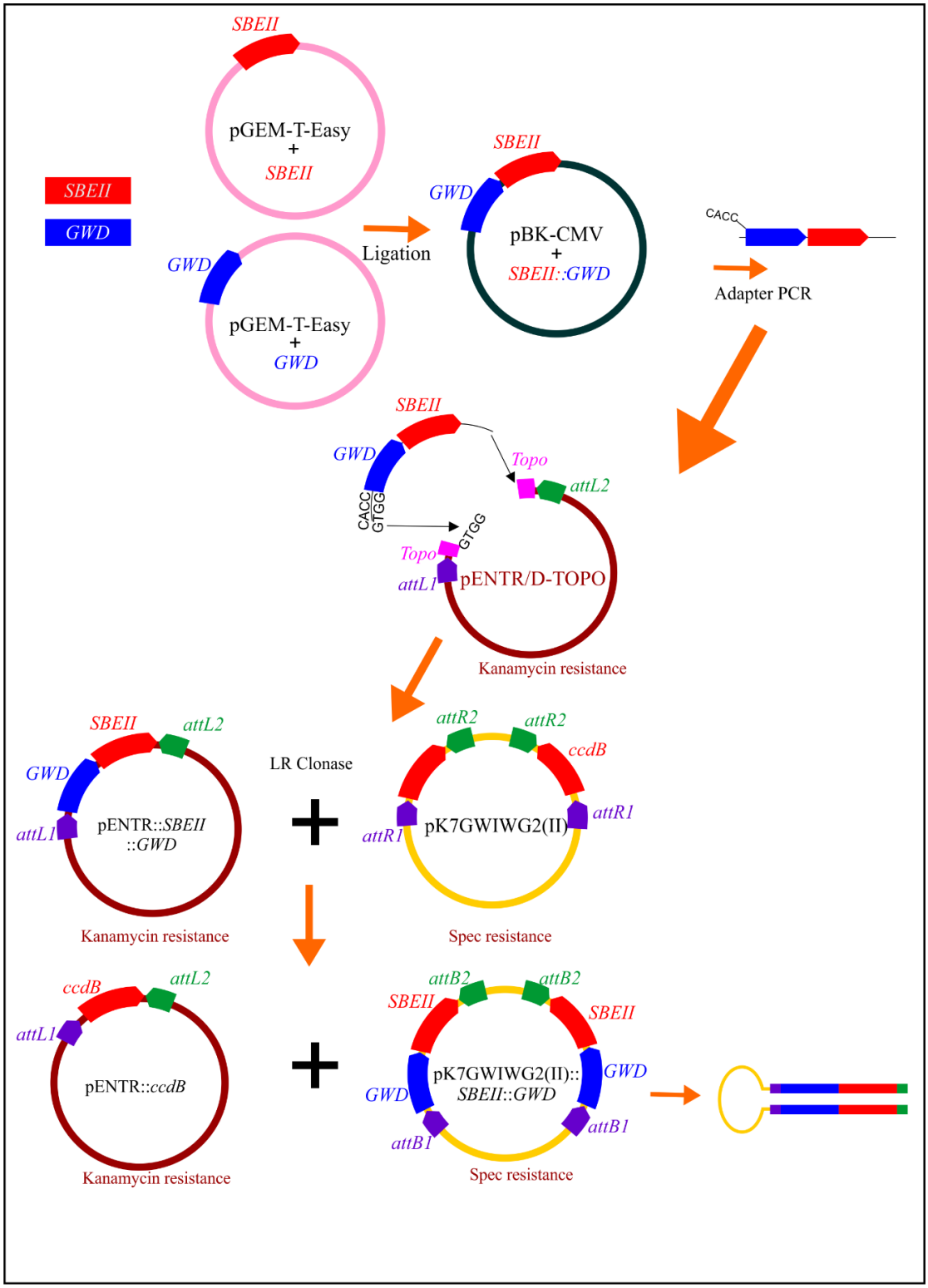


Supplementary figure 2 | Plasmid map of entry vector (pENTR/D-TOPO)

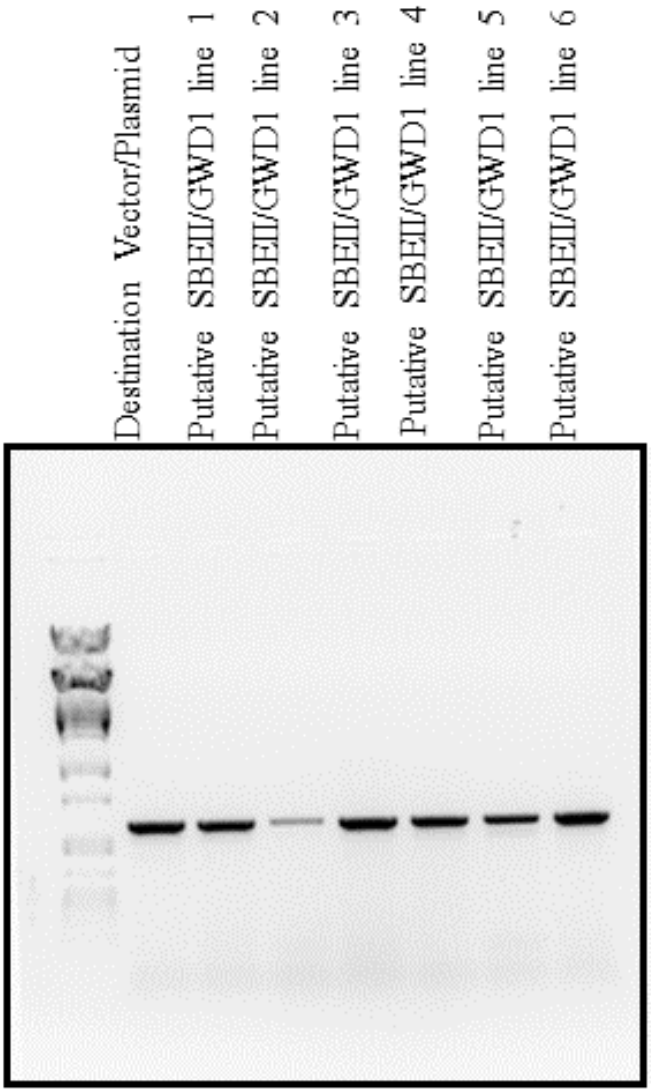




Supplementary figure 3 | Plasmid maps of destination vectors [pK7GWIWG2 (II) and pHELLSGATE]



Supplementary figure 4 | A general Illustration of the strategy used in the construction of the RNAi silencing vectors. (A) PCR amplification of *SBEI, SBEII* and *GWD*. (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 Topo adapter with PCR for the recombination reaction (E) Insertion of PCR generated gene fragment into TOPO vector (F) Recombination of the fragments into the pK7GWIWG2 (II) vector. (G) hairpin structures that form in plants after successful transformation.



Supplementary figure 5 | Gel showing the insert of construct into the genome of transgenic SBEII/GWD1 lines using PCR