Production of Amylopectin and High-Amylose Starch in Separate Potato Genotypes

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Abstract

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Starch is one of the most important processed products from agriculture. Two main outlets can be identified; starch is either enzymatically processed for the production of sweeteners and as raw material for fermentation or channelled to various applications as dry starch. Native or chemically modified starches are utilized in food as well as non-food applications, where the specific physicochemical properties are main determinants for their respective use. Starch consists of two different molecules, amylose and amylopectin. To be able to take the full benefit of the unique properties of either component it is of interest to divide the production of amylose and amylopectin into separate plant genotypes.
In the presented work, potatoes producing either amylopectin or high-amylose starch were achieved using genetic modification. For potato transformation a highly efficient protocol was developed for a herbicide selection gene instead of the commonly used *nptII* antibiotic selection gene. In order to achieve respective starch qualities, the expression of genes important for amylopectin or amylose synthesis was silenced. Antisense technology as well as the expression of dsRNA was investigated where the expression of dsRNA was determined to be at least ten-fold more efficient for gene silencing. An added benefit of dsRNA expression was that a higher fraction of silenced transgenic lines compared to the use antisense were associated with single copy T-DNA integrations.
One amylopectin potato line was furthermore characterized regarding genetic and chemical composition. The T-DNA was found integrated as an inverted repeat with the inverted repeat region extending into potato chromosomal DNA. This transgenic locus was found to be more consistent with integration into a double-stranded chromosomal break than insertion by a mechanism nicking one strand of the locus. The high-amylose trait generally resulted in a higher tuber fresh weight yield, much elevated sugar levels and a decreased starch content. Amylose levels were obtained where very limited amounts of material recognizable as amylopectin could be found. The production of amylopectin and amylose was divided into separate genotypes but additional factors are needed to be able to produce amylose at levels comparable to starch contents of cultivated potatoes.

Keywords: *Solanum tuberosum*, gene inhibition, amylose, amylopectin, granule bound starch synthase, starch branching enzyme, selection gene.

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Appendix

Papers I-V

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

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

II. Andersson, M., Melander, M., Pojmark, P., Larsson, H. Bülow, L., Hofvander, P. Targeted gene suppression by RNA interference: an efficient method for production of high amylose potato lines. (Submitted)

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

IV. Andersson, M., Turesson, H., Strömdahl, A.-C., Pojmark, P., Bülow, L., Larsson, H. and Hofvander, P. Molecular and compositional analysis of an amylopectin potato. (Submitted)

V. Andersson, M., Trifanova, A., Andersson, A.-B., Johansson, M., Bülow, L. and Hofvander, P. 2003. A novel selection system for potato transformation using a mutated AHAS gene. *Plant Cell Reports 22*, 261-267.

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Additional publication

Hofvander, P and Andersson, M. 2004. Enhanced amylose production in plants. PCT document WO 2004078983. Geneva: WIPO

Abbreviations

AGPase	ADPglucose pyrophosphorylase
AHAS	acetohydroxyacid synthase
bp	basepairs
CaMV	Cauliflower Mosaic Virus
CL	chain length
DBE	debranching enzyme
DP	degree of polymerization
DSB	double-strand break
dsRNA	double-stranded RNA
GBSS	granule bound starch synthase
GWD	α-glucan,water dikinase
kb	kilo basepairs
miRNA	micro RNA
MOS	malto-oligosaccharides
NHEJ	non-homologous end-joining
NptII	neomycin phosphotransferase II
PCR	polymerase chain reaction
Pi	inorganic phosphate
Ppi	pyrophosphate
PTGS	post transcriptional gene silencing
RISC	RNA-induced silencing complex
RNAi	RNA interference
SBE	starch branching enzyme
SEC	size exclusion chromatography
siRNA	small interfering RNA
SS	soluble starch synthase
StGH	Solanum tuberosum glycogenin homologue
TGS	transcriptional gene silencing
3-PGA	3-phosphoglyceric acid

Introduction

Starch is the second most abundant compound produced in higher plants after cellulose. While cellulose is a structural component of plants, starch mainly serves as a compound to temporarily store energy that can be accessed at a later time point. Generally starch is deposited as semi-crystalline granules, which consist of two types of molecules, amylose and amylopectin. Both molecules are polymers of glucose residues and contain the same chemical linkages. Amylopectin is a much larger molecule than amylose and consists of shorter chains of α-1,4 linked glucose residues that are connected by α-1,6 glycosidic linkages resulting in a branched structure. Amylose is an essentially linear molecule of α-1,4 linked glucose with few branches. However the α-1,4 glucan chains have a much higher average degree of polymerisation as compared to amylopectin.

In photosynthetic tissues, starch is accumulated in a diurnal fashion to store sugars produced from photosynthesis. During the dark period the starch is degraded for use as energy source and growth. Starch is also deposited and stored for longer periods in tuberous tissues and seeds where it is used as an energy source for the formation of the next plant generation. Starch granules are also produced by photosynthetic eukaryotes like green algae. Red algae accumulate floridean starch, which is deposited as granules but consists mainly of an amylopectin-like polysaccharide. By analogy another compound used to store energy is glycogen. Glycogen is composed of the same glycosidic linkages as starch and is produced in bacteria as well as fungi and animals. Glycogen is similar to amylopectin with some important differences such as a more homogeneous branching structure and shorter chain length that renders it soluble in contrast to amylopectin that form crystalline structures and is a structural component of starch granules.

Glycogen formation in fungi and animals share some components with bacteria and plants like the branching enzyme forming the α-1,6 linkages, but other steps are handled by entirely different enzymes performing analogous reactions, exemplified by the synthase forming the α-1,4 linkages. The widespread nature of glycogen and starch demonstrates the importance of α-1,6 branched α-1,4 glucans as a largely osmotically inert structure to store energy for a longer or shorter time. In some context it is conserved throughout living organisms and must be of very early evolutionary origin.

However, while these glucose polymers are biologically important to store energy, starch is also of central importance to man as a food source, who also has put the extracted component to use in many different applications. Enormous amounts of starch is processed from cultivated crops annually and subsequently used for food as well as non-food purposes. In 2000 around 48.5 million tons was produced worldwide with the most important crop plants for extraction being corn, wheat and potato (LMC International, 2002). Corn is by far the biggest crop corresponding to 80% of total starch production. The main part of the produce is converted into syrups of sugars to be used as sweeteners or substrate for fermentation reactions where the end product may be ethanol and more recently

lactic acid for the production of PLA (polylactic acid) (Corn Refiners Association, 2003; Colman, 2003).

Starch is used in both food and non-food applications. As an ingredient in foods its main function is as thickener to add viscosity and texture to food products but there are also other uses such as coatings and extruded products (Jobling, 2004). In non-food applications the larger part of the starch is consumed in the paper industry (Röper, 2002). It has uses in the wet end parts as a retention aid and to bind the cellulose fibres but is also applied for coating of paper. Other non-food uses are for example adhesives and in oil drilling.

Different applications put different demands on the properties and specifications of the starch used. Starches from the main crops are sufficiently different to make them more or less suitable for certain uses. Cereal starches in general contain more protein and lipids compared to tuber starches which affect the properties and taste of the starch (Swinkels, 1985). For food products potato starch has an advantage for some applications in that it has a bland taste and is thus neutral in relation to the product. On the other hand potato starch granules are much larger than cereal starch granules, which can be of disadvantage since this yields a more grainy texture to the product. There are also differences in molecular size of the amylose component and branching structure of the amylopectin, where potato starch contain amylose with much higher degrees of polymerization and amylopectin with longer branches compared to other sources of starch. A higher degree of polymerization is desired for applications where polymeric properties are of importance such as for film formation, while shorter side chains of amylopectin may yield improved properties of thickeners in products that are frozen or cooled. A longer chain length may cause synerises and a separation of water from the product, which is undesirable.

One type of natural chemical starch modification is found *in planta*, which is the attachment of phosphate groups to the starch backbone where potato contains considerably more phosphate than cereal starch (Swinkels, 1985). Phosphate groups yield a slightly anionic character to the starch and make it preferable for paper production. Chemical and physical modification of starches has been developed to enhance or change properties of starch or to mask undesired properties.

Starch is not very homogeneous in physical functionality since the two starch components are so different in molecular size as well as structure. A first step of improvement would be to separate amylose and amylopectin to be able to take the full benefit of either component without having to modify the starch in view of the other. Technically a separation is possible but is expensive and also has an impact on the molecular structure of the molecules. A preferred system would be to have crop plants that produce only amylopectin or amylose in a more or less pure form. This has been achieved by the identification of mutants especially in maize with first the *waxy* mutants that contain starch with only amylopectin and later *amylose extender* (*ae*) mutants that have reduced levels of branching and thereby increased levels of amylose like starch.

Technology advances including the abilities to transfer genetic material to plants for expression or inhibition of genes and the identification of individual enzymes and genes participating in the formation of starch has extended the scope of starch modification considerably. For two of the main starch crops, potato and wheat, it has been very difficult to work efficiently using mutational breeding due to the tetraploid nature of potato and hexaploidity of wheat. Especially for potato, genetic engineering has been put to use on one hand in basic research to elucidate the pathways of starch biosynthesis and the function of individual enzymes and on the other hand to produce useful modifications to starch that are of commercial importance.

Presented in this thesis is the development of tools for genetic modification of potato as well as the production of genetically modified potato lines containing either amylopectin starch or starch mainly consisting of amylose. **Paper I** and **Paper IV** are concerned with the production of high-amylose potatoes and amylopectin potatoes respectively by using antisense technology. **Paper II** further expands on the technology of gene inhibition by employing RNA interference for the production of high-amylose potatoes. **Paper III** describes the identification of a second branching enzyme of potato that was of great importance for amylopectin branching. Potato genotypes producing amylopectin or high-amylose starch are eventually intended to be released on the European market. In **Paper IV** is outlined the characterization of an amylopectin potato line regarding molecular and chemical composition which are essential parts of a European market notification. **Paper V** describes the use of a novel selection gene for highly efficient potato transformation that could replace the questioned antibiotic resistance gene *nptII*. Further discussed are secondary effects from the separation of the biosynthesis of amylopectin and amylose into separate potato lines. Emphasis regarding work by others discussed in the different chapters is on potato as this is the plant species used for the modifications. Details are also discussed regarding comparable questions in maize as this is the most important crop for starch production in the world and pea, which is representative of another starch storing tissue than tubers or endosperm. Much important work in elucidating starch biosynthesis and starch structure has been performed in rice but maize has been chosen as a representative for endosperm starch storage due to its importance as a crop for starch extraction. More recently great progress has been made regarding starch biosynthesis in wheat although wheat is also a very complicated plant species with three different genomes. In some sections reference is made to the green algae *Chlamydomonas reinhardtii*, which has been used for elucidating functionality of starch enzymes in a series of mutant studies.

Starch Structure

General

A widespread molecular structure for temporary or longer term storage of energy is α-1,6 branched α-1,4 glucan. This is also a way to accumulate sugar in a largely osmotically inert form that does not influence important metabolic pathways as free sugars would. Different types of α-1,6 branched α-1,4 glucans can be found in nature. In plants and green algae amylose and amylopectin collectively called starch is found. In red algae there is a special form of amylopectin called floridean starch and in bacteria, fungi and animals glycogen is the comparable molecule. Amylose, amylopectin, floridean starch and glycogen are molecules with a progressively increased degree of branching affecting structure and solubility. Starch and floridean starch are both found as water insoluble granules. Although while starch is synthesized in the plastids, red algae have their synthesis located to the cytoplasm. Glycogen is a water-soluble molecule due to dense branching and can be defined as a spherical dendrimeric molecule (Alonso *et al.*, 1995).

The starch granule

Storage starch of plants displays an extraordinary similarity regarding deposition, structure and composition. Generally starch is deposited as granules and consists of two components, amylopectin and amylose. Amylopectin and amylose differ considerably in molecular weight and structure The ratio between the two components is rather conserved among non-mutant green algae and higher plants with 20-30% by mass being amylose and consequently 70-80% being amylopectin.
Further the amylopectin of organisms studied displays a polymodal size distribution of α-1,4-glucan chains. Starch granules are laid down in plastids which in storage organs such as endosperm, embryo or tuber are non-photosynthetic and called amyloplasts.

However there are also quite some differences among plants. Starch granules come in different sizes and shapes depending on plant species and starch storage tissue. Amyloplasts may contain a single large granule as in potato or many smaller granules as in rice. Jane *et al.* (1994) have published a rather extensive collection of scanning electron microscopy pictures of starches from various sources including estimations of granule size distributions. Starch from the three main crop plants for starch extraction represent different typical starch granules. Granules of potato tubers are among the largest known (15-100μm) and representative for tuber and root starches. They are in shape, round for smaller to oval for larger granules and have a smooth surface with larger granules appearing mussel-like when viewed under the microscope due to the so-called growth rings. Maize endosperm starch granules are smaller than potato (5-20μm) and tend to be polyhedral in shape. Wheat endosperm displays a bimodal size distribution of starch granules that are termed A-granules (22-36μm on the long axis), which are disc shaped and B-granules (2-3μm), which are rounded.

Starch granules are semi-crystalline and contain crystalline as well as amorphous regions. The use of X-ray diffraction has defined a number of diffraction patterns that are common to different sources of starch. The A-type pattern is characteristic for cereal starches while the B-type pattern is typical for tuber starches (Imberty *et al.*, 1991). Legume starches mainly show a C-type pattern, which is a mixture between A- and B-type patterns. It is generally accepted that amylopectin is the component that determines the structure and shape of the starch granule since mutants containing starch solely composed of amylopectin morphologically and in size are the same as wild type granules.

Amylopectin

Amylopectin is very large molecule with a molecular weight of 10^7 to 10^8, which consist of α-1,6 linked α-1,4 glucan chains. 4-6% of the glycosidic linkages are of α-1,6 type. The branched structure has been defined as consisting of three classes of chains termed A, B and C (Peat, Whelan & Thomas, 1952). A-chains are linked to inner B-chains and have no glycosidically linked chains. B-chains have glycosidically linked A-chains and B-chains and are themselves linked to other B-chains or a C-chain. There is only one C-chain in one amylopectin molecule, which thus has the sole reducing end of the amylopectin molecule.

Debranched amylopectins display a polymodal distribution of chain lengths consisting of overlapping distribution curves contrasting to the unimodal size distribution of chains observed in glycogen. Polymodal size distribution has been assumed to contain discrete normally distributed size fractions of chains. The interpreted classification, number of peaks and size distribution around peaks as well as chain length at peaks, differ to some extent depending on source of starch and experimental conditions used. The similarities are more important than the differences though. The polymodal chain length distribution can be resolved into a short chain fraction of DP 12 to 17 depending on species, a largely overlapping fraction with a peak at DP 18 to 24, a third peak at DP 40 to 50 and a fourth at around DP 70 (Hizukuri, 1986; Blennow *et al.*, 2000). Generally there is a periodicity to the peaks and an amylopectin with a peak at the lower end of the size range consistently are at the lower end of the range for the other peaks as well. This indicates that even though peaks are overlapping considerably there is a non-random nature to the molar distribution of chain lengths in amylopectin. Potato is generally considered to contain fractions of longer amylopectin side chains than cereal amylopectins (Kalichevsky, Orford & Ring, 1990).

Amylose

Amylose has a molecular weight of 10^5 to 10^6 and is thus several orders of magnitude smaller than amylopectin. Determined molecular weights of amyloses from different sources vary considerably but potato starch is considered to contain an amylose of higher degree of polymerization with longer α-1,4 glucan chains as compared to cereal starches. Amylose is regarded as an essentially linear polymer of α-1,4 linked glucose residues although it does contain a limited amount of

branching, which may range from 0.1 to 1.0% depending on method of purification and analysis. Most likely the degree of branching on real amylose is in the lower range of figures given. There may be different populations of amylose with different molecular weights and degrees of branching. Amylose is a rather difficult molecule to study as there are no plant mutants producing only amylose and it thus needs to be separated by different means from amylopectin. Although even if the possibility of coexistence of amyloses with different properties is taken into account there is no structural continuum with amylopectin (Buléon *et al.*, 1998).

Starch organization

Starch granules from mutant plants that are devoid of amylose have the same size and the same three-dimensional structure as well as are undistinguishable morphologically from starch granules containing amylose. It can therefore be concluded that in non-mutant plants amylose contributes very little if at all to the structure and three-dimensional organization of the granule. Amylose is considered to be present in the amorphous regions of the starch granule, filling out space in the amylopectin three-dimensional structure.

The most accepted model for amylopectin is the cluster model, which has been drawn in a variety of similar configurations (Robin *et al.*, 1974; Manners & Matheson, 1981; French, 1984). According to this model, side chains are arranged into clusters where two adjacent chains are associated in a double helix configuration. At the base of each cluster is a branching region. It is believed that 9-10 nm repeats of alternating crystalline and amorphous lamellas observed in starch granules are related to the cluster model (Jenkins, Cameron & Donald, 1993). The clusters of side chains would then form the crystalline lamella and the branching regions would be present in the amorphous lamella. Assuming a double helical configuration of the chains in the cluster, crystalline lamella of 5-6 nm would fit rather well with DP determined for the short chain length fraction of debranched amylopectin. This would mean that longer chain length fractions of the polymodal size distribution could span more than one crystalline lamella and thus could link several branching regions and clusters as well as participate in double helices of clusters. Much focus has been on aligning the observations of non-random size distributions of the side chains to the cluster model but the non-random nature of branching clusters and positioning of branch points has also been used for incorporation into the model (Thompson, 2000). The alternating crystalline and amorphous regions have also been interpreted to represent a superhelical organization of the amylopectin molecule with clusters being constituents of the super helices and the amorphous regions representing the space between the turns of individual super helices (Oostergetel & van Bruggen, 1993).

At a next level of organization the shell structure seen in dissected starch granules interpreted as alternating 120-420 nm thick amorphous and semi-crystalline shells has been suggested to be formed by more or less spherical "blocklets" that are stacked sideways and on top of each other with smaller and larger "blocklets" being constituents of amorphous and semi-crystalline shells

respectively (Gallant, Bouchet & Baldwin, 1997). Each “blocklet” would then contain a number of crystalline lamella with the 9-10 nm spacing. It was also noted that the determined “blocklet” size of 20-500 nm in diameter (depending on species and location in the starch granule) coincides with estimation for the size range of amylopectin molecules. The shell or growth ring structure has also been speculated to be a consequence of that the highly ordered lamellar structure at a certain level becomes energetically less favourable and a transition of further growth into an amorphous state occurs (Pilling & Smith, 2003). In the amorphous zone the build up of a new ordered lamellar structure could subsequently form the next semi-crystalline shell.

What is described are still models and interpretations of experimental results, conclusive evidence and definitive proof of the detailed structural organization of the starch granule remains to be sought for. A general representation of starch structure and granule organization is shown in Figure 1. What is clear though is that the starch granule is a highly organized structure where the amylopectin structure allows dense packing of polysaccharide material. Amylose does not contribute to this structure but is evolutionary conserved among green algae and higher plants. Since amylose is believed to accommodate amorphous spaces of the granule and perhaps also to some extent participate in the crystalline regions it allows a further higher density of polysaccharide storage material to be contained within a certain volume.

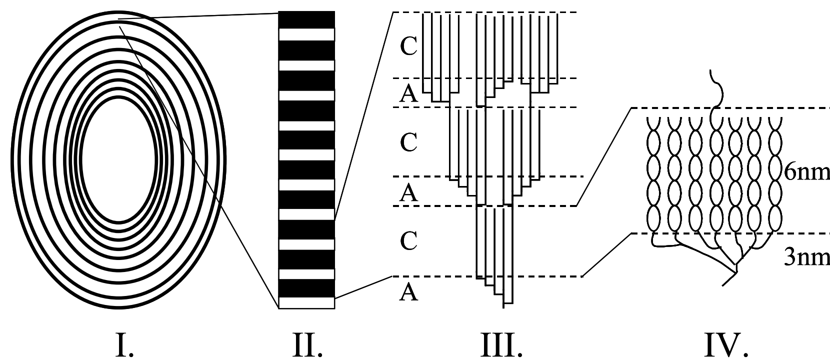


Figure 1. General representation of amylopectin structure and granule organization. I. Starch granule with crystalline shells or growth rings. II. Section of crystalline shell with concentric alternating amorphous and crystalline lamellae. III. A section of the amylopectin molecule with branching regions in the amorphous lamellae (A) and α-1,4 glucan chains clustered in the crystalline lamellae (B). IV. A branching region at the base of a cluster with helical α-1,4 glucan chains.

The enzymes of starch biosynthesis

General

A common feature among starch produced by green algae and higher plants, floridean starch produced by red algae and glycogen produced by bacteria, fungi and animals is that an activated sugar nucleotide is polymerized into an α-1,4 glucan, which subsequently is branched with α-1,4 glucan chains attached with α-1,6 linkages. There are at least two distinct enzymatic systems building the basic glucan structure where ADP-glucose is utilized by bacteria, green algae and plants, while fungi and animals use UDP-glucose as the activated sugar nucleotide for biosynthesis. Red algae most likely use UDP-glucose for the formation of floridean starch (Nyvall *et al.*, 1999).

The enzyme synthesizing the α-1,4 glucan chains from the activated sugar nucleotide share homology among higher plants, green algae and bacteria as one group and fungi and animals as a second group but there are no recognizable homologies between the two groups. A gene coding for a synthase from red algae has so far not been presented and it is thus not known, which group this enzyme belong to from a structural point of view. The enzyme responsible for the α-1,6 branches is likely to be of the same evolutionary origin as they share significant homology among all organisms.

In bacteria, which share the same evolutionary origin of genes as higher plants for α-1,4, α-1,6 glucan synthesis and form the analogous molecule glycogen there is diversity among different species regarding the organization of the genes participating in the biosynthesis and degradation of glycogen. Interestingly genes for glycogen synthesis as well as degradation are genetically linked in *Escherichia coli* and *Agrobacterium tumefaciens* (Romeo, Kumar & Preiss, 1988; Ugalde *et al.*, 1998). The genes encoding ADPglucose pyrophosphorylase (*glgC*), glycogen synthase (*glgA*) and branching enzyme (*glgB*) are in close operons together with the genes coding for the degrading enzymes, glycogen phosphorylase (*glgP*) and debranching enzyme (*glgX* or *glgDB*). In *A. tumefaciens* phosphoglucomutase (*pgm*) is also associated with the glycogen operon. Phosphoglucomutase is crucial for glycogen as well as starch biosynthesis in that it produces the substrate glucose-1-phosphate utilized by ADPglucose pyrophosphorylase (AGPase) (Harrison *et al.*, 2000; Marroquí *et al.*, 2001). In other bacteria such as *Bacillus subtilis* fewer of the genes are associated at the same locus and in Cyanobacteria there is no genetic linkage among the genes at all. Higher plants are believed to have an evolutional relationship to Cyanobacteria or similar photosynthetic bacteria in the photosynthetic chloroplasts that are thought to be remnants of invading bacteria.

The key enzymes for starch biosynthesis are thus AGPase, starch synthases and branching enzymes controlling the three basic steps required for the formation of starch; Generation of the sugar nucleotide ADP-glucose by AGPase, the polymerization of glucose residues by starch synthases forming α-1,4 glucans and branching enzymes that cleaves α-1,4 glucans and reattach the cleaved chain to an α-1,4 glucan chain by an α-1,6 glycosidic linkage thereby forming a branch.

ADPglucose pyrophosphorylase

ADPglucose pyrophosphorylase (AGPase) is responsible for the first committed step leading to starch synthesis. ADP-glucose is formed from glucose-1-phosphate in an ATP consuming reaction. This reaction releases pyrophosphate (PPi), which subsequently is degraded to two phosphates by inorganic pyrophosphatase.

AGPase is at least in some forms and plants subjected to a tight control by metabolites where inorganic phosphate (Pi) inhibits the enzyme and 3-phosphoglyceric acid (3-PGA) activates it (Ghosh & Preiss, 1966). Bacterial ADPglucose phosphorylases generally are regulated by other metabolites with fructose-1,6-bisphosphate being an activator and AMP an inhibitor although the AGPase of photosynthetic Cyanobacteria is regulated in a similar fashion as in higher plants (Iglesias, Kakefuda & Preiss, 1991). The regulation by 3-PGA being an activator and Pi being an inhibitor most likely is an evolutionary tuning effect where 3-PGA is one of the first products of photosynthesis and Pi is used for ATP generation and thus serves as important indicators for the physiological and metabolic state of the cells. Endosperm AGPases of cereals as maize and barley seem to be less regulated as compared to dicot AGPases of leaves and fruits.

Overall there is likely a tissue specificity affecting the properties of different forms of AGPases expressed. In cereal endosperm the enzyme activity is almost exclusively extraplastidial (Thorbjørnsen *et al.*, 1996; Denyer *et al.*, 1996) while in in all tissues examined of dicots, AGPase activity is localized to the chloroplasts or amyloplasts. Upon closer examination of activation and inhibition patterns of plant AGPases there is a variation among species and tissues where activation by 3-PGA can be on its own or in the presence of Pi (Ballicora, Iglesias & Preiss, 2004). For potatoes, AGPase has also been suggested to be regulated by redox modulation where higher sucrose concentration in the tuber correlates with activation of AGPase (Tiessen *et al.*, 2002).

Plant AGPases are heterotetramers consisting of two small and two large subunits. The difference in size is very small (50-55kDa for small subunit and 51-60kDa for large subunit depending on plant species) and genes coding for both types of subunits are of the same evolutionary origin. This is also evident from the structure of the prokaryotic enzyme, which is of homotetrameric nature. The specific plant subunits are also active as homotetramers although not very efficient as shown by expression in *E. coli* of the large and small potato subunits respectively (Ballicora *et al.*, 1995). Various attempts have been made to attribute specific functions or a functional divergence between the large and small subunits but it now seems as if both subunits contribute to both catalytic and allosteric properties of the enzyme (Cross *et al.*, 2004).

Mutations in genes encoding either subunit greatly impact the starch formation in the organ where the particular gene is expressed. In maize *brittle-2* (*Bt2*) and *shrunken-2* (*Sh2*) mutants, affecting the small and large subunit respectively, both result in 90-95% reduction in AGPase activity in endosperm and a concomitant 75% reduction in starch. In potato, transgenic studies have been performed using antisense inhibition to reduce the enzyme activity (Müller-Röber, Sonnewald & Willmitzer, 1992). The AGPase activity in potato tubers could be reduced to 2% of control levels, which as in maize had a great impact on starch deposition and was reduced down to 5% of control levels in tubers. Other effects observed were more but smaller tubers produced and a large increase in sucrose and glucose (Müller-Röber, Sonnewald & Willmitzer, 1992). In subsequent experiments on potato it was shown that inhibition by antisense technology also resulted in smaller starch granules and a reduction of amylose content of the starch (Lloyd et al., 1999a). Most likely this is an effect of the reduced substrate supply for starch synthases where GBSS has a high K_m for ADP-glucose. These results are also in agreement with other studies on the amylose content of mutants for genes coding for AGPase subunits in *Chlamydomonas* (Van den Koornhuyse *et al.*, 1996) and pea where amylose content could be modulated by exogenously applied ADP-glucose (Clarke *et al.*, 1999).

Since this enzyme is so important for starch synthesis and considered by some to be rate-limiting, attempts have been made to enhance the enzyme activity by expressing a mutant *E. coli* AGPase (*glgC16*) that is less dependent on activation and less sensitive to inhibition (Stark *et al.*, 1992). Starch content in potato variety Russet Burbank could be increased by more than 30% when localizing the enzyme to plastids. To be noted though is that the starch levels achieved are still moderate in comparison with what is possible with conventional breeding. It is not clear though whether this was specific for the experimental set up and potato variety transformed since other experiments in potato using the same gene did not yield an increased starch content although showing an increased rate of starch synthesis (Sweetlove, Burell & ap Rees, 1996a,b). Most likely there are other enzymatic activities in starch storing tissues that are of higher importance for the control of flux into starch.

An interesting aspect of AGPase is its apparent evolution from the homotetrameric state and allosteric properties of bacterial enzymes in general to the changed allosteric properties of Cyanobacteria that are more useful for a photosynthetic organism. Furthermore in plants it has possibly migrated to the nuclear genome and through gene duplication assumed a heterotetrameric nature with two subunits of different size but with a signal peptide directing the protein product to chloroplasts and amyloplasts. Finally we see the cytoplasmic localisation of the enzyme in cereal endosperm and a loss of allosteric properties.

Granule bound starch synthase

At least four isoforms of starch synthases can be distinguished in plants based on their molecular weight, domain structure and amino acid sequence (Kossmann & Lloyd, 2000). All starch synthases have some degree of affinity for starch and can be found associated with starch although they can be divided into those that mainly are in the soluble fraction and one isoform that exclusively is found bound to the starch granules. Granule bound starch synthase (GBSS) was early implicated as responsible for the synthesis of amylose in endosperm storage starch (Nelson & Rines, 1962). Plants defective for this enzyme are easily identified by iodine staining of starch, which turns reddish-brown while wild type starch stains bluish-violet. Alternatively null mutants may be identified by the lack of the major starch granule bound protein, as GBSS constitutes about 80% of the protein in the starch granule. Endosperm mutants for GBSS (*waxy*) producing solely amylopectin starch have been known for a long time in important crop plants as maize and rice. More recently mutants have also been identified in potato (*amf*) and pea (*lam*) (Hovenkamp-Hermelink *et al.*, 1987; Denyer *et al.*, 1995). In wheat, the waxy genotype has been accomplished by the identification of mutants for GBSS of the individual genomes and subsequent cross breeding to achieve homozygosity for all the mutant genes (Nakamura *et al.*, 1995).

In maize, pea, rice and wheat mutants for GBSS, amylose is still produced in leaves suggesting a different genetic locus responsible for amylose synthesis in leaf tissue. For pea a second gene with a deduced amino acid sequence highly similar to GBSS has been isolated and characterized (Denyer *et al.*, 1997). This form has been named GBSSIb and is thought to be responsible for the amylose synthesized in leaf. In potato only one gene coding for GBSS has been identified and in the *amf* mutant, amylose is reported to be lacking in leaf as well as tuber starch (Jacobsen *et al.*, 1989).

Due to the tetraploid nature of potato and its genetic complexity, studies by reverse genetics have mostly employed antisense or related technologies for the silencing of genes. Technology for the production of potato lines with tuber starch consisting solely of amylopectin has been developed using genetic engineering (Visser *et al.*, 1991; Hofvander *et al.*, 1992). **Paper IV** describes the characterization of an amylopectin potato line that was produced using antisense technology and which has proceeded to development for a notification to be released on the market in Europe. For the development of this and other transgenic potato lines a genomic clone corresponding to the locus of the *gbss* gene was isolated from a potato genomic library. Fragments corresponding to different regions of the *gbss* gene were used to generate antisense constructs for determination of the region best suited for inhibition of the *gbss* gene. Three fragments found effective are shown in Figure 2. CaMV 35S and potato *gbss* promoters were used to control the expression of the three antisense fragments in various combinations (Hofvander *et al.*, 1992).

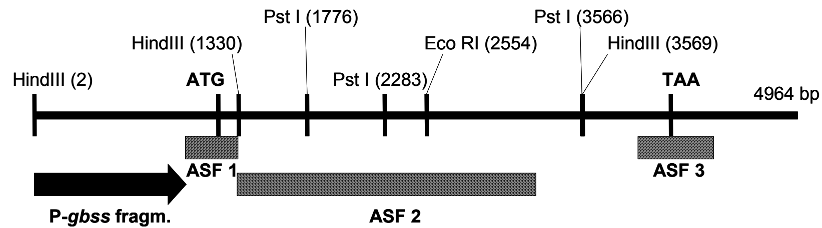


Figure 2. Map of gbss genomic locus with gbss promoter (P-gbss fragm.) and fragments expressed in antisense antisense (ASF 1, ASF 2 and ASF 3) indicated.

After transformation of the gene constructs to several potato varieties the efficiency of inhibiting gene expression was investigated. Starch from tissue culture microtubers were either stained with iodine or determined for amylose content. All three antisense fragments were found to induce inhibition of *gbss* gene expression although the degree of inhibition, stability and also the ratio of regenerated potato lines displaying inhibition differed among the promoter and antisense fragment combinations tested. In our hands the use of CaMV 35S and *gbss* promoters resulted in lines with a high degree of silencing in tubers. A difference could be seen between the use of CaMV 35S and *gbss* promoter in that for CaMV 35S many lines that showed a complete amylopectin phenotype in tissue culture had a less pronounced phenotype in the field while when using the *gbss* promoter the fraction of starch being amylopectin improved from tissue culture to field conditions. In contrast to when using the CaMV 35S promoter for antisense expression, expression by the *gbss* promoter never provided an unstable phenotype. Although all antisense fragments could provide complete silencing the ratio of lines with a high degree of silencing and thus containing starch essentially amylose free was better with fragments ASF2 and ASF3 (Figure 2.). Subsequently a cDNA fragment of *gbss* was also used for antisense inhibition using the *gbss* promoter as controlling element. Results from these studies were generally in agreement with what has been published by Kuipers *et al.* (1995) although in our hands also genomic fragments from the central region of the *gbss* gene readily yielded lines with complete inhibition of which potato line EH92-527-1 (**Paper IV**) is one representative. Notably this line did not produce a blue core, containing supposedly amylose-like material, upon staining with iodine (Figure 3A). Further no peak representing amylose could be detected when performing Size Exclusion Chromatography (SEC) of debranched starch derived from the potato line as shown in Figure 3B. The blue core has been reported to be characteristic for transgenic lines in comparison to the *amf* mutant, which stains homogenously reddish brown, suggesting that a 100% complete inhibition cannot be achieved using transformation of antisense constructs (Kuipers, Jacobsen & Visser, 1994).

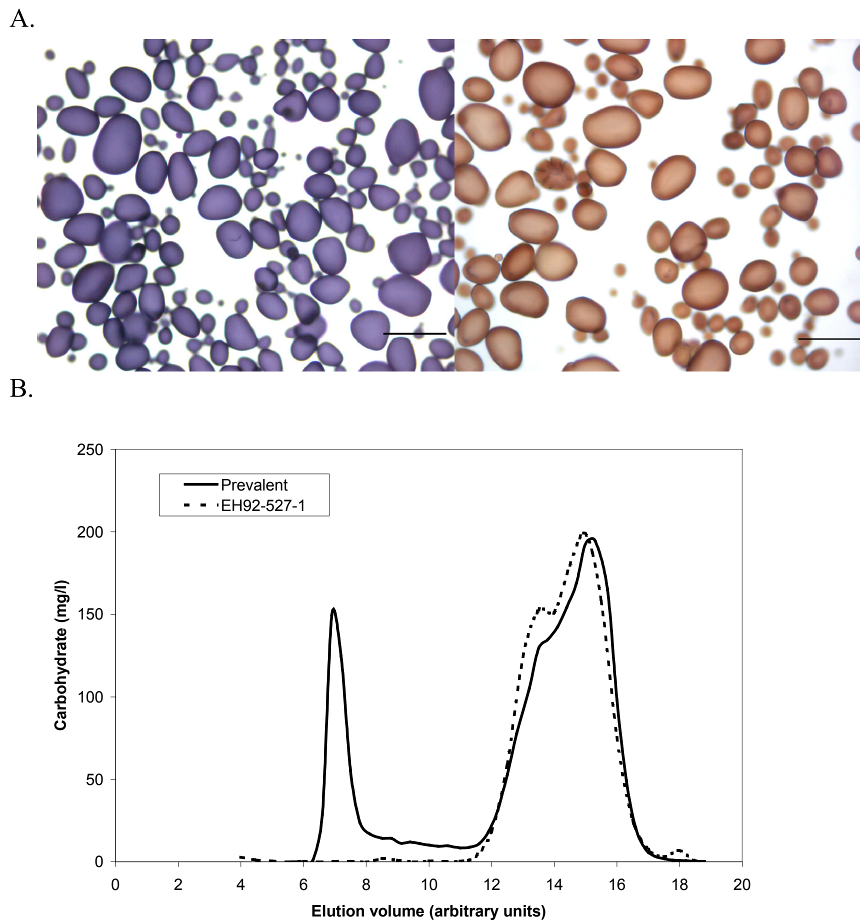


Figure 3. A. Starch granules stained with Iodine, size bar is 100 µm. Left hand picture: Ordinary potato starch. Right hand picture: Starch of transgenic high amylopectin line EH92-527-1. *B.* Size exclusion chromatography (SEC) diagram of debranched starch from EH92-527-1 and Prevalent showing the lack of amylose in starch from EH92-527-1.

The studied potato line of **Paper IV** did not reveal any significant reductions in starch content. A deficiency in amylose synthesis hence seems to largely be compensated by additional amylopectin production. The concentration of soluble sugars was significantly increased with around 30%, which may be related to the loss in starch biosynthetic capacity. Curiously vitamin C in the tuber is also increased by around 30-40%. Most probably the increase in simple sugars, which are at the root of ascorbic acid production, stimulate the production of increased levels of vitamin C.

Potato amylopectin starch derived from the potato line of **Paper IV** as well as from transgenic potato lines produced by others has been the subject of characterization and application development by many scientific groups and companies (Nilsson *et al.*, 1996; Visser *et al.*, 1997; Fredriksson *et al.*, 1998; McPherson & Jane, 1999; Svegmark *et al.*, 2002). The amylopectin chain length distribution of potato line EH92-527-1 indicated that it is similar to its parental

variety as well as other potato varieties (Fredriksson *et al.*, 1998) although slight differences in chain length peak maxima could be found. This suggests that GBSS has very limited impact if any on amylopectin chain length distribution in potato. The degree of branching for starch isolated from EH92-527-1 has been determined to 4.2%, which was similar to that of purified amylopectin (4.1%) from a commercial source (Nilsson *et al.*, 1996).

The link between GBSS and amylose synthesis has firmly been established but the biochemical properties of the enzyme have been found difficult to definitely determine. One problem experienced is that GBSS has very poor or even no detectable activity if not embedded in a starch context. It has been demonstrated that potato GBSS activity is strongly dependent on amylopectin concentration while potato soluble starch synthase II (SSII) activity is not (Edwards *et al.*, 1999). This characteristic makes it necessary to study amylose synthesis in isolated starch granules or other mixed environments. Another character that distinguishes GBSS from at least SSII is the processive elongation of malto-oligosaccharides (MOS) by GBSS (Denyer *et al.*, 1999a,b). That GBSS in contrast to SSII can add consecutive glucose residues to the α-1,4 glucan chain without dissociating from it may hold one of the keys to the ability of GBSS to synthesize very long chains. In the elucidation of amylose synthesis two different perspectives have been proposed in recent times. First is the perspective that amylose synthesis by GBSS starts as very long branches of amylopectin, which are then processed by some unknown hydrolytic activity to be further used in formation of the molecule known as amylose (van de Wal *et al.*, 1998). Second is the perspective of small MOS and ADP-glucose that diffuse into the starch granule where GBSS resides and the MOS are then processively elongated to form amylose (Denyer *et al.*, 1999a,b). Data in support of both perspectives have been presented in a number of publications although definitive proof for by what mechanism and substrates GBSS synthesizes amylose *in vivo* still remains to be presented.

In general agreement among publications is that at least in higher plants GBSS contributes under normal conditions very little if at all to the final amylopectin molecular structure. Under some conditions it may be that GBSS interferes with normal amylopectin biosynthesis though. GBSS has a much higher K_m for ADP-glucose in comparison with soluble starch synthases. The concentration of ADP-glucose has been estimated for pea embryos and was found to be much below saturating levels for GBSS (Clarke *et al.*, 1999). This may affect GBSS activity in plants that carry mutations for other genes involved in starch biosynthesis. Increases or decreases of ADP-glucose most likely leads to an altered contribution by GBSS to starch synthesis and maybe also to amylopectin structure as described under “Soluble starch synthases”.

Interestingly, even though conditions are not optimal for GBSS, it is responsible for around 25% of the total starch, produced in an essentially linear form in the presence of starch branching enzymes. There may be several different explanations or combinations of them as to how GBSS can achieve this. GBSS is believed to function inside the starch granule and this may be an environment where starch branching enzymes can either not access the growing amylose polymer or has poor activity. GBSS could also protect the amylose being

synthesized from branching by the strong affinity it has for starch. Possibly GBSS makes amylose when inside the granule while it acts also in amylopectin biosynthesis when it is closer to the granule surface. There are indications from mutants that this could be the case as when more open structures with lower crystallinity exists there is an impact on amylopectin with longer chains being contained within the amylopectin fraction. So even though it is perfectly clear what happens if GBSS is not present in the amyloplast, much remains to be learned concerning by what mechanism this enzyme manages to synthesize 25% of the total starch amount if present.

Soluble starch synthases

At least three distinguishable isoforms of soluble starch synthases (SS) exist in plants. The three different groups, SSI, SSII and SSIII, can be defined according to amino acid sequence homology and molecular weight. Their role can be assigned to the formation of α-1,4 glucan chains of the amylopectin biosynthesis. All have to a lesser or greater extent affinity for starch and can be found among the proteins entrapped or bound to the starch granule. Their respective distribution between the soluble phase or starch granule has led to some speculation and questions on whether in some plant species they should be assigned to the group of granule bound starch synthases or soluble starch synthases. However all three groups in all plant species are now referred to as soluble starch synthases.

Soluble starch synthases contain an N-terminal domain predicted to be flexible, characterized by proline amino acids at the flexible joint (Marshall *et al.*, 1996). The function of this flexible extension is still unknown although several suggestions have been made such as being related to substrate affinity, a role for how long chains are produced or being of importance for the interaction with other enzymes such as starch branching enzymes. Interestingly also one of the starch branching enzyme isoforms has been noted to contain a predicted flexible extension (Burton *et al.*, 1995).

Representatives of all three groups have been studied in potatoes regarding expression patterns and transgenic plants where antisense inhibition have been used to silence respective genes. Reverse genetics has been applied for silencing soluble starch synthase genes individually as well as in various combinations. From studies on tuber starch from transgenic plants it has been possible to assign what functional importance respective isoforms have for starch structure and deposition. SSI of potatoes seems to be of minor importance for amylopectin in tuber starch and is mainly expressed in photosynthetic tissues (Kossmann *et al.*, 1999). Antisense inhibition of SSI resulted in no significant changes of the amylopectin in potato tuber starch. Potato tuber SSII accounts for approximately 10 to 15% of total tuber soluble starch synthase activity and antisense experiments inhibiting the expression of SSII did not yield any significant alterations to the amylose/amylopectin ratio (Edwards *et al.*, 1995). The major soluble starch synthase of potato tubers has been determined to the SSSIII form, which constitutes 80 to 85% of the total soluble starch synthase activity (Abel *et al.*, 1996 Marshall *et al.*, 1996). Inhibition of SSIII by antisense technology leads to

interesting effects on starch granule formation. Starch granules either had cracks and fissures or were present as clusters of small granules. No structural changes to the chain length distribution could be determined and amylose content was not altered. In one of the publications a 70% increase of starch phosphate was noted. It was also reported that starch content was unaffected. However it is unlikely that this could be determined from the experimental set-up since tests were restricted to greenhouse grown tubers. Upon further investigation an altered chain length pattern was found in the amylopectin of SSII or SSIII inhibited potato tubers (Edwards *et al.*, 1999; Lloyd, Landschütze & Kossmann, 1999b). Lines where both SSII and SSIII had been inhibited were also characterized. In SSIII inhibited potato lines the amylopectin was a considerable increase in DP 6 chains while in SSII inhibited lines it was enriched in DP 8-12 chains and reduced DP 15-25 chains. The effect was amplified when both starch synthases were inhibited with a concomitant decrease of intermediate chains and increase of a longer chain length fraction.

In pea embryo it is the SSII form that is responsible for the most of soluble starch synthase activity and accounts for 60-70% of the activity (Denyer *et al.*, 1992). Mutants in the locus coding for SSII, *rug5* (*rugosus5*) yields similar effects on the starch as is seen in the antisense experiments of combined inhibitions of potato SSII and SSIII. Starch granule morphology is altered resulting in multilobed granules. Further the fraction of intermediate chains is decreased while the short chain fraction is increased. An increase in the long chain fraction of amylopectin could also be detected (Craig *et al.*, 1998). For maize one mutation locus, *dull1*, has conclusively been identified that corresponds to a soluble starch synthase (Gao *et al.*, 1998). There are also other mutations that affect soluble starch synthase activity although these have either been shown to be attributed to other genes and are thus secondary effects or the gene corresponding to a specific locus so far remains unidentified. The *dull1* locus encodes a soluble starch synthase that regarding amino acid sequence homologies can be grouped among the SSIII enzymes of potato and pea (Gao *et al.*, 1998). Starch granule morphology is not altered although it has been noted that granules are smaller. Upon dissection of starch composition and structure it was found that the apparent amylose content was elevated and the starch contained a substantial amount of intermediary material, which was suggested to be lower molecular weight amylopectin with extended chains. Debranched amylopectin displayed an increase in the short chain fraction and a decrease in the intermediate fraction of amylopectin chains (Wang *et al.*, 1993a,b). The soluble starch synthase activity in maize can be separated into two activity peaks where SSIII belongs to the second peak. Another major starch synthase activity exists in maize of which an SSI enzyme form is responsible (Mu *et al.*, 1994; Knight *et al.*, 1998). In the soluble fraction of maize endosperm SSIII accounts for 17 to 24% while SSI was reported to be responsible for 47 to 66% of the soluble starch synthase activity. It was concluded that these two isoforms are responsible for nearly all soluble starch synthase activity found in maize endosperm (Cao *et al.*, 1999). The definitive roles of respective enzyme forms in maize still remains to be elucidated as no mutants, so far, have been reported for SSI. Soluble starch synthases belonging to the other

groups SSI and SSIII of pea and SSII of maize have been identified although they have not been determined as important for amylopectin structure in storage tissue.

Interestingly different isoforms seem to be the main contributor to total soluble starch synthase activity in different starch storing plant species. In maize SSI accounts for the major part of endosperm soluble starch synthase activity while in the pea embryo SSII and in potato tuber SSIII are the major soluble starch synthases. Mutations or inhibition by transgenic techniques that impact soluble starch synthases, which significantly contribute to the total activity, more or less results in similar patterns, with an increased proportion of short and long chains while the fraction of intermediate chains is reduced. An interesting question is whether this is an effect of the reduced overall enzymatic activity in these plant organs or if specific isoforms are contributing more or less to specific chain length fractions. The sharp peak at CL 6 of SSIII inhibited lines in potato could be a indication of that total activity has decreased to a point where it cannot balance starch branching enzyme activity. Starch branching enzymes that are allowed access to substrates without interference from other enzymes tend to yield a final product with a significant CL 6 fraction. However if combined, the results obtained in the SSII and SSIII antisense experiments in potato would suggest a different probability spectrum of chain length synthesis for SSII and SSIII respectively.

It has been suggested that some of the observations made regarding resulting starch structure in SS mutants or antisense lines might be attributed to activity by GBSS (Craig *et al.*, 1998). The increased concentration of sugars that is likely to occur in this background of reduced starch formation may affect the activity of GBSS that has a comparably high K_m for ADP-glucose or GBSS activity may benefit from the altered starch structure. This is supported by that if expression of GBSS is inhibited in combination with SSIII in potato then starch granules visually assumes a normal morphology (Fulton *et al.*, 2002). When combining SSII, SSIII and GBSS inhibition similar results were obtained with a restored granule structure and increase in shorter chains (Jobling *et al.*, 2002). Another plausible explanation could be that the alteration in starch synthesis makes amylose chains more accessible to branching enzymes, which could explain the amylopectin molecules of lower molecular weight observed in some mutants.

Conflicting with that total starch synthase activity is the main factor in determining chain length distribution are results from the heterologous expression of the *E. coli* glycogen synthase (*glgA*) in potato tubers (Shewmaker *et al.*, 1994). The transgenic potatoes had lower starch content, lower amylose content, increased short chains and decreased intermediate chains of amylopectin. This would imply that the increased soluble synthase activity has an impact on starch structure due to the specific enzymatic properties of the introduced bacterial glycogen synthase possibly in combination with the ability of potato starch branching enzymes to interact with the α-1,4 glucan chains produced.

A picture emerges where starch formation is a fine tuned enzyme concert of activities where an imbalance could result in abnormal starch granule formation implicating GBSS as responsible for granule structure changes.

Branching enzymes

Starch branching enzymes belong to a larger family of starch hydrolytic enzymes that contain a number of recognizable motifs and which coding genes most likely are distantly related. To this group of enzymes belong among others various amylases, branching enzymes and various debranching enzymes. They all contain a catalytic (β/α)8-barrel domain composed of eight parallel β-strands interlinked with eight parallel α-helices where length of motifs, distances between and specific amino acids may be important for respective functions and specificities (Jespersen *et al.*, 1993).

Two major isoforms of starch branching enzymes have been identified in higher plants. A consistent naming of these forms has been difficult since different nomenclature have been applied depending on plant species studied. For example SBEI from maize is comparable to SBEII from pea. More recently a suggestion has been made to use class A and class B, where class A would consist of SBEII types of maize and potato, SBEI of pea and RBE3 of rice while class B would consist of SBEI types of maize, potato and rice and SBEII of pea. However in recent publications the nomenclature of rice genes seems to have been adapted to the maize nomenclature and further new genes isolated from wheat have been assigned using the maize nomenclature. In the discussion below the maize numbering of starch branching enzymes is used except for pea where class A and B is used in order to not confuse comparisons.

Defects in branching enzyme activity are associated with an increased ratio of amylose in the starch and a modified amylopectin with a reduced branching frequency (Boyer & Preiss, 1978; Smith, 1988). Invariably this has been associated with the SBEII (class A) isoform of starch branching enzyme (Boyer & Preiss, 1978, Bhattacharyya *et al.*, 1990; Mizuno *et al.*, 1993). In maize two genes have been identified that correspond to the SBEII isoform, *Sbe2a* and *Sbe2b*, and they are thus expressed independently (Gao *et al.*, 1997). It is mutations in *Sbe2b* that are associated with an increased amylose content of maize endosperm (Boyer & Preiss, 1978). Although there are also pleiotropic effects of the mutation affecting the activity of several other enzymes associated with starch biosynthesis. Comparable mutants have also been identified in rice (*ae*) and as the *rugosus* (*r*) mutant of pea. The consequences of the lack of the SBEII (class A) isoform in these plant species are to a large extent similar. Starch production is reduced by 30 to 40%, furthermore starch granules are smaller and have an irregular fissured appearance. Apparent amylose content is increased to levels between 50 and 70% of the starch. However some of this apparent increase is due to the production of a modified amylopectin with longer chains, which may be detected as amylose. In *amylose extender* (*ae*) maize starch an intermediate material of lower molecular weight than normal amylopectin can be found and the fraction of short chains in the amylopectin is decreased while the fraction of long chain material is increased (Takeda, Guan & Preiss, 1993, Wang *et al.*, 1993b). For maize endosperm and pea embryo experimental evidence shows that the branching enzyme lacking in *ae* and *r* mutants respectively provides the absolute majority of total branching enzyme activity. In *ae* mutants branching enzyme activity is reported as 82 % lower than in the wild type (Singletary *et al.*, 1997). In pea *r* mutants only 10 to 20% residual

starch branching enzyme activity remains in the embryo (Edwards, Green & ap Rees, 1988).

An insertional mutation in maize at the locus coding for SBEIIa altered leaf starch drastically but there was no impact on endosperm starch suggesting this enzyme to be of importance for starch biosynthesis of photosynthetic tissues (Blauth *et al.*, 2001). Recently *Sbe1* mutants have been identified in maize (Blauth *et al.*, 2002) and rice (Satoh *et al.*, 2003). Endosperm starch isolated from the maize mutant revealed no changes in starch structure and chain length distribution. This also applied to starch isolated from leaf. In rice however some consistent but rather moderate changes could be detected with increases in the molar distribution of chains below DP 10 and in the range 24<DP<34 with the concomitant decrease in the range 24<DP<34 and above DP 37. Starch isolated from the rice *Sbe1* mutant was also reported to display altered physicochemical properties.

For quite some time only the isoform corresponding to SBEI could be identified in potato tubers and was noted in scientific papers (Borovsky, Smith & Whelan, 1975; Vos-Scheperkeuter *et al.*, 1989; Kossmann *et al.*, 1991; Flipse *et al.*, 1996). Using antisense technology for the inhibition of the gene it was possible to reduce total branching enzyme activity in potato tuber by 90% (Kossmann, 1992). However this did not lead to any significant changes in the amylose/amylopectin ratio although some effects on soluble carbohydrate extracts was noted. When performing antisense inhibition of *Sbe1* in the *amf* mutant of potato a blue core was visible in iodine stained granules indicating a fraction of longer chain material (Flipse *et al.*, 1996). The difference in staining between transgene and control does indicate that the branching pattern of at least one polysaccharide component in the tuber is affected by the inhibition of *Sbe1*. When investigating the starch structure no significant changes could be found although there were some changes in physicochemical properties. Later an up to 98% reduction of total starch branching enzyme activity in potato has been reported using antisense directed to *Sbe1* with similar results (Safford *et al.*, 1998). A change in physicochemical properties could be found when branching enzyme activity was reduced by at least 95% but there were no alterations in amylose content or branching structure of amylopectin. One chemical change could be detected though; phosphorous content of starch was increased by 50-100%, which might explain the changes in physicochemical properties observed.

In **Paper III** is described the digestion and subsequent sequencing of peptides derived from starch granule bound or entrapped proteins of a potato line with *gbss* expression inhibited as well as from an ordinary table potato cultivar. From these experiments peptide sequences could be identified that were not consistent with the previously isolated starch branching enzyme of potato but were more similar to amino acid sequences of the class A branching enzyme of pea. The subsequent isolation of a cDNA fragment by PCR using peptide sequences for the generation of degenerate primers conclusively showed that potato in alignment with other studied plants species contained a gene coding for a branching enzyme of the SBEII type. In **Paper III** it is shown the value of identifying proteins that have an enzymatic role in starch biosynthesis by focusing on proteins that are associated with the starch. Further enhancement of the protein resolution was achieved by

using a potato line where the GBSS protein had been eliminated by the use of antisense technology to inhibit the coding gene. As the GBSS protein constitutes approximately 80% of the protein in the starch granules other proteins become more visible and contaminations by this protein in peptide fractions is reduced. In a parallel approach another cDNA fragment was isolated corresponding to the SBEII isoform by using PCR and designing degenerate primers based on conserved regions when comparing the amino acid sequence of SBEII of maize and SBEI of pea, as shown in Figure 4.

Peptide 3:	YNAVQIMA
Primer BE2.3:	5´-TAYAAYGCIGTICARATHATGGC-3´
Peptide 4:	AFWLMDKD
Primer BE2.4:	5´-TCYTTRTCCATIARCCARAAIGC-3´

Figure 4. Conserved peptides between pea and maize SBEII isoforms used for the design of degenerate primers BE2.3 and BE2.4 for cDNA fragment isolation in potato.

Despite extensive efforts it was not possible to isolate a full-length cDNA from a potato cDNA library despite it could be shown to be present by PCR. Most likely it is a feature of this particular cDNA where either DNA sequence motifs as such or the expression of the protein causes problem in the *E. coli* background. Problems with the expression of the gene coding for *Sbe2* in *E. coli* have also been noted in another report when studying the biochemical activity of heterologously expressed *Sbe2* assembled from PCR fragments (Jobling *et al.*, 1999). The *Sbe2* gene is expressed to a much less degree in potato tubers on an RNA level as compared to *Sbe1*, which is also reflected in relative concentrations of SBEI and SBEII by Western Blotting (Larsson *et al.*, 1998, Jobling *et al.*, 1999). In leaf tissue *Sbe2* was more highly expressed than *Sbe1*. When investigating expression levels using quantitative RT-PCR we have found *Sbe2* to be expressed around 200-fold less than *Sbe1* in potato tuber while expressed almost ten-fold higher in leaf as compared to *Sbe1*.

A 1.3kb fragment derived from the 3´end of the *Sbe2* cDNA was used for antisense inhibition in potato using the *gbss* promoter as controlling element. Tuber starch was extracted from field grown lines derived from transformation of two different starch varieties. Starch contents and granule morphology of the potato tubers were unaffected. Very small effects on apparent amylose content and chain length fractions were found, as seen in Figure 5. The antisense inhibition of *Sbe2* in potato has also been studied by Jobling *et al.* (1999) but in contrast to this study we could find no significant changes in phosphate content of the starch. A so far unexplained effect we have found of *Sbe2* inhibition in the potato tuber is that while parental lines fall to pieces upon cooking, possibly due to high starch contents, tubers of all transgenic lines remain intact, as shown in Figure 6.

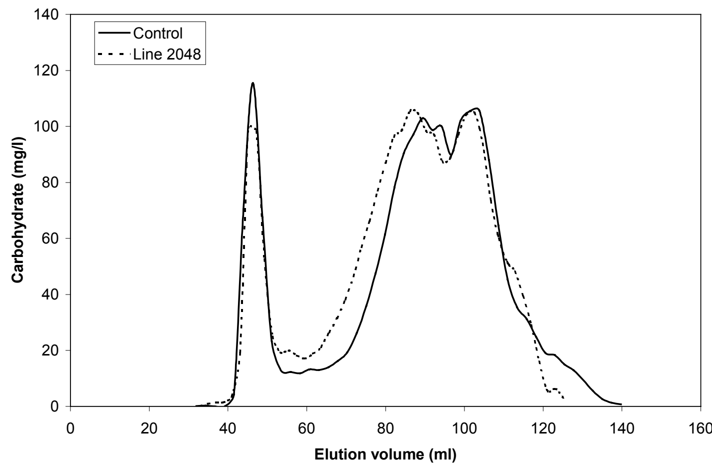


Figure 5. Size Exclusion Chromatography (SEC) diagram of debranched starch from *Sbe2* inhibited potato line 2048 and parental variety indicating a slightly altered chain length distribution of amylopectin.

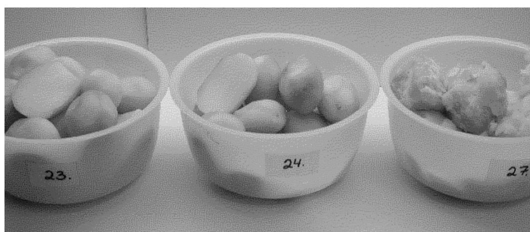


Figure 6. Boiled potatoes of two *Sbe2* inhibited lines and their parental variety. Potatoes of the two transgenic lines on the left are firm while the control potatoes on the right have disintegrated.

Even though there are some discrepancies between what has been published in the literature on consequences of antisense downregulation of *Sbe2* and our own studies it is clear that the impact for storage starch is much less severe in potatoes than for maize and pea mutations affecting the comparable enzyme. There might be a difference between mutants and antisense technology in that antisense technology produces a somewhat leaky phenotype in this case. However a more plausible explanation would be that SBEI fully can complement for the loss of SBEII in potato tuber and the slight impact on the starch is a reflection of the different biochemical properties of SBEI in that it transfers longer chains. SBEI of potato is accounted for at least 95% of the tuber starch branching enzyme activity (Safford *et al.*, 1998). In maize endosperm and pea embryo the SBEII (class A) isoform accounts for more than 80% of the starch branching enzyme activity (Edwards, Green & ap Rees, 1988; Singletary *et al.*, 1997).

In **Paper I** is described the inhibition of both known potato branching enzyme genes and the impact of this inhibition on field grown potato in general and tuber starch in particular. The gene construct used for inhibition of gene expression contained 3´ends of cDNA fragments derived from *Sbe2* (1.3 kb) and *Sbe1* (1.8 kb) cloned in tandem and in antisense direction in relation to the *gbss* promoter of potato.

Originally the selection of the high-amylose lines of the study was based on selection by amylose content of starch extracted from tissue culture microtubers. We have at a later point also determined the expression of *Sbe1* and *Sbe2* in the transgenic lines using quantitative RT-PCR as described in **Paper II**, confirming the down regulation of respective genes (results not shown). In tubers there was a severe reduction of expression of both genes. However it was difficult to draw the same conclusions in leaf tissue suggesting a level of organ or tissue specific down regulation by the *gbss* promoter. This indicates that the *gbss* promoter is not sufficiently expressed in leaf tissue to induce silencing and a silencing signal is not transported from tubers to provide a high degree of silencing in leaf tissue.

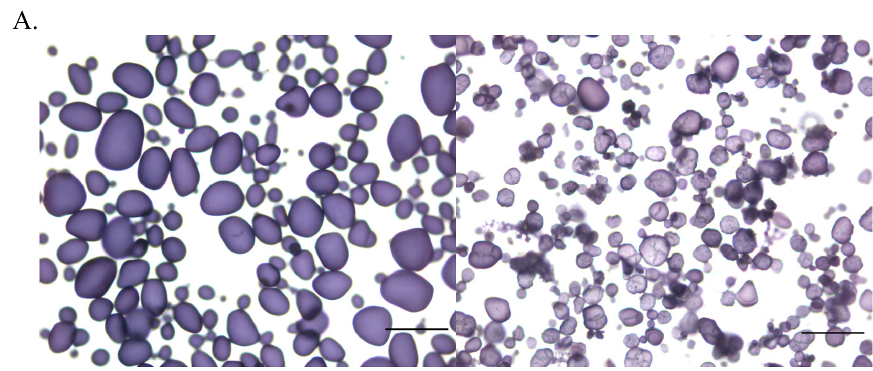
Growth and phenotypic appearance in the field was similar to parental genotypes for most of the lines investigated. However for the line with the most reduced degree of branching, line 715, a stunted growth was apparent and plants never reached the same height and volume of canopy as the parental genotype. High-amylose lines, except line 715, all had a 15 to 50% higher fresh weight tuber yield than their parental genotypes. Tubers harvested from high-amylose lines were elongated in shape with extent of elongation being correlated with amylose content. Total starch contents were reduced in all high amylose lines (Table 1.). Another effect of the modification is significantly increased contents of glucose and fructose in the tubers (Table 1.). Elongated tubers of transgenic potatoes have been observed previously (Sonnewald *et al.*, 1997; Tjaden *et al.*, 1998). An increased sugar content of tubers is a common factor among these experiments, which imply that the tuber shape may be related to this.

Table 1. *Data presented in **Paper I** on characterization of starch derived from eight high-amylose lines and respective parental varieties. Data are the mean values of two analyses*

Potato line/variety	Granule size (µm±1)	Starch content (% of FW±0.3)	Glucose/Fructose (% of FW±0.1)	Amylose SEC (%±3)	Degree of branching (%±0.04)	Phosphate content (%±0.01)
Producent	48	21.9	1.1	23	2.27	0.05
418	29	16.6	1.6	66	1.16	0.27
Prevalent	49	21.0	0.8	23	2.27	0.07
301	34	12.4	1.3	61	1.10	0.28
342	33	11.8	1.5	64	0.80	0.28
Dinamo	56	22.7	0.7	22	2.23	0.09
643	37	13.6	1.2	56	1.18	0.33
676	32	12.8	1.5	75	0.59	0.28
715	28	6.1	1.3	78	0.31	0.24
Kuras	45	21.8	1.1	21	2.36	0.07
910	32	14.5	2.3	56	1.31	0.24
970	35	14.4	2.5	58	1.58	0.25

Starch granule morphology of the high-amylose potatoes is greatly altered with seemingly collapsed interiors and being much reduced in size, as is shown in Figure 7A. In immature tubers, starch granules were very disorganized and had long protruding extensions. This demonstrate that some organization of the granule develops with maturity but the factor behind this is yet unknown. Assuming similar densities of starch granules in potato starch and high amylose potato starch, the reduction in total volume of starch does not parallel the reduction in starch content indicating an increased number of starch granules present in high amylose potatoes. Alterations in starch granule morphology are consistent with another study in potato (Schwall *et al.*, 2000) and also of maize and pea high amylose mutants. Most likely the collapsed granule interior and smaller size is due to the disruption of the ordered structure induced by amylopectin. Odd shapes of granules in association with longer chain length fractions are also seen in starches of potato tubers inhibited for soluble starch synthases SSII and SSIII.

A.



B.

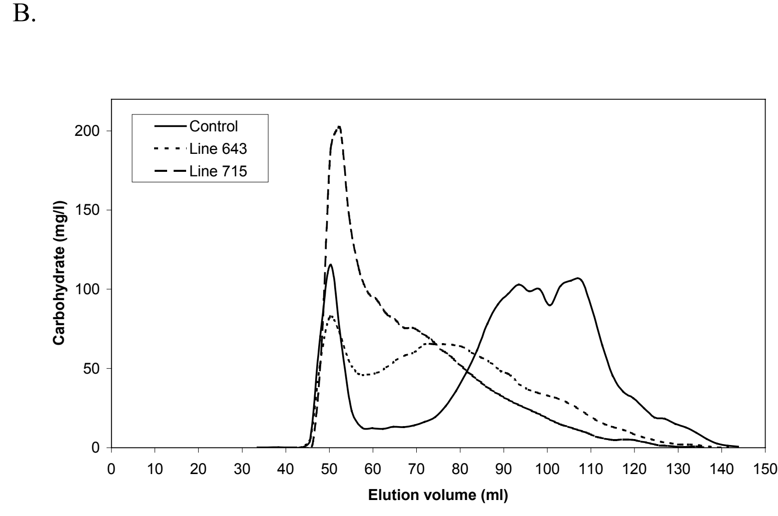


Figure 7. A. Starch granules stained with Iodine, size bar is 100µm. Left hand picture: Ordinary potato starch. Right hand picture: Starch of transgenic high-amylose line 715. *B.* Size exclusion chromatography (SEC) diagram of debranched starch from Dinamo, line 643 and line 715.

Lines producing starches with different ratios of apparent amylose content and degree of branching were among the ones studied. The amylose/amylopectin ratio can be determined by several different methods. The most convenient methods are commonly based on the differential binding of iodine by different chain lengths. A drawback of this approach is that it does not distinguish between amylose and long chains of amylopectin. To yield a more direct picture of the chain length size distribution, size exclusion chromatography (SEC) of debranched starches was used. In the lines containing a starch of around 50 to 70% amylose as determined by SEC evaluation, there was a significant intermediate fraction of what is likely a modified amylopectin. In lines containing more than 70% amylose an almost continuous peak with material migrating as amylose was evident as is seen in Figure 7B. In none of the lines normal amylopectin could be detected. Determined increases in amylose content was also reflected in values obtained for degree of branching, which was decreased up to seven-fold or down to 0,3% (Table 1.). Interestingly, there was a direct correlation between degree of branching and granule size suggesting a link between starch structure and the ability of that structure to form a starch granule as is shown in Figure 8.

Phosphorous content of the starch was increased 3-5 fold in the high-amylose lines which is consistent with that reported by Schwall *et al.*, (2000). Interestingly lines with higher determined amylose contents had a lower phosphorous content. This suggests that the increased phosphorous content is rather associated with a modified amylopectin with longer chains than with the increased amylose content. This observation is in line with that amylopectin contains the phosphate esters and that longer chains of amylopectin are preferentially phosphorylated (Mikkelsen, Baunsgaard & Blennow, 2004).

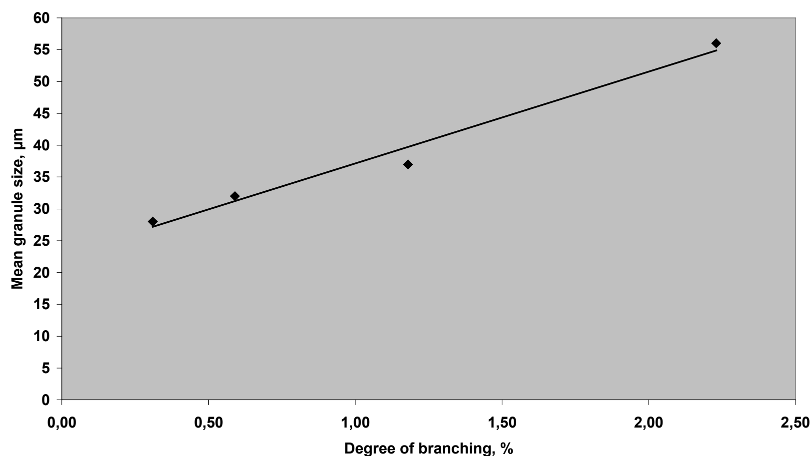


Figure 8. Correlation between starch degree of branching and granule size. The series shown is by decreasing order of degree of branching Dinamo, line 643, line 676 and line 715. Transgenic lines are all derived from Dinamo.

Line 715 showed the most dramatic changes in starch structure and composition while also displaying some undesirable properties such as a stunted plant phenotype and reductions in tuber yield as well as starch content. If starch output per area of land is calculated then line 715 produced 20% of its parental genotype while the figures for the other high amylose lines were 70-80%. Either this line is impacted in some unrelated epigenetic way as compared to the other transgenic lines or it has passed a threshold where residual starch produced cannot support a normal plant development perhaps in relation to metabolical implications for transient starch turnover.

The enzymatic action of plant branching enzymes has been studied *in vitro* and by heterologous expression in microorganisms. In a study of purified maize branching enzymes, all three enzymes (SBEI, SBEIIa, SBEIIb) displayed no activity with glucans of an average CL of 25. Action on reduced amylose resulted in similar average chain lengths of 20-25. SBEI was found to preferentially transfer longer chains compared to SBEIIa and SBEIIb (Takeda, Guan & Preiss, 1993). In a similar study it was further concluded that SBEI had a 20-fold higher rate of branching amylose than amylopectin. SBEII on the other hand had a two-fold higher rate of branching of amylopectin than amylose and a six-fold higher rate of branching amylopectin than SBEI (Guan & Preiss, 1993).

Expressed in a *glgB*$^{-}$ strain of *E. coli* lacking branching enzyme activity, both SBEI and SBEII produced a soluble glucan with a broad chain length distribution. A sharp peak at CL 6 could be identified and a peak around CL 11 for the broad size distribution range. Average CL was 14 for SBEI, 16 for SBEII and 12 for SBEI+SBEII (Guan *et al.*, 1995). Minimum CL for branching was 16 for SBEI and 12 for SBEII. This minimum requirement was also mirrored in the size distribution of amylose branched by respective branching enzymes where shorter chain were much more abundant in SBEII branched material. It was also noted that the minimum chain length for branching was within the range for the crystalline lamella of amylopectin cluster structure (Guan *et al.*, 1997). The different starch branching enzymes of maize have also been expressed in yeast with somewhat different results (Seo *et al.*, 2002). SBEI did not complement the yeast branching enzyme mutant although both SBEII forms did. It was suggested that glycogen synthase might not provide sufficiently long chains for SBEI to act upon.

Heterologously expressed and purified branching enzymes derived from potato have also been studied (Rydberg *et al.*, 2001). No increase in molecular weight was found, indicating mainly intra molecular branching. SBEI was more active on longer chain material than SBEII. Debranching of glucan after action of branching enzyme revealed dual peaks for SBEI centred around CL 11-12 and 29-30, additionally there was a peak around CL 6. SBEII showed one peak around CL 13-14 and a significant spike at CL 6. Results for the debranched material was strikingly similar to the actions of maize SBEI and SBEII on reduced amylose reported in Takeda, Guan & Preiss (1993) with SBEI displaying a stepwise size distribution range with peaks at CL 70, 31 and 11-14 over time while SBEII displayed a more uniformly migrating size distribution range indicating a transfer of shorter chains. That SBEI is more likely to transfer longer chains as compared

to SBEII might explain the small differences seen between ordinary potato starch and starch isolated from *Sbe2* inhibited lines where longer chains in amylopectin could be observed.

Potato is different from pea and maize in that it contains an overwhelming activity of SBEI in the tuber although it is likely that SBEII determines the structure of amylopectin as there is little impact on starch deposition or structure if no SBEI is present in the tuber. In pea and maize where total branching enzyme activity activity is much more reduced when SBEII is lacking, a more pronounced effect is seen on the starch in comparison to potato where SBEII activity had been reduced by antisense inhibition. The SBEII isoform contain a N-terminal domain not found in the SBEI isoform, which is predicted to be flexible with some analogy to the N-terminal domains described for soluble starch synthases (Marshall *et al.*, 1996). Although the functional importance of the N-terminal domain has not been determined it is of interest to note that it is associated with enzymes determined to be of importance for amylopectin synthesis. It is almost as if SBEI and GBSS are evolutionary remnants where GBSS has a new role in starch biosynthesis by allowing more dense packing of carbohydrate in starch granules. SBEI does not have an apparent role in starch synthesis other than possibly, as a backup should SBEII not function. In *Arabidopsis thaliana* there is even no evidence of any *Sbe1* gene, instead two genes corresponding to *Sbe2* can be found (Khoshnoodi *et al.*, 1998). In potato tubers SBEI can fully complement for the loss of SBEII activity. To the question on how much starch branching enzyme activity is needed in potato tubers to produce normal amounts of normally structured amylopectin it can be concluded that the answer is; Not much or at least there is far more than needed.

Starch phosphorylation

The only known in vivo chemical substitution of starch are phosphate monoesters linked to α-1,4 glucan chains. Several other compounds are contained in the starch but they are complex bound rather than covalently attached to the sugar backbone. Starches of most crop plants, as cereals, contain low levels of covalently bound phosphate that in many cases is below detection levels. Potato starch is the only commercially produced starch, which contain significant amounts of phosphate where around 0.1 % of glucose residues have attached phosphate groups (Swinkels, 1985).

It was long debated how this substitution was made but from a proteomic approach a cDNA corresponding to a putitative phosphorylating enzyme called R1 was isolated. The inhibition of R1 by antisense expression in potatoes resulted in reduced levels of phosphate as well as the heterologous expression of the same cDNA in *E. coli* led to elevated levels of phosphate in glycogen (Lorberth *et al.*, 1998). Subsequently an assay for the enzyme was developed and the R1 protein was determined to be an α-glucan,water dikinase (GWD) where a phosphate from ATP is transferred to the C-3 or C-6 carbon of the glucosyl residue in starch (Ritte *et al.*, 2002). Amylopectin has been determined to be the molecule of starch that is phosphorylated and amylose has in *in vitro* experiments been shown to be a very

poor acceptor molecule for the reaction. Generally it seems as very long chain glucans as in amylose and very short chain glucans as in glycogen are poorly phosphorylated by GWD (Mikkelsen, Baunsgaard & Blennow, 2004).

Genes coding for this enzyme can be found in higher plant genomes independent of phosphate concentration in starch. Interestingly there is also no clear correlation between amount of GWD protein present in various species and organs and the phosphate content of starch (Ritte *et al.*, 2000). The role of GWD is somewhat obscure but general observations have been made that makes indirect predictions regarding functionality possible. Mutants of the gene in *Arabidopsis thaliana* show a leaf starch excess phenotype and transgenic potato with the GWD gene antisense inhibited is less prone to cold sweetening, which suggests an importance in starch degradation (Lorberth *et al.*, 1998, Yu, T.-S. *et al.*, 2001). Combined antisense lines that are inhibited for SBEI and GWD activity are reported to produce high amylose starch (Kossmann & Lorberth, 1997). This may implicate the phosphate groups as important for optimal SBEII branching activity in potato although the effect has not been reported elsewhere. The phosphate groups may thus serve as markers or recognition sites for degrading enzymes and possibly also for branching. Structurally some of the degrading enzymes and branching enzymes have similar features and share motifs (Jespersen et al., 1993).

There is a strong correlation between events and enzyme actions leading to the synthesis of amylopectin and amylose on one hand and phosphorylation on the other. As can be seen in **Paper I**, interestingly the phosphate content of high amylose potato starch seem to peak at some starch structural level and then become lower as branching is further reduced. The level is the highest when there is a large proportion of altered amylopectin. When several results are taken into account it could be hypothesized that GWD, at least in potato, is most active on moderate chain lengths. Amylose is not substituted to any significant degree. More speculative is that phosphate groups may serve as markers for branching and degradation.

Other enzymes

Several enzymes have been implicated in both the biosynthesis of starch and in starch degradation. The bacterial glycogen operons encode enzymes, which operate in the degradation of glycogen in addition to the basic enzymes needed in formation of glycogen. Glycogen phospohorylase has the ability to sequentially degrade α-1,4 glucan chains to malto-tetraose stubs upon release of glucose-1-phosphate. The plant homolog, starch phosphorylase, has been found in a cytosolic as well as a plastidic form. Starch phosphorylase can operate in a synthesis or degrading direction depending on concentration and balance of metabolites. In the synthetic direction glucose from glucose-1-phosphate is transferred to an α-1,4 glucan while in the hydrolytic direction inorganic phosphate is used to yield a glucose-1-phosphate.

This enzyme activity was originally suggested to be the main chain elongating activity in plants but upon the finding and description of starch synthases this picture has been revised. It has been difficult to assign any function of importance

for this enzyme in plants even though the comparable activity is central in glycogen degradation. The protein and enzymatic activity is easily detectable and abundant in starch storing tissues. Speculation has been both in the direction of a biosynthetic and degrading function but its specific importance for starch biosynthesis or degradation has yet to be proven (Yu, Y. *et al.*, 2001). Recently a study of *Arabidopsis* mutants for the plastidic form revealed no difference in comparison with wild type regarding starch composition or degradation in leaf (Zeeman *et al.*, 2004).

Another gene found in the bacterial glycogen operons codes for a debranching enzyme (DBE), which hydrolyses α-1,6 linkages of exterior chains that have been fully recessed by glycogen phosphorylase. In bacteria this enzyme activity has exclusively been linked to the degrading pathway of glycogen. In plants there are two different types of the enzyme, the pullulanase type that is efficient towards pullulan and amylopectin and the isoamylase type that is efficient towards glycogen and amylopectin.

The isoamylase type enzyme has been shown to be of crucial importance for normal starch deposition and its biochemical importance in starch synthesis was first described for *sugary-1* (*Su1*) mutants of maize endosperm (Pan & Nelson, 1984, James, Robertson & Myers, 1995). Endosperm of these mutants accumulates phytoglycogen, which is a more branched form of amylopectin that is similar in structure to glycogen. Additionally accumulation of starch is severely reduced. The most dramatic effect is seen in *Chlamydomonas* where no granular starch is accumulated at all and only phytoglycogen is produced (Dauvillée *et al.*, 2001).

Observations of impact on starch formation in isoamylase mutants have led to new models for starch biosynthesis being suggested. In the glucan trimming model, debranching activity is needed for amylopectin to assume its clustering and subsequent crystallization. Branch points of amylopectin are suggested to be hydrolyzed in the amorphous lamella of the growing starch granule and remaining branches can then assume a more ordered structure (Ball *et al.*, 1996). An alternative model and role for isoamylase has been suggested from observations of an *Arabidopsis* mutant (Zeeman *et al.*, 1998). In this mutant a 90% reduction of starch was found which was accompanied by accumulation of phytoglycogen with a resulting 40% less total glucan produced. Normal starch as well as phytoglycogen accumulated in the same chloroplasts. Starch degrading enzymes would according to this model play a role to prevent soluble glucans from being accumulated. A mutation in the debranching activity would then result in soluble glucan being accessible for soluble starch synthases and branching enzymes that result in the formation of phytoglycogen and concomitant reduced starch content. More recently the potato isoamylase genes have been inhibited by antisense expression (Bustos *et al.*, 2004). Starch granules of an apparently normal composition were formed in tubers of silenced lines. However the number of granules formed was greatly increased and many of the granules were very small. These results would support that isoamylase has a role in limiting the number of molecules that can give rise to new granules. On the other hand it could, in view of the large number of small granules, also be argued that growth of starch

granules is terminated prematurely because of reduced organization at the surface of the starch granule.

In maize a mutant for the pullulanase type debranching activity has also been identified. Starch accumulation and structure of this mutant is the same as for the control. However combinations of genetic backgrounds suggests that the isoamylase and pullulanase may have some overlapping roles in starch biosynthesis although the pullulanase type enzyme has limited capacity to compensate for a deficiency in the isoamylase type of enzyme (Dinges *et al.*, 2003).

Even if the role of isoamylase in starch biosynthesis is not fully elucidated it is clear that debranching enzyme has an important function in facilitating a structure which makes starch formation more effective. It could very well be that amylopectin processing and scavenging of soluble glucans are parallel functions of isoamylase in plants. Otherwise it must be assumed that there is a fundamental difference in accessibility among glucans in the plastid and whether they can be used for the formation of a starch granule. It seems likely that the specificity of plant debranching enzyme as compared to bacterial debranching enzyme and its presence during starch deposition is a main difference between glycogen accumulation of bacteria and starch formation as a granular structure in plants.

Disproportionation enzyme (D-enzyme) removes part of an α-1,4 glucan chain and transfer it to another α-1,4 glucan chain of at least three glucose residues (maltotriose) thus not changing the average chain length of an α-1,4 glucan population. Malto-oligosaccharides as well as starch can be used as both donor and acceptor (Takaha *et al.*, 1996). Data from mutants of D-enzyme have implicated a role in starch formation as well as starch degradation. D-enzyme mutants in *Chlamydomonas* accumulates significantly less starch with a higher amylose content than the wild type and at the same time accumulates linear malto-oligosaccharides under photosynthetic as well as starch storing (nitrogen limiting) conditions (Colleoni *et al.*, 1999, Wattebled *et al.*, 2003). On the other hand *Arabidopsis* mutants for D-enzyme accumulate more starch in photosynthetic tissues or rather the degradation of accumulated starch is impaired (Critchley *et al.*, 2001). The *Arabidopsis* D-enzyme mutants also contain higher amounts of amylose and an increased MOS concentration. Possibly the increased amylose content is a result of the increased MOS accumulation where GBSS responsible for amylose synthesis has been shown to be stimulated by malto-oligosaccharides.

Results from mutants of *Arabidopsis* and *Chlamydomonas* are both from photosynthetic tissues, which could be important for the observations made. Antisense experiments where a starch storing tissue was targeted did not reveal any changes in starch composition even with a reduction to 1% of control activity in potato tubers although a slower production of sprouts from tubers was noted (Takaha *et al.*, 1998). In summary the studies on mutants and inhibition in transgenes have been performed on a limited number of species that are quite diverse including the tissues and conditions studied. Therefore a definitive function of D-enzyme for starch biosynthesis and/or degradation is not yet clear.

Initiation of starch synthesis

Although it is clear what enzymes participate for formation of the general starch structure, how the synthesis of starch is initiated is still largely unresolved. For glycogen synthesis in fungi and animals it has conclusively been shown that the formation of the α-1,4 glucan is absolutely dependent on a glucan primer. The enzyme responsible for this activity has been termed glycogenin (Rodriguez & Whelan, 1985).

Glycogenin utilizes UDP-glucose in an Mn^{2+} dependent self-glycosylation reaction where 5 to 13 glucose residues are attached as a short α-1,4 glucan chain (Alonso *et al.*, 1994). In yeast there are two enzymes with a function related to the production of primers for glycogen synthesis. A yeast mutant with knockouts for both genes produces no glycogen (Cheng *et al.*, 1995). From the various biochemical studies performed, invariably a tyrosin is the amino acid, which is glycosylated. In animals one specific tyrosine has been determined to be the site for the glycosylation but in yeast several tyrosins need to be mutated to completely eliminate the glycosylation acceptor site of the enzymes (Cao *et al.*, 1993; Mu, Cheng & Roach, 1996).

In plants an analogous enzymatic activity was identified where the protein was of a molecular weight in the same range as glycogenin (Ardila & Tandecarz, 1992; Rothschild & Tandecarz, 1994). A protein corresponding to the enzymatic activity studied was partially sequenced and given the name amylogenin implying a function for starch initiation being analogous to glycogenin for glycogen (Singh *et al.*, 1995). Amylogenin shared no sequence homology with glycogenin though. Subsequent work has shown that it is unlikely that amylogenin from corn and corresponding enzymes in other plant species have a role in starch synthesis (Bocca *et al.*, 1999). Localization, substrate range and a β-configuration of the glycosidic linkage point more in the direction of a role for cell wall polysaccharides.

To be remembered is that bacteria and plants have a completely different enzyme performing the α-1,4 glucan synthesis as compared to fungi and animals and that the starch/ glycogen synthase may have a role in initiation as well as in elongation. Results have recently been presented that indicate such a function for glycogen synthase in *Agrobacterium tumefaciens* (Ugalde, Parodi & Ugalde, 2003). In the model suggested, glycogen synthase is first priming the formation of α-1,4 glucan by covalent binding of a glucose residue and then assuming the well-known elongation role. In plants unprimed activity of starch synthases has been investigated although it has only been possible to detect in vitro using very artificial conditions with high concentrations of citrate in the reaction mixture.

Interestingly gene sequences with homology in some translated domains to glycogenin can be found in plants even if absent from most bacterial species. This of course raises the question regarding whether any of these genes are important for starch biosynthesis. In *Arabidopsis thaliana* at least thirteen expressed or predicted genes will produce significant hits after translation when comparing with human glycogenin. The predicted protein products of these gene sequences can be grouped based on molecular weight of protein and homology. Of the thirteen gene

sequences from *Arabidopsis*, eight could be determined to belong to the galactinol synthase group of amino acid sequences and could thus be excluded from further discussion.

Two full-length potato cDNAs named StGH1 and StGH2 respectively were isolated from a potato tuber cDNA library using short EST sequence information (Hofvander & Andersson, 2004). The isolated genes were upon translation found to share a number of recognizable motifs with animal glycogenin. Overall homology on a DNA or protein level was not significant and limited to the mentioned motifs. Extensive searches for similar potato sequences in EST and other databases did not reveal any other genes or gene sequences of significant homology to the isolated cDNAs or glycogenin. Not all five *Arabidopsis* sequences have been shown to be expressed as ESTs or isolated cDNAs. This may provide an explanation on the greater number of genes observed in *Arabidopsis*. From a structural point of view the deduced amino acid sequences can be divided into three groups based on homology, molecular weight, predicted signal sequences and membrane spanning regions. TargetP and TMHMM2.0 were used for the prediction of signal sequences and membrane spanning regions respectively (Sonnhammer, von Heijne & Krogh, 1998; Emanuelsson *et al.*, 2000). The translated StGH1 protein belongs to a group with a predicted signal peptide and five predicted C-terminal membrane spanning regions. StGH2 belongs to a group where the corresponding translated *Arabidopsis* sequence is predicted to contain a chloroplast transit peptide but no membrane spanning regions. The core region of homology to glycogenins of the two groups where the potato cDNAs have been isolated are shown in Figure 9.

In glycogenins a number of conserved amino acids have been suggested to be of catalytical importance (Lomako, Lomako & Whelan, 2004). The DXD motif is conserved in both StGH1 (Asp-132, Asp-134) and StGH2 (Asp-410, Asp-412) and is thought to be involved in the binding of Mn^{2+}. A DXXD motif is considered to be a potential nucleotide acceptor for the glucose residue before binding to the tyrosine residue. The second Asp of the motif was concluded to be the intermediate acceptor site. This motif is conserved in StGH1 (Asp-189, Asp-192) while in StGH2 the first Asp in the motif is an Asn (Asn-466). The tyrosine, which is glucosylated is conserved among animal glycogenins but not between the animal and yeast enzyme forms where more than one tyrosine residue can function as the glucosylation site. In StGH1 a number of Tyr-residues can be found in the corresponding region (Tyr-234, Tyr-235, Tyr-236), which is glucosylated in animal and yeast glycogenins. StGH2 lacks a tyrosine in this region although tyrosins can be found both in the close proximity of the intermediate acceptor site (Tyr-465) as well as close to the conserved KPW motif (Tyr-521).

Transcript expression levels of potato genes StGH1 and StGH2 were determined in tuber and leaf tissue by quantitative RT-PCR (results not shown). StGH1 is expressed at approximately the same level in tuber as in leaf while StGH2 is expressed at the same level as StGH1 in leaf but expressed several fold higher in potato tuber. Neither of the two transcripts is highly expressed in any potato tissue examined although a high expression would not be of importance for an involvement in the initiation of starch synthesis.

A.

```
StGH1      (115) NKLYAWSLVNYDRVVMLDADNL-----FLQKTDELFQCG--QFCAVFIN-
NP_922675  (142) NKLYAWTLVDYERVVMIDSDNI-----FLQKTDELFQCG--QFCAVFIN-
AtGH1      (144) NKLYAWSLSDYDRVVMLDVDNL-----FLKNTDELFQCG--QFCAVFIN-
HsGLG1      (85) TKLHCWSLTQYSKCVFMDADTL-----VLANIDDLFDRE--ELSAAPDPG
ScGLG1     (105) IKARLWELTQFEQVLYLDSDTLPLNKEFLKLFDIMSKQTTSQVGAIADIG
ScGLG2     (103) LKARLWELVQFDQVLFLDADTLPLNKEFFEILRLYPEQTRFQIAAVPDIG

StGH1      (157) -PCIFHTGLFVLQPSKKVFNDMIHEIEIGRENQDGADQGFIGGHFPDLLD
NP_922675  (184) -PCYFHTGLFVLQPSMDVFKGMLHDLEIGRANSDGADQGFLVGCYPDLLD
AtGH1      (186) -PCIFHTGLFVLQPSMEVFRDMLHELEVKRDNPDGADQGFLVSYFSDLLN
HsGLG1     (128) WPDCFNSGVFVYQPSVETYNQLLH-LASEQGSFDGGDQGILNTFFSSWAT
ScGLG1     (155) WPDMFNSGVMMLIPDADTAS-VLQNYIFENTSIDGSDQGILNQFFNQNCC
ScGLG2     (153) WPDMFNTGVLLLIPDLDMAT-SLQDFLIKTVSIDGADQGIFNQFFNPICN

StGH1      (206) RPMFHPP-LNGTQLQGSYRLPLGYQMDASYYYLKLHWSVPCGPNSVITFP
NP_922675  (233) RPMFHPP-ENGSKLNGTYRLPLGYQMDASYYYLKLHWHVPCGPNSVITFP
AtGH1      (235) QPLFRPPPDNRTALKGHFRLPLGYQMDASYYYLKLRWNVPCGPNSVITFP
HsGLG1     (177) TD-------------IRKHLPFIYNLS-SISIYSYLPAFKVFGASAKVVH
ScGLG1     (204) TDELV----KDSFSREWVQLSFTYNVTIPNLGYQSSPAMNYFKPSIKLIH
ScGLG2     (202) YSKEVLH--KVSPLMEWIRLPFTYNVTMPNYGYQSSPAMNFFQQHIRLIH

StGH1      (255) GAPWLKPW
NP_922675  (282) SAPWFKPW
AtGH1      (285) GAVWLKPW
HsGLG1     (213) FLGRVKPW
ScGLG1     (250) FIGKHKPW
ScGLG2     (250) FIGTFKPW
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B.

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StGH2      (393) SKFRLWQLTDYDKIIFIDADLL-----ILRNIDFLFEMP--EITAIGNN-
NP_915047  (367) SKFRLWQLTDYDKIIFIDADLL-----ILRNVDFLFAMP--EITATGNN-
NP_566615  (395) SKFRLWQLTDYDKIIFIDADLL-----ILRNIDFLFSMP--EISATGNN-
HsGLG1      (85) TKLHCWSLTQYSKCVFMDADTL-----VLANIDDLFDRE--ELSAAPDPG
ScGLG1     (105) IKARLWELTQFEQVLYLDSDTLPLNKEFLKLFDIMSKQTTSQVGAIADIG
ScGLG2     (103) LKARLWELVQFDQVLFLDADTLPLNKEFFEILRLYPEQTRFQIAAVPDIG

StGH2      (435) -ATLFNSGVMVVEPSNCTFQLLMDHINEIESYNGGDQGYLNEIFTWWHR-
NP_915047  (409) -ATLFNSGVMVIEPSNCTFQLLMDHINEITSYNGGDQGYLNEIFTWWHR-
NP_566615  (437) -GTLFNSGVMVIEPCNCTFQLLMEHINEIESYNGGDQGYLNEVFTWWHR-
HsGLG1     (128) WPDCFNSGVFVYQPSVETYNQLLHLASEQGSFDGGDQGILNTFFSSWATT
ScGLG1     (155) WPDMFNSGVMMLIPDADTASVLQNYIFENTSIDGSDQGILNQFFNQNCCT
ScGLG2     (153) WPDMFNTGVLLLIPDLDMATSLQDFLIKTVSIDGADQGIFNQFFNPICNY

StGH2      (483) ------------IPKHMNFLKHYWEGDEEEKKQMKTRLFGADPPVLYVLH
NP_915047  (457) ------------IPKHMNFLKHFWEGDEEEVKVKKTRLFGADPPILYVLH
NP_566615  (485) ------------IPKHMNFLKHFWIGDEDDAKRKKTELFGAEPPVLYVLH
HsGLG1     (178) D-----------IRKHLPFIYNLS-SISIYSYLPAFKVFGA---SAKVVH
ScGLG1     (205) DELV—-KDSFSREWVQLSFTYNVTIPNLGYQSSPAMNYFKP---SIKLIH
ScGLG2     (203) SKEVLHKVSPLMEWIRLPFTYNVTMPNYGYQSSPAMNFFQQ---HIRLIH

StGH2      (521) YLG-LKPW
NP_915047  (495) YLG-LKPW
NP_566615  (523) YLG-MKPW
HsGLG1     (213) FLGRVKPW
ScGLG1     (250) FIGKHKPW
ScGLG2     (250) FIGTFKPW
```

Figure 9. Comparison of core region of homology between deduced amino acid sequence of plant genes with glycogenins of human and yeast. *A.* StGH1 origin potato cDNA, NP_922675 origin rice GenBank accession, AtHG1 origin *Arabidopsis thaliana* deduced from genomic sequence, HsGLG1 human glycogenin-1, ScGLG1 *Saccharomyces cerevisiae* Glg1, ScGLG2 *Saccharomyces cerevisiae* Glg2 *B.* StGH2 origin potato cDNA, NP_915047 origin rice GenBank accession NP_566615 origin *Arabidopsis thaliana* GenBank accession. Homology is scored by plant sequences as a group compared with primarily human glycogenin first and yeast glycogenins second. Tyrosins proven to be targets for glucosylation are underlined.

Potato enabling technology

Potato transformation

Breeding by mutations has been a powerful technology for the modification of several crop plants, examples are starch mutants in maize and low-erucic, low-glucosinolate rapeseed. For potato possibilities of mutational breeding have been much more limited due to the tetraploid genome, which has a buffering effect and make mutations more difficult to recognize. A notable exception being the amylose-free potato produced in the late eighties (Hovenkamp-Hermelink *et al.*, 1987). Furthermore potato varieties are essentially vegetatively propagated hybrids. This means that extensive breeding is needed even if only one particular trait is intended to be introduced by sexual crossing. For potato, genetic engineering is a very useful technology to introduce new traits by either the inhibition of endogenous genes or the expression of foreign or endogenous genes.

Many different technologies for the introduction of foreign genetic material into plants has been developed and used over the years. The two most important and employed technologies have been either to use the soil bacterium *Agrobacterium tumefaciens*, which in nature transfers a specific part of a plasmid (T-DNA) into the plant cell or by discharging DNA into the plant cells mostly attached on inert metal spheres. *Agrobacterium* transformation is the most commonly applied method for potato and other dicotyledonous plants. Monocotyledonous plants as maize, rice and wheat have largely been recalcitrant to *Agrobacterium* transformation and direct DNA transfer has been the method of choice. However in recent years this mode of transformation has been successfully adapted for the transformation of rice and maize and is now also the most used method for these plant species (Komari *et al.*, 1998).

Agrobacterium tumefaciens contains a large plasmid (Ti-plasmid) where a section called T-DNA that is delimited by an imperfect repeat (border repeats) is transferred to the plant cell and integrated into its genome (reviewed in Zupan *et al.*, 2000). The T-DNA contains genes that redirect plant metabolism to facilitate *Agrobacterium* growth but does not contain any functions that are involved in the transfer and integration of the T-DNA. *Agrobacterium* strains have been engineered to provide a suitable system for the transfer of genes to plant cells by replacing DNA in between the border repeats by genetic material of choice. A major step in the development of a transformation vehicle was to delete the entire T-DNA from the Ti-plasmid and instead contain this region in a smaller plasmid called a binary vector (Hoekema *et al.*, 1983). Potato was one of the earlier plant species that could be transformed using *Agrobacterium tumefaciens* and a binary vector (An, Watson & Chiang, 1986). Potato is generally recognized as one of the more transformable plant species and most often the *nptII* gene providing resistance to the antibiotics kanamycin and neomycin has been used to enable the propagation of transgenic shoots on selective medium.

In recent years the use of antibiotic resistance as a selection tool for transgenic plant cells has been questioned in view of theoretical possibilities of a transkingdom migration of genetic material providing antibiotic resistance genes back to bacteria. Even though neither the *nptII* gene product nor the resistance provided raise any health or environmental concerns from a scientific perspective, there is still the desire to replace it by alternative technology. Many alternative selection schemes have been developed in recent years (Joersbo, 2001; Erikson, Hertzberg & Näsholm, 2004). Much effort has also been devoted to the possibility of eliminating DNA segments such as the selection gene post transformation by the action of recombinases but this is not yet generally applied on a larger scale. Another approach would be to leave out the selection gene completely which recently has been applied in potatoes (de Vetten *et al.*, 2003), although this multiplies screening efforts to identify transgenic shoots considerably. Herbicide tolerance is the most widely commercially used trait derived from the transformation of plants (James, 2003). Genes coding for herbicide tolerance provide a trait, which also can be adapted as a selection tool.

In **Paper V** is described the implementation of a mutated *Arabidopsis thaliana* gene encoding acetohydroxyacid synthase (AHAS) for the use as a selection gene in potato transformation. AHAS is an enzyme catalyzing he first step in the synthesis of branched-chain amino acids and is the target of several classes of herbicides. The mutation utilized, S653N, confers resistance to imidazolinone herbicides (Chang & Duggleby, 1998). The main findings in this paper is that we could adapt a transformation protocol to the use of *AHAS* as selection gene and surprisingly were able to establish a system with higher transformation efficiency than previously noted with other selection genes as for example when using the *nptII* gene. After transformation of four different potato varieties with a gene construct containing a reporter gene (*uidA*) and selection at 0.5µM Imazamox, 93-100% of regenerated plants expressed the reporter gene depending on variety transformed. The potato varieties tested were of table potato type as well as starch varieties, which shows the general applicability of *AHAS* and Imazamox for selection of transgenic potato cells. The new selection system has furthermore been successfully integrated into a transformation program for the development of high-amylopectin potatoes that is a commercially relevant trait (**Paper V**).

The transfer of T-DNA to the plant cell and subsequent integration into the genome is a process that is known in a general context but many questions in particular regarding the integration and the respective roles of *Agrobacterium* and plant genetic factors remains to be elucidated. The study of individual integration events can be used to establish general patterns and provide clues as how the T-DNA is integrated and the locus subsequently repaired (Somers & Makarevitch, 2004). In the approval phase of a transgenic plant that is intended to be released on the market, the transgene locus is investigated regarding inserted DNA and in what chromosomal environment it is integrated. In **Paper IV** the insert of a transgenic event is characterized. This potato line is one of several high-amylopectin events produced using the same or similar gene constructs but were not characterized to such detail regarding the inserted DNA. However, it should be noted that this is not a randomly selected event but is chosen for its particular properties of producing tuber starch consisting essentially of amylopectin while

containing one transgene locus. Until now many different T-DNA insertions have been characterized. If border junctions towards the plant chromosomal DNA are investigated then a typical T-DNA insert is often predictably restricted at the right border repeat while usually shorter or longer deletions can be noted towards the left border integration (Tinland et al., 1995). Potato line EH92-527-1 described in **Paper IV** contains a very atypical T-DNA insert as shown in Figure 10. This insert consists of two right border repeats at the junctions towards potato chromosomal DNA and no left border junction. At the right border junctions the complete right border repeat and two further bases are deleted. This junction compares well with right border junctions that are suggested as repair of double-strand breaks (DSB) (Kumar & Fladung, 2002).

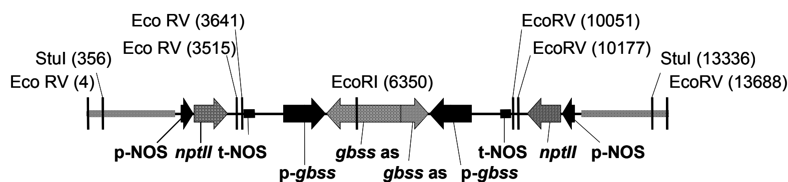

Figure 10. Determined structure of T-DNA insert in EH92-527-1. Segments left and right of the two p-NOS segments is potato chromosomal DNA flanking the insert. p-NOS (nopaline synthase promoter), *nptII* (neomycin phosphotransferase II), t-NOS (nopalin synthase terminator), p-*gbss* (potato *gbss* promoter), *gbss* as (truncated *gbss* antisense fragments).

Most likely this insert has resulted from the occurrence of two consecutive events as shown in Figure 11. First, internal microhomologies and DNA repair have resulted in a duplication of parts of the T-DNA. This caused T-DNA sequences towards the left border after the region of microhomology to be deleted. Second, the DNA repair synthesis has further extended into potato chromosomal DNA using the protruding 3´end at the DSB as a template. A replication loop like structure has thereafter resulted in the separation of the newly synthesised and the template strand. Since the T-DNA insertion as described in **Paper IV**, most likely is situated in an imperfect inverted repeat region of the potato chromosome, the imperfect repeat has then guided the final repair steps resulting in a perfect inverted repeat structure of the insert extending into the flanking potato chromosomal DNA sequence. Repair of DSB in plants is commonly achieved by non-homologous end joining (NHEJ) that recognize and align microhomologies between different strands of DNA (Gorbunova & Levy, 1999). Frequently this is further aided by single-strand annealing (SSA) and subsequent DNA repair synthesis. A two-step single-strand annealing as part of a non-homologous end joining explains the structure of the insert and flanking sequence in potato line EH92-527-1. Recently DSB has been suggested to be a major pathway for the integration of T-DNA into the plant genome (Tzfira *et al.*, 2004), which would make the atypical aspects of the EH92-527-1 insert a function of specific T-DNA attributes as the microhomology in the *gbss* antisense fragment and the specific chromosomal locus of integration that is likely present as an imperfect inverted repeat.

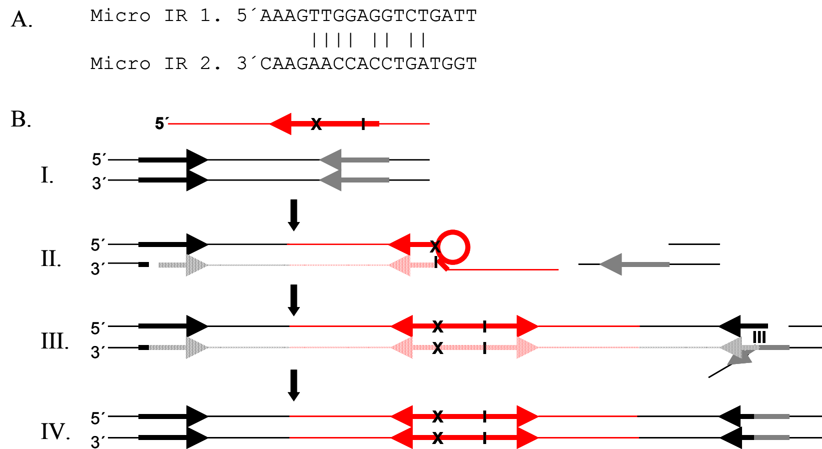

Figure 11. A. Patches of inverted repeat microhomology (Micro IR) available on the *gbss* antisense fragment of pHoxwG. Micro IR 1. is represented by X under *B.* and Micro IR 2. is represented by I under *B. B.* Schematic representation of SSA and DSB-repair leading to the insert structure of potato line EH92-527-1. Potato chromosomal DNA is shown in black and grey. T-DNA is shown in red. Newly synthesized DNA is shaded according to template used for synthesis. *I.* The T-DNA of pHoxwG is assumed to exist as single-stranded DNA in the transformed potato cell. The *gbss* antisense fragment is shown as a red arrow. In the potato chromosomal locus for integration there is an imperfect inverted repeat represented by black and grey arrows respectively. *II.* Upon repair of the DSB microhomology is used to align DNA strands. The alignment of microhomology in the gbss antisense fragment and subsequent DNA synthesis using the region towards the right border as a template causes the resynthesis of T-DNA which then proceeds into the recessed end of the potato DSB using one of the potato inverted repeats as template. Unaligned T-DNA 3´of the annealing structure is digested by nuclease activity. The replication bubble of the T-DNA then will cause the release of the newly synthesized strand from the template. *III.* In the second part of the repair synthesized potato DNA will find stretches of homology and align to the 3´protruding end of the other DSB end. Nuclease activity processes the 3´protruding end until the alignment structure and DNA repair synthesis can proceed to seal the DSB. *IV.* The resulting structure of the T-DNA insertion and subsequent repair is an inverted repeat, which includes T-DNA sequences as well as potato chromosomal DNA sequences. The first SSA event using microhomology of the gbss antisense fragment results in two truncated antisense fragments being part of the insert with one fragment more truncated than the other. The second SSA event yields the same flanking sequence being present on both sides of the T-DNA insert.

Gene inhibition by RNA expression

Reverse genetics, utilizing and screening for mutations have successfully been used in many plant species in research as well as in conventional breeding programs. However for genetically complex plant species as potato or where information is available about the actual gene sequence allowing more targeted approaches, the emergence of genetic modification has provided other tools for reverse genetics. Various technologies for the inhibition or silencing of gene expression by genetic modification have been developed. Antisense inhibition, where the complementary strand to a known transcript is expressed to cause the recognition and silencing of a target gene has been extensively used as a tool for reverse genetics in plants. The origin of this technology comes from the mimicking of a naturally occurring gene regulation system known from bacteria and phages. Later it was also found that over expressing the transcript or parts of the transcript could also yield gene silencing (Napoli, Lemieux & Jorgensen, 1990). Gene silencing by over expression has generally been called co-suppression. More recently both these approaches have been combined into one where a sense transcript and an antisense transcript are simultaneously expressed from introduced foreign genetic material, which can then form a double-stranded RNA (dsRNA) (Waterhouse, Graham & Wang, 1998).

It is not possible to further discuss these technologies without first reviewing what the current status of knowledge is regarding naturally occurring regulation of gene expression by small RNAs. In many higher organisms a population of small RNAs (nt 21-24) have been identified and some of these are termed as micro RNAs (miRNA) (reviewed by Mallory & Vaucheret, 2004; Murchison & Hannon, 2004). The origin of miRNAs can be found among longer transcribed genes that are subsequently processed to the size distribution observed. Generally the transcripts are characterized by secondary structures forming stems and loops that are important for the processing. The transcripts may contain several discrete secondary structures that each results in separate miRNAs, thus there is a polycistronic nature to some of these genes. A larger transcript pri-miRNA is processed in the nucleus by Drosha, an RNaseIII enzyme, yielding one or several pre-miRNAs that are about 70 nt in length that are exported into the cytoplasm by exportin-5. Pre-miRNAs are further processed in the cytoplasm by Dicer, an RNase III enzyme, resulting in the mature miRNA. Drosha has not been identified in plants and it is suggested that a nucleus located Dicer-like enzyme is responsible for the processing. Many miRNAs have been implicated for having a role in important developmental steps in the biogenesis of organisms. Different miRNAs display complementarities to various transcripts coding for transcription factors and controlling the translation of them either by a translational arrest or by guiding the degradation of its complementary transcript. In animals the predominant mechanism is translational inhibition by binding to the 3´untranslated region of an mRNA while in plants miRNAs mainly direct the cleavage of transcripts. The miRNA is assembled into a nucleotide protein complex, RISC, where the miRNA serves as a guide directing the complex to its transcript target. Commonly one strand of the miRNA is used by RISC, which is determined by the duplex stability towards the ends of the small dsRNA. A characteristic of miRNAs

is that the genomic locus of the miRNA is distinct from its transcript target. Sometimes the miRNA nucleotide protein complex is referred to as miRNP when controlling translation. Most likely the same or a very similar system is responsible for the generation of another group of small RNAs termed small interfering RNAs (siRNAs) that are processed from longer dsRNAs. Several siRNAs are produced from the same dsRNA and they may be derived from the same locus or source as they are controlling. Transposons and chromosomal repeat regions as well as virus infections in plants have been suggested as targets for siRNAs. The latter observation has been implemented to deliberately express dsRNAs in a variety of organisms to achieve a highly efficient tool for gene silencing. Another complex, RITS, has been implicated for having a role in regulating chromatin status by the use of small RNAs as guides, thus there are likely two systems dependent on small RNAs as guides for controlling steps in either the nucleus or the cytoplasm. What determines whether a particular small RNA will be recruited for chromatin modification resulting in transcriptional gene silencing (TGS) or post transcriptional gene silencing (PTGS) by transcript degradation or translational inhibition is currently not known. Collectively the phenomenon of small RNA regulation is called RNA interference (RNAi).

Antisense inhibition and co-suppression have extensively been applied to silence the manifestation of target genes in plants. There have been many hypothesises regarding the functional mechanism for these technologies although now from the observation of small RNAs derived from successfully silenced genes in transgenic plants points in the direction of similar or same pathways being involved as for RNAi (Hamilton & Baulcombe., 1999). When transgene gene silencing in plants occurs it has frequently been observed that complete or near complete silencing of genes are associated with multiple gene copies inserted into the genome and complex inserts with multiple copies in tandem or as inverted repeats in one locus (Stam *et al.*, 2000; de Buck *et al.*, 2001).

In **Paper IV**, investigation of the locus of a transgenic line transformed with an antisense construct directed against the *gbss* gene is described. In this line no amylose is detectable in the tuber starch as determined by Size Exclusion Chromatography (SEC) and the starch granules are completely stained reddish brown by iodine. Southern analysis indicated a one-copy insert but further experiments revealed an inverted repeat structure consistent with two partial T-DNAs. The original antisense fragment is then present as an asymmetric inverted repeat flanked by *gbss* promoters that are oriented towards the centre of the insert (Figure 10). Interestingly this particular context of the insert has several features that may have implications for the efficiency of silencing the *gbss* gene. First expressed from either promoter the produced transcript will be able to form a dsRNA with a loop. This type of structure has been found to be highly efficient in plants for regulation of gene expression by PTGS (Wang *et al.*, 2001). Second there are two *gbss* promoters that are directed towards each other, which could effect a TGS response in the plant. It has been shown that expression of non-coding sequences, as promoters can be effective for the transcriptional gene silencing in plants (Mette *et al.*, 2000).

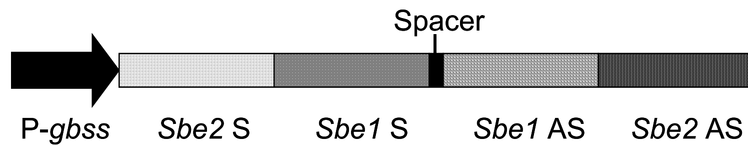

Figure 12. Construct design for dsRNA expression to inhibit *Sbe* genes. P-*gbss* (potato *gbss* promoter), *Sbe2* S (*Sbe2* sense block), *Sbe1* S (*Sbe1* sense block), *Sbe1* AS (*Sbe1* antisense block) and *Sbe2* AS (*Sbe2* antisense block).

With current knowledge regarding the impact of dsRNA structures on gene regulation, transgenic gene silencing has largely evolved from antisense and co-suppression to the deliberate design of gene constructs that express inverted repeat structures where the resulting transcript can fold into dsRNA. We have investigated whether RNAi technology could be used to increase the ratio of silenced genotypes (**Paper II**). From experiments described in **Paper I** we knew what gene regions could be used for silencing of potato *Sbe* genes. However this would result in very large gene constructs where an increased risk might be experienced of including DNA sequence information that could result in early transcript termination or otherwise affect the transcription. Therefore computer modelling was applied to find regions in *Sbe* transcripts that could be predicted to be efficiently accessed by siRNAs. Three different building blocks of 50, 100 and 200 bp were designed for use in a silencing structure as shown in Figure 12.

Cloning of the fragments into an inverted repeat structure proved to be an extremely difficult task. Using a recombination deficient *E. coli* strain and extending the spacer sequence between the repeated DNA elements solved this. In the original design a spacer intended to form the loop structure of the expressed dsRNA was 5 nucleotides long but this was found impossible to assemble into the desired structure. Increasing the spacer length to approximately 35 nucleotides considerably improved the likelihood cloning the fragments as an inverted repeat. Efficiency and rate of transgenic lines showing gene silencing and thereby a high amylose phenotype was greatly increased in comparison to the use of antisense technology. An at least ten-fold improvement in ratio of transgenic lines being of high-amylose type was observed in comparison to the use of antisense technology (Table 2.).

Table 2. *Transgenic lines produced with the dsRNA gene constructs pHAS2 (100 bp blocks), pHAS3 (200 bp blocks) and the antisense construct pHAS4. * Frequency of lines with increased amylose content is based on lines without backbone integration*

Construct	Parental variety	Number of transgenic shoots	Frequency of backbone-free lines (%)	Frequency of lines with increased amylose content* (%)
pHAS3	Dinamo	403	43	55
pHAS3	Kuras	527	40	50
pHAS2	Dinamo	54	33	0
pHAS4	Dinamo	110	45	3
pHAS4	Kuras	51	63	0

A second observation was that up to 88% of the high-amylose lines contained a single copy of the gene construct, which is in contrast with previous studies of transgenic lines for high-amylose and also for amylopectin lines. We believe that the increase in rate of single copy lines showing silencing phenotype, on one hand is related to the higher efficiency of dsRNA expression as compared to expressing an antisense transcript but also to the observation that very high amylose lines are growing more slowly than wild type potato material and have phenotypical aberrations. Thus it may be difficult to regenerate true null *Sbe* expressing lines and if there is an expression relation between level and silencing effect it may be that it is the moderate dsRNA expressors that are isolated which in turn disfavours multicopy events. There is no information on the regeneration behaviour of antisense AGPase lines, which could give information as to how important starch formation is during the regeneration process. However there may also be an effect from the structures generated by non-branched α-1,4 glucan material that imposes other deleterious effects on cell structure or metabolism. Another finding was that the use of 200 bp blocks corresponding to *Sbe1* and *Sbe2* were much more efficient than the 100 bp blocks in the silencing of respective genes (**Paper II**). The nucleotide sequence of the 200 bp blocks comprise the sequence of the 100 bp blocks and the cause of the higher efficiency of the 200 bp blocks so far remain undetermined. It may be that more efficient siRNAs can be derived from the nucleotide sequence lacking in the 100 bp blocks or there may be a simple threshold from more siRNAs being generated from the larger expressed hairpin RNA.

The reduced complexity of T-DNA integrations with only one T-DNA copy inserted is beneficial for regulatory studies where a transgenic event is produced in order to release it on the market. Multiple copy events are generally undesirable as it adds complexity to the detailed characterization of the plant line that is intended to be released on the market.

Conclusions

It was possible to largely separate the biosynthesis of amylopectin and amylose into different potato genotypes. The complete inhibition of amylose synthesis in potato had virtually no impact on the performance and phenotypic appearance of the potato plant. A limited number of changes in tuber chemical composition could be found although some of these might be related to the specific transgenic potato line studied and may not be a general consequence of the lack of GBSS activity. Inhibition of the two known branching enzyme genes had in addition to the altered starch composition a much higher impact on several other characters. At a very high amylose content, plants showed a stunted growth and an overall reduced biomass production, which suggests that some branching is needed for normal plant development. There seem to be a critical point regarding the branching structure of the starch produced where the plant is more severely affected. Most likely this transition point is related to the fraction of real amylose present in relation to the modified amylopectin dependent on degree of *Sbe* gene inhibition. High-amylose tuber starch was always associated with a great increase in soluble sugar content which indicates the importance of branching for maximum starch biosynthetic efficiency. Potato is different from other studied plant species in that both branching enzyme isoforms can more or less fully complement for the lack of the other. Down regulation of either activity alone does not alter starch granule morphology or tuber starch content.

Two potato cDNAs were isolated that when translated contain a core region with motifs very similar to conserved motifs determined or hypothesized to be of importance for the catalytic function of glycogenin in yeast and animals. A use of these genes could be for the over expression in high-amylose potatoes in order to utilize the increased sugar content for production of additional α-1,4 glucan.

For large-scale transformation and production of transgenic lines it is highly desirable to optimize several steps in what is generally referred to as enabling technology. To this category belong *Agrobacterium* transformation, gene expression and gene inhibition. A transformation protocol for potato was developed where the widely used but questioned *nptII* antibiotic selection gene could be replaced by a mutant *Arabidopsis AHAS* gene. The transformation efficiency obtained was higher than has been reported for other selection genes. For most of the work presented, antisense technology was used for gene inhibition. When using the more recently applied RNAi technology the fraction of transgenic potato lines producing high-amylose starch could be increased ten-fold. However this was valid for one specific gene construct. Another gene construct targeting a smaller region of respective *Sbe* gene produced high-amylose lines to a much lesser degree.

The transgenic insert was determined for the amylopectin potato line EH92-527-1. A structure was found that is consistent with integration into a chromosomal double strand break. Repair of the double strand break had utilized microhomology and single strand annealing for DNA synthesis in two steps resulting in an inverted repeat structure extending into the flanking chromosomal DNA. The resulting structure of the transgenic locus could have implications for transcriptional gene silencing from the opposing gbss promoter fragments as well as post-transcriptional gene silencing from the inverted repeat gbss gene sequence.

Future perspectives

High-amylose potato starch is a product that may find many areas of use in food as well as non-food applications. A drawback with potatoes containing a very high amylose content is that plant growth and starch deposition is affected. For future use and production of this starch from potatoes it would be highly desirable increase the starch content. Further knowledge on how and under what conditions GBSS operate but also how starch synthesis is initiated will be very important in order to utilize the increased sugar content in high-amylose potato lines for the production of amylose starch. Interestingly, starch granules are formed even at very high amylose levels. The most accepted models for starch granule organization does not apply to these granules as no amylopectin with chain length fractions that could form clusters are present. Studies on starch structure and organization in high-amylose starch granules would thus be of great interest to determine what new level of organization is taking place and what physical constraints may be related to this.

It is interesting to note that starch granules are formed under conditions that physiologically must be considered to be quite diverse among plant species and plant tissue types. Furthermore granules are even formed when several of the important enzymatic activities are lacking. Yet it has so far not been possible to form granules in an organism producing glycogen by the heterologous expression of genes involved in starch biosynthesis enzymes. Hence it remains to be proven what the minimum conditions in higher plants are to form a starch granule.

Developments in the area of plant transformation and selection of transgenic cells are of interest to all whom for research purposes or otherwise want to propagate transgenic plants under field conditions in Europe. Even in light of no scientific evidence that selection genes used in transformation could cause concerns for food and environmental safety there may for legislative and public reasons be a need for new schemes of plant transformation. Recently the frequency of potato transformation without using any selection gene has been reported (de Vetten et al., 2003). Although this report does not contain any new technology it is still of interest since it applies regeneration under no selection pressure on a quite large scale with the subsequent determination of transgenic status. In another study, plant genomic loci with DNA sequence similar to the T-DNA border repeats was utilized to design a T-DNA containing only plant DNA, which can be recognized and transferred to the potato genome by *Agrobacterium tumefaciens* (Rommens et al., 2004). In further developments it would be highly desirable to be able to efficiently use the mechanism of homologous transformation for the modification of plant genomes. A challenge for all these directions in the modification of plant genomes is to increase the fraction of cells being transformed without increasing the number of inserts in the plant cells. Furthermore the single locus of modification should be in a desirable genetic context.

Technologies for gene inhibition have progressed greatly during the last years. In this context it is of great interest to follow up on if specific genes or gene structures are more prone to silencing. It is likely that there are mechanisms regulating this on several different levels although how remains to be elucidated. This is important in order to reduce the amount of effort spent with regenerating and characterizing transgenic lines that are not silenced and thus of no importance for further studies.

It will be very interesting to see if, when and where the produced potato starch qualities will be used in different applications (Figure 13.). Physical studies and tests are ongoing and needed to assess respective product potentials.

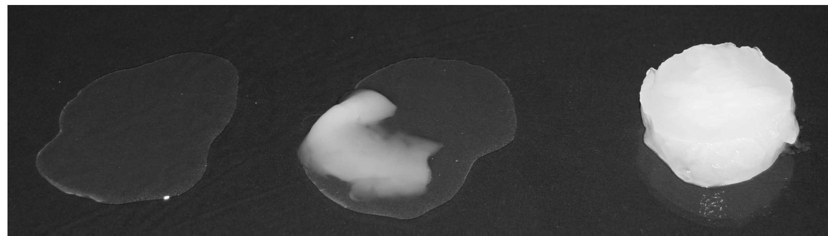

Figure 13. 5% starch w/v in water solution autoclaved for 20 minutes at 120°C and allowed to cool to room temperature. From left to right; amylopectin starch, ordinary potato starch and high-amylose potato starch.

The span from genes to product properties is one of the exiting aspects of studies related to starch. First, α-1,4 α-1,6 glucans as glycogen and starch are at the very basic root of evolution being represented in most living organisms. Studies comprise how living organisms have evolved the storage of energy as a largely inert compound and also the genetic regulation, biosynthetic production and molecular organization of this product. Second, starch is a very important compound for the human being as a source of energy from crop plants and furthermore as a material for an extensive range of applications in the food as well as in the non-food sector.

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