Modification of Potato Starch Granule Structure and Morphology *in planta* by Expression of Starch Binding Domain Fusion Proteins

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Content

Chapter 1

General Introduction

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Carbohydrates are the most abundant bio-compounds on Earth. More than 100 billion metric tons of CO_2 and H_2O are converted into carbohydrates and other plant compounds by green plants during the process of photosynthesis every year (Nelson and Cox, 2004). Most carbohydrates found in nature occur as polysaccharides containing from twenty to hundreds or thousands of monosaccharide units. Different kinds of polysaccharides differ in the recurring monosaccharide units, the length of chains, the types of bonds linking the units, and the degree of branching. Carbohydrates have different biological functions such as energy storage, like starch and glycogen, structural components (such as cellulose, hemicellulose, pectin and chitin) and play important roles in the lubrication of skeletal joints and participate in recognition and adhesion between cells. Due to these functional diversities, polysaccharides are exploited both in food and non-food industries (Ramesh *et al.*, 2003).

Starch structure

Starch is the most abundant storage carbohydrate in higher plants. It can be found in different plant organs such as cereal grains, roots, tubers, fruits and leaves and when it is stored in amyloplasts it is in a granular form. Starch is a versatile and very useful carbon source because it is abundant, cheap and renewable. Starch synthesized in the chloroplasts of green leaves as transitory starch during the day can provide carbon for non-photosynthetic metabolism at night. Starch produced in amyloplasts of storage tissues has a carbon store function.

Starch granules (ranging in size from 1 μm to 100 μm, depending on the source) consist of an amorphous and a semi-crystalline region (Myers *et al.*, 2000), and have a complex structure with hierarchical order composed of amylose and amylopectin. Amylose is an essentially linear molecule of α-1,4 linked glucose residues, and it is the main component of the amorphous layers of the granules and typically constitutes about 20-25% of the granule mass. In wild type cereals the amylose content can go up to 30%. Amylopectin locates in the semi-crystalline region of the granules having the same α-1,4 linked glucan backbone as amylose but with 5-6% of α-1,6 bonds at the branch points and

accounts for the other 70% or more of the sugar polymers in starch granules. Amylopectin (molecular weight of 10^{7-8} Dalton) is by far a larger molecule than amylose (molecular weight of 5 x 10^5 Dalton). Amylopectin can form double helices which are the basis of the semi-crystalline structure of the starch granules.

Starch biosynthesis in storage organs

The synthesis of starch granules takes place in the chloroplasts and amyloplasts of higher plants and involves three major steps (i) the transport of Glc-6-P into the plastids, (ii) the synthesis of ADP-glucose from Glc-1-P and (iii) the synthesis of starch from ADP-Glc, where a plethora of starch enzymes are acting together (Figure 1). Sucrose transported by the phloem through the whole plant is the source of carbon for starch biosynthesis which is converted into hexose-phosphate sugars and transported into the amyloplast to supply Glc-6-P (Kammerer *et al.*, 1998). Sucrose can enter the cell via the apoplast, either by sucrose transporters or hexose transporters after hydrolysis, or via plasmodesmata (symplastic transport). Once inside the cell, sucrose can be transported into the vacuole and/or hydrolysed by acid and alkaline invertases to glucose and fructose. Alternatively, sucrose synthase (SuSy) can convert sucrose into UDP-glucose and fructose in the cytosol. The hexoses formed in the vacuole can be transported into the cytosol and phosphorylated. The first committed step in the biosynthesis of both transient starch in chloroplasts and storage starch in amyloplasts is the synthesis of ADP-Glucose from Glc-1-P and ATP which is catalyzed by ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27). Starch synthase enzymes (SS; EC 2.4.1.21) transfer glucose from ADP-Glc to the non-reducing end of an α-1,4 glucan to synthesize the insoluble glucan polymers amylose and amylopectin. Based on sequence similarities in *Arabidopsis thaliana*, five isoforms of starch synthases have been predicted in plants: granule-bond starch synthases (GBSSI) and soluble starch synthases (SSI, SSII, SSIII and SSIV) (Ball *et al.*, 1998). The granule-bound starch synthase (GBSSI) in storage tissues, in leaves, and in other non-storage tissues, catalyzes the synthesis of amylose. Soluble starch synthases are involved in the synthesis of amylopectin. The starch branching enzymes (SBEI and SBEII; EC 2.4.1.18) creates α-1,6 linkages by cleaving α-1,4 linkages to generate the

branched structure of the amylopectin molecule. Subsequently, amylopectin is crystallized into starch granules by concerted efforts of starch debranching enzymes (DBE; EC 2.4.1.41) and disproportionating enzyme (D-enzyme; EC 2.4.1.25) (Ball *et al.*, 1998).

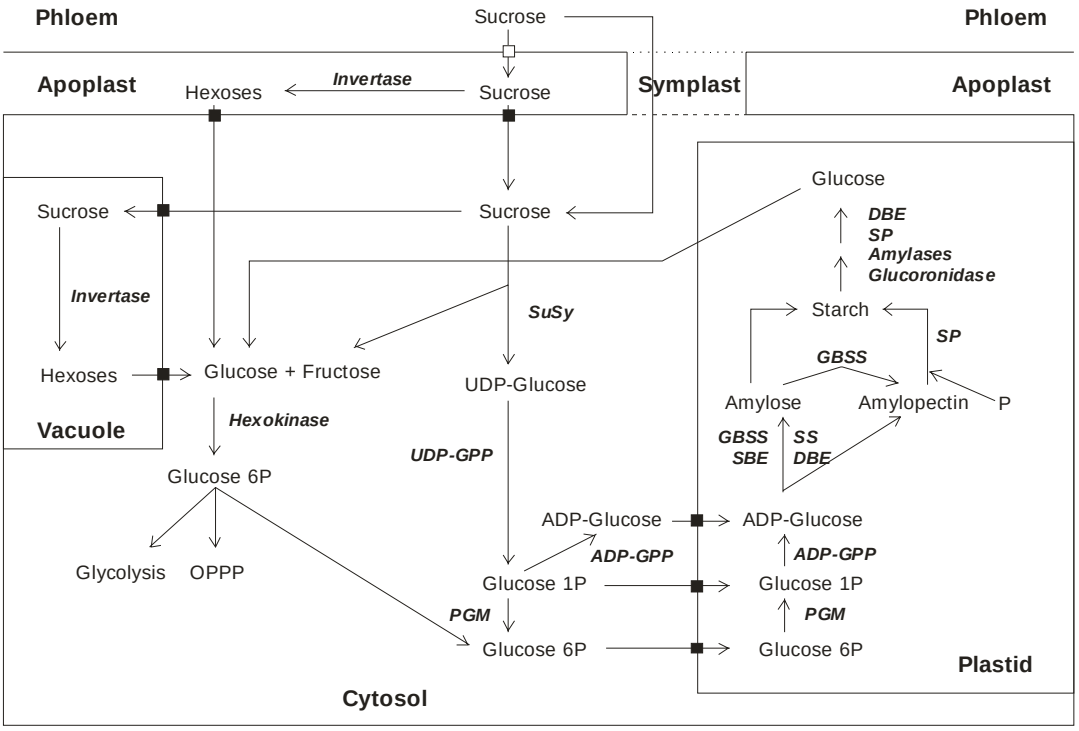

Figure 1. Starch biosynthetic pathway. Sucrose synthase (SuSy), oxidative pentose phosphate pathway (OPPP), glucose-1-phosphate (Glucose-1-P), UDP-glucose pyrophosphorylase (UDP-GPP), glucose-6-phosphate (Glucose-6-P), phosphoglucomutase (PGM), ADP-glucose pyrophosphorylase (ADP-GPP), granule bound starch synthase (GBSS), starch synthases (SS), starch branching enzymes (SBE), phosphate monoesters (P), starch phosphorylases (SP), debranching enzymes (DBE), starch phosphorylases (SP).

From an industrial and environmental point of view, starch is a very interesting resource as it is renewable and biodegradable and it can be modified to a diverse range of products. With the better understanding of the starch biosynthesis pathway, it is becoming more and more clear that producing novel starches through genetic modification of plants may be realized. The engineering of starch biosynthesis may lead to modification of starch granule shape, amylose to amylopectin ratio, amylopectin chain length, phosphorylation, and lipid content. Starches with modified properties are expected

to be used more efficiently and for a wider range of applications in both food and nonfood industries.

Native starch has a limited number of uses because it does not have most of the desired characteristics demanded by industry. But it can be modified by physical treatment, chemical reagents or enzymes to suit various applications and to meet the specific needs of end users. Recently, novel starches that have improved functionalities have been produced *in planta* through genetic modification techniques. Starch with altered amylose/amylopectin ratio and a different branch degree of amylopectin have been achieved in potato (Kuipers *et al.*, 1994, Schwall *et al.*, 2000, Jobling *et al.*, 2002). In these mutants, genes involved in starch biosynthesis have been knocked out/down.

Alteration of starch composition through the modeling of plant genes

During the last 15 years the scientific community has been occupied with the increasing request for carbohydrates with novel properties by industry. Several different strategies have been used to study the starch biosynthesis pathway in higher plants and to produce novel products with new functional properties. One of these strategies was to modulate the starch biosynthetic pathway by knocking out or over-expressing endogenous genes involved in this pathway. A second strategy involved the expression of heterologous genes encoding biosynthetic or modifying enzymes from other organisms, including bacterial, fungal and animal enzymes. Although the activity of several of these enzymes was initially identified in other carbohydrates than starch including glycogen, other glucans or fructans, most of these enzymes have shown an effect on different aspects of starch such as composition, granule morphology and starch properties.

Amylose-free starch

The first genetically modified amylose-free (*amf*) starch in potato has been generated by the silencing of the GBSS gene by using antisense RNA (Visser *et al.*, 1991). Besides these transformed lines an induced mutant by X-irradiated was also obtained (Hovenkamp-Hermelink *et al.*, 1987). Both the GM line and the natural GBSS

mutant genotypes have improved paste clarity and stability (Visser *et al.*, 1997). The naturally occurring mutant has been explored commercially for different applications by the Dutch company AVEBE under the brand name ELIANE^{TM}. A different amylose-free starch with short-chain amylopectin showing an extremely high freeze−thaw stability was created by simultaneous knock down of three starch synthase genes (GBSS, SSII and SSIII) (Jobling *et al.*, 2002).

High-amylose starch

High-amylose starches are of great interest for the starch industry such as the development of starch-based films for its unique functional properties (van Soest and Borger, 1997; Rindlav-Westling *et al.*, 1998). One of the strategies used for the generation of starch with an increased level of amylose was the down regulation of genes involved in the synthesis of amylopectin (Jobling *et al.*, 1999). Inhibition of SBE A and B in potato lines has resulted in starch granules with more than 60% of amylose (Schwall *et al.*, 2000). One other strategy for the increase of amylose content in starch granules was the tagging of antibodies to enzymes involved in amylopectin synthesis. A single-domain antibody directed against SBE Ⅱ in potato was successfully used to produce starches with more than 50% amylose (Jobling *et al.*, 2003).

Modification of starch properties by expression of bacterial proteins in plants

Glycogen is a highly branched water-soluble glucan molecule like amylopectin, but it has a shorter average length of the side chains (Myers *et al.*, 2000). The formation of α-(1→6) branch points is catalyzed by branching enzymes (BE) and starch branching enzymes (SBEs) in plants, and glycogen branching enzymes in *E. coli* (Nakamura, 2002). In order to increase the branching degree of potato tuber starch, several genes encoding branching enzymes involved in glycogen biosynthesis in *E. coli* were expressed in amylose-containing or/and amylose-free potato (Stark *et al.*, 1992; Shewmaker *et al.*, 1994; Kortstee *et al.*, 1996).

A mutant *E. coli* ADP-Glc pyrophosphorylase (*glgC*) has been expressed in potato tuber amyloplasts resulting in increased starch accumulation in the tubers (Stark *et al.*, 1992). Expression of *E. coli* glycogen synthase (*glgA*) in potato tuber amyloplasts leads to a number of changes in the starch composition: a decreased specific gravity, a reduction in the percentage of starch, a decline in amylose/amylopectin ratio and a reduced phosphorous content. The starches obtained from these transgenic potato lines have a lower viscosity value, reduced enthalpy and gelatinization properties when compared with starches of control (Shewmaker *et al.*, 1994).

The expression of the *E. coli* branching enzyme (*glgB*) in the amylose-free potato mutant resulted in more branched starch structure and up to 25% increase in the branching degree (DE) of amylopectin (Kortstee *et al.*, 1996). Except for a slightly higher amylopectin percentage and a small difference in granule surface morphology, the expression of *glgB* in amylose-containing background did not show any significant changes in starch composition and properties (Krohn *et al.*, 1994).

In order to increase the acetylation of starch, an amyloplast-targeted *E. coli* maltose acetyltransferase gene (MAT) was expressed in tubers of wild type (Kardal) and mutant amylose-free (*amf*) potato plants. Acetyl groups were found in the starch granules of transgenic plants, although at a low concentration (Nazarian *et al.*, 2007a).

Use of carbohydrate binding domain to modify starch *in planta*

Microbial hydrolytic enzymes, which catalyze the degradation of starch or other insoluble polysaccharides, typically have a distinct domain referred to as carbohydrate-binding modules (CBMs). CBMs from different enzymes are about 90-130 amino acids long and have well conserved amino acids sequences (Janecek *et al.*, 1999). To date, various types of carbohydrate-binding modules (CBMs) have already been recognized and classified into 59 different CBM families (Cantarel *et al.*, 2009, Guillén *et al.*, 2010) based on their amino acid similarity. Starch binding domains (SBDs) are CBMs with the affinity to bind to starch granules. There are nine CBM families, termed 20, 21, 25, 26, 34, 41, 45, 48, and 53, which have been reported to contain SBDs (Christiansen *et al.*, 2009). Interestingly, SBDs normally can be found as a single unit at the C- or N-

terminus of catalytic domains, but sometimes they also can be found as multiple repeats, for example, an α, β-amylase from *Paenibacillus manihotivorans* contains a tandem CBM25 domain between the α-amylase domain and the β-amylase domain (Cantarel *et al.*, 2009).

Although there are 9 CBM families which contain SBDs, until now only SBDs from CBM family 20 have been well studied (Christiansen *et al.*, 2009) and introduced into plants successfully (Ji *et al.*, 2004, Howitt *et al.*, 2006). It is still unknown whether the SBDs from other CBM families can be used to target proteins into starch granules and whether they have other different biochemical properties relatively to CBM20s.

SBDs can be used as tools to target effector proteins to starch granules during the biosynthesis process because SBDs do not lose the binding ability to starch granules when they are separated from the natural proteins (Lin *et al.*, 2003) and enzymes can acquire affinity to starch granules by fusing to SBDs (Ohdan *et al.*, 2000). In order to use SBDs as anchors for targeting effector enzymes to generate potato starches with new or improved properties, a family 20 SBD domain from cyclodextin glycosyltransferase (CGTase) of *Bacillus circulans* was successfully incorporated into starch granules during starch biosynthesis without any effects on starch properties (Ji *et al.*, 2003). Several different enzymes from bacteria, such as the *E. coli* maltose acetyltransferase (MAT) and a truncated mutansucrase (GtfICAT), have been introduced into starch granules using this SBD technique and resulted in morphologically altered starch granules (Nazarian *et al.*, 2007 a, b).

Scope of this thesis

The aim of this thesis was to modulate starch *in planta* by expressing different bacterial proteins fused to an SBD from CBM family 20 in potato and to explore the possibility of using SBDs from other CBM families for applications in starch bioengineering.

Producing starches with altered composition, structure and novel physico-chemical properties to meet specific needs for different applications by manipulating the enzymes which are involved in starch metabolism has huge advantages in starch industry. It can

not only broaden the range of starch applications but also reduce the costs of the post-harvest starch modification, which is mainly done by using chemicals and is high energy-consuming, expensive, and often environmentally unfriendly. *In planta* modification of starch offers a great opportunity for producing so-called "tailored starches" by using the SBD technique (Ji *et al.*, 2003). First of all, starch biosynthesis has been much better understood in the last two decades which makes it far more easy to manipulate or interfere with the particular enzymes for producing novel starches. For instance, starch with altered amylose/amylopectin ratio and altered branching degree of amylopectin have been achieved in plants by down-regulating the native genes such as GBSSI and Branching Enzymes. Secondly, the fused SBD facilitates the catalytic activity of enzymes because it brings the enzyme and substrate closer to each other. Moreover, the *E. coli* MAT fused with SBD acetylates starch granules during starch biosynthesis (Nazarian *et al.*, 2007a) and makes this a successful example of using SBD with other enzymes.

In this thesis, two different microbial enzymes, *E. coli* glycogen branching enzyme (GlgB) and *Neisseria polysaccharea* amylosucrase, were chosen to modify starch composition or structure by fusing with an SBD. Furthermore, the GlgB/SBD and Amylosucarse/SBD transformants were analyzed at the transcriptional level by using the POCI array to investigate whether and how the expression of these heterologous genes affect the expression levels of genes which are involved in the starch biosynthesis pathway. A number of questions were tried to be answered in this thesis research: (i) How does the expression of MAT/SBD fusion protein affect starch biosynthesis and give rise to amalgamated starch granules? (ii) Is it possible to increase the amylopectin branching degree by a GlgB/SBD fusion protein? (iii) Is it possible to synthesize amylose-like polymers in potato by amyloscurase fused with SBD? and (iv) Is it possible to use SBDs from other CBM families for starch engineering?

Previous study showed that the expression of an *E.coli* maltose acetyltransferase (MAT) fused with a starch binding domain (SBD) resulted in amalgamated starch granules. In Chapter 2, the starch granule morphology at different stages during potato tuber development was analyzed to elucidate when and how the deposition of MAT/SBD fusion protein interferes with starch biosynthesis, and the transcriptomic analysis of

tubers from the controls and transformants with altered morphology of the granules was performed.

Branching degree of amylopectin plays an important role in the physico-chemical behavior of starch. In Chapter 3, a glycogen branching enzyme (*glgB*) from *E. coli* fused to an SBD was expressed in both amylose containing potato background (Kardal) and an amylose free potato mutant (*amf*) in order to increase the amylopectin branching degree of potato starch. The POCI array was used to investigate how the expression of *glgB* interfered with the transcription level of other genes in the starch biosynthesis pathway.

Amylosucrase is a glucansucrase belonging to glysoside-hydrolase (GH) family 13 which catalyzes the synthesis of an amylose-like polymer from sucrose. In Chapter 4, an amylosucrase from *Neisseria polysaccharea* fused to an SBD was introduced in two potato genetic backgrounds (wild type and amylose-free mutant) to modify amylose/amylopectin ratio, and thereby, broaden starch applications. Moreover, transcription profiling of genes in an Amylosucrase/SBD transformant was analyzed by using the POCI array.

Besides the CBM20, there are eight CBM families have the affinity for starch granules. In Chapter 5, a tandem CBM25 domain of α-amylase from *Microbacterium aurum* was introduced in potato to assess whether it is suitable for applications in starch bioengineering.

In Chapter 6, results from all above chapters are discussed. Furthermore, future perspectives on starch modification *in planta* are discussed.

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Chapter 2

Transcriptional profiling of potato expressing an *E. coli* maltose acetyltransferase gene

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Abstract

It has been shown previously that the expression of an *E.coli* maltose acetyltransferase (MAT) fused with a starch binding domain (SBD) in potato amyloplasts resulted in amalgamated starch granules. Two representative transformants, KDMS-30 and *amf*MS-15, which showed a high MAT/SBD protein accumulation level in starch granules and both had the highest percentage (approximately 12%) of amalgamated starch granules were chosen for further studies. Starch granule morphology at five tuber developmental stages was analyzed in both MAT/SBD transformants and untransformed control tubers. The results showed that the amalgamated starch granules appeared in the MAT/SBD transformant tubers when the tuber had a size of more than 1cm in diameter. Gene expression profiling of MAT/SBD transformant tubers was performed using POCI array. The transcriptomic analysis showed that the expression of MAT/SBD fusion protein in Kardal and *amf* potato tubers did not affect the transcript level of other genes in the starch biosynthetic pathway. However, many genes involved in lipid metabolism were down-regulated in KDMS-30 transformant tubers. This was consistent with the fact that in this transformant a small increase in acetylation degree of the starch was obtained.

Keywords: *E.coli* maltose acetyltransferase, SBD, starch morphology, starch acetylation, lipid metabolism

Introduction

Starch is an abundant, renewable, and biodegradable polymer in many plants such as rice, corn, wheat, tapioca, and potato as a source of stored energy (Buléon *et al.*, 1998). It is an important human ingredient since it contributes more than half of the energy in human diet. It is also widely used as a raw material for many industrial applications such as paper and cardboard, packing material, adhesives, pharmaceuticals, textiles, bioplastics, and oil drilling (Ellis *et al.*, 1998; Roper, 2002). Although starch granules mainly consist of two α-glucan macromolecules: amylose and amylopectin, they have different size, shape, and chemical content depending on their botanical origin. Normally potato starch granules contain amylose (20-25%), amylopectin (75-80%), and other minor compositions such as lipids and phosphorus (less than 1%) (Ellis *et al.*, 1998). Potato starch granules are oval or round in shape, have smooth granule surface, and range from 1 to 110 μm in size (Hoover *et al.*, 2001). As well as other native starches, potato starch also has limitations such as low shear, acid and thermal resistance, and high tendency towards retrogradation that reduce their uses in industrial applications (Singh *et al.*, 2004). To meet the specific needs of the end uses, native starches are often modified by chemical or physical treatments (Singh *et al.*, 2009).

Acetylated starch is widely used in the food industry to overcome undesirable changes in product texture and appearance caused by retrogradation during processing and storage (Miyazaki *et al.*, 2006). Among the many kinds of chemically modified starches, acetylation of starch has been applied for more than a century to improve starch functional properties since acetylated starch has typically physico-chemical characteristics such as low gelatinization temperature, high solubility, good cooking and storage stabilities (Miyazaki *et al.*, 2006). During acetylation, three free hydroxyl groups at the C2, C3 and C6 position of the glucose molecule within the starch can be substituted with acetyl groups. A degree of substitution (DS) ranging from 0.01 to 0.2 is considered best for food and used to improve binding, thickening, stability and texturing (De Graaf *et al.*, 1998). Acetylated starch with low DS is commonly obtained by esterification of native starch with acetic anhydride in aqueous medium in the presence of an alkaline catalyst. However, the drawback of this chemical treatment is that the industrial wastes

which are a consequence of using these (toxic) chemicals are environmentally unfriendly.

As a biological alternative to chemical derivatization, the *E. coli* maltose acetyltransferase gene (MAT) fused with a starch-binding domain (SBD) has been expressed in potato to produce acetylated starches *in planta* (Nazarian *et al.*, 2007a). The starch granules in transgenic plants in the wildtype (amylose containing) background contain a low degree of acetyl groups and the enzyme MAT residing inside the starch granules had post-harvest activity (Nazarian *et al.*, 2007a). Moreover, the starch granule morphology is altered in transgenic plants in both (amylose-free and -containing) genetic backgrounds. Although in the last several decades many enzymes were recognized which are involved in starch biosynthesis, the mechanisms that determine starch granule morphology, size, and number are not well understood (Zeeman *et al.*, 2010). The starch morphological alteration in MAT/SBD transformants suggests that the expression of the MAT enzyme may have interfered with starch biosynthesis or the starch granule assembling process.

To elucidate when and how the deposition of MAT/SBD fusion protein interferes with starch biosynthesis pathway and the key genes involved in this process, we have investigated the starch granule size distribution and morphology at different stages during potato tuber development and carried out transcriptomic analysis of the tubers with altered morphology of the granules in selected representative MAT/SBD transformants.

Results

Alteration in starch granule morphology occurs at or after tuber developmental stage 6

In a previous study it was shown that the expression of *E. coli* maltose acetyl transferase fused with SBD (MAT/SBD) in potato resulted in starch acetylation at a low level in Kardal background. In *amf* background, however, no starch acetylation was observed (Nazarian *et al.*, 2007a). The other effect of accumulation of MAT/SBD fusion protein in starch is the alteration of starch granule morphology. Amalgamated starch granules were observed in transformed mature potato tubers in both Kardal and *amf*

backgrounds (Nazarian *et al.*, 2007a and Fig. 2 F and H). In order to investigate at which time point during potato tuber development the accumulation of MAT/SBD fusion protein gave rise to the starch morphological alterations, starches were isolated from two representative transformants, KDMS-30 and *amf*MS-15. as well as from the untransformed potato (Kardal and *amf*) tubers at five tuber developmental stages corresponding to potato tuber developmental stages 3, 4, 5, 6, and 7 as described in Kloosterman (Kloosterman *et al.*, 2005; Fig. 1 A). The KDMS-30 and *amf*MS-15 transformants showed a high MAT/SBD protein accumulation level in starch granules and had a high percentage (approximately 12%) of amalgamated starch granules. The median granule size and starch granule size distribution were determined by a Coulter Multisizer.

The results of this analysis showed that the distribution pattern of starch granules at different stages were similar in all potato clones but shifted from small to large in particle size from tuber developmental stages 4 through 7 (Fig. 1 B). No starch size distribution data at tuber developmental stage 3 is shown because not enough material was available to perform the analysis. The median granule size increased from 7.7 ($\pm$ 0.4) μm in *amf* and 6.9 ($\pm$0.6) in Kardal untransformed control tubers at tuber stage 4 (potato tuber size is about 0.2 cm in diameter) to 14.1 ($\pm$ 0.5) μm in *amf* and 16.9 ($\pm$ 0.6) μm in Kardal at tuber stage 7 (about 2 cm in tuber size) (Fig. 1 B).

The morphology of the starch granules from KDMS-30, *amf*MS-15, and untransformed controls at different tuber development stages was analyzed with scanning electron microscopy (SEM). The SEM analysis showed that the starch granules were oval or spherical with smooth granule surface in untransformed controls, Kardal and *amf*, at all tuber developmental stages. In other words, besides the change in size of the starch granules no other changes in starch granule shape and/or surface could be observed.

Starch morphological alterations were detectable at tuber stage 6 (tuber size about 1 cm) in MAT/SBD transformants in Kardal and *amf* backgrounds. The starch granule morphology of both KDMS-30 and *amf*MS-15 tubers were similar to that of untransformed control starches (Kardal and *amf*) at stage 3, 4, and 5. As can be seen in Figure 2, many polygonal and irregularly shaped small starch granules were visible in KDMS-30 and *amf*MS-15 stage 6 tubers which were not observed in Kardal and *amf* untransformed tubers (Compare Fig. 2 A with E and C with G). In the mature tubers,

these polygonal small granules turned into grape-looking amalgamated starch granules which were observed in previous study (Nazarian *et al.*, 2007a) in both Kardal and *amf* backgrounds (Compare Fig. 2 B with F and D with H). This phenomenon indicated that the alteration of starch granules morphology caused by the expression of MAT/SBD fusion protein in starch granules can be detected when the KDMS-30 and *amf*MS-15 tubers are about 1 cm in size.

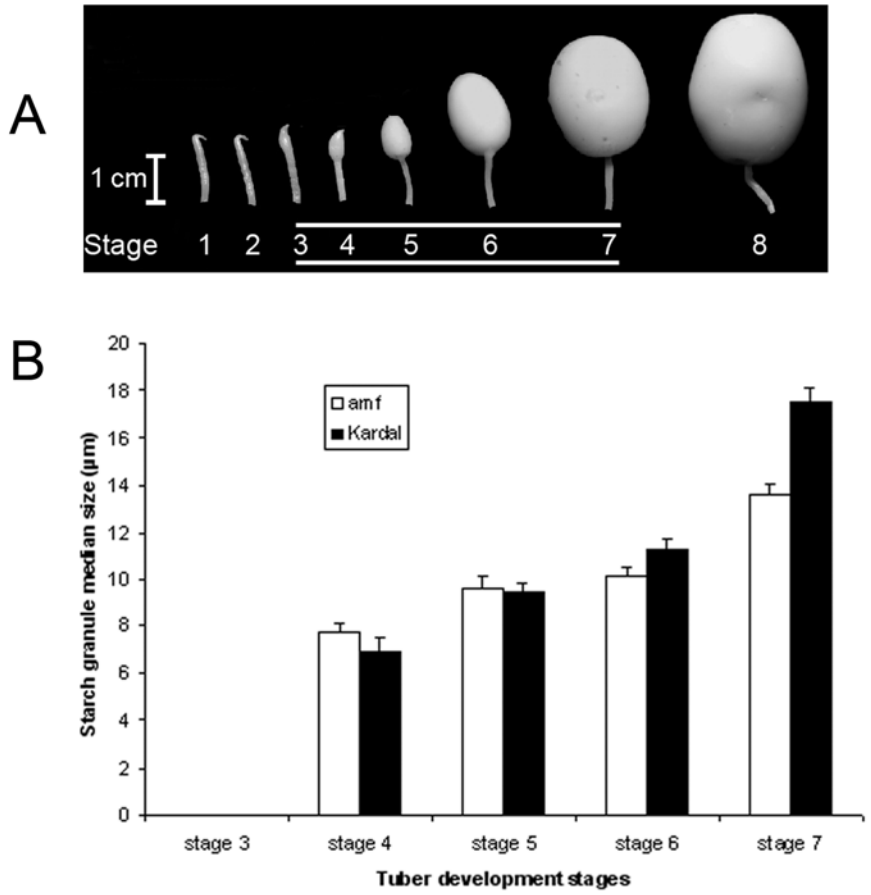

Figure 1. Panel A: the potato tuber developmental stages (Kloosterman *et al.*, 2005); Panel B: the starch granule median size of Kardal and *amf* untransformed controls at different tuber developmental stages (stage 3 to 7).

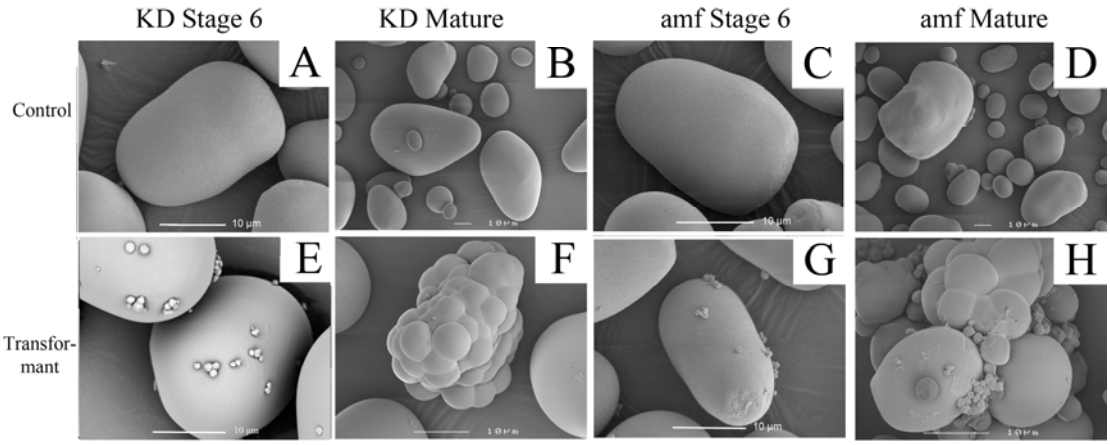

Figure 2. SEM analyses of starch granules from KDMS-30 and *amf*MS-15 in comparison with that of the untransformed controls at different magnifications. KD-UT Stage 6 (A), KD-UT Mature (B), *amf*-UT Stage 6 (C), *amf*-UT Mature (D), KDMS-30 Stage 6 (E), KDMS-30 Mature (F), *amf*MS-15 Stage 6 (G), and *amf*MS-15 Mature (H). The scale bar is shown in each image.

Apparently normal granules are modified in their inner structure

In mature tubers, besides the amalgamated starch granules, there were starch granules in normal size and appearance but containing many small granules as a cluster inside the granule in both KDMS-30 and *amf*MS-15 transformants when the starch granules were investigated by light microscopy under polarized light (Fig. 3 A, E and B, F). As can be seen in Figure 3, these altered starch granules did not show the characteristic “Maltese cross” as the normal assembled starch granules.

Starch granule sections were analyzed with the light microscopy to investigate the internal structure of starch granules from KDMS-30 and *amf*MS-15 as well as their corresponding controls. The internally amalgamated starch granules were observed in MAT/SBD transformants in both genetic backgrounds. Furthermore, the section images showed that there were many polygonal irregular tiny granules inside big normal-looking granules (Fig. 3 C, G and D) and the grape-looking amalgamated starch granule was composed of many small granules inside too (Fig. 3 H). These internally amalgamated starch granules still showed growth rings, however, the growth rings were not distributed as regular as that of untransformed starch granules (Fig. 3 D). These observations suggest that the amalgamated phenotype start at an early stage of starch granule formation.

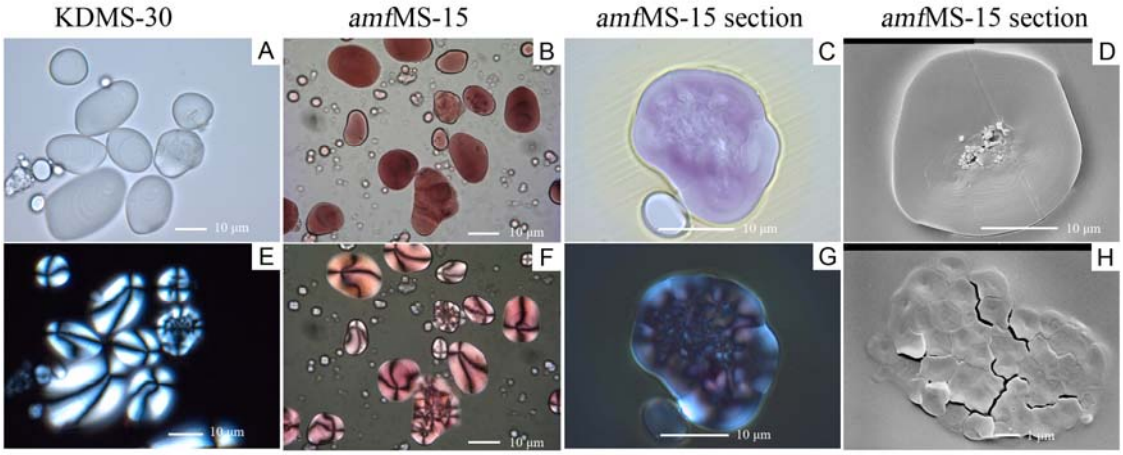

Figure 3. Micrographs showing the whole granule images of KDMS-30 (A, E) and *amf*MS-15 (B, F) under Light Microscope normal (A, B) and polarized light (E, F) and section images of *amf*MS-15 (C, D, G, and H) using Light Microscope (C, G) and SEM (D, H). The scale bar is showed in each image.

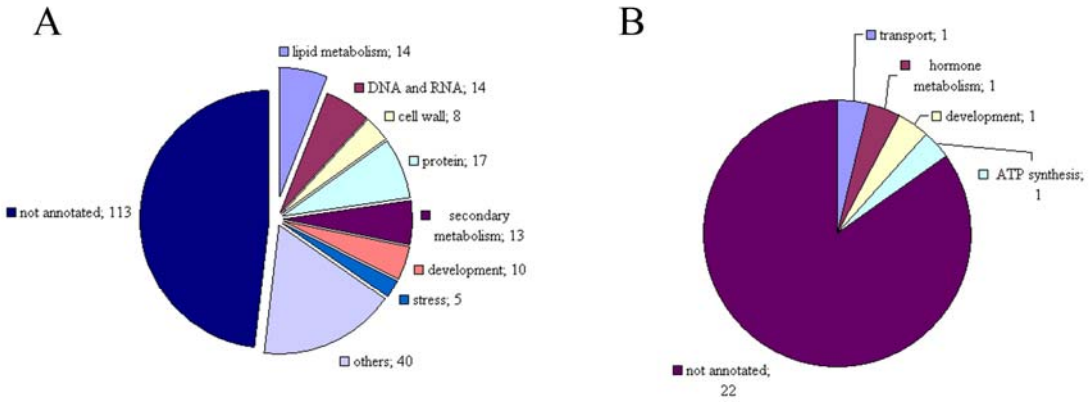

Figure 4. Classification of the differentially expressed genes in KDMS-30 stage 6 tubers (**A**) and *amf*MS-15 stage 6 tubers (**B**) in comparison with their untransformed control stage 6 tubers. The differentially expressed genes were classified into different biological processes by MapMan tool.

Expression of MAT/SBD fusion protein did not affect the expression of genes directly involved in starch metabolism

Expression of *E. coli* maltose acetyltransferase fused with SBD in Kardal and *amf* gave rise to starch granule morphology alterations from potato developmental stage 6 (Fig. 2 E and G), which indicates that the effect of expression of MAT/SBD fusion protein on starch granule morphology in potato tubers showed up when tubers are around

1cm in size. In order to understand gene expression in tubers containing the MAT/SBD fusion protein and how it interfered with starch biosynthetic pathway or the starch granule assembling process, a high-throughput transcriptomic profiling of potato stage 6 tubers (approximately 1cm in tuber size) from KDMS-30 and *amf*MS-15 transformants as well as the Kardal and *amf* untransformed controls was performed using the POCI array (Kloosterman *et al.*, 2008). Microarray data analysis was performed in which genes were considered to be differentially expressed when they showed an expression ratio ≥ 2-fold or ≤ 0.5-fold with a statistical significance of $p < 0.05$.

The microarray results showed that there were 229 genes (55 genes up-regulated and 174 down-regulated) that had at least a 2-fold change in expression level with a statistic p value below 0.05 when KDMS-30 tubers were compared with untransformed Kardal tubers. From these 229 differentially expressed genes, more than 50% were without annotation. However, in *amf* background, there were only 26 genes which were differentially expressed in *amf*MS-15 tubers in comparison with the *amf* untransformed tubers (21 genes were induced and 5 were repressed). Furthermore, only three genes, bf_mxflxxxx_0013h06.t3m.scf, MICRO.17222.C1, and MICRO.15836.C1, were shared in both Kardal and *amf* background. However, all these three genes have no functional annotation.

The MapMan tool (Thimm *et al.*, 2004) was used to identify the biological processes in which the genes with altered expression level were involved in. It showed that expression of MAT/SBD fusion protein in Kardal background interfered with the expression of many genes involved in multiple processes such as protein synthesis and degradation, DNA and RNA regulation, cell wall biosynthesis, lipid metabolism, plant development, secondary metabolism and so on (Fig. 4 A). However, the effects on these biological processes were not observed in *amf*MS-15 tubers (Fig. 4 B).

The genes encoding enzymes directly involve in starch and sucrose metabolism such as ADP-glucose pyrophosphorylases (AGPase), soluble and granule-bound starch synthases (SSs and GBSS), branching enzymes (SBEs), debranching enzymes: isoamylases and limit dextrinase (ISAs and LDA), α-glucan water dikinases (PWD and GWDs), disproportionating enzymes (DPEs), α-amylases (AMYs) and β-amylases (BAMs), did not show any changes in both KDMS-30 and *amf*MS-15 tubers in

comparison with untransformed control tubers.

Table 1. Differentially expressed genes in lipid metabolism pathway in KDMS-30 stage 6 tubers

Gene Name	Description	CLASS	p value	Folds
MICRO.14875.C1	acyltransferase	lipid metabolism	0.001	2
MICRO.10131.C1	acyltransferase	lipid metabolism	0.001	2
MICRO.14341.C1	hypothetical protein	lipid metabolism	0.009	2
STMIG79TV	acyl CoA reductase	lipid metabolism	0.002	2
MICRO.3996.C1	hypothetical protein	lipid metabolism	0.001	3
MICRO.8605.C1	putative acyl-CoA synthetase	lipid metabolism	0.003	3
MICRO.16325.C3	hypothetical protein	lipid metabolism	0.004	3
MICRO.8619.C1	hypothetical protein	lipid metabolism	0.010	3
MICRO.6234.C1	hypothetical protein	lipid metabolism	0.004	3
bf_mxflxxxx_0039g06.t3m.scf	No description	lipid metabolism	0.001	3
MICRO.670.C1	acyltransferase	lipid metabolism	0.001	3
MICRO.10325.C1	3-ketoacyl-CoA synthase	lipid metabolism	0.002	3
POCBC15TV	putative O-linked N-acetyl glucosamine transferase	lipid metabolism	0.001	5
MICRO.5582.C2	hypothetical protein	lipid metabolism	0.001	6

Noteworthy, the expression level of 14 genes in lipid metabolism was affected by the expression of MAT/SBD fusion protein in starch granules in KDMS-30 transformant in comparison with the untransformed control, as is shown in Figure 4. However, these were no changes in expression of these genes *amf*MS-15 tubers. The full details of these differentially expressed genes are presented in Table 1. Interestingly, there are several acyltransferases which have a similar function to the MAT and all these genes were down-regulated in KDMS-30 tubers.

Discussion

In this study, the starch granule morphology of MAT/SBD transformants and untransformed control plants at different stages during potato tuber development was investigated. The transcriptomic analysis of the tubers with altered morphology of the granules showed that the deposition of MAT/SBD fusion protein did not affect the transcript level of genes in starch biosynthesis or degradation, but down-regulated many

genes in lipid metabolism.

The investigations on starch morphology and properties during potato tuber development rarely have been studied, especially from the potato tuber initiation stages. Many studies showed that the size, chemical composition, and physico-chemical properties of starches vary during starch granule growth in various plant species such as sweet potato and wheat (Noda *et al.*, 1992; Bechtel and Wilson 2003; Zhang *et al.*, 2010). Studies showed that the starch chemical composition (such as amylose content and phosphorous content) and physico-chemical properties vary during granule growth. However, further studies on the relationship between physical changes (such as the organization of starch internal structure and the chain length of amylopectin) and the physico-chemical properties of starch during granule growth are needed to provide precise information on the mechanism of the determinants of starch functionalities.

The MAT/SBD fusion protein could cross-link initiating starch granules to form the amalgamated starch phenotype. The expression of MAT/SBD fusion protein in both Kardal and *amf* potato tubers gave rise to the amalgamated starch granules (Nazarian *et al.*, 2007a), however it did not affect the transcript level of genes which are involved in starch metabolism. These results suggest that the starch morphological alteration most likely was caused by the physical effects of MAT/SBD fusion protein interfering with the starch assembling process rather than the interference with the expression level of genes which are involved in starch biosynthetic pathway. This result supports the proposed mechanism that the amalgamated starches in the KDMS and *amf*MS transformants are caused by the physical effects of the MAT/SBD fusion protein. As was discussed previously, the MAT/SBD fusion protein contains more than three binding sites to α-glucans and has higher affinity for starch granules than SBD2 and SBD3 (Nazarian *et al.*, 2007b). In this study, besides the amalgamated starch granules, starch granules which are normal-looking but contain many small granules inside were observed in both KDMS-30 and *amf*MS-15 transformants suggesting that the MAT/SBD cause effects on starch granules already from the early stages of granule formation. This MAT/SBD protein complex could cross-link many different starch granule nucleation sites by the exposed three binding sites and then the amalgamated clusters may be formed at the center of granule and grow to one normal-looking starch granule which contains an

amalgamated granule cluster as shown in Figure 3 at the starch granules initiation stage. Moreover, the MAT/SBD fusion protein could also combine many single small granules and result in big grape-looking amalgamated cluster at later stages of potato tuber development (see Fig. 2 F and H).

The results of transcript analysis of the MAT/SBD transformant tubers in this study would be helpful to unveil the starch granule formation process. Although the transcript level of genes which are involved in starch metabolism did not show changes in MAT/SBD transformants in both Kardal and *amf* backgrounds, the starch granule morphology was severely altered. Up till now, the mechanism of how the starch granule formation initiates and what controls the granule morphology and size in plants is not well understood (Zeeman *et al.*, 2010). The highly ordered organization structure of amylopectin and amylose in starch granules seems mainly due to the physical process. We found that 26 genes out of the 28,427 were differentially expressed in *amf*MS transformant tubers and there were only 3 genes shared in both Kardal and *amf* backgrounds in this study. Although the function of these differentially expressed genes during starch granule formation is not clear at the moment, further study of these genes, especially the 3 genes which are shared in both Kardal and *amf* backgrounds, might be helpful to uncover genes involved in the mechanism of the granule formation.

The MAT might reduce the supply of acetyl-CoA for other biological processes such as lipid metabolism in plant because it could compete with other acetyl transferase for using the acetyl-CoA in other biological processes. This study shows that the expression of MAT/SBD fusion protein is accompanied by severe changes in the expression of genes involved in multiple processes especially in the lipid metabolism pathway in KDMS-30 tubers. Interestingly, all these genes are down-regulated in Kardal background but not in the *amf* potato which is consistent with the earlier findings (Nazarian *et al.*, 2007a) that only in the wild type Kardal background a very low degree of acetylation was found. Acetyl-CoA is an intermediate product to a variety of metabolic processes (Fatland *et al.*, 2005). For instance, acetyl-CoA is the precursor for *de novo* fatty acid biosynthesis in plastids (Nikolau *et al.*, 2003). The *E. coli* maltose acetyl transferase (MAT) cleaves acetyl group from acetyl-CoA and transfers it to the C6 position of a glucosyl residue (Leggio *et al.*, 2003). The results of transcript level analysis suggest that the expression of

the heterologous acetyltransferase in wild type potato suppresses the endogenous acyltransferases in lipid metabolism pathway (Table 1). It might be because the MAT competes for the substrate acetyl-CoA with other similar functioning enzymes in potato amyloplasts.

Potato starch contains a very low amount of lipid (around 0.1%), and it is difficult to extract lipids from a small amount of potato starches as the ones available in this study. In future studies, a large amount of starches from MAT/SBD transformants and untransformed controls should be prepared for lipid extraction and analysis to investigate whether and how the MAT affects lipid content and composition in MAT/SBD transformants. Taken the limited supply of acetyl-CoA in amyloplast into consideration, it might be possible to produce a higher degree of acetylation of starches in potato by pumping in more acetyl-CoA in amyloplast of potato. Another option for producing higher degrees of acetyled starches is worth to try by expressing the MAT in plants such as corn and wheat which contain higher lipid content (about 1%) in starch granules than potato starch and should supply more acetyl-CoA for MAT to acetylate starches. However, this awaits further experiments.

Materials and methods

Plant materials

For the potato tuber developmental series, approximately 50 *in vitro* potato plants of KDMS-30 and *amf*MS-15 transformants and two potato backgrounds (Kardal and amylose-free potato mutant) were maintained on Murashige and Skoog medium (Murashige and Skoog, 1962) for 2 weeks under controlled conditions with a 16 h light period at 22 °C. *In vitro* plants were transferred to greenhouse and maintained in a climate chamber for a period of 10 weeks. Then all tubers have been collected and divided into 5 developing tuber stages according to size and were putted in liquid nitrogen immediately. All stages were harvested 3–4 h into the light period. There are three biological replicates for each stage sample and each specific sample was harvested

from at least 5 individual plants.

Isolation of tuber starch

All tubers were collected from greenhouse and separated to 5 stages according to tuber size. Tubers at each stage from five plants were pooled and cut in pieces and putted in small centrifuge tubes. 1.0ml 3% rapidase in 0.2M NaAc with 0.5g/l Na_2SO_3 (pH=5.5) was added to tubes and incubated overnight at 30 ºC. Starches were collected by centrifuging 2 min (rpm>8000) and removing the cell wall rests by using tweezers. The starch sediment was washed three times with 30mM Phosphate buffer (pH=7.0), one time with demi-water, one time with acetone and finally air-dried at room temperature.

Analysis of physicochemical properties of starch granules

Average granule size and granule size distribution of the starches were determined with a Coulter Multisizer II, equipped with an orifice tube of 200 μm (Beckman-Coulter, UK). Approximately 10 mg of starch were dispersed in 160 mL of Isoton II. The granule size distribution was recorded by counting approximately 50,500 (±500) particles. The coincidence (the frequency of two granules entering the tube at the same time, and consequently being counted as one) was set at 10%.

Starch samples were prepared and sections were cut according to the method described by Ridout *et al.*, (2004). The sections were investigated by light microcopy and SEM.

Starch granule morphology was investigated by light microscopy (LM, Axiophot, West Germany) and Scanning Electron Microscopy (SEM, JEOL 6300F, Japan). For LM, starch granules were stained with a 20× diluted Lugol's solution (1% I_2/KI).

Scanning electron microscopy

The starch granules were mounted onto brass holders with double-sided sticky carbon tape (EMS, Washington, U.S.A.). The sample holder was placed in a dedicated

preparation chamber (Oxford Instruments CT 1500 HF, Eynsham, England), and sputter coated with 10 nm Platinum. Specimens were analyzed with a field emission scanning electron microscope (JEOL 6300 F, Tokyo, Japan) at room temperature at a working distance of 15 mm, with SE detection at 2.5 kV. All images were recorded digitally (Orion, 6 E.L.I. sprl., Belgium) at different scan rates depending on the sample charging. The images were optimized and resized with Adobe Photoshop CS.

Sample preparation and hybridization for microarray and data analysis

RNA extraction and POCI microarray hybridization were performed as described by Kloosterman *et al.* (2008). Differences between averages and p-values for the t-tests were performed in Microsoft Excel. Log_2 ratios for all hybridizations were exported into Genstat® v11 for statistical analysis and calculation of expression estimates and standard errors for each feature. Calculated estimates and standard errors of 28,427 features were finally imported into the GenemathsXT 1.6 (Applied Maths) software package for additional analysis.

Genes with a log2 expression ratio ≥ 1(expression ratio 2-fold) or ≤ -1 (expression ratio 0.5-fold) in combination with a p-value < 0.05 were considered significantly different.

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Chapter 3

Expression of an engineered granule-bound *E. coli* glycogen branching enzyme in potato results in severe morphological changes of starch granules

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Abstract

In order to increase the branching degree of potato tuber starch, the *E. coli* glycogen branching enzyme (*glgB*) was fused to either the C- or N-terminus of a starch binding domain (SBD) and was expressed in the amylose-free mutant (*amf*) and an amylose-containing (Kardal) potato genetic background. Regardless of background or construct used, a large amount of GlgB/SBD fusion protein was accumulated inside the starch granules, however without an increase in branching. The presence of GlgB/SBD fusion proteins did result in altered morphology of the starch granules in both genetic backgrounds. In the *amf* genetic background, the starch granules showed both amalgamated granules and porous starch granules, whereas in Kardal background the starch granules showed an irregular rough surface. The altered starch granules in both *amf* and Kardal backgrounds were visible from the initial stage of potato tuber development. High-throughput transcriptomic analysis showed that expression of GlgB/SBD fusion protein in potato tubers did not affect the expression level of most genes directly involved in the starch biosynthesis except for the up-regulation of a beta-amylase gene. The over-expression of the beta-amylase could be responsible for degradation of extra branches potentially introduced by GlgB.

Keywords: starch, *E. coli* glycogen branching enzyme, GlgB, transgenic potato, SBD

Introduction

Starch is the major storage carbohydrate in plants and is present in almost all plant organs such as leaves, stems, fruits, roots, and tubers at certain time points during plant development. This unique glucan biopolymer in the form of discrete granules is an important storage of energy that is captured by plants using sunlight, water and carbon dioxide. Depending on the organs and species in which it is produced, the size and morphology of starch granules can vary widely. Starch granules are approximately 0.5-110 μm in diameter and spherical, elliptical or polyhedral in shape. The two α-glucan polymers, amylose and amylopectin, are assembled together to form a semi-crystalline starch granule (Thompson, 2000). Starch is one of the most important plant raw materials for both food and non-food applications. It is a staple in the diet of most of the world's population and broadly used in paper, textile, adhesive and pharmacy industries (Ellis *et al.*, 1998).

As an abundant, renewable and biodegradable polymer, starch is becoming increasingly attractive for industrial uses because of the environmental concerns about the industrial waste and greenhouse gases generated from petroleum products. Native starches are often modified by chemical or physical processing to meet the specific needs of the end uses as it does not perform ideally in a range of industrial applications. Modification of starch biosynthetic pathway holds an enormous potential for tailoring granules or polymers with new functionalities and decreasing the quantity of post-harvest modifications currently being used which are environmental damaging (Kok-Jacon *et al.*, 2003). By manipulating the expression level of genes involved in starch biosynthesis such as knocking out or over-expressing key genes, starches with altered properties have been obtained in potato (Kuipers *et al.*, 1994; Schwall *et al.*, 2000; Jobling *et al.*, 2002). Most of those approaches focused on the alteration of the amylose/amylopectin ratio. Another strategy involves the expression of heterologous genes from other organisms, including bacteria, fungi and animals, which encode biosynthetic or modifying enzymes for producing starches with novel properties. For instance, expression of *E. coli* glycogen synthase (*glgA*) resulted in an increased branching degree of amylopectin in potato (Shewmaker *et al.*, 1994).

It is known that the long and unbranched α-glucans such as amylose have a higher tendency to retrograde than highly branched and much shorter-chain amylopectin (Jobling *et al.*, 2002). It has also been shown that amylose-free starches have better freeze-thaw stability than amylose-containing genotypes (Zheng and Sosulski, 1998). To improve freeze–thaw stability of starch, an alternative is to manipulate the structure of starch components (amylose and amylopectin) through genetic engineering. In principle, it may be possible to create starches with high freeze-thaw stability by (i) shortening of long chains of amylose, (ii) reducing the branch chain length of amylopectin and (iii) increasing the number of short branch chains of amylopectin. For instance, down regulation of three starch synthase genes (GBSSI, SSII and SSIII) simultaneously by antisense in potato led to reduction in the chain length of the amylopectin branches. As a result, a significant improvement of freeze-thaw stability of potato starch was achieved in which freeze-thaw stable starches could maintain their stability through five freeze-thaw cycles (Jobling *et al.*, 2002).

For producing starches with higher branching degree of amylopectin and novel properties *in planta*, the *E. coli* glycogen branching enzyme (GlgB) was fused to SBD at either carboxyl or amino terminus (Ji *et al.*, 2003), and was introduced in amylose containing (cv. Kardal) and amylose-free mutant (*amf*) genetic backgrounds. The SBD should help to bring GlgB in more intimate contact with its substrate and acceptor molecules, and thereby act more effectively than GlgB alone. In this study, the influence of an engineered GlgB on structure and properties of potato starch is discussed.

Results

GlgB transformants do not show a visible phenotype in plant architecture and tuber morphology

Two constructs, the pBIN19/*glgB*-SBD and pBIN19/SBD-*glgB* plasmids were used for the expression of the GlgB-SBD and SBD-GlgB fusion proteins in potato plants (Kardal and *amf*), respectively (Fig. 1). Transformed potato plants in this study are referred to as *amf*GS, *amf*SG, KDGS and KDSG, in which GS and SG stand for the

GlgB-SBD and SBD-GlgB fusion proteins, respectively. Besides, untransformed genotypes are referred to as *amf*-UT and KD-UT. Thirty kanamycin-resistant, independent transformant clones from each transformation were grown to maturity in the greenhouse. None of the transgenic plants showed visible differences in the architecture of the plants or the morphology of the tubers, or in the tuber-yield in comparison with that of the control plants (data not shown).

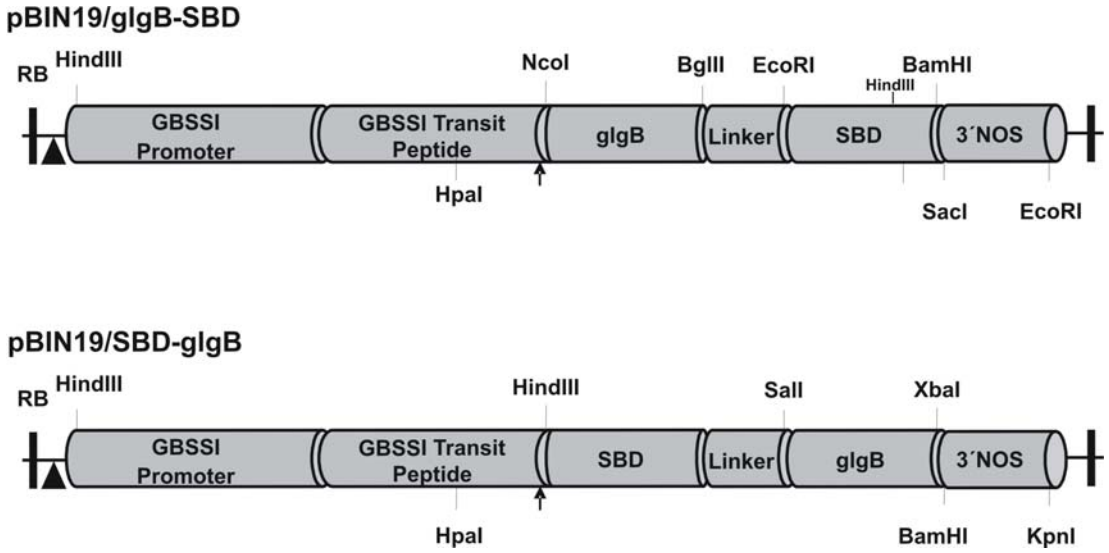

Figure 1. Schematic depiction of two different binary vector constructs used for the transformation of potato plants. Genes were in frame with GBSSI transit peptide to allow amyloplast targeting and were driven by GBSSI promoter for tuber expression. RB and LB, right and left borders, respectively. *glgB*, SBD, 3´Nos and linker stand for *E.coli* glycogen branching enzyme, starch binding domain of cyclodextrin glycosyltransferase from *B.circulans,* nos terminator and Proline-Threonine rich stretch, respectively. ▲ and ↑ signs represent kanamycin resistant gene and cleavage site of the transit peptide.

GlgB fusion protein is incorporated into starch granules

The accumulation levels of GlgB/SBD fusion protein in starch granules of *amf*GS, *amf*SG, KDGS and KDSG transformant plant series were quantified by Western dot blot analysis according to Ji *et al*. (2003) in which the numbers from 0+ to 6+ were assigned, with 0+ meaning that no fusion protein could be detected, and 6+ the highest amount of fusion protein. It was observed that more than 90% of the *amf* transformants (96% and 92% of GlgB-SBD and SBD-GlgB transformants, respectively) and 85% of Kardal

transformants (86% and 89% of GlgB-SBD and SBD-GlgB, respectively) were classified as 4+ through 6+ classes (Fig. 2). Moreover, almost half of the transformants accumulated the highest amount of the fusion proteins (6+) regardless of constructs and backgrounds.

In order to determine whether GlgB/SBD fusion protein was present in the soluble phase of the potato tubers, potato juices of tubers from transformants were subjected to Western dot blot analysis too. As can be seen in Table 1, a relatively low (2+) amount of protein was found in the soluble part of 6+ transformants, and no fusion protein was found in the soluble fractions of any of the other classes.

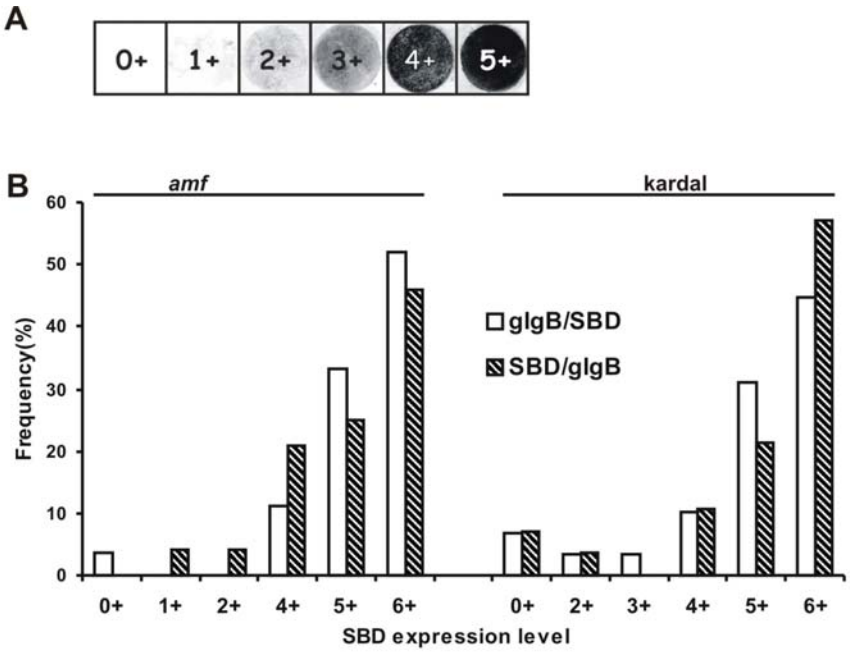

Figure 2. Comparison of the starch-binding domain (SBD)-containing fusion protein content of starch granules in the *amf* and Kardal background. (A) Classes of SBD accumulation in potato starch granules. This classification is based on the results of a Western dot blot analysis with various starch samples. 0+ represents the class with no fusion protein, and 6+ the class with the largest amount of fusion protein. The 6+ category represents the transgenic granules which gave a similar signal in Western dot blot analysis as the 5+ class with half the amount of starch. (B) Distribution of the individual transformants over the seven classes of SBD accumulation in both backgrounds.

SDS-PAGE followed by immunoblotting with both anti-SBD and anti-GlgB antibodies revealed that GlgB/SBD fusion proteins were accumulated in the starch granules of the transgenic plants. The observed mass of about 100 kDa corresponded well

with the predicted molecular mass of the fusion proteins which were detected with both anti-SBD and anti-GlgB (Fig. 3) antibodies, indicating that the observed proteins in starch granules are indeed fusion proteins. These results showed that correctly processed GlgB/SBD fusion proteins were successfully targeted to the starch granules.

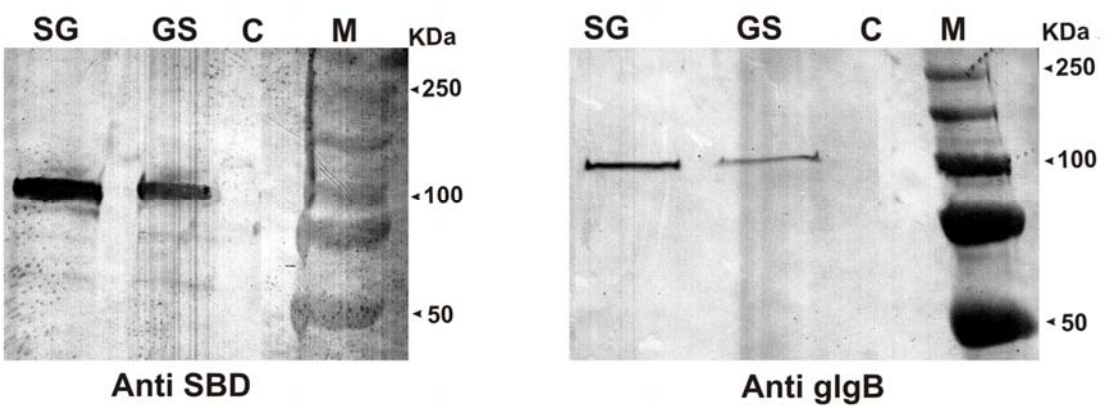

Figure 3. SDS-PAGE/Immunoblot analyses of starch granule-bound proteins of *amf*GS26 and *amf*SG7. Migration of marker proteins (M) is shown to the right in each panel. SG and GS represent SBD-glgB and glgB-SBD fusion proteins respectively. Lanes designated with "C" represent starches from untransformed control plants.

Table 1. Summary of different starch characteristics in relationship with amount of fusion proteins accumulation. Starch apparent amylose content (%AM) median granule size(d_{50}) and starch gelatinization temperature are shown. Western dot blot analysis for selected starches and their corresponding juice fractions are shown. %AM, d_{50} and T onset data are average of three independent measurements.

Clone	W.B		%AM	T onset	d_{50}
	Granule	juice			
amf-UT	NP	NP	3.0 (±0.4)	69.4±(0.0)	19.3 (±0.2)
*amf*GS26	6+	2+	3.0 (±0.3)	70.7±(0.2)	10.7 (±0.4)
*amf*SG12	6+	2+	3.4 (±0.1)	69.2±(0.1)	18.9 (±0.3)
KDUT	NP	NP	18.6 (±0.4)	63.1±(0.1)	25.2 (±0.1)
KDGS26	6+	2+	17.9 (±0.5)	61.8±(0.1)	22.8 (±0.3)
KDSG25	6+	2+	18.3 (±0.3)	61.9±(0.1)	14.2 (±0.2)

W.B= Western dot blot, NP= Not present

Starch granule morphology is affected by the accumulation of GlgB fusion protein

To investigate the effects of the accumulation of GlgB/SBD fusion proteins on starch granule morphology, both light microscopy (LM) and scanning electron

microscopy (SEM) were used to check the morphology of various starches from the transgenic plants. The LM analysis showed that starches from the transgenic plants have different phenotypes in comparison to starches from the untransformed controls. SEM analysis revealed that the morphology of starches from transgenic plants was severely affected as a result of the expression of the fusion proteins in both genetic backgrounds (Figs. 4 and 5). Starches from transgenic amylose-containing plants exhibit irregular bumpy surfaces (Fig. 4 B, C, E, and F). Starches from amylose-free transgenic plants show both amalgamated starch granules and porous starch granules with many deep holes (Fig. 5 B, C, E, and F) which sometimes resulted in starch granules in which cracks connect different holes (Fig. 5 E). With respect to the SBD position in the fusion proteins, N as well as C terminal fusion proteins resulted in the same phenotype in each particular genetic background. These starch phenotypes can be seen in different transformants irrespective of their level of GlgB/SBD fusion protein accumulation. Quantification of such altered starch granule phenotypes as shown in Figure 4 and 5 revealed that the higher the amount of granule-bound fusion proteins, the more altered starch granules (Fig. 6).

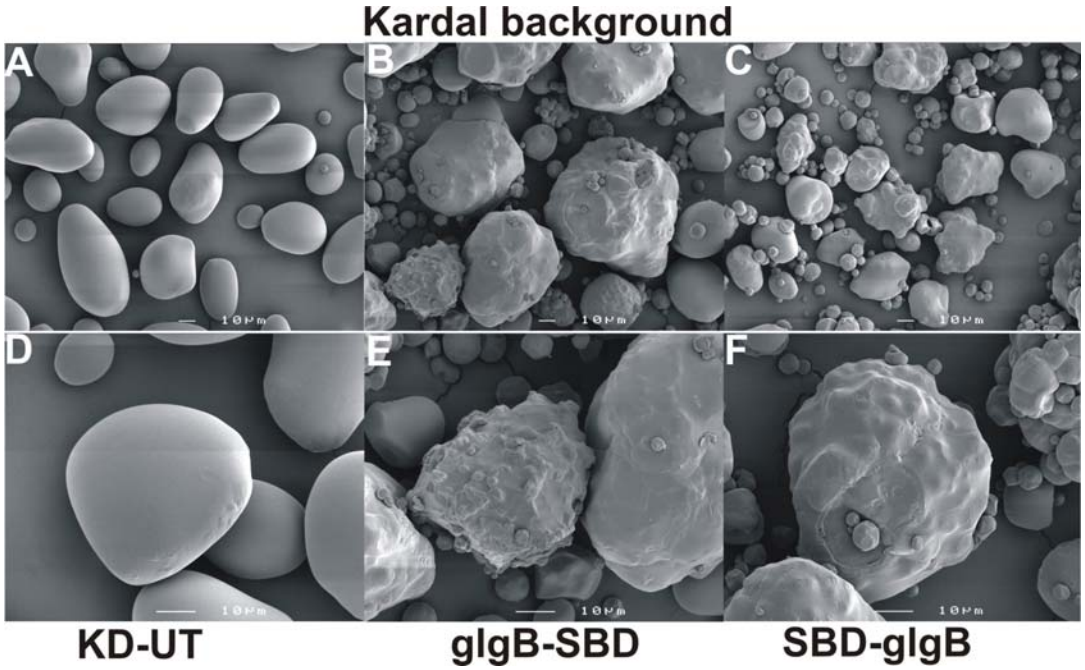

Figure 4. SEM analyses of starch granules from Kardal-UT (A and D) in comparison with that of the different selected transformants at different magnifications. KDGS26 (6+) (B and E) and KDSG25 (6+) (C and F). Magnifications are; A (×250), B and C (×500) D, E and F (×2000).

To investigate whether the deposition of GlgB/SBD fusion proteins affected the crystallization and the internal structure of the starch granules, sections of starch granules from two high expressor transformants; *amf*GS26 and KDGS26 as well as their corresponding controls were prepared as described by Ridout and coworkers (Ridout *et al.*, 2004) and images were taken by using light microscopy. The starch granule section analysis showed that both the rough surface granules and porous granules showed the well known growth rings (Fig 7). But the growth rings in the rough and porous granules were not distributed as evenly as in control starch granules and the growth rings in the outer layer of porous granules were broken by the holes in the porous granules (compare Fig. 7 A with D and 7 B with E). Moreover, the holes in the porous granules were only superficial and did not penetrate through the whole granule (Fig. 7 E). Furthermore, there were starch granules which did not show the concentric growth rings of normally assembled granules but contained multiple small granules inside (Fig. 7 C and F).

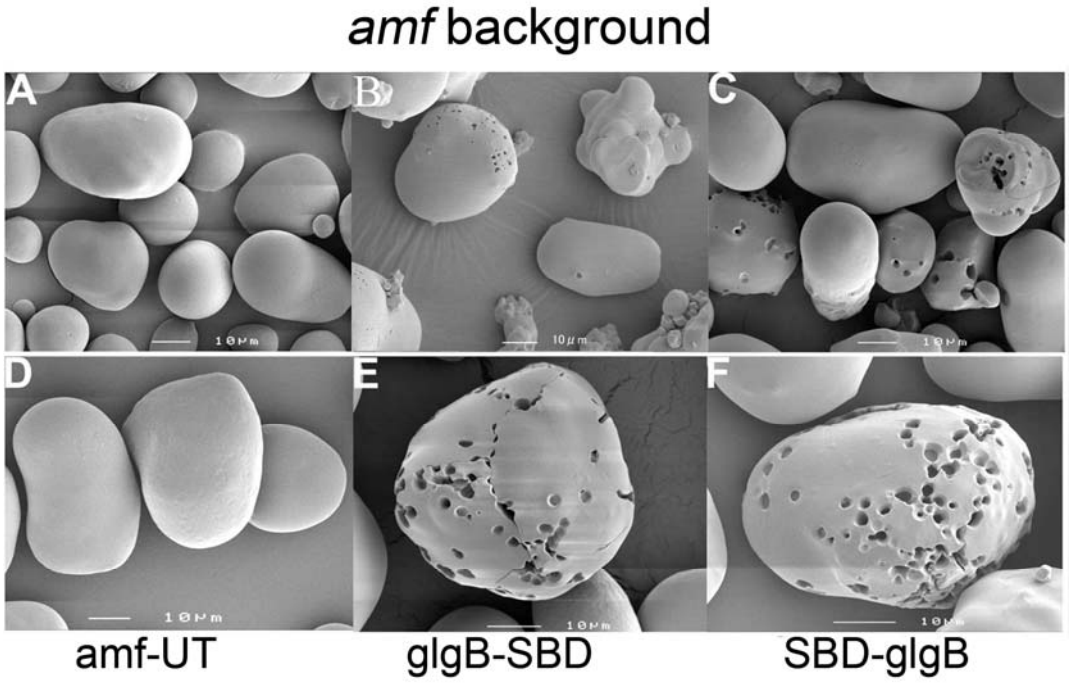

Figure 5. SEM analyses of starch granules from *amf*-UT (A and D) in comparison with that of the different selected transformants at different magnifications. *amf*GS26 (6+) (B and E) and *amf*SG7 (6+) (C and F). Magnifications are; A (×250), B and C (×500), D (×2000), E and F (×4500).

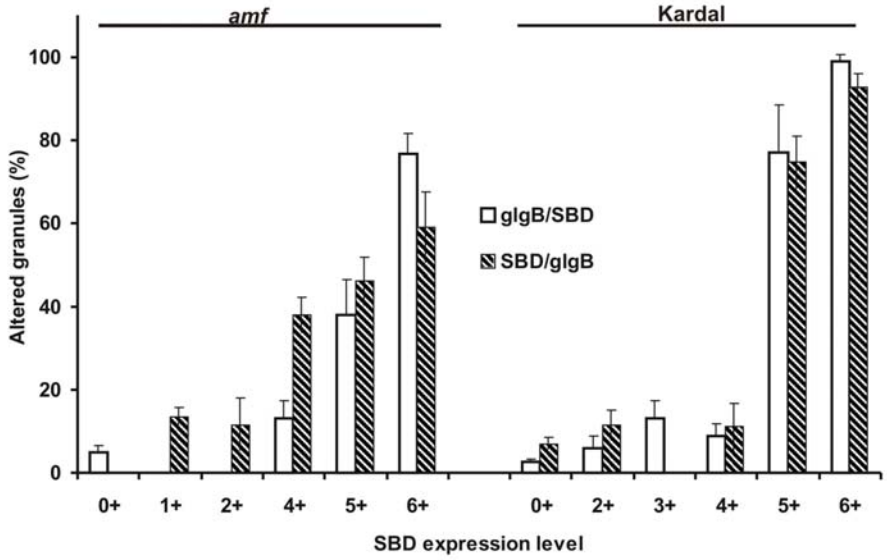

Figure 6. Percentage of granules with altered morphology for the various classes of SBD-containing fusion proteins. A population of 100 starch granules in triplicate was counted. Bars indicate mean ±SD.

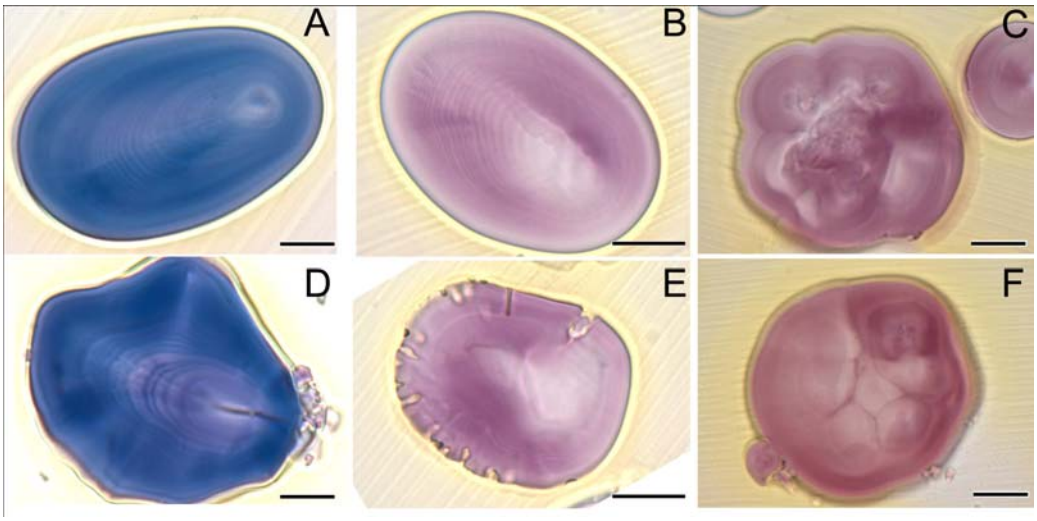

Figure 7. Micrographs showing the section images of starch granules of Kardal (A), KDGS26 (D), *amf* (B) and *amf*GS26 (C, E, F). Starch granule sections were stained with a 10× diluted Lugol solution. The black scale bar indicates 5μm in all images.

Altered starch granules are detectable from the initial stage of potato tuber development

The presence of GlgB/SBD fusion protein resulted in porous and amalgamated starch granules in amylose-free (*amf*) background and irregular bumpy surface granules

in amylose-containing (Kardal) background. In order to investigate at which time point during potato tuber development the accumulation of GlgB/SBD fusion protein affected starch granule morphology, starches from *amf*GS26 and KDGS26 transformants were isolated at five tuber developmental stages according to potato tuber size, ranging from swelling stolons and four different tuber growth stages which correspond to potato tuber developmental stages 3, 4, 5, 6, 7 in Kloosterman's study (Kloosterman *et al.*, 2005). The morphology of the starch granules was analyzed with scanning electron microscopy (SEM) and the results are shown in Figure 8.

The SEM analysis showed that starch granule morphological phenotypes caused by expression of GlgB/SBD fusion proteins were different in the Kardal and *amf* backgrounds at different stages of tuber development. Starches harvested from both *amf*GS26 and KDGS26 transformants showed altered phenotypes from tuber stage 3 on (swelling stolon) (Fig. 8 A-D and E-H). The starch morphology in *amf*GS26 appeared to follow a transient pattern. There were 50 $\pm$ 2% of starch granules that showed deep pores at tuber development stage 3 and 4; then the starch morphology changed to porous (20 $\pm$ 1%) with amalgamated granules (~2%) co-existing from stage 5 onwards (Fig. 8 A to D). Starches from KDGS26 transformant had more than 90% altered granules with small shallow pores on the granules surface at tuber developmental stages 3 and 4 (Fig. 8 E and F), then these pores seemed to be filled with amylose and turned to irregular granules from stage 5 on (Fig. 8 G and H).

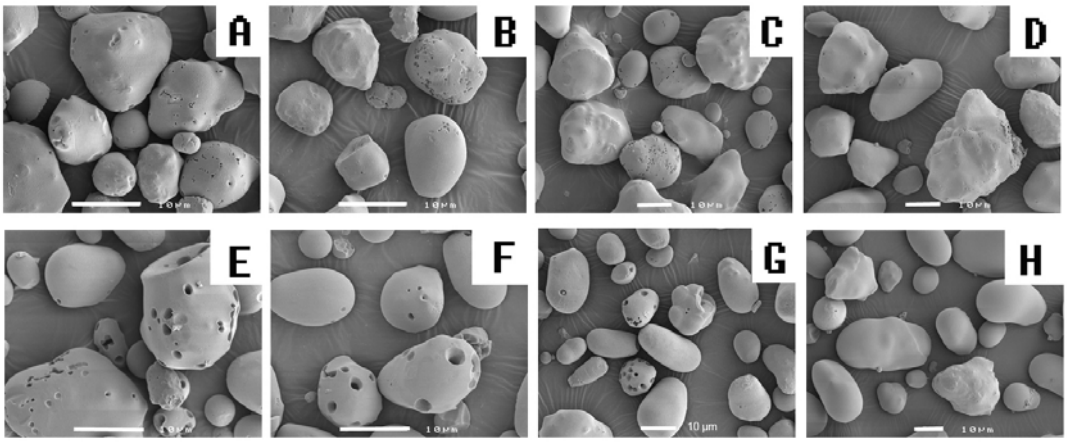

Figure 8. SEM analyses of starch granules from 4 different tuber development stages (stage 3, 4, 5, 6) of KDGS26 (A-D) and *amf*GS26 (E-H) at different magnifications. The scale bar indicates 10μm in all images. Magnifications are; A, B, E and F (×3500), C, D, G and H (×600).

Gene expression profiles in GlgB-SBD transformants

Altered starch granules are detectable in GlgB transformant tubers from potato tuber development stage three to mature tubers regardless of the genetic backgrounds, which indicated that the expression of GlgB/SBD fusion protein in potato tubers might affect the activity of enzymes which are involved in starch biosynthesis and/or in the process of starch granule assembly. To better understand how GlgB/SBD fusion protein interfered with the starch biosynthesis pathway, high-throughput transcriptomic analysis of potato tubers from KDGS26 and *amf*GS26 transformants in comparison with their untransformed controls was performed using the POCI array (Kloosterman, *et al.*, 2008). Furthermore, analysis of differential expressed genes using the MapMan tool (Thimm, *et al.*, 2004) (http://gabi.rzpd.de/projects/MapMan/) was conducted to understand biological processes which were affected by expression of GlgB/SBD fusion protein. As shown in Figure 9, there were 307 genes (229 upregulated and 78 down regulated) that had at least a 2-fold change in expression level with a statistic p value below 0.05 when KDGS tubers were compared with KDUT tubers. Meanwhile, there were 96 genes differentially expressed significantly in *amf*GS transformant (82 genes were induced and 16 were repressed). Seventeen genes were shared between both Kardal and *amf* backgrounds (Table 2). Of these differentially expressed genes, about 50% did not have an annotation.

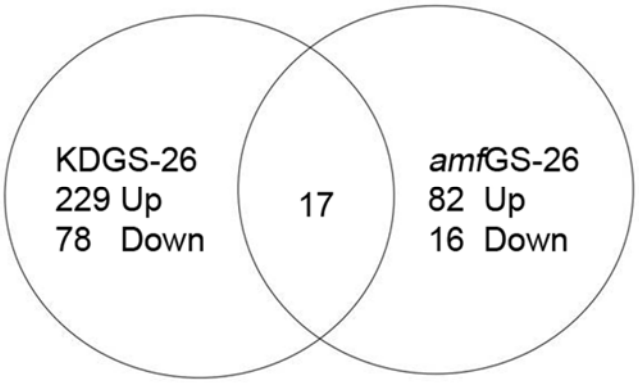

Figure 9. Differentially expressed genes in KDGS26 and *amf*GS26 in comparison with their untransformed control tubers. The number in the overlapping area indicates the shared number of genes in both KDGS26 and *amf*GS26. Numbers outside the overlapping area represent genes specifically up or down regulated in KDGS26 and *amf*GS26.

The microarray and Mapman analysis results also showed that the expression of GlgB/SBD fusion protein interfered with the expression of genes involved in multiple processes such as protein synthesis and degradation, DNA and RNA regulation, hormone metabolism, secondary metabolism and so on. However, most of genes which code for enzymes directly involved in starch and sucrose metabolism such as ADP-glucose pyrophosphorylases (AGPase), starch synthases (SSs and GBSS), starch-branching enzymes (SBEs) and α-glucan water dikinases (PWD and GWDs) did not show any changes in KDGS26 and *amf*GS26 tubers in comparison with the expression in tubers of their untransformed controls. Noteworthy, there was a beta-amylase gene (Micro. 13368. C1) which showed a more than 2-fold up-regulation in KDGS26 transformant tubers in comparison to Kardal untransformed tubers, but this difference was not observed in *amf* transformant tubers.

Quantitative real-time PCR (qRT-PCR) was performed on the beta-amylase gene (Micro. 13368. C1) in KDGS26 and *amf*GS26 transformants as well as the untransformed control tubers. The results showed that the beta-amylase gene had similar expression patterns as the microarray analysis (data not shown).

Starch properties remain unaltered

In order to investigate the expected increase in branching, chain length distribution was determined by both HPSEC and HPAEC. No differences were observed between starches of transgenic plants and their corresponding control plants after complete debranching by isoamylase treatment. Furthermore, the HPAEC profiles of the debranched starches from the tarnsformants after treatment with isoamylase did not deviate from starches of untransformed controls (data not shown).

Other starch characteristics such as starch gelatinization behavior, apparent amylose content (%AM), and starch granule size distribution, were also investigated (Table 1). No significant differences were found in these analyses in comparison with the control plants.

Table 2. Differentially expressed genes shared in both KDGS26 and *amf*GS26 transformant tubers relatively to the untransformed control tubers.

	*amf*GS26		KDGS26			
GeneName	p value	Fold	p value	Fold	Description	CLASS
SDBN004E09u.scf	0.002	+2	0.001	+6	NA	not assigned
MICRO.6763.C1	0.001	+6	0.001	+5	putative chalcone isomerase 4 [Glycine max]	secondary metabolism
bf_arrayxxx_0055d11.t7m.scf	0.001	+4	0.001	+4	NA	not assigned
STMES26TV	0.001	+3	0.001	+3	patatin A gene, patatin B gene [Solanum tuberosum]	not assigned
MICRO.2037.C6	0.018	+4	0.045	+3	homologue to UP|Q40151_LYCES (Q40151) Hsc70, partial (32%)	protein folding
SSBN002H22u.scf	0.001	+3	0.001	+3	weakly similar to UP|Q9M621_ARATH (Q9M621), partial (17%)	protein degradation
MICRO.7062.C1	0.011	+2	0.001	+3	weakly similar to UP|Q2V405_ARATH (Q2V405), partial (36%)	not assigned.glycine rich proteins
POABI58TV	0.034	+2	0.003	+3	S-adenosylmethionine synthetase 2 (AdoMet synthetase 2) , partial (65%)	amino acid metabolism
MICRO.11846.C1	0.001	+3	0.001	+2	NA	not assigned
bf_mxlfxxxx_0054a11.t3m.scf	0.001	+3	0.002	+2	NA	not assigned
MICRO.4281.C1	0.008	+2	0.003	+2	S-adenosylmethionine synthetase 2 (AdoMet synthetase 2) , partial (98%)	amino acid metabolism
MICRO.1805.C2	0.007	+2	0.001	+2	weakly similar to UP|Q60EC7_ORYSA (Q60EC7), partial (19%)	not assigned
bf_ivrootxx_0003a09.t3m.scf	0.015	+2	0.004	+2	NA	not assigned
bf_arrayxxx_0003a04.t7m.scf	0.006	+2	0.007	+2	NA	not assigned
BF_TUBSXXXX_0038F01_T3M.SCF	0.025	+2	0.014	+2	NA	not assigned
MICRO.15472.C1	0.001	+2	0.001	+2	NA	not assigned
MICRO.15185.C1	0.001	-2	0.001	-3	similar to MADS-box transcription factor CDM36, partial (48%)	mitochondrial electron transport / ATP synthesis

"+" represents up-regulated and "-" represents down-regulation. NA means No Annotation

Discussion

The *E. coli glgB* gene fused with SBD has been targeted into both Kardal and *amf* potato starches during starch biosynthesis. The objective of expressing the *glgB* gene in potato was to introduce more α (1→6) linkages during amylopectin synthesis which then would result in an altered amylopectin structure with higher branching degree in potato starch. The results showed that the chain length profiles of starches from transgenic plants were comparable to the untransformed control plants which indicates that GlgB fused to SBD either does not introduce more α (1→6) linkages or that the number of newly introduced side chains is too less to be detected. However, our findings confirmed a previous finding that the expression of the *E. coli glgB* gene under control of the patatin promoter did not increase the amylopectin branching degree in amylose-containing genetic background (Krohn *et al.*, 1994).

The accumulation of GlgB/SBD fusion proteins in this study resulted in altered starch morphology (Figs. 4 and 5) which is similar as the previous study in wild type potato (Krohn *et al.*, 1994), despite the fact that no starch morphology alterations were reported in the expression of the *glgB* gene alone in potato amyloplasts of the amylose-free (*amf*) mutant (Kortstee *et al.*, 1996) and rice caryopsis (Kim *et al.*, 2005). The porous and amalgamated starch granules are found in *amf* genetic background and the irregular bumpy surfaces with protrusions appeared in Kardal transformants (Figs. 4 and 5). However, the physico-chemical properties of the starches from GlgB/SBD transformants are not affected (Table 1). The gelatinization behavior was unaltered, as well as the granule size distribution and the apparent amylose content.

The GlgB/SBD fusion protein has high affinity to starch granules. In this study, regardless of the background (Kardal or *amf*) and construct (GlgB-SBD or SBD-GlgB) used, more than 85% of the transformants accumulated the highest amount of the fusion proteins (4+ and higher) (Fig. 2). It shows that the affinity to starch granules of GlgB/SBD fusion protein is higher than other proteins which were fused with the SBD such as *GtfI*CAT/SBD and MAT/SBD (Nazarian *et al.*, 2007a, b); even higher than multiple SBDs (Nazarian *et al.*, 2007c) and in previous studies. The *E. coli* GlgB belongs to the glycoside hydrolase (GH) family 13 and catalyzes the formation of α-1,6 branch

points in either glycogen or starch. Previous studies on the structure of GlgB enzyme showed that it consists of three major domains: an amino-terminal domain, a carboxyl-terminal domain, and a central α/β-barrel domain containing the enzyme active site (Abad *et al.*, 2002). The *E. coli glgB* gene was expressed in an amylose-free potato mutant (*amf*) and the GlgB protein was detectable in potato starches from transgenic plants with Western blot analysis (Kortstee *et al.*, 1996) which proved that the GlgB enzyme has affinity for starch granules on its own. It was doubted that the amino-terminal domain of GlgB plays an important role in the enzyme affinity for starch granules and recent studies confirmed that the amino-terminal domain belongs to the carbohydrate binding module family 48 (CBM48) which is a starch binding domain (Koay *et al.*, 2007; Palomo *et al.*, 2009). Although the 3D structure and binding site of CBM48 have not been characterized thoroughly, it is possible that the two starch binding domains in GlgB/SBD fusion protein (CBM48 and SBD) could bind starch granules similarly as the double SBD. Previous study showed that the tandem SBDs has much higher affinity to starch granules than a single SBD (Ji *et al.*, 2004) which suggesting the high accumulation level of GlgB/SBD fusion proteins in the starch granules of transgenic plants might be explained by the joint effects of the CBM48 domain of the GlgB enzyme with the extra fused SBD domain.

The alteration of starch granule morphology is unlikely caused by the activity of the GlgB enzyme on glucan branches but should be the result of the deposition of GlgB/SBD fusion protein in starch granules which interferes with the normal assembly of amylopectin during amylopectin biosynthesis. As we discussed before, the GlgB/SBD fusion protein has three domains, a CBM48 domain, an SBD domain, and the active site of GlgB enzyme, which can bind on the glucan branches during starch synthesis. The extra SBD domain fused with GlgB could make the GlgB/SBD fusion protein comparable to a double SBD during starch biosynthesis. Based on the mode which was proposed by Nazarian and coworkers (Nazarian *et al.*, 2007c), the exposed SBD domain of the double SBD can cross-link different granule nucleation sites and eventually result in amalgamated starch granules at a higher concentration. That the GlgB/SBD fusion protein might also work similarly as a double SBD can be seen from the comparable starch phenotypes (like amalgamated starch granules and multiple small starch granules

in the center of the big granules in the starch granule section images; Fig. 7 C and F). Although the porous starch granules in *amf* background and the irregular starch granules in Kardal background show growth rings, the distribution of the growth rings is not as evenly as in the untransfomed control starches (Fig. 7 D). It indicates that the GlgB/SBD fusion protein did not affect the crystallization of amylopectin but the distribution of amylopetin structure in the granules during the starch granule formation process. The deposition of the GlgB/SBD fusion proteins on the starch surface which could be released later on and give rise to the observation of starch granules with a lot of holes on the starch surface. These starch morphological phenotypes can be detected clearly from the tubers of transformants already at the initial stages of potato tuber development. During tuber development and starch granule size growing, the holes are being filled with amylose later on in the amylose-containing genetic background such that amylose is protruding from these holes and some of amylose is deposited on the granule surface which eventually gives rise to bumpy surfaces as can be seen in Figure 4, whereas the holes turn deeper in amylose-free background because of the absence of amylose.

Although the starch morphology has been severely altered in this study, the expression of GlgB/SBD fusion protein did not affect the expression of most of the genes which code for enzymes directly involved in starch and sucrose metabolism. The transcription level of starch synthases (SSs and GBSS), starch-branching enzymes (SBEs) and starch de-branching enzymes (Isoamylases) did not show any changes in KDGS26 and *amf*GS26 transformant tubers. Interestingly, a β-amylase gene was up-regulated in KDGS transformant tubers but not in *amf*GS transformant tubers. The β-amylase is an exohydrolase that catalyses the liberation of β-maltose from the non-reducing end of α-1,4-glucans, leaving a β-limit dextrin (Baba and Kainuma, 1987). A primary role of β-amylase is in hydrolytic transitory starch degradation (Scheidig *et al.*, 2002; Weise *et al.*, 2005; Fulton *et al.*, 2008). Besides the important role in starch degradation, β-amylase is also involved in amylopectin biosynthesis process by hydrolyzing α-1,4-glucans before they form in crystallization structure (Delatte *et al.*, 2005). Streb and coworkers proposed a model which suggests that the isoamylases remove miss positioned branches to make crystallize starch granules during amylopectin synthesis and the miss positioned branches which are cleaved off by isoamylases are degraded into maltose by

beta-amylase and re-incorporated in starch biosynthesis pathway (Streb *et al.*, 2008). Based on the fact that no extra introduced branches were found in starches from transgenic plants, the β-amylase was up-regulated in KDGS transformant tubers and the debranching enzymes (isoamylase 1 and isoamyase 2) are highly expressed in both transformant tubers and untransformed control tubers, and the existing knowledge of glucan-modifying enzymes in amylopectin formation, it could be speculated that on the one hand GlgB brings extra branches on the amylopectin backbone structure during amylopectin biosynthesis, but on the other hand the debranching enzymes remove these extra positioned branches which are introduced by GlgB activity at the same time which result in no changes of amylopectin branching degree or alternatively there are too little extra introduced branches to be detected in the starch granules of transgenic plants.

Materials and methods

Preparation of constructs

The *E. coli* GlgB was fused in frame to the SBD of *Bacillus circulans* cyclodextrin glycosyltransferase (*CGTase*) gene at both its carboxyl (C) and amino (N) terminus. Potato GBSSI promoter segment and its transit peptide (TP) sequence were used for tuber-specific expression and amyloplast entry of the proteins (Fig. 1). Two constructs, the pBIN19/*glgB*-SBD and pBIN19/SBD-*glgB* plasmids were used for the expression of the *glgB*-SBD and SBD-*glgB* fusion proteins in potato plants (Kardal and *amf*), respectively.

In order to generate the pBIN19/glgB-SBD plasmid, a *glgB*-encoding fragment was obtained by PCR amplification with the primers 5'-CAACCATGGCCGATCGTATAG-3' and 5'-TTAGATCTCTCTGCCTCCCGAAC-3', which contained *Nco*I and *Bgl*II sites at their 5' ends, respectively. This amplified *glgB* fragment was used to replace the first SBD fragment (also *Nco*I-*Bgl*II) in the pUC19/SBD2 (Ji *et al.*, 2003), giving the pUC19/*glgB*-SBD plasmid. After digestion of this plasmid with *Hpa*I and *Sac*I, the *Hpa*I-*Sac*I fragment was inserted into the corresponding sites of the pBIN19/SBD2 to generate the pBIN19/*glgB*-SBD. The predicted molecular mass of the *glgB*-SBD

produced in plants is 98,424 Da, excluding the transit peptide. The pBNI19/SBD-*glgB* plasmid was assembled from below fragments: (1) GBSSI promoter, GBSSI transit peptide and SBD-linker fragment (*Hind*III-*Sa*lI) obtained from pUC19/SBD2 plasmid, (2) a *glgB* fragment (*Sal*I-*Xba*I), and (3) a NOS terminator (*BamH*I-*Kpn*I). The *glgB* fragment was amplified by PCR with 5'-GTGTCGACCATGGCCGATC-3' and 5'-TCTAGAGTCATTCAGCCTCCCG-3' as primers, which contained *Sal*I and *Xba*I at their 5' ends. The predicted molecular mass of the SBD-*glgB* produced in plants is 98,089 Da, excluding the transit peptide. In all amplification procedures high fidelity Pfu polymerase (Epo571, Fermentas) was used. Furthermore, all constructs were sequenced to verify their correctness and integrity.

Potato transformation

The two different constructs were transformed into electro-competent cells of *Agrobactrium tunefaciens* strain LBA4404 by electroporation. Furthermore, two different potato genotypes, namely wild type potato (cv. Kardal, tetraploid) and the mutant amylose-free (*amf,* diploid) were used for *Agrobacterium*–mediated transformation. Potato transformation procedures were carried out according to Visser *et al.* (1991). For each construct 30 independent transformants were harvested from MS30 selection medium (Murashige and Skoog, 1962), including kanamycin (100 mg/l) and later each was propagated to 5 plants to get enough tubers.

Plant materials

In vitro plantlets of all different transformants, KDGS, KDSG, *amf*GS, and *amf*SG, and their untransformed controls (Kardal and *amf*) were grown in MS medium for 2 weeks and transferred to the greenhouse and maintained in a climate chamber for tuber development. For the potato tuber developmental series, selected transformant tubers were collected and classified into 5 developing tuber stages according to size which correspondingly similar to stage 3 to 7 in Kloosterman's tuber development stages (Kloosterman *et al.*, 2005) and be put in liquid nitrogen immediately. Tubers from all

stages were harvested 3–4 h into the light period. There are three biological replicates for each stage sample and each specific sample was harvested from at least 5 individual plants.

Isolation of tuber starches

All tubers form each individual transformant (five clones) were peeled, mixed and ground using a Sanamat rotor (Spangenberg, The Netherlands) to which 0.01% of sodium metabisulphite ($Na_2S_2O_5$) was added. Collected juices were taken to 4° C to settle for at least 3 hours. A part of the juice was kept and frozen at -20 ° C for further analysis. The settled starch was washed at least 3 times and passed through a Whattman filter paper on a Buchner funnel. All starch samples were later air dried at room temperature and powdered with a sieve shaker (Retsch, Germany).

For the potato tuber developmental series, tubers at each stage from five plants were pooled and cut in pieces and putted in small centrifuge tubes. 1.0ml 3% rapidase in 0.2M NaAc with 0.5g/l Na_2SO_3 (pH=5.5) was added to tubes and incubated overnight at 30 ºC. Starches were collected by centrifuging 2 min (rpm>8000) and removing the cell wall rests by using tweezers. The starch sediment was washed three times with 30mM Phosphate buffer (pH=7.0), one time with demi-water, one time with acetone and finally air-dried at room temperature.

Western dot blot analysis

Western dot blot analyses, using gelatinized starch were done for SBD containing fusion proteins. The amount of accumulated protein was quantified as described by Ji *et al*. (2003). The soluble fractions were also subjected to Western dot blot analyses according to Ji *et al.* (2003).

Immunoblot analysis of granule bound fusion proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was

carried out as described by Nazarian *et al*. (2007a). Fifty milligrams of starch sample from selected transformants were solubilized in 1 ml SDS sample buffer [final concentrations: 1M Tris-HCL, pH 6.8, 2 %(w/v) SDS, 10% (v/v) glycerol, 3% (v/v) β-mercaptoethanol] and incubated at boiling temperature for 5 min before being analyzed on a gel. Samples were centrifuged and initially, equal amounts of control and transformants (25 µl supernatant) were loaded onto lanes of a 12% polyacrylamide separating gels (145mm x95mm x3mm, BioRad, UK). The Precision Plus Protein (Bio-Rad) ladder was used as size standard. Proteins were then transferred onto a hybond ECL (Amersham, Amersham, UK) membrane and immunobloted using either anti-SBD or anti-GlgB antibodies as described by Nazarian *et al*. (2007a).

Analysis of physico-chemical properties of different transformants

Average granule size and granule size distribution of the starches were determined with a Coulter Multisizer II, equipped with an orifice tube of 200 µm (Beckman-Coulter, UK). Approximately 10 mg of starch were dispersed in 160 mL of Isoton II. The granule size distribution was recorded by counting approximately 50,500 (±500) particles. The coincidence (the frequency of two granules entering the tube at the same time, and consequently being counted as one) was set at 10%.

Starch granule morphology was investigated by light microscopy (LM, Axiophot, West Germany) and Scanning Electron Microscopy (SEM, JEOL 6300F, Japan). For LM, starch granules were stained with a 20× diluted Lugol's solution (1% I_2/KI).

The starch granules were mounted onto brass holders with double-sided sticky carbon tape (EMS, Washington, U.S.A.), The sample holder was placed in a dedicated preparation chamber (Oxford Instruments CT 1500 HF, Eynsham, England), and sputter coated with 10 nm Platinum. Specimens were analyzed with a field emission scanning electron microscope (JEOL 6300 F, Tokyo, Japan) at room temperature at a working distance of 15 mm, with SE detection at 2.5 kV. All images were recorded digitally (Orion, 6 E.L.I. sprl., Belgium) at different scan rates depending on the sample charging. The images were optimized and resized with Adobe Photoshop CS.

To test the chain length distribution, 5 mg of starch from transgenic potatoes was

suspended in 250 µl of DMSO and gelatinized for 10 min at boiling temperature. After cooling down to the ambient temperature, 700 µl of 50 mM NaAc buffer pH 4.0 was added. A sufficient amount of isoamylase (Hayashibara Biochemical laboratories, Okayama, Japan; 59,000 U/mg protein) to debranch the starch polymers completely was added to the mixture, which was incubated for 2 h at 40º C. After inactivation of the enzyme for 10 min at boiling temperature, 1ml of 25% DMSO was added. For high-performance size-exclusion chromatography (HPSEC), the samples were used as such (HPSEC). The HPSEC was performed on a p680HPLC pump system (Dionex, Sunnyvale, Cal. USA) equipped with three TSKgel SWXL columns in series (one G3000 and two G2000: 300mmx7.5mm; Montgomeryville, USA) in combination with a TSKgel SWXl guard column (40mmx6mm) at 35ºC. Aliquots of 100 µl were injected using a dionex ASI-100 Automated Sample Injector, and subsequently eluted with 10 mM NaAc buffer (pH 5.0) at a flow rate of 0.35 ml/min (3h run). The effluent was monitored using a RID-6A refractometer (Shimadzu, Kyoto, Japan). The system was calibrated using dextran standards (10, 40, 70, 500 kDa; Pharmacia, Uppsala, Sweden). Dionex Chromelon software version 6.50 SP4 Build 1000 was used for controlling the HPLC system and data processing.

High-performance anion-exchange chromatography (HPAEC) was used to obtain a better separation of the smaller amylopectin side chains (in the range of 2 to 45 glucose residues). HPAEC was performed on a GP40 gradient pump system (Dionex) equipped with a carboPac PA 100 column (4mmx250mm; Dionex) at 35º C. The flow rate was 1.0 ml/min and 20 µl samples were injected with Dionex AS3500 automated sampler. Two eluents were used, eluent A (100mm NaOH) and eluent B (1 M NaAc in 100 mm NaOH), for mixing the following gradient: 0→5 min, 100% eluent B (rinsing phase); 5→20 min, 100% eluent A (conditioning phase); 20→25 min, linear gradient from 0→20% eluent B (100→80% eluent A); 25→50 min, linear gradient from 20→35% eluent B (80→65% eluent A); 50→55 min, linear gradient from 35→50% eluent B (65→50% eluent A); 55→60min , 50% eluent B (50% eluent A). The sample was injected at 20 min. The eluent was monitored by ED40 electrochemical detector in the pulsed amperometric mode (Dionex).

Post harvest experiments

Five mg of starch from selected transformants was incubated in two different ways. Assay (a): Starches were incubated in 100 mM phosphate buffer pH=7.5 and incubated for 5 days at 40º C and 55º C. Phosphate buffer was removed and isoamylase treatment for HPSEC and HPAEC analyses were done as mentioned in the previous section. Assay (b): In order to create enough space for the granule-bound GlgB, five milligrams starch was solubilized in 250µl DMSO, 800µl of 10mM phosphate buffer was added and the mixture was incubated for 48 hours at 30º C. 100 µl of 1 M NaAC was added followed with sufficient amount of isoamylase (Hayashibara Biochemical laboratories, Japan; 59,000 U/mg protein). To keep the DMSO at 25% final concentration, 550µl distilled water and 250µl DMSO were added. The HPSEC and HPAEC analyses were performed as mentioned earlier.

Sample preparation and hybridization for microarray and data analysis

RNA extraction and POCI microarray hybridization were performed as described by Kloosterman *et al.* (2008). Differences between averages and p-values for the t-tests were performed in Microsoft Excel. Log_2 ratios for all hybridizations were exported into Genstat® v11 for statistical analysis and calculation of expression estimates and standard errors for each feature. Calculated estimates and standard errors of each feature were finally imported into the GenemathsXT 1.6 (Applied Maths) software package for additional analysis.

Genes with a log2 expression ratio ≥ 1 (expression ratio 2-fold) or ≤ -1 (expression ratio 0.5-fold) in combination with a p-value < 0.05 were considered significantly different.

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Chapter 4

Expression of an engineered amylosucrase in potato results in larger starch granules

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Submitted

Abstract

Starch is the most important carbohydrate in many food and non-food applications. The ratio of amylose and amylopectin, the two most important constituents in starch granules, plays a key role in starch physico-chemical properties. To modify this ratio, and thereby broaden starch applications, an amylosucrase from *Neisseria polysaccharea* fused to a starch binding domain (SBD) was introduced in two potato genetic backgrounds, an amylose-containing cultivar (Kardal) and an amylose-free mutant (*amf*). Expression of SBD-amylosucrase fusion protein in the amylose-containing potato resulted in starch granules with a rough surface, with a 2-fold increase in median granule size, and altered physico-chemical properties including higher end viscosity and higher enzymatic digestibility. High-throughput transcriptomic analysis (using the POCI 44K microarray) showed that expression of SBD-amylosucrase in potato tubers resulted in increased expression of a glucose-6-phosphate/phosphate translocator 2 (GPT2) gene and a beta-amylase gene.

Keywords: Amylosucrase, SBD, starch, bigger starch granules, digestibility, viscosity

Introduction

Starch is the second most abundant polysaccharide on earth and is the most important carbohydrate used for many food and non-food applications (Jobling, 2004). It is composed of two α-glucan biopolymers, an essentially linear amylose and an highly branched amylopectin, which are deposited as insoluble, semi-crystalline granules in chloroplasts of green leaves and in amyloplasts of storage organs (Kossmann and Lloyd, 2000; Copeland *et al.*, 2009). Starch physico-chemical properties are mainly influenced by the ratio of linear amylose and branched amylopectin (Schwall *et al.*, 2000; Parker and Ring, 2001) as well as its non-starch components such as lipids, proteins, and phosphorous (Madsen and Christensen, 1996; Ellis *et al.*, 1998). Besides, starch granule size and uniformity are determinants for applications (Kaur *et al.*, 2007). It has been shown that amylose content, swelling power, peak viscosity, solubility, enzymatic digestibility, wet-milling and water binding capacity of starch are significantly correlated with the starch granule size and uniformity (Singh and Singh, 2001; Roper, 2002; Kaur *et al.*, 2007).

Since the percentage of amylose greatly influences the range of starch applications, finding ways to manipulate the amylose/amylopectin ratio in starch is an important goal. Although the function of many genes encoding starch biosynthesis enzymes is known, the question how starch granule size and properties are genetically controlled is still a matter of debate. To date, conventional breeding and antisense technology (Visser *et al.*, 1991; Safford *et al.*, 1998; Edwards *et al.*, 1999; Lloyd *et al.*, 1999; Schwall *et al.*, 2000; Jobling *et al.*, 2002) have been used to achieve *in planta* modification of the amylose to amylopectin ratio.

Amylosucrase (EC 2.4.1.4) from *Neisseria polysaccharea* is a glucansucrase belonging to glysoside-hydrolase (GH) family 13 and catalyzes the synthesis of an amylose-like polymer from sucrose. Amylosucrase is clearly unique in GH family 13 which mainly contains starch-degrading enzymes; it is the only member displaying polymerase activity. In comparison to the other enzymes responsible for α-glucan synthesis, amylosucrase produces α-(1, 4)-glucan without an α-D-glucosyl-nucleoside-diphosphate such as ADP- or UDP-glucose. Instead, it uses the

energy produced by the cleavage of the glycosidic linkage of sucrose for the synthesis of other glycosidic linkages (Hehre *et al.*, 1949). This property gives amylosucrase an advantage for the use in numerous industrial applications.

Synthesizing amylose-like polymers from sucrose as sole substrate (Hehre *et al.*, 1949; Potocki de Montalk *et al.*, 2000), accommodating maltooligosaccharides (MOS) longer than maltopentaose as recipient (Albenne *et al.*, 2002) and more importantly the presence of sucrose in potato amyloplasts (Gerrits *et al.*, 2001) have potentially made the enzyme amylosucrase an interesting target enzyme to introduce into potato and produce amylose. The synthesis of amylose by this enzyme can, in theory, be monitored relatively easy in the amylose-free potato (*amf*) plants which lack granule-bound starch synthase I (GBSSI) activity (Kuipers *et al.*, 1994). Moreover, modulation of amylose/amylopectin ratio will broaden starch applications if amylosucrase cooperates with GBSSI in a wild type potato genetic background.

In an attempt to synthesize amylose-like polymers in potato starch tubers by a different approach rather than modulating endogenous starch biosynthetic enzymes, the amylosucrase gene either alone or fused to a starch binding domain has been introduced in two potato genetic backgrounds, Kardal and *amf* mutant.

Results

Amylosucrase is accumulated in the amyloplasts

Amylosucrase (EC 2.4.1.4) from *Neisseria polysaccharea* alone or fused to starch binding domain (SBD) at both its carboxyl (C) and amino (N) terminus was cloned in frame with the plastidic protein targeting sequence (FD), and driven by the strong tuber-expressed patatin promoter (Fig. 1). These constructs were introduced into amylose-free (*amf*) and amylose-containing (Kardal) potato genetic backgrounds, yielding the series KDA, *amf*A, KDAS, *amf*AS, KDSA and *amf*SA in which A, AS and SA stand for the amylosucrase alone, amylosucrase with an SBD at the C terminus and amylosucrase with an SBD at the N terminus, respectively. KD-UT and *amf*-UT represent

the untransformed Kardal and *amf* control, respectively.

Green house grown transgenic plants did not show any visible phenotypic difference relative to untransformed control plants. Tuber yield and starch content in amylose-containing genetic background did not change in comparison with the wild-type control plants. In the *amf* background starch yield penalty was observed in some of the transformants (data not shown).

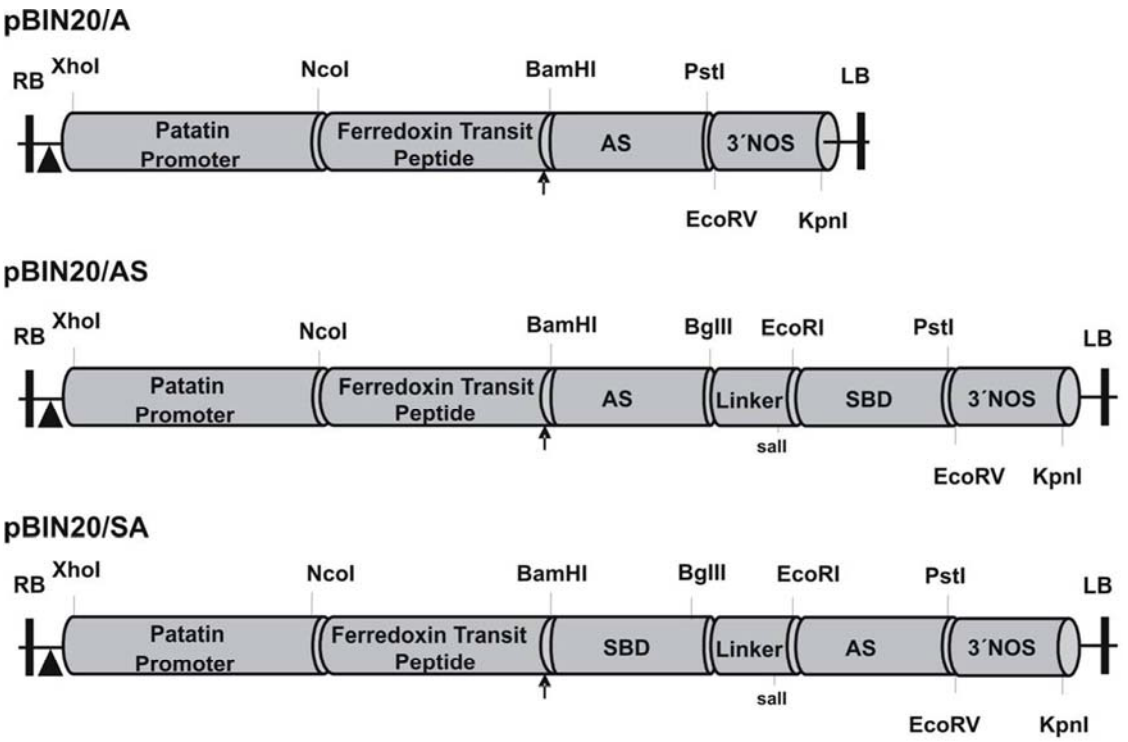

Figure 1. Schematic representation of three different binary vector constructs used for the transformation of potato plants. Genes were in frame with the ferredoxin transit peptide to allow amyloplast targeting and were driven by patatin promoter for tuber expression. RB and LB, right and left borders, respectively. AS, SBD, 3´Nos and linker stand for amylosucrase from *Neisseria polysaccharea*, starch-binding domain of cyclodextrin glycosyltransferase from *B. circulans,* nos terminator and proline-threonine rich stretch, respectively. ▲ and ↑ signs represent kanamycin resistance gene and cleavage site of the transit peptide.

The transgenic line of KDAS and KDSA series were classified and divided into separate classes according to the amount of fusion protein associated with starch granules (Ji *et al.*, 2003) (Fig. 2 A). Figure 2 B shows that the number of transformants with higher amounts (>4+) of fusion protein accumulation in starch is significantly higher than those with less fusion protein accumulation. Based on Western dot blot analysis and for the Kardal series, there were no transformants in KDSA series which belonged to the 1+ to

3+ protein classes and 56.7% from the KDAS transformants and 86.9% from the KDSA transformants showed a dot intensity of 4+ or higher protein accumulation classes. These figures were 75.0% and 82.0% for *amf* background, respectively (data not shown).

Western dot blot analysis of fusion proteins in the soluble fraction of the tubers was also conducted. It was found that for Kardal series in the soluble fractions of the tubers, fusion proteins can be found in all classes tested for both the AS and the SA transgenic plants (Fig. 2 C). In the *amf* background, the amount of fusion proteins in the potato juice is higher for the SA series than AS series. No correlation between the fusion protein content in the granules and that in the juice was observed.

As no antibody was available for amylosucrase, Northern blot analysis was used for classification of the transgenic clones expressing amylosucrase alone. Based on mRNA transcript levels, four different categories (none like KDA5, low like KDA 28, medium like KDA3 and 26 and high like KDA27) were recognized (Fig. 2 D, upper panel). Sixteen, twelve, sixteen and fifty six percent of transformants were classified as high (H), medium (M), low (L) and no (N) expressers, respectively.

In order to check correct processing of fusion proteins accumulated in starch granules of potato plants, one representative of each fusion protein with the highest accumulation (KDAS2 and KDSA2, *amf*AS3 and *amf*SA1) were extracted from starch granules and analyzed by SDS-PAGE followed by immunoblotting detection using anti-SBD (supplementary Figure S1). Proteins of about 85 kDa in all selected transformants were found, which corresponds well to the predicted molecular mass of 86.1 kDa and 86.5 kDa for SBD-AS and AS-SBD fusion proteins, respectively. These results clearly show that proteins encoded by AS and SA constructs were successfully targeted to the starch granules.

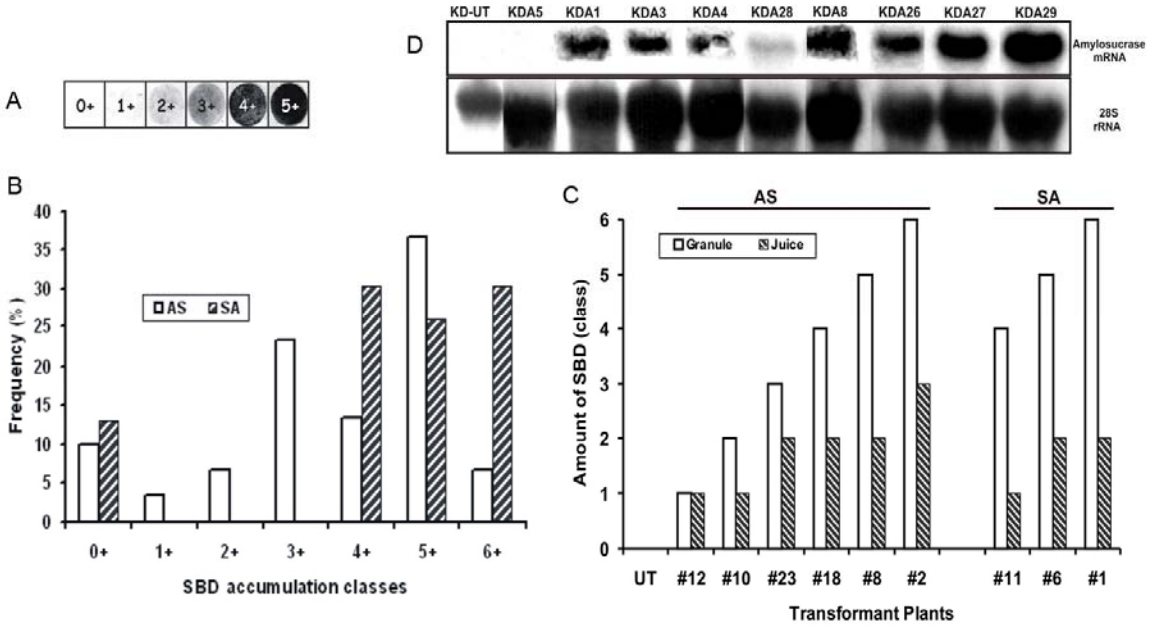

Figure 2. Comparison of the starch-binding domain (SBD)-containing fusion protein content of starch granules in Kardal background. (A) Classes of SBD accumulation in potato starch granules. This classification is based on the results of a Western dot blot analysis with various starch samples. 0+ represents the class with no fusion protein, and 6+ the class with the largest amount of fusion protein. The 6+ category represents the transgenic granules which gave a similar signal in Western dot blot analysis as the 5+ class with half the amount of starch. (B) Distribution of the individual transformants over the seven classes of SBD accumulation in Kardal background. (C) Accumulation levels of SBD fusion protein in starch granules and juices of transformants in Kardal background. (D) Northern blot analysis of amylosucrase expression in selected transgenic lines of KDA transformants.

Amylosucrase expression in the amyloplast results in morphological alteration of starch granules

Preliminary observations with light microscopy showed that starch granule phenotype was altered, especially in the highest expressors (Fig. 3 A-D). Starch granules showed a rough surface in all three series in both *amf* and Kardal genetic background. For better visualisation, transgenic starches were also analyzed with scanning electron microscopy (SEM). This confirmed that starch granule morphology was severely affected. The presence of A, AS and SA fusion proteins in the amylose-containing and amylose-free starch granules resulted in different starch phenotypes (Fig. 3 E-H and I-L). Surprisingly, accumulation of amylosucrase alone or fused to the SBD in Kardal background gave granules with different surface morphology (Fig. 3 compare F to G and

H). A cratered surface, resembling a golf ball appearance, in KDA series (Fig. 3 F), a discontinuous thin layer in KDAS series (Fig. 3 G), and a particulate but thick layer in KDSA series (Fig. 3 H) can clearly be observed in the different starches from the transformed plants. In order to quantify the number of such altered starch granules in different classes of fusion proteins as well as different transformants, a population of three hundred granules from different transformants was counted. In transformants containing the amylosucrase alone, no altered granules were found in low amylosucrase expressers and 15.0% (±1.2) and 6.0% (±2.1) of altered granules were found in Kardal genetic background for the high (H) and intermediate (M) amylosucrase expressers, respectively (data not shown). There was a positive correlation between the altered starch granule percentage and the protein accumulation level up to level 4+ in KDAS transformants. In both KDAS and KDSA series, transformants with the highest number of altered starch granules, 56.5% (±4.9) in KDAS and 75.0% (±7.1) in KDSA, belonged to protein level 4+. Moreover, the further accumulation of the fusion protein more than 4+ protein level did not have an effect on the number of altered starch granules, indicating that at 4+ the maximum number of altered starch granules was reached (data not shown).

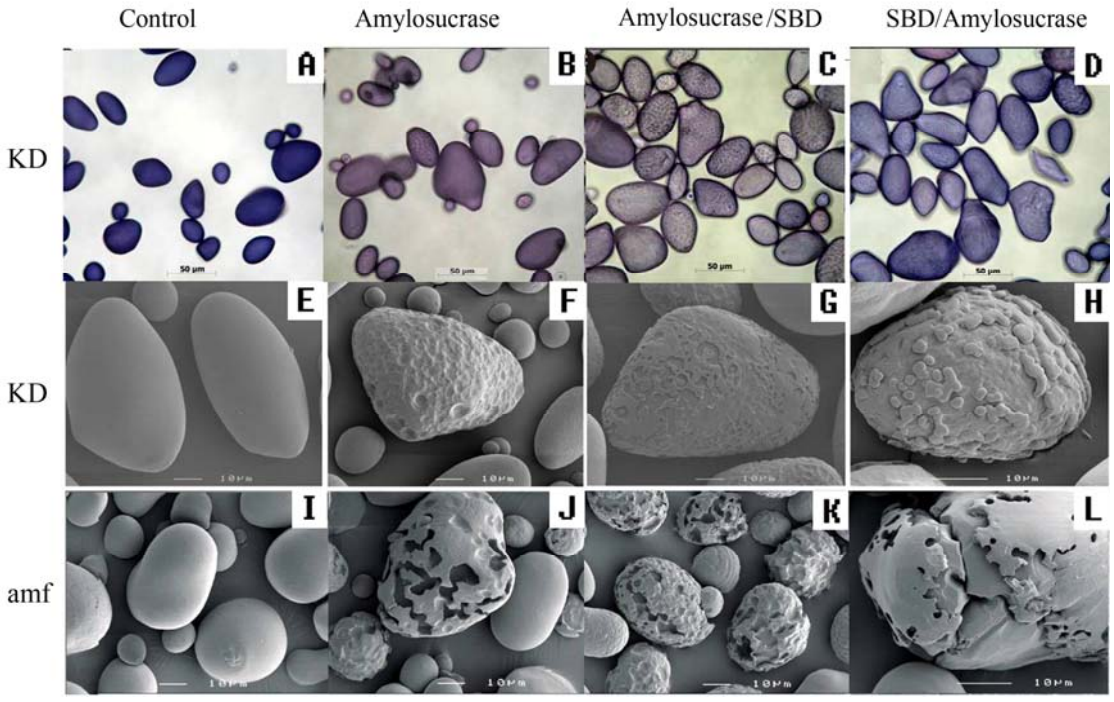

Figure 3. Light micrographs and SEM analyses showing the starch granule morphology of KD-UT (A, E), KDA (B, F), KDAS (C, G), KDSA (D, H), *amf*UT (I), *amf*A (J), *amf*AS (K), and *amf*SA (L). Starch granules were stained with a 20× diluted Lugol solution for light microscopy.

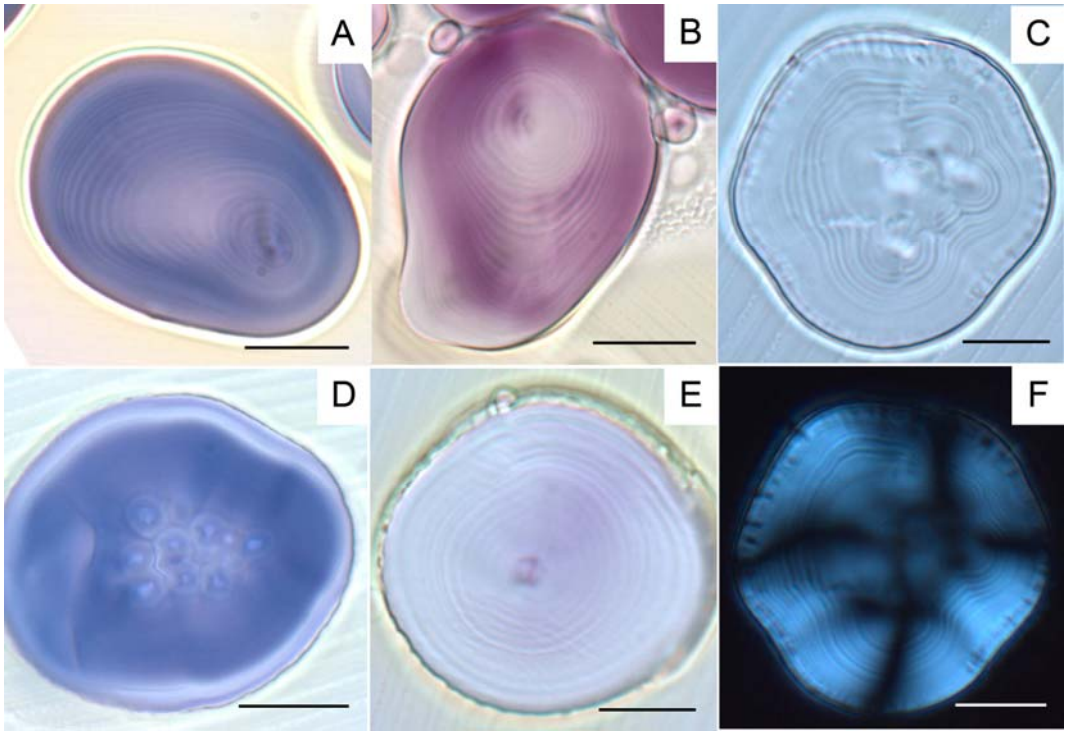

Figure 4. Section images showing the starch granule internal structure of KD-UT (A), KDSA1 (D), *amf*UT (B), and *amf*SA1 (C, E, F). The scale bar indicates 10μm in each image.

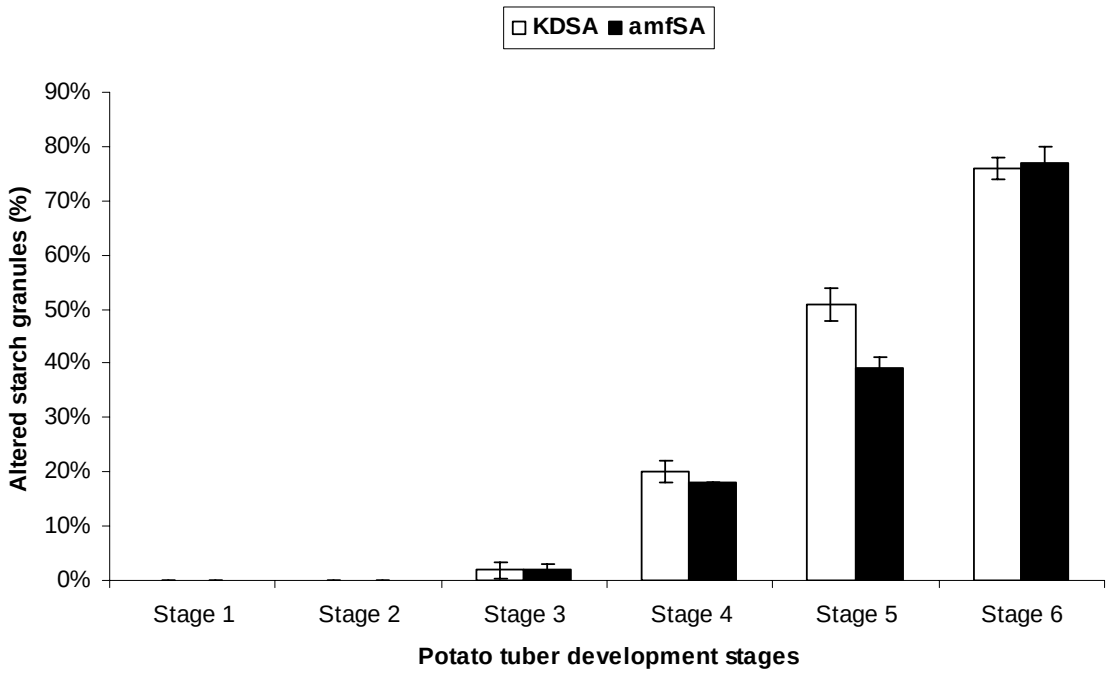

Figure 5. Percentage of starch granules with altered morphology of KDSA1 and *amf*SA1 transformant lines during potato tuber development stages. A population of 100 granules was counted in triplicates.

Starch granules of KDSA1, *amf*SA1, and corresponding controls were sectioned and visualized under light microscope. As shown in Figure 4 E, the rough surface phenotype of the starch granules was only superficial. Furthermore, there were starch granules which did not show the concentric growth rings of normally assembled granules but contained multiple small granules inside in both KDSA1 and *amf*SA1 transformants (Fig. 4 C, D and F).

Altered starch granules can be detected at early tuber developmental stage

In order to investigate starch granule phenotypes during tuber development, starches from KDSA1 and *amf*SA1, which had the highest percentage of altered granules, were isolated from five tuber developmental stages. The tuber development stages were classified according to tuber size, corresponding to stages 3, 4, 5, 6, 7 in Kloosterman's study (Kloosterman *et al.*, 2005), ranging from swelling stolons and four different tuber growth stages.

The morphology of the starch granules during tuber development was analyzed by scanning electron microscopy (SEM) and the results are shown in Figure 5. The SEM analysis shows that the surface of the granules was altered both in KDSA1 and *amf*SA1 transformants from developmental stage 6 (corresponding to around 1 cm in tuber size) onwards, and the percentage of altered granules in the amylosucrase lines increased during potato tuber development (Fig. 5).

Expression of amylosucrase fusion proteins in Kardal results in larger starch granules

Potato starch granules have differences in size within the same tuber even among the ones synthesized in the same cell; therefore starch granule size is always expressed as a distribution of sizes which is characteristic for each particular plant species and in some cases depends on cultivars. Granule size distribution analysis of different Kardal transformants clearly showed that starches with fusion proteins had larger granules (Fig. 6). Although the expression of amylosucrase alone changed starch granule morphology

severely (Fig. 3F and 3J), it did not result in any significant difference in granule size. In contrast, expression of both AS and SA fusion proteins in Kardal increased the starch granule median size significantly. In KDAS and KDSA series, 83% (25 out of 30) and 70% (16 out of 23) showed a median granule size significantly larger than that of the control plants, respectively. Starch granule size increased significantly up to two fold in KDAS and KDSA transformants which belong to the protein accumulation 2+ or higher level. But, no significantly increase in starch granule size in the transformants which protein level below 2+. However, no positive correlation between the protein accumulation level from level 2+ through 6+ and the increase of starch granule median size were found. With respect to the SBD orientation, there was no significant difference between AS and SA transformant series in starch granule size (one way ANOVA F=1.09, P=0.343). The position of SBD in the fusion protein did not matter in this respect.

In spite of the fact that all three transformants, *amf*A, *amf*AS and *amf*SA, generated rough surface granules (Fig. 3), there was no change of the granule size distribution in all the transformants (data not shown).

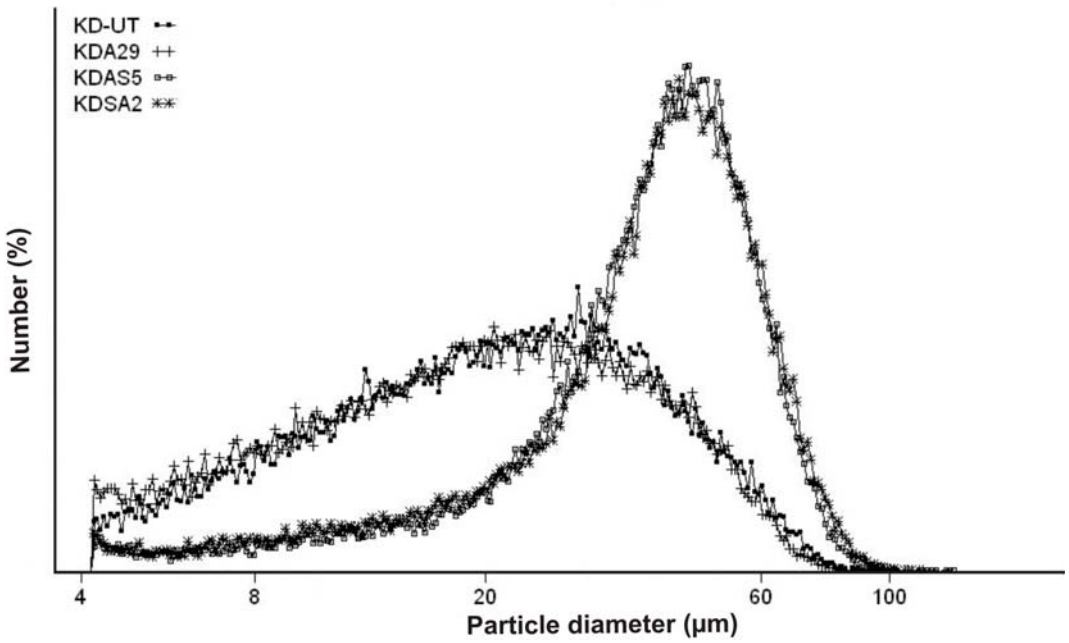

Figure 6. Average particle size distribution of the transgenic starches of three transformants in Kardal background. Each starch sample was analyzed in triplicates.

Expression of amylosucrase does not affect the expression of many genes directly involved in starch metabolism

Expression of amylosucrase fused with SBD in Kardal potato leads to an increase in starch granule size and to a different (rough granule) surface morphology of the granule. To better understand this phenomenon, high-throughput transcriptomic analysis of tubers from KDSA1 and *amf*SA1 transformants and their untransformed controls was performed using the POCI 44K 60-mer oligo array (Kloosterman *et al.*, 2008).

The results showed that the expression of amylosucrase/SBD fusion protein did not affect the expression level of most of the genes which code for enzymes directly involved in starch and sucrose metabolism such as ADP-glucose pyrophosphorylases, starch synthases, starch-branching enzymes, de-branching enzymes, α-glucan water dikinases in both KDSA1 and *amf*SA1 transformants tubers in comparison with their untransformed control tubers. Noteworthy, the expression of SBD-amylosucrase in Kardal potato tubers resulted in enhanced expression of a glucose-6-phosphate/phosphate translocator 2 gene (Probe ID: MICRO.16386.C1, a 9-fold increase) and of a beta-amylase gene (Probe ID: MICRO.13368.C1, a 3-fold increase). These results were confirmed by quantitative real-time PCR (qRT-PCR) showing that the expression of both genes was clearly higher in the KDSA1 transformant tubers than in KD-UT tubers (data not shown). In short, both microarray and qRT-PCR results showed that the expression of SBD-amylosucrase in Kardal resulted in up-regulation of glucose-6-phosphate/phosphate translocator 2 (GPT2) and beta-amylase.

Starches from transgenic plants with larger granules have different rheologic behavior

The starch viscosity profile upon a heating/cooling cycle of a number of transformants was tested (Table 1). Starch viscometric analyses showed that transgenic starches with larger granules had a different behavior than the control. The end viscosity (the viscosity of starch pastes after cooling) of all transgenic starches was much higher than that of the control starches (Table 1). The T_{onset} (at this temperature starch granules

start to gelatinize) and the peak viscosity (the highest viscosity reached during the gelatinization of starch usually corresponding to the point where all the granules are swollen to their maximum level) did not show consistent differences in starches from the transgenic plants in comparison to that of the untransformed control plants.

Other starch properties such as apparent amylose content (%AM) (Table 1) and chain length distribution profile which was determined by both HPSEC and HPAEC have been measured. No significant changes were observed in transgenic lines relatively to untransformed controls.

Table 1. Overview of various parameters determined for the transgenic starches in Kardal background.

Clone	Protein class	AM(%)	d50(µm)	T-onset (°C)	PV (PaS)	EV (PaS)
KD-UT	-	20.0+(0.1)	20.8+(0.3)	69.6 ± 0.3	300 ± 10	80 ± 10
KDAS-15	6+	20.6+(0.3)	27.4+(0.0)	68.5 ± 0.3	290 ± 10	150 ± 10
KDAS-2	6+	21.6+(0.4)	38.0+(0.5)	71.5 ± 0.5	350 ± 10	275 ± 10
KDAS-6	3+	19.9+(0.3)	47.4+(0.1)	69.9 ± 0.4	335 ± 20	150 ± 10
KDSA-12	6+	19.9+(0.5)	26.5+(1.5)	67.8 ± 0.3	300 ± 20	185 ± 5
KDSA-1	6+	19.9+(0.1)	37.4+(0.7)	68.6 ± 0.5	265 ± 15	135 ± 15
KDSA-2	6+	20.0+(0.1)	40.9+(0.1)	69.7 ± 0.1	280 ± 20	140 ± 15

AM, apparent amylose content; d50(µm), median value of the granule size distribution; T-onset (°C), temperature of onset of starch gelatinization; PV (PaS), peak viscosity; EV (PaS), end viscosity.

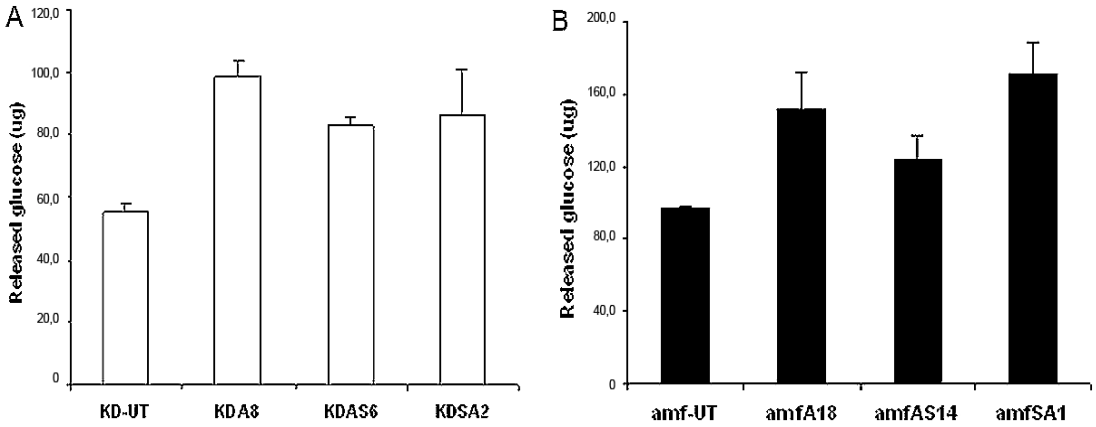

Figure 7. Starch digestibility analysis of transgenic starches of various transformants. Each starch sample was treated with α-amylase and measured in triplicates.

Modified starches show a higher digestibility after α-amylase treatment

Native potato starch has B-type crystallinity resulting in much less susceptibility to hydrolytic enzymes than other starches with A-type crystallinity. In order to investigate whether the accumulation of amylosucrase protein in potato starch granules affected starch digestibility, starches from each selected transformant and untransformed controls were treated with α-amylase. The results showed that more glucose was released from starches of all selected transformants than from starches of untransformed controls in both Kardal and *amf* backgrounds, indicating that the starches from all transformants are more susceptible to α-amylase than starches from untransformed controls regardless of constructs and backgrounds (Fig. 7).

Discussion

Expression of amylosucrase resulted in alteration of the starch granule morphology. The dendritic surface of the altered starch granules (Fig. 3) resembles the surface of small crystallites at the surface of glycogen particles when amylosucrase was incubated with a mixture of sucrose/glycogen in which sucrose was present at a much lower concentrations (Putaux *et al.*, 2006). With the fact that the amount of sucrose in potato amyloplasts is limiting (Gerrits *et al.*, 2001), it could mean that a very low amylosucrase activity (because it could not be measured) could be the reason for the observed small crystallites deposited on certain transformants starch granules. Amylosucrase 3D structure analysis shows that the enzymes possesses five domains (named N, A, B, B′, and C domain) (Skov *et al.*, 2001) and it contains extra three oligosaccharide binding sites besides the active site of $(\beta/\alpha)_8$-barrel catalytic A-domain which seem to be involved in positioning the glycogen external chains during the elongation process (Albenne, *et al.*, 2007). The presence of these polysaccharides binding sites makes amylosucrase with high affinity for amylopectin and consequently this enzyme might act as a double SBD with α-glucan side chains of amylopectin in the process of the double-helical structure assembling. Moreover, the extra fused SBD in amylosucrase/SBD fusion protein enhances the binding ability of amylosucrase to amylopectin. As described by Nazarian

and co-workers (Nazarian *et al.*, 2007a), the double SBD binds at the end of amylopectin double helix and cross-links different starch nucleation sites, whereas amylosucrase has a larger contact area (possibly lateral) and therefore also a larger impact on starch assembly resulting in the craters and grooves on the starch granule surface. Furthermore, due to the presence of amylose, these holes on the starch granule surface are filled up to a certain extent with amylose in Kardal background which give rise to the less severe starch morphological alteration in Kardal background than in *amf* background as showed in Figure 3. Interestingly, the expression of a mutansucrase in potato plants also resulted in starch granules with rough surfaces in wild type potato starches in which possible interaction between the amylopectin side chains and mutansucrase catalytic domain might have led to perturbation at the granule surface (Nazarian *et al.*, 2007b). However, in comparison with mutansucrase, amylosucrase has more binding sites which may capture α-glucan side chains of amylopectin in a more effective way and leads to the more pronounced rough starch granule surface phenotypes than mutansucrase.

The expression of fusion proteins comprising amylosucrase resulted in a major shift in starch granule size distribution regardless of the SBD orientation. All the transformants showed a significant increased in granule size, resulting in a two-fold increase of the median starch granule size in some of the transformants (Table 1). Alteration of starch granule size and distribution has been reported in a number of studies (Ji *et al.*, 2004; De Pater *et al.*, 2006; Howitt *et al.*, 2006). Starch granule size has a profound effect on starch functionalities in such a way that the size and distribution of starch granules can determine starch specific applications. Over-expression of a gene involved in chloroplast division (*FtsZ1*) in potato plants led to less but larger starch granules(De Pater *et al.*, 2006). Ji *et al*. (2004) were able to produce starches with a severe decrease in granule size in an amylose-free genetic background, through the expression of a tandem starch binding domain of cyclodextrin glycosyltransferase (CGTase) of *Bacillus circulans.* Interestingly, expression of tandem SBDs from the an orthologue gene from *Thermoanaerobacterium thermosulfurigenes* in Arabidopsis plants increased starch granule size in the starch excess mutant (*sex 1-1*), but not in the wild type (Howitt *et al.*, 2006). As we discussed before, the amylosucrase/SBD fusion protein may behave as the double SBD which aggregates different granule nucleation sites

resulting in the multiple small granules in one apparent granule (Fig. 4). These aggregated small granules could be united and leads to larger granules in Kardal transformants because of the presence of amylose, but not in *amf* transformants.

Besides the physical interactions between the amylosucrase/SBD fusion protein and the α-glucans in starch granules, the up-regulation of GPT2 gene could contribute to the larger starch granules in Kardal transformants. Starch synthesis in photosynthetic plastids such as chloroplast does not need the import of carbon units or energy as these are directly obtained from photosynthesis. In contrast, starch synthesis which takes place in non-green plastids like amyloplasts relies on the import of glucose-6-phosphate (Glc-6-P) and adenosine triphosphate (ATP) from cytosol into the stroma. Two transporters, the glucose-6-phosphate/phosphate translocator (GPT) and the adenylate translocator (nucleotide translocator, NTT), are involved in these import processes. Niewiadomski *et al.* proved that both GPT1 and GPT2 are functional as glucose-6-phosphate transporter in *Arabidopsis thaliana* by the fact that GPT1 is ubiquitously expressed and GPT2 is expressed at a lower level in most tissues (Niewiadomski *et al.*, 2005). Starch content and tuber yield within amyloplasts is co-limited by substrate supplying (Glc-6-P and ATP) for AGPase. Studies showed that altering adenylate pools and double over-express GPT and NTT can enhance starch content and tuber yield (Regierer *et al.*, 2002; Zhang *et al.*, 2008). But none of the studies mentioned whether starch granule size has been changed. Theoretically, the over-expressing GPT2 gene would lead to an increase in transport of Glc-6-P into the amyloplasts for the synthesis of ADP-Glucose which is the substrate for starch synthases and represents the first committed precursor for starch synthesis. This would offer a potential possibility of synthesis of larger granules. Moreover, the starch granule size in *amf* tranformants does not increase which is consistent with the observation of no expression changing of the GPT2. However, the reason for the up-regulation of GPT2 in Kardal transformants is not clear. Nowadays, although there is increased interest in characterization of the enzymes that are involved in starch biosynthesis and more and more function of enzymes have been identified in starch biosynthesis pathway, the mechanisms underlying starch granule initiation, starch granule size and distribution remain unknown (Zeeman *et al.*, 2010). In this paper, we have shown that the expression

of engineered amylosucrase allows the synthesis of starch granules twice as big as in the wild type potato. Manipulation of GPT2 gene expression level could provide a possibility to change starch granule size and even shed more lights on how starch granules size and distribution are controlled during starch biosynthesis.

Starches with bigger granules in Kardal transformants have different viscosity behavior and digestibility. Pasting and thermal properties of starch are affected by amylose, lipid, phosphorous content, and starch granule size. De Pater *et al*. (2006) found almost no significant change in peak viscosity for larger starch granules in comparison to the small as well as the control starches, whereas they found higher end viscosity for bigger starch granules. In contrast our starches with larger starch granule size also did not show difference on peak viscosity and the end viscosity of our larger starch granules was higher for all transformants. Although in general it is accepted that a low amount of amylose leads to a higher end viscosity (Blennow *et al*., 2003), the apparent amylose percentage of bigger granules did not change which is in agreement with results from those larger granules obtained with modulated plastidial division (De Pater *et al.*, 2006). Several studies showed that the starch granule size is an important factor affecting the digestibility of raw starch by amylase, for instance, large starch granules are digested slower than smaller granule because they have a smaller surface area than smaller granules (Cottrell *et al.*, 1995; Noda *et al.*, 2005; Noda *et al.*, 2008, Kasemwong *et al.*, 2008). Interestingly, we observed that starch granules with larger granule size are easier digested than untransformed starches in this study. Although the starch granule size of transformant plants are bigger than that of granules from untransformed potato, but comparing with smooth surface granules, the rough surface granules have much bigger surface area which result in the larger rough surface granules are easier to be caught and digested by amylase.

Moreover, the starches from transformants not only show bigger in size but also have different viscosity behavior which can find specific applications in industry. First of all, the potatoes with larger starch granules have a great advantage on starch isolation in the potato starch industry. Generally, starch granules are isolated from potato tubers during several processes such as tubers grinding, starches and fibers separating, and starches drying. Normally, the total starch recovery is up to 96% which means there are at

least 4% of the starch granules lost mainly because smaller granules are easily lost during the tubers grinding and the starches cleaning processes (Grommers and van der Krogt, 2009). The increase of starch granules size in tubers would decrease the lost of starches during the isolation processes which would bring a great benefit to the potato starch industry with the fact that the big amount of potato starches (2.5 million tons) produced in the world (Grommers and van der Krogt, 2009). Secondly, the different viscosity behavior of the larger starches also gives advantages for the starch application since the potato starch is widely used in textiles, paper, adhesives and food uses (Treadway, 1952). One of the most frequent uses is to make thickeners and binders in food and confectionery industry because potato starches make clear and translucent effect while starches from wheat or corn gives more muddy appearance. The higher end viscosity of bigger size starch could make it more suitable because less starch will need to be added in order to meet the end viscosity in these applications. This would have an extra advantage in the diet foods because of less calories intake.

Experimental Protocol

Preparation of constructs

Three different constructs were made in this study, consisting of the *Amylosucrase* gene from *Neisseria polysaccharea* ($G_{EN}B_{ANK}$ accession number AY099335) and a starch binding domain (SBD) encoding region of cyclodextrin glycosyltransferase from *Bacillus circulans* (Fig. 1). The pBIN20/AS and pBIN20/SA plasmids were used for the expression of the Amylosucrase-SBD and SBD-Amylosucrase fusion proteins in the amylose-containing (c.v. Kardal) and amylose-free (*amf*) potato plants respectively. The pBIN20/A plasmid was used as a control in which amylosucrase was directed to the amyloplast without granule-targeting sequence (SBD).

The pBIN20/AS plasmid was constructed as follows. An expression cassette for the AS gene was first assembled in a pBlueScript vector from three DNA fragments: (1) the

tuber-specific patatin promoter(Wenzler *et al.*, 1989) and a ferredoxin signal sequence (Pilon *et al*., 1995) (XhoI-BamHI), (2) an AS fragment (BamHI-PstI), and (3) the NOS terminator sequence (EcoRV-KpnI). The AS fragment (BamHI-PstI) was amplified from the pTrcHisB/AS plasmid by PCR using the following primers: 5′–CGAAAA**GGATCC**CCCGAAT–3′ and 5′–TCT**CTGCAG**CGCCAAAACA–3′, which contained BamHI and PstI sites, respectively. The pBlueScript/AS was digested with XhoI and KpnI, and the fragment (XhoI-KpnI) was inserted into the corresponding sites of the pBIN20 vector, giving plasmid pBIN20/AS (Fig. 1). The size of the expected AS fusion protein accumulated in potato tubers has a predicted molecular mass of 86,468 Da, excluding the transit peptide.

For making the pBIN20/SA construct, a SA fragment (BamHI-PstI) was amplified from the pTrcHisB/SA plasmid by PCR with primers 5′–TGACGAAAA**GGATCC**ATTGTC–3′ and 5′–TA**CTGCAG**GCATTTGGGAA–3′, which contained BamHI and PstI sites, respectively. The amplified SA fragment (BamHI-PstI) was used to replace the AS fragment in the pBlueScript/AS plasmid to generate plasmid pBlueScript/SA. The rest of the procedure was conducted in the same as described for pBIN20/AS. The resultant plasmid is referred to as pBIN20/SA (Fig. 1). The SA fusion protein accumulated in potato tubers has a predicted molecular mass of 86,110 Da, excluding the transit peptide.

For making the pBIN20/A plasmid, an amylosucrase-encoding fragment was obtained by PCR amplification using the amylosucrase as a template with the primers 5′–CGAAAA**GGATCC**CCCGAAT–3′ and 5′–TA**CTGCAG**GCATTTGGGAA–3′, containing BamHI and PstI sites, respectively. The plant-expressed amylosucrase has a predicted molecular mass of 72,466 Da, excluding the transit peptide. All constructs were verified by DNA sequencing.

Plant transformation and regeneration

The pBIN20/A, pBIN20/AS, and pBIN20/SA plasmids were transformed into *Agrobacterium tumefaciens*, according to the three-way mating protocol described by Visser (1991). Internodal stem segments from Kardal and *amf* potato were used for

Agrobacterium-mediated transfomation containing the plasmid (Visser *et al.*, 1991). More than 50 independent shoots from each construct were harvested. Shoots were tested for root growth on a kanamycin-containing (100 mg/L) MS30 medium (Murashige and Skoog 1962). Thirty transgenic, root-forming, shoots from each construct were multiplied and transferred to the greenhouse for tuber development. In addition, 10 untransformed controls were grown in the greenhouse. In this study obtained transformant series are referred to as KDA*xx*, *amf*A*xx*, KDAS*xx*, *amf*AS*xx*, KDSA*xx* and *amf*SA*xx* in which A, AS and SA stand for the amylosucrase, amylosucrase-SBD and SBD-amylosucrase fusion proteins, respectively, xx represents the clone number. Besides, KD-UT and *amf*-UT represent the untransformed control plant.

In vitro plantlets of the different transformants KDSA, *amf*SA and their untransformed controls (Kardal and *amf*) were grown in MS medium for 2 weeks and transferred to the greenhouse and maintained in a climate chamber for a period of 10 weeks for the checking of starch morphology and transcriptional analysis at different potato tuber development stages. All tubers were collected and classified into 5 developing tuber stages according size (stage 1; tuber initiation about 0.2cm in diameter. stage 2; tuber size 0.2-0.5cm. stage 3; tuber size about 0.5cm. stage 4; tuber size about 1cm. stage 5; tuber size around 2cm) which correspondingly similar to stage 3 to 7 in Kloosterman's tuber development stages (Kloosterman *et al.*, 2005) and be put in liquid nitrogen immediately. Tubers from all stages were harvested 3–4 h into the light period. For each developmental stage, tubers were harvested from at least 5 individual plants.

Isolation of tuber starch

All tubers derived from five plants of each greenhouse-grown clone were combined, and their peels were removed in an *IMC Peeler* (Spangenberg, The Netherlands). The peeled tubers were homogenized in a *Sanamat Rotor* (Spangenberg), and filtered through a sieve to remove particulate material. The resulting homogenate was allowed to settle for 20 min at 4 °C, and the tuber juice was collected for later use, and stored at −20 °C. The starch sediment was washed three times with water, and finally air-dried at room temperature (Ji *et al.*, 2004).

For isolate starch from different developmental stage tubers, tubers were harvested, cut in pieces and putted in small centrifuge tubes. One ml 3% rapidase in 0.2M NaAc with 0.5g/l Na_2SO_3 (pH=5.5) was added to tubes and incubated overnight at 30 ºC. Starches were collected by centrifuging 2 min (rpm>8000) and removing the cell wall rests by using tweezers. The starch sediment was washed three times with 30mM Phosphate buffer (pH=7.0), one time with demi-water, one time with acetone and finally air-dried at room temperature.

Determination of AS gene transcript levels

Total RNA was prepared from frozen plant material using 5 g (fresh weight) of potato tuber material according to Kuipers *et al*. (1994). Following electrophoresis (1.5% (w/v) agarose, 15% formaldehyde (w/v)), RNA was transferred to nylon membranes (Hybond-N, Amersham, UK) overnight. The Northern blots were hybridized overnight at 65°C in modified church buffer containing 7% (w/v) SDS, 0.5% sodium phosphate (w/v) buffer and 1 mM EDTA (pH=7.2) with a [^{32}P]-labeled amylosucrase DNA fragment (BamHI-PstI) probe. The labeling was performed using a *redi*prime^{TM}II kit (Amersham, England), according to the instructions of the manufacturer. The blots were washed at 65°C once with 2 x SSC for 5 minutes and 30 minutes in 1×SSC, each containing 0.1% (w/v) SDS.

Determination of AS and SA protein content of transgenic starches by Western dot blot analysis

A 12.5% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel (50 × 50 × 3 mm) with 9, equally-spaced, holes (∅ = 9 mm) was placed in contact with a similarly-sized Hybond ECL nitrocellulose membrane (Amersham, Pharmacia Biotech, UK). Twenty mg of (transgenic) starch were boiled for 5 min with 200 µL of a 2× SDS sample buffer (Laemmli, 1970). After cooling to room temperature, the starch gel was transferred into the holes. The proteins from transgenic starch gels were blotted to the membrane using a Phast System (Pharmacia, Sweden; 20 V, 25 mA, 15 °C, 45 min). The

AS and SA fusion proteins were identified with anti-SBD antibodies according to the method described by Ji *et al*. (2003).

The AS and SA fusion proteins in the soluble fraction were also determined as follows. Five-hundred micro litter of tuber juice were freeze-dried and then dissolved in 200 μL of 2× SDS sample buffer, boiled for 5 min in the presence of 20 mg starch from the control samples. The forth-coming starch gel was applied to one of the holes in the SDS-PAGE gel. The rest of the procedure was conducted in the same way as described for the granule-bound proteins.

SDS-PAGE and immunoblotting analysis of granule bound fusion proteins

Western blotting of the starch granule-bound proteins was carried out as described by Nazarian *et al.*, (2007a), using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fifty milligrams of dry starch sample from KDAS2 and KDSA2 were solubilized in 1 mL SDS sample buffer (final concentrations: 1M Tris-HCL, pH 6.8, 2% SDS (w/v), 10% glycerol (w/v), 3% β-mercaptoethanol (w/v)) and boiled for 2 min prior to analysis on a gel. 25 microliters were loaded onto lanes of 12% polyacrylamide gel (145mm x95mm x3mm, Bio Rad, UK). Proteins were transferred onto a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, UK) and immunobloted using anti SBD as described in previous sections.

Analysis of morphology and physicochemical properties of starch granules

Average granule size and granule size distribution of the starches were determined with a Coulter Multisizer II, equipped with an orifice tube of 200 μm (Beckman-Coulter, UK). Approximately 10 mg of starch were dispersed in 160 mL of Isoton II. The granule size distribution was recorded by counting approximately 50,500 (±500) particles. The coincidence (the frequency of two granules entering the tube at the same time, and consequently being counted as one) was set at 10%.

Starch granule morphology was investigated by light microscopy (LM, Axiophot, West Germany) and Scanning Electron Microscopy (SEM, JEOL 6300F, Japan). For LM,

starch granules were stained with a 20× diluted Lugol's solution (1% I_2/KI). For SEM, dried starch granules were mounted onto brass holders with double-sided sticky carbon tape (EMS, Washington, U.S.A.), sputter coated with cold, (Edwards S150B, Crawley, England) and subsequently transferred into a FESEM (JEOL JSM-6300F, Tokyo, Japan). The samples were analyzed and recorded with SE detection at 5 kV and a working distance of 15 mm. All images were recorded digitally (Orion, 6 E.L.I. sprl, Belgium) at a scan rate of 100 seconds (full frame) at the size of 2855 x 2154, 8 bit.

The apparent amylose content was determined according to the method described by Hovenkamp-Hermelink *et al.* (1988). Starch viscosity parameters were measured from a 5% (w/v) and 2% (w/v) starch suspension by employing a Thermo Haake rheoscope (Thermo Haake, Germany). The machine was equipped with parallel plate geometry (type C70/1 Ti) and the gap size was 0.1 mm. Oscillating shear deformation was recorded at a frequency of 1 Hz. The pasting profile of starch suspensions (5% and 2%) was obtained by subjecting the suspensions from 40° C to 90° C at 2° C per min, 15 min at 90° C and cooling down to 20° C at 2° C/min, a constant temperature of 20° C for 15 min. Starch properties such as T_g (start gelatinization temperature, T_p (peak temperature) and the corresponding temperature were measured. Subsequently, to check the linearity of the measurement in a linear viscoelastic region, the sample was subjected to an amplitude sweep region, in which the amplitudes of stress and strain are proportional to each other.

Determination of starch content

Approximately 50 mg of potato tuber material were dissolved in 0.5 mL of 25% HCl and 2 mL of dimethylsulfoxid (DMSO) for 1 h at 60 °C. After incubation the mixture was neutralized with 5 M NaOH and diluted in 0.1 M citrate buffer (pH 4.6) to a final volume of 10 mL. Twenty µL of the hydrolyzed starch sample were analyzed enzymatically using a test kit (Boehringer, Mannheim Germany), according to the manufacturer instructions.

Chain length distribution of transgenic starch

To test the chain length distribution, 5 mg of starch from transgenic potatoes were suspended in 250 µl of DMSO and gelatinized for 10 min at boiling temperature. After cooling down to the ambient temperature, 700 µl of 50 mM NaAc buffer PH 4.0 were added. A sufficient amount of isoamylase (Hayashibara Biochemical laboratories, Japan; 59,000 U/mg protein) to debranch the starch polymers completely was added to the mixture, and was incubated for 2 h at 40 ° C. After inactivation of the enzyme for 10 min at boiling temperature, 1 ml of 25% DMSO was added. For High-Performance Size-Exclusion Chromatography (HPSEC), the samples were used as such (HPSEC). The HPSEC was performed on a p680HPLC pump system (Dionex, Sunnyvale, Cal. USA) equipped with three TSKgel SWXL columns in series (one G3000 and two G2000: 300mmx7.5mm; Montgomeryville, USA) in combination with a TSKgel SWXl guard column (40mmx6mm) at 35°C. Aliquots of 100 µl were injected using a dionex ASI-100 Automated Sample Injector, and subsequently eluted with 10 mM NaAc buffer (ph 5.0) at a flow rate of 0.35 ml/min (3h run). The effluent was monitored using a RID-6A refractometer (Shimadzu, The Netherlands). The system was calibrated using dextran standards (10, 40, 70, 500 kDa; Pharmacia). Dionex chromelon software version 6.50 SP4 Build 1000 was used for controlling the HPLC system and data processing.

HPAEC was used to obtain a better separation of the smaller amylopectin side chains (in the range of 245 glucose residues). HPAEC was performed on a GP40 gradient pump system (Dionex) equipped with a carboPac PA 100 column (4mmx250mm; Dionex) at 35 ∘C. The flow rate was 1.0ml/min and 20 µl samples were injected with Dionex AS3500 automated sampler. Two eluents were used, eluent A (100mm NaOH) and eluent B (1 M NaAc in 100 mm NaOH), for mixing the following gradient: 0→5 min,100% eluent B(rinsing phase);5→20 min, 100% eluent A (conditioning phase);20→25 min, linear gradient from 0→20% eluent B (100→80% eluent A);25→50 min, linear gradient from 20→35% eluent B (80→65% eluent A);50→55 min, linear gradient from 35→50% eluent B(65→50% eluent A);55→60min , 50% eluent B (50% eluent A). The sample was injected at 20 min. The eluent was monitored by ED40 electrochemical detector in the pulsed amperometric mode (Dionex).

Determination of digestibility of starch granules by α-amylase treatment

Twenty mg of (transgenic) starch were suspended in 1 mL of 50 mM phosphate buffer (pH 6.9) and treated with 5 u of α-amylase (porcine pancreas, Sigma, The Netherlands) for 4 h at 25 °C. A sample without α-amylase addition served as control. After incubation, the samples were centrifuged (7,500×*g*, 10 min). The supernatants were analyzed by using D-Glucose measurement Kit (R-biopharm) for the release of glucose.

Sample preparation and hybridization for microarray and data analysis

RNA extraction and POCI microarray hybridizaition were performed as described by Kloosterman *et al.* (2008). Differences between averages and p-values for the t-tests were performed in Microsoft Excel. Log_2 ratios for all hybridizations were exported into Genstat® v11 for statistical analysis and calculation of expression estimates and standard errors for each feature. Calculated estimates and standard errors of 28,427 features were finally imported into the GenemathsXT 1.6 (Applied Maths) software package for additional analysis.

Genes with a log2 expression ratio ≥ 1(expression ratio 2-fold) or ≤ -1 (expression ratio 0.5-fold) in combination with a q-value < 0.05 were considered significantly different.

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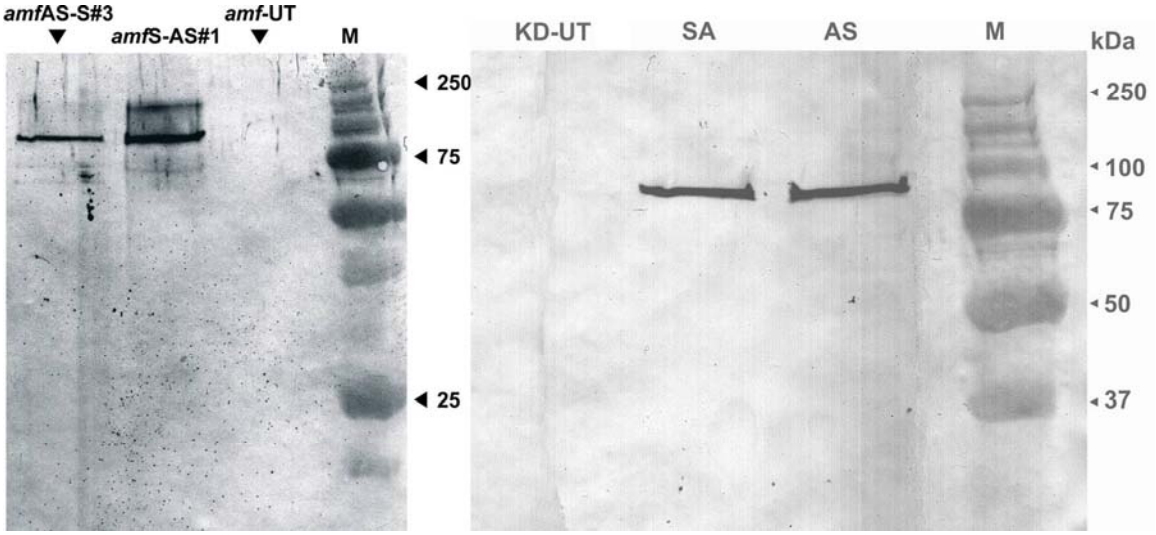

Supplementary Figure S1. SDS-PAGE/Immunoblot analyses of starch granule bound proteins of AS and SA in Kardal and *amf* backgrounds.

Chapter 5

A tandem CBM25 domain of α-amylase from *Microbacterium aurum* is a novel tool for targeting proteins to starch granules

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Abstract

Starch-binding domains (SBDs) from Carbohydrate Binding Module (CBM) family 20 have been used as a tool for starch engineering. In this study, the possibility of using SBDs from another CBM family in the modification of starch biosynthesis *in planta* was investigated. Two fragments, the tandem CBM25 domain (2CBM25) and the tandem CBM25 with multiple fibronectin type III (FN3) domains of the α-amylase enzyme from *Microbacterium aurum*, were expressed in the tubers of a wild type potato cultivar (cv. Kardal) and an amylose-free (*amf*) potato mutant. The 2CBM25 and FN3 protein accumulated in starch granules of both Kardal and *amf* transformants. The accumulation of 2CBM25 did not result in starch morphological alterations in Kardal but gave rise to rough starch granules in *amf*, while the FN3 gave morphological changes of starch granules (helical starch granules in Kardal and rough surface granules in *amf*) but only in a very low frequency. The starches of the different transformants did not show significant differences in starch size distribution, apparent amylose content, and physico-chemical properties in comparison with that of untransformed controls which suggests that the tandem CBM25 can be used as a novel tool for targeting proteins to granules during starch biosynthesis without side-effects on starch morphology, composition and properties.

Keywords: starch, transgenic potato, SBD, CBM20, CBM25

Introduction

Starch is the most important and abundant storage carbohydrate in plants and exists in the form of discrete granules in higher plants such as wheat, rice, maize, potato, and pea. Starch granules are mainly composed of two α-glucan polymers, linear amylose (20-30%) and highly branched amylopectin (70-80%) (Kossmann and Lloyd, 2000). Starches are used in food and many other industrial applications. But for most of the industrial applications, starch normally needs to be modified to overcome certain physico-chemical limitations such as low shear and thermal resistance, and a high tendency towards retrogradation (Singh *et al.*, 2007). Although starch modification can be conducted by chemical or physical treatments, these treatments are time and money consuming or they generate environmental pollution. Starches with novel properties can be produced by modification of the starch biosynthetic pathway. Nowadays, using modern molecular tools it becomes possible to generate plants which can produce so-called "tailor-made" starches that can be used directly for specific industrial uses after harvest from plants without an extra treatment. For example, starches with altered amylose/amylopecin ratio have been successfully achieved by controlling the expression levels of genes which are involved in starch biosynthesis (Kuipers *et al.*, 1994; Schwall *et al.*, 2000).

Microbial extracellular hydrolytic enzymes that catalyze the degradation of insoluble polysaccharides, such as glycogen, starch, and cellulose, typically possess a modular architecture referred to as carbohydrate-binding modules (CBMs) which target cognate catalytic modules to specific polysaccharide structure (Christiansen *et al.*, 2009; Guillén *et al.*, 2010). To date, various types of CBMs have been recognized and classified into 59 different CBM families based on their amino acid similarity (Cantarel *et al.*, 2009). CBMs which show affinity to starch granules are called Starch-Binding Domains (SBDs). Currently, there are nine CBM families, named CBM20, CBM21, CBM25, CBM26, CBM34, CBM41, CBM45, CBM48, and CBM53, which have been reported to contain SBDs. SBDs are about 100 amino acids long and have well conserved amino acid sequences among different families (Svensson *et al.*, 1989; Machovic and Janecek, 2006). It is known that SBDs play three known roles: they allow the interaction between the

insoluble substrate and the enzyme in solution; they help the catalytic domain of enzymes to catch the substrate; and in some cases they have a disruptive function on the starch granule surface (Rodríguez-Sanoja *et al.*, 2005).

Until now, only SBDs from CBM family 20 have been well studied (Christiansen *et al.*, 2009) and introduced into plants successfully (Ji *et al.*, 2004; Howitt *et al.*, 2006). For instance, using the SBD as an anchoring tool, an active luciferase-SBD fusion protein was accumulated inside starch granules, whereas luciferase alone could not (Ji *et al.*, 2003). It is still unknown whether the SBDs of other CBM families can be used to target proteins to starch granules and whether they have other different biochemical properties in comparison with CBM20s. A multi-domain α-amylase enzyme composed of a catalytic α-amylase domain, multiple fibronectin type III domains, and a tandem carbohydrate binding domain belonging to the CBM family 25, was isolated from *Microbacterium aurum*. This α-amylase enzyme is responsible for the formation of numerous small pores in starch granules when it was incubated with potato starch (Eeuwema *et al.*, 2005). The tandem CBM25 domain and the repeated fibronectin III domain are responsible for α-amylase domain catching and working on starch granules by the fact that deletion of the fibronectin type III and the tandem CBM25 resulted in loss of α-amylase function (Eeuwema *et al.*, 2005).

In this study, the effects of the tandem CBM25 domain on starch granules were studied and the potentiality of using this tandem CBM25 domain as tool for anchoring proteins into starch granules during starch biosynthesis was assessed.

Results

No visible phenotype in plant architecture and tuber morphology

Two constructs (Fig. 1), which are referred to as pBIN19/2CBM25 (the tandem CBM25 domain sequence) and pBIN19/FN3 (tandem CBM25 with multiple fibronectin type III domains) were transformed into wild type potato (Kardal) and amylose-free potato (*amf*), respectively. Thirty independent kanamycin-resistant transformant clones from each transformation were transferred to the greenhouse for tuberization and further

analysis. During the growth in the greenhouse, the morphology of transgenic plants did not show detectable differences when compared with that of untransformed control plants. After tuber harvest and starch isolation, the morphology of tubers, the tuber yield, and the starch yield of all transformants were comparable with that of untransformed controls (data not shown).

Figure 1. Schematic representation of the full amino acid sequences of the two different constructs used in this study for potato transformation. Recombinant genes are driven by potato granule-bound starch synthase I (GBSSI) promoter and the amyloplast targeting is ensured by potato GBSSI transit peptide.

Different mRNA expression levels in 2CBM25 and FN3 transformants

Expression of the two gene fragments, 2CBM25 and FN3, in potato tubers was determined by semi-quantitative RT-PCR. Results showed that tubers of transgenic plants possess the mRNA transcripts of the expressed fusion proteins (Fig. 2). Moreover, the intensity of semi-qRT-PCR products varied in different transformants. Based on the band intensity of the PCR products, the transformants were divided into four different classes (-, +, ++, and +++ representing transformants with no, low, medium and high levels of mRNA, respectively).

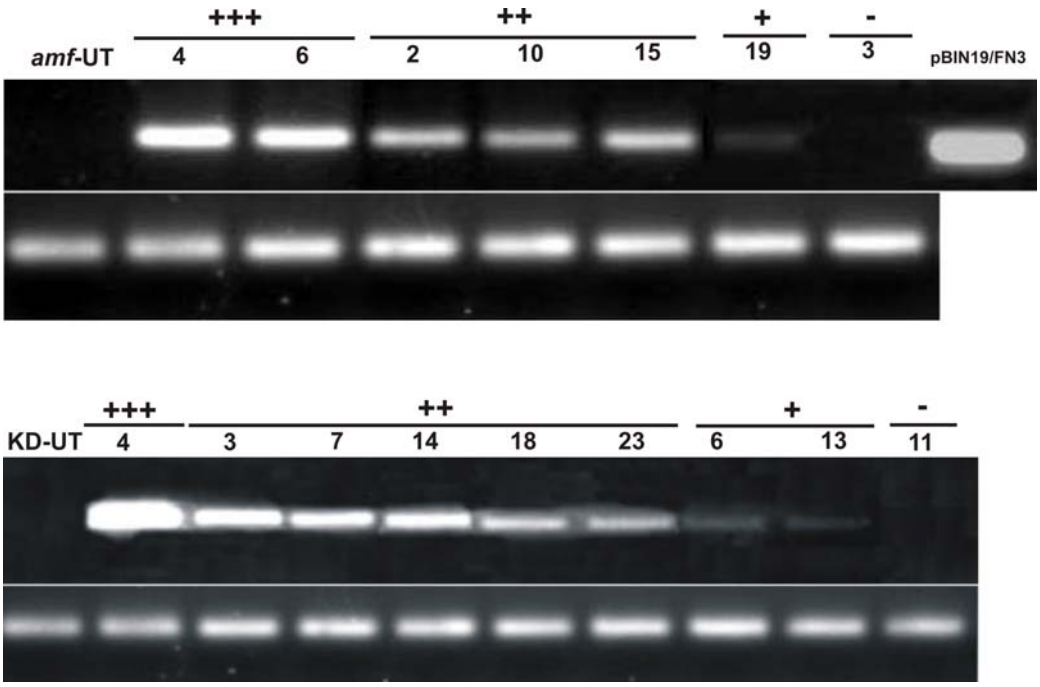

Figure 2. Semi-quantitative RT-PCR with specific primer pairs for KD-FN3 and *amf*-FN3 transformant series. The lower panel shows PCR product of the internal control gene Ubiquitin (Ubi3).

2CBM25 and FN3 proteins are present in starch granules

The expression of 2CBM25 and FN3 proteins in both Kardal and *amf* potato starches was analyzed by Western dot blot. An N-terminally engineered RGS(His)$_6$-tag allows the detection and classification of the accumulation of the His-tagged fusion proteins in starch granules from transformants expressing different constructs. The Western dot blot results showed that the His-tag fusion proteins can be detected in 2CBM25 and FN3 transformant series both in Kardal and *amf* backgrounds (data not shown).

As predicted the molecular masses of the His-tagged fusion proteins were 22,954 Dalton and 59,283 Dalton for 2CBM25 and FN3 respectively. Starch granule bound proteins were extracted from different transformants and were analyzed with SDS-PAGE and subsequent Western blotting immuno-detection with anti-RGS(His)$_6$ antibodies. As shown in Figure 3, bands with the right size were present in starch granules of 2CBM25 and FN3 transformed plants and bands were undetectable in both untransformed control

Kardal and *amf* starches. These results indicate that 2CBM25 fused with poly-His-tag and FN3 fused with poly-His-tag fusion proteins have been targeted to the starch granules successfully.

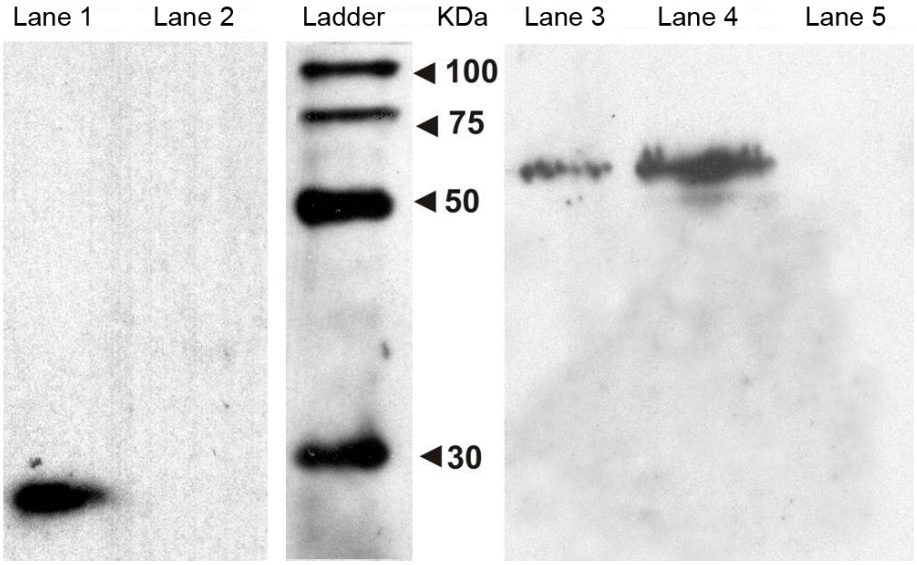

Figure 3. Western blot analysis of the highest expressor of 2CBM25 and FN3 transformants. Total proteins were extracted from starch granules of controls (*amf* and Kardal) and one representative of each series of lines. Lane 1, 2, 3, 4, 5 represent *amf*-2CBM25-29, *amf* control, KD-FN3-04, *amf*-FN3-15, and Kardal control, respectively. Western blot analysis was performed by using an anti-RGS(His)$_6$ antibody. The molecular mass ladder is indicated.

Starch granule morphology remains unaltered in most transformants

The 2CBM25 and FN3 proteins were accumulated in starch granules of transformants in both Kardal and *amf* backgrounds. In order to investigate whether the accumulation of fusion proteins in starch granules affect the starch granule morphology, starch granules of each transformant were investigated by both light microscopy and electron scanning microscopy (SEM).

Light micrographs showed that the accumulation of 2CBM25 and FN3 proteins did not affect starch granule morphology in Kardal background. Most of the transformants did not show any starch granule morphological alteration in KD-2CBM25 and KD-FN3 transformant series in comparison with that of untransformed control. Interestingly, 2 out of 30 KD-FN3 transformants, KD-FN3-04 and KD-FN3-07, showed a strange starch phenotype. Starch granules from these transformed plants showed a helical shell-shaped

structure at a very low frequency which was observed when starch was stained with iodine. Further investigations with SEM revealed the feature of these structures in more detail (Fig. 4 D). The two transformants KD-FN3-04 and KD-FN3-07 belong to high level and medium level expressors in the mRNA transcript analysis of FN3 (Fig. 2).

In *amf* background, the expression of both proteins did not cause severe effects of starch granule morphology. The light micrographs showed that the *amf*-2CBM25 and *amf*-FN3 transformants did not show starch morphological alterations (data not shown). However, SEM microscopy revealed that the surface of starch granules from both *amf*-2CBM25 and *amf*-FN3 transformants were rough, especially in the higher expressors (Fig. 4 C and E). There were 5 out of 32 transformants in *amf*-2CBM25 and 13 out of 18 transformants in *amf*-FN3 that showed rough surface granules albeit at a low percentage. Moreover, there was one transformant (*amf*-FN3-15) that showed amalgamated starch granules (Fig. 4 F).

To investigate the frequency of starch granules with altered morphology, the number of altered granules was scored by counting a population of 300 starch granules in triplicate for selected transformants which have the highest fusion protein accumulation level. The results from these quantification analysis showed that the helical starch granules were present at a very low percentage (less than 1%) in KD-FN3-04 and KD-FN3-07 transformants. The starch granules with rough surface in *amf*-2CBM25 and *amf*-FN3 transformants were also present at a low frequency (about 5%) in the highest expressors (*amf*-2CBM25-29 and *amf*-FN3-04) (data not shown).

In order to investigate at which time point during potato tuber development the helical shell-shaped starches in transformant KD-FN3-07 and the starches with rough surface in *amf* transformants can be detected, the morphology of the starch granules in 5 potato tuber developmental stages was analyzed with scanning electron microscopy (SEM). The SEM analysis showed that helical starch granules can be detected in the swelling stolons of the tranformant clone KD-FN3-07 and rough surface granules and the amalgamated starch granules can only be detected in mature potato tubers of the selected transformants (*amf*-FN3-10 and *amf*-FN3-15).

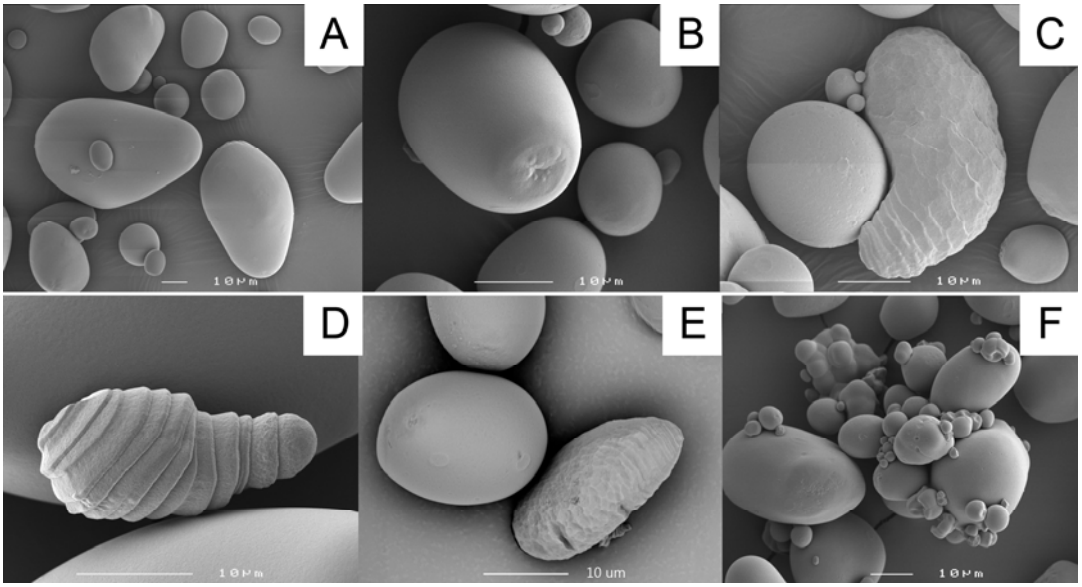

Figure 4. SEM analyses of starch granules from KD-UT (A) and *amf* (B) in comparison with that of the different selected transformants: *amf*-2CBM25-29 (C), KD-FN3-07 (D), *amf*-FN3-10 (E), and *amf*-FN3-15 (F). The scale bar in each picture indicates different magnifications.

Table 1. Summary of different starch characteristics in relationship with class of semi-qRT-PCR.

Code	semi-qPCR class	AM(%)	d50(μm)	ΔH (kJ/g)	Tonset(ºC)
KD-UT	-	22,5	22,2	13,6	64,7
KD-FN3-11	0	21,3	17,3	12,2	65,5
KD-FN3-13	1	21,0	19,5	12,9	65,0
KD-FN3-03	2	22,2	20,0	13,6	65,3
KD-FN3-07	2	21.8	18.2	11.9	66.7
KD-FN3-04	3	21,3	19,0	12,5	66,4
amf-UT	-	3,3	10,5	13,7	70,2
amf-FN3-03	0	3,9	8,1	12,3	69,5
amf-FN3-19	1	3,4	9,3	14,1	69,0
amf-FN3-10	2	3,6	7,0	10,7	69,2
amf-FN3-15	2	3,9	8,6	13,4	69,2
amf-FN3-06	3	3,6	8,7	12,1	69,6
amf-UT	-	2,9	9,1	16,0	73,3
amf-2CBM25-06	0	3,8	7,6	16,5	73,5
amf-2CBM25-23	1	3,4	9,1	16,9	72,7
amf-2CBM25-12	2	3,0	8,2	15,8	73,6
amf-2CBM25-29	3	3,2	8,2	16,8	73,3

Starch apparent amylose content (%AM), median granule size(d_{50}), and starch gelatinization temperature are shown. %AM, $d_{50,}$ Δ H, and T onset data are average of three independent measurements.

Starch granule size distribution, gelatinization behavior, and amylose content remain unchanged

The impact of CBM25 and FN3 protein accumulation in starch granules on starch physico-chemical properties, such as apparent amylose content, mean granule size and granule size distribution, and starch granule melting behavior (Tonset and ΔH), was measured. These results are summarized in Table 1. As shown in Table 1, no significant differences were observed between the starches of *amf*-2CBM25, KD-FN3, and *amf*-FN3 transformants and their corresponding controls.

Discussion

In this study, the tandem CBM25 sequence and the tandem CBM25 with repeated fibronectin type III domain sequence from *M. aurum* were expressed in amylose containing and amylose-free potatoes, respectively, to test the potentiality of the tandem CBM25 domain as a tool to target proteins into the starch granules during starch biosynthesis.

The tandem CBM25 domain has been successfully incorporated into potato starch granules during starch biosynthesis in both Kardal and in *amf* genetic backgrounds in this study (Fig. 3). Starch-binding domains from CBM family 20 have been well studied and previous studies found the SBD from CBM20 accumulated inside the starch granules in transgenic plants without any detectable side effects on starch properties (Ji *et al.*, 2003). Another study showed that both a single SBD and an engineered repeat SBD of the CGTase from *Thermoanaerobacterium thermosulfurigenes* have been incorporated in the chloroplast of *Arabidopsis thaliana* wild type and the starch excess mutant (*sex 1-1*) (Howitt *et al.*, 2006). Above mentioned studies demonstrated that the SBDs can be used as tools for targeting proteins into starch granules. Furthermore, the SBD from CBM20 has been successfully used as tool to introduce enzymes or enzyme domains, which do not have affinity for starch granules, into starch granules to modify starch structure and physicochemical properties (Nazarian *et al.*, 2007 b, c). This technique provides a promising future for starch modification *in planta* by using SBDs. In this study, the

expression of the tandem CBM25 did not affect starch composition (apparent amylose content) and starch physico-chemical properties (Table 1). It is in agreement with results of previous studies except for the size distribution of starch granules which did not change in this study as was the case when tandem CBM20 SBDs were expressed in potato tubers or Arabidopsis leaves (Ji *et al.*, 2004; Howitt *et al.*, 2006).

The accumulation of the tandem CBM25 domain in starch granules neither lead to severe starch morphology alterations nor affected the starch granule size distribution, but there are some altered starch granules at a very low frequency were observed in this study. For instance, the helical starch granules were only observed in two out of thirty KD-FN3 transformant plants at a very low frequency (less than 1%) and the rough surface granules (approximately 5%) showed in both *amf*-2CBM25 and *amf*-FN3 transformants. The rough surface granule phenotype in this study and the amalgamated starch granules which only observed in *amf*-FN3-15 are similar to the starch morphological phenotypes which are reported in the double CBM20 tranformants in *amf* background (Ji *et al.*, 2004). These starch morphological alterations suggest that the 2CBM25 and FN3 protein might interfere in the starch granule assembling process to some degree to give rise to starch morphological alterations, though at a very low rate.

In the nine CBM families which contain SBDs, the structure and property of CBM20 and CBM25 have been thoroughly characterized (Sigurskjold *et al.*, 1994; Sorimachi *et al.*, 1997; Boraston *et al.*, 2006). Although both CBM20 and CBM25 have affinity for starch granules, they do not interact with α-glucans in the same way. First of all, the SBD from CBM20 has two binding sites with distinct affinities for malto-oligosaccharides, whereas the CBM25 only have one binding site. Furthermore, the CBM20 only binds the non-reducing termini of two parallel side chains of amylopectin (Morris *et al.*, 2005), but CBM25 binds the linear single α-glucan chain in both amylose single helix and amylopectin double helix (Boraston *et al.*, 2006). Taken the structure and mechanism of CBMs binding α-glucans into consideration, the interaction of tandem CBM25 used in this study with α-glucans in starch granules is much like the single CBM20 which contains two binding sites. Moreover, because the CBM25 binds single α-glucan chain, it likely binds with both amylose and amylopectin during starch biosynthesis and therefore can be present in both crystalline and non-crystalline regions

of starch.

The absence of amalgamated granules may be due to the fact that the double CBM25 has less binding sites than the double CBM20. It was proposed by Nazarian and co-workers (Nazarian *et al.*, 2007a) that the tandem CBM20 can cross-link different starch granule nucleation sites and lead to the amalgamated granules which are observed in the high 2CBM20 expressers. Since the tandem CBM25 which only have two binding sites in total would work similarly as a single SBD (2 binding sites), it is no surprise that most of the transformants did not show amalgamated starch granules which are observed in the double CBM20 transformants in both Kardal and *amf* backgrounds (Ji *et al.*, 2004). However, there is one transformant *amf*-FN3-15 which has similar amalgamated starch morphologyical phenotype. It is suggesting that another reason for absence of amalgamated granules could be that the accumulation level of 2CBM25 is lower than 2CBM20, although this cannot be confirmed by the comparison of these two proteins accumulation level two different antibodies were used for Western Blotting detection. Moreover, the helical granules which were observed in two KD-FN3 transformants have never been observed before in any of the transgenic potato starches. Since the mRNA expression level of FN3 in both transformants is quite high comparing with that of other transformants, the expression of FN3 could affect genes involved in starch assembling process and give rise to these helical starches, though at a very low rate. However, this needs to be investigated further.

Since the incorporation of the tandem CBM25 in starch does not affect any properties of starch and it is supposed to deposit in all the regions of the starch granule, the tandem CBM25 domain can be a more ideal tool than CBM20 to anchor proteins into starch granules to maximize the potential for the effector proteins. This study gives another option for using starch binding domains as a tool to anchor effector proteins into starch granules for starch bioengineering.

Experimental procedures

Construction of different expression vectors

Two DNA fragments, the $(CBM25)_2$ tandem sequence and the $(CBM25)_2$ with repeated fibronectin type III domain sequence of the α-amylase from *M. aurum*., were cloned in frame to the GBSSI promoter and its transit peptide sequence with a $RGS(His)_6$-tag to facilitate antibody detection The two constructs, named pBIN19/2CBM25 and pBIN19/FN3 were made in pBIN19 expression binary vector. These constructs were made to express 2CBM25 and FN3-2CBM25-3FN3 fusion proteins in potato plants. To obtain the pBIN19/2CBM25 plasmid (Figure 1), a 2CBM25-encoding fragment was obtained by PCR amplification with the primers 5'-ACCCATGGCAGAGAAGCAGGCGTCGACGTC -3' and 5'-GGGATCCGGCATCACGGGTCCTTCACCGACAC -3', which contained *Nco*I and *BamH*I sites at their 5' ends, respectively. *Mictrobacterium aurum* DNA was used as a template for PCR amplification. The amplified fragment was used to replace the SBD2 fragment (also *Nco*I-*BamH*I) in the pUC19/SBD2 plasmid (Ji *et al*., 2004), giving rise to the pUC19/2CBM25 plasmid. Similarly, the other fragment was cloned into the same pUC19/SBD2 plasmid with the primers 5'-AGCCCATGGTGCGTGCACCGAC -3' and 5'-ATCGGATCCTTACGCCGAAGTACCTGCC -3', for FN3-2CBM25-3FN3 amplifications. The 5' end of the coding regions in all constructs carried a $RGS(His)_6$ tag to facilitate protein detection and purification. All constructs in pUC19 were later digested with *Hpa*I-*BamH*I and cloned into the same position in pBIN19 vector containing potato granule-bound starch synthase I (GBSSI) promoter and its transit peptide for tuber expression and amyloplast targeting and a NOS terminator, respectively. The pBIN19/2CBM25 and pBIN19/FN3 were assembled from the following fragments: (1) GBSSI promoter and transit peptide, (2) $RGS(His)_6$-$(CBM25)_2$ or $RGS(His)_6$-FN3-$(CBM25)_2$-$(FN3)_3$, and (3) a NOS terminator. The predicted molecular masses of the $RGS(His)_6$ containing proteins were 22,954 Dalton and 59,283 Dalton for $RGS(His)_6$-$(CBM25)_2$ and $RGS(His)_6$-FN3 respectively, excluding transit peptide. All constructs were sequenced to verify their correctness.

Potato transformation

Agrobacterium tumefaciens strains LBA4404 harboring the expression cassette

constructs (Figure 1) was used for transformation according to the three-way method by Visser *et al*. (1991). Murashige and Skoog medium (MS30) containing100 mg/l kanamycin were used in tissue culture experiments (Murashige and Skoog, 1962), Amylose-free potato mutant (*amf*) and amylose-containing potato plants (wild-type) were used for transformations. Two constructs (Figure 1), which are referred to as pBIN19/2CBM25 and pBIN19/FN3 were transformed in Kardal and *amf*, respectively. Thirty kanamycin-resistant individual transformant clones from each transformation were transferred to the greenhouse for tuberization and further analysis. 2CBM25 refers to the $(CBM25)_2$ domain; FN3 refers to $(CBM25)_2$ with repeated fibronectin type III domain. KD-UT and *amf*-UT refers to two untransformed controls respectively, and xx stands for the clone number. Plantlets grown on MS30 selection medium were harvested, multiplied and 5 plantlets of each clone were subsequently taken to the greenhouse to generate tubers.

Starch isolation

All tubers form each individual transformant (five plants) were peeled, mixed and ground using a Sanamat rotor (Spangenberg, The Netherlands) to which 0.01% of sodium metabisulphite ($Na_2S_2O_5$) was added. Collected juices were taken to 4 °C to settle for at least 3 hours. A part of the juice was kept and frozen at -20 °C for further analysis. The settled starch was washed at least 3 times and passed through a Whattman filter paper on a Buchner funnel. All starch samples were later air dried at room temperature and powdered with a sieve shaker (Retsch, Germany).

For the potato tuber developmental series, tubers at each stage from five plants were pooled and cut in pieces and putted in small centrifuge tubes. 1.0ml 3% rapidase in 0.2M NaAc with 0.5g/l Na_2SO_3 (pH=5.5) was added to tubes and incubated overnight at 30 ºC. Starches were collected by centrifuging 2 min (rpm>8000) and removing the cell wall rests by using tweezers. The starch sediment was washed three times with 30mM Phosphate buffer (pH=7.0), one time with demi-water, one time with acetone and finally air-dried at room temperature.

Determination of the content of fusion protein in starch granules

The amount of RGS(His)$_6$-tag fusion proteins accumulated in transgenic starch granules was quantified by Western dot blot analysis. For determining the amount of fusion proteins 50 mg of (transgenic/control) starches was boiled for 5 min with 1mL of 2× SDS sample buffer containing 5% β-mercaptoethanol (v/v) (Laemmli 1970). After cooling to room temperature, equal amount of the supernatants (500 µl) was applied to a Hybond ECL transfer membrane (Amersham, UK) using the S&S manifold-I dot blot system (Schleicher & Schuell, Keene, NH, USA). To detect expression of recombinant His-tagged proteins, a monoclonal anti-RGS(His)$_6$-tag antibody was obtained from Qiagen (91974 Courtaboeuf Cedex). Protein fractions were electroblotted onto nitrocellulose membrane. Probing and detection of Western-dot blots were performed as described by Ji *et al.* (2003). Primary antibodies were used at a dilution of 1:1000 in Tris-buffer-saline (TBS)-Tween (2mM Tris, 15mM NaCl, Tween 20 0.05%, pH7.6).

Electrophoretic separation of granule proteins

SDS-extracted proteins were obtained with the following steps. Approximately 50 mg of potato starch was mixed and incubated at 70 ºC for 5 min in 1 mL of SDS lysis buffer (4% w/v SDS, 0.3% w/v DTT, 50 mM sodium phosphate, pH 7.0). The homogenate was then cooled on ice for 15 min before centrifugation (15 min, 13000*g*, 4 ºC). The resulting supernatant(s) was/were centrifuged (20 min, 13000*g*, 4 ºC) again using the same parameters before precipitation using 4 mL of 10 mM DTT in cold acetone for an hour in -20 ºC. The protein pellet was washed twice with 2 mL of 10 mM DTT in cold acetone. The washed pellet was then air-dried for 30 min at RT and then solubilized in 50 µL of SDS sample buffer. Twenty five µL of supernatant was applied onto a 12% polyacrylamide gel (145 mm x 95 mm x 3 mm) and the proteins were separated by electrophoresis. Proteins were transferred onto a Hybond ECL membrane (Amersham, UK) and detected using anti-RGS(His)$_6$-tag (Qiagen) according to instruction of manufacturer.

RT-PCR analysis

Relative expression of mRNA was determined by Semi-quantitative RT-PCR analysis as described by Nazarian *et al*. (2007a). Total RNA from 5 g of ground tubers was isolated with adding 1 ml Trizol® reagent (Invitrogen) and the manufacturer's protocol was followed. The RNA pellet was air dried and dissolved in 100 µL RNase-free water, and solubilization was achieved by incubating at 55ºC for 10 min. Samples were quantified and visualized. Based on spectrophotometric RNA determination, similar amounts of total RNA were used for RT-PCR according to Nazarian *et al*. (2007a). The ubiquitin gene (L22576) was used as an internal control (Garbarino and Belknap, 1994)

Analysis of physiochemical properties of starches from different transformants

A combination of light microscopy (LM; Axiophot, Germany) and scanning electron microscopy (SEM; JEOL 6300F, Japan) were used to investigate the granule morphology of starch granules. For LM pictures, a solution of a 20× diluted Lugol's solution (1% I_2/KI) was used to stain starch granules. For SEM, 1 mg of dried starch was mounted onto brass holders with double-sided sticky carbon tapes (EMS, Washington, USA), sputter coated with gold, (Edwards S150B, Crawley, England), and subsequently transferred into a FESEM (JEOL JSM-6300F, Tokyo, Japan). The samples were analyzed and recorded with SE detection at 5 kV and a working distance of 15 mm. All images were recorded digitally (Orion, 6 E.L.I. sprl, Belgium) at a scan rate of 100 seconds (full frame) and a size of 2855 x 2154 (8 bits).

The apparent amylose content was determined according to the method described by Hovenkamp-Hermelink *et al*. (1989).

The onset temperature (Tonset) of starch granule melting were measured by differential scanning calorimetry (DSC), using Perkin-Elmer Pyris 6 (Perkin-Elmer, The Netherlands), as described by Nazarian *et al*. (2007a). The T_{onset} and the enthalpy released (ΔH) were calculated automatically.

Granule size distributions were measured with a Coulter Counter Multisizer III, equipped with an orifice tubes of 140 µm (Beckman-Coulter, UK). Approximately 10mg

of starch was suspended in about 160ml isotone solution and the granule size distribution was recorded by counting about 30,000 particles. Each sample was measured at least in triplicate.

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Chapter 6

General Discussion

Introduction

The modification of the structure or composition of native starches by regulating the expression level of genes involved in starch biosynthesis gives a promising potentiality for generating starches with specific properties to be used more efficiently and in a wider range of applications without chemical or physical post-harvest modification (Jobling *et al.*, 2002). In our laboratory, the *in planta* modification of potato starch granules by expressing different engineered enzymes has been extensively explored (Kok-Jacon *et al.*, 2003). The Starch Binding Domain (SBD) from *Bacillus circulans CGTase* has been used as an anchor for targeting different enzymes during starch biosynthesis successfully (Ji *et al.*, 2003). To produce starches with altered composition and novel structure such as altered amylose/amylopectin ratio and higher branching degree of amylopectin, two different microbial enzymes, the *GlgB* from *E. coli* and the *amylosucrase* from *Neisseria polysaccharea*, fused with the SBD were introduced into potato amyloplasts (This thesis Chapters 3 and 4). Furthermore, the effects of introducing a tandem CBM25 domain from *Microbacterium aurum* on starch granules was studied and the potentiality of using this tandem CBM25 domain as a novel tool for anchoring proteins into starch granules during starch biosynthesis was assessed (Chapter 5). The effects of the expression of these heterologous enzymes on starch granule morphology, the expression level of genes involved in starch biosynthesis pathway, on starch physico-chemical properties, and the future of starch modification *in planta* are discussed below.

Starch granule morphology is severely altered by the accumulation of fusion proteins

Several proteins involved in carbohydrate metabolism and degradation have been targeted to potato starch granules and resulted in severe changes in the starch morphology as described in this thesis (Chapters 3 and 4) and previous researches (Nazarian *et al.*, 2007 a, b, and c; Kok-Jacon *et al.*, 2005 a and b; Kok-Jacon *et al.*, 2007). Several kinds of starch granule morphological phenotypes such as amalgamated starch granules, rough

surface granules and porous granules, have been observed in different transformants expressing proteins fused with SBD in potato. Furthermore, these starch morphological phenotypes appear at different stages during potato tuber development suggesting that these heterologous enzymes interact with the α-glucan chains in different ways in the amyloplast during potato tuber development.

Although the pathway of starch metabolism has been extensively studied and a significant number of enzymes have been identified in the starch biosynthetic pathway, the variations in starch granule size, morphology, and structure among different botanic sources remain largely unknown (Ball and Morell 2003; Zeeman *et al.*, 2010). The above mentioned starch morphological alterations suggest that the starch granule formation in transformants was affected by the accumulation of different SBD fusion proteins in the starch granule assembling process or by interfering with the expression level of enzymes involved in the starch biosynthetic pathway. Another possible reason could be that the introduced heterologous enzymes competing with the enzymes involved in starch biosynthesis or altering the affinity of these enzymes for the starch during starch granule formation process. However, the transcriptomic analysis using the POCI array showed that the expression level of most of the genes involved in sucrose and starch metabolism pathway did not show any changes in the different transformant tubers (SA/SBD, GS/SBD and MAT/SBD) relatively to the untransformed controls, although the starch morphology was severely changed. We propose that the starch morphological alteration was most likely caused by the physical effects of the deposition of different fusion proteins in the starch assembling process rather than the interference with the expression level of genes involved in starch biosynthesis.

The starch granule morphology was altered in GlgB/SBD transformants (Chapter 3). The 3D structure of both the *E. coli.* GlgB and the *Neisseria polysaccharea* Amylosucrase is well characterized (Abad *et al.*, 2002; Skov *et al.*, 2001). It is well known that the Glycoside Hydrolase family 13 (GH13) are multi-domain proteins normally containing the main catalytic domain in the form of a parallel $(\beta/\alpha)_8$-barrel (domain A) and two other distinct domains (domain B and C) (Janeček *et al.*, 1997). As a member of GH13, the GlgB enzyme consists of three major domains, an amino-terminal domain which is a SBD belonging to CBM family 48, a carboxyl-terminal domain, and

the central $(\beta/\alpha)_8$-barrel catalytic domain (Abad *et al.*, 2002). Taken the structure of GlgB/SBD fusion protein into consideration, it contains at least three domains, the CBM48, the catalytic A domain, and the extra fused SBD, which can bind the α-glucans of amylopectin during starch biosynthesis.

In amylosucrase/SBD transformants, two different rough surface starch morphology phenotypes were observed in Kardal and *amf* potato respectively (Chapter 4). The study of the amylosucrase enzyme structure showed that besides the three common A, B, C domains in GH13, the amylosucrase contains two other domains including an N-terminal domain and a B′-domain (Skov *et al.*, 2001). The active site of amylosucrase is at the bottom of a pocket at the molecular surface rather than in the bottom of a large cleft of other enzymes in GH13. The B′-domain is suggested to be important for the polymer synthase activity of the enzyme and has an active site which could be used specifically for the malto-oligosaccharides/glucan polymer to get in and out of the active site pocket. With respect to the oligosaccharide binding sites, the amylosucrase contains three extra binding sites besides the active site A-domain suggesting that the amylosucrase enzyme has affinity for starch granules by itself and this was confirmed by the fact that expression of amylosucrase alone in amyloplasts also resulted in starch granule morphological alteration, though to a lower degree than that of amylosucrase/SBD transformants.

The SBD in GlgB/SBD and amylosucrase/SBD fusion proteins enhances the ability of these proteins to bind the α-glucan chains of amylopectin molecules, thereby, may facilitate the interaction of these enzymes with amylopectin chains. As was discussed in a previous study, the mutansucrase fused with SBD (GtfICAT/SBD) could interact with the lateral chains of amylopectin and lead to spongeous roughness at the granule surface (Nazarian *et al.*, 2007a). During starch granule biosynthesis, the SBD in GlgB/SBD and amylosucrase/SBD fusion protein binds at the termini of the amylopectin double helix chains and the GlgB and amylosucrase, which have more glucan binding sites, might interact with the lateral amylopecin chains. Since both enzymes have multiple binding sites this might give a larger impact on starch assembly resulting in the craters and grooves on the granule surface.

Fusion proteins cross-link different granule nucleation sites resulting in amalgamated starch granule clusters

The amalgamated starch granules which were observed in the MAT/SBD transformants are phenotypically similar to the starch morphological phenotype in multiple SBDs transformants observed in previous studies (Ji *et al.*, 2004a; Nazarian *et al.*, 2007a, b). Furthermore, the expression of MAT/SBD fusion protein did not affect the transcript level of genes directly involved in amylose and amylopectin biosynthesis. Interestingly, besides the grape-like cluster amalgamated starch granules, starch granules with a normal-looking but containing many small granules inside were also observed in MAT/SBD transformants. Furthermore, similar internally amalgamated granules were also detected in GlgB/SBD and Amylosucrase/SBD transformants. In potato tubers, it has been described that one amyloplast normally contains only one starch granule (Ohad *et al.*, 1971); however the mechanism of starch granule initiation is not yet fully understood. We suggest that one of the reasons for the formation of amalgamated starches in MAT/SBD transformants could be because of the physical interaction of the MAT/SBD fusion protein with α-glucan chains in the granule. Another reason could be that the expression of MAT/SBD fusion protein interferes with the signal of nucleation site formation resulting in the formation of more granules in one amyloplast. A better understanding of the starch granule initiation in the amyloplast would be very helpful to explain the amalgamated starch phenotype. With the fact that all these fusion proteins contain more than two domains which can interact with the α-glucan chains, they could cross-link different starch granules at the initial stage of granule formation as the multiple SBDs work during starch biosynthesis resulting in amalgamated and internally amalgamated granules (Nazarian *et al.*, 2007a).

Amylose plays an important role in starch granule morphology in the transformants

The presence or absence of amylose affects starch granule morphology phenotypes in transformants because starch granules showed different phenotypes depending on the potato genetic background. In our studies, two potato genetic backgrounds, an

amylose-containing (cultivar Kardal) and an amylose-free mutant (*amf*), were used. Amylose-free starch granules have similar morphology as wild type starches, but there is almost no amylose present in *amf* starch granules because of the loss of activity of GBSSI in comparison with wild type potato starches, which contain about 25% amylose (Kuipers *et al.*, 1994; Visser *et al.*, 1997). It is likely that the amylose plays an important role in the starch granule morphological alteration in GlgB/SBD and Amylosucrase/SBD transformants by the fact that different starch morphological phenotypes were observed when expressing the same construct in Kardal and *amf* backgrounds (Chapters 3 and 4). Interestingly, the starch morphological alteration which was caused by the expression of the same fusion protein in amylose-free background is more severe than that in wild type background. For instance, in the GlgB/SBD transformants, the starch granules with a lot of deep holes on the surface were observed in *amf* background, these holes were probably filled by amylose to some degree resulting in irregular starch granules with rough bumpy surface in Kardal background. Likewise, in the amylosucrase/SBD transformants, the rough surface granules in *amf* background are more severe than in Kardal background. We suppose that the amylose might work as 'cement' in wild type starch granules and make the phenotype less severe than in amylose free granules. To further investigate the mechanism of the amylose affecting the starch phenotype, a high amylose containing potato background should be used for expressing these fusion proteins.

The expression level of most genes directly involved in starch biosynthesis remain unaltered

The expression of fusion proteins does not affect the expression level of most genes directly involved in starch biosynthesis in our studies (Chapters 2, 3, and 4). There are at least four groups of genes that are required for normal starch biosynthesis which encode ADP-glucose pyrophosphorylases (AGPase), starch synthases (SSs), starch-branching enzymes (SBEs), and starch-debranching enzymes (DBEs) (Martin and Smith 1995; Morell and Myers, 2005; Zeeman *et al.*, 2010). Although the pathway of starch metabolism seems relatively straight forward, the significant number of enzymes and the different isoforms make it a complicated process. Moreover, the mechanism of how the

starch granule formation initiates and what controls the granule number, size, and morphology in plants is not understood (Zeeman *et al.*, 2010). In this study, the alterations of starch morphology were not accompanied with changes in transcript level of enzymes which are recognized so far as being directly involved in the starch biosynthetic pathway, although many genes involved in other biological processes were drastically affected.

In this study, the same construct in two different genetic backgrounds (Kardal and *amf*) showed great differences in the transcriptomic analysis by the fact that all these three transformants (GlgB/SBD, MAT/SBD, and Amylosucrase/SBD) in Kardal background have more differentially expressed genes compared to in *amf* background (Table 1). Moreover, more up-regulated genes than down-regulated genes showed up in all transformants except for the MAT/SBD in Kardal background. These differences can be partly explained by the fact that Kardal is a tetraploid variety and *amf* is a diploid mutant from different genetic backgrounds. Although different starch morphological phenotypes were apparent in these three different constructs in the same backgrounds, 8 differentially expressed genes were shared in the *amf* background and 25 in Kardal background in all these three transformants (Table 1). However, most of these shared genes do not have an annotation. Since there is very limited information about the genetic mechanism of controlling starch granule morphology so far and the change of the transcript level of these genes is accompanied with starch morphological alteration in this study, further studies on the function of these genes might be helpful to unveil how the accumulation of fusion proteins gives rise to the granule morphological alteration and the mechanism of controlling starch granule morphology.

Interestingly, the expression level of the glucose-6-phosphate/phosphate translocator 2 (GPT2) which transports glucose-6-phosphate into the amyloplast for starch biosynthesis was induced in KDSA transformant tubers which have bigger granules suggesting that the GPT2 might play a role in changes of starch granules size (Chapter 3). The manipulation of this gene's expression level could shed more light on how starch granule morphology is regulated during starch biosynthesis. Furthermore, many genes involved in the lipid metabolism pathway were down-regulated which is consistent with the observation that starches were acetylated at a low degree in the

MAT/SBD transformant in Kardal background (Nazarian *et al.*, 2007b and Chapter 2 in this thesis). Noteworthy, these differentially genes in lipid pathway did not show changes in *amf*MS transformant tubers in which no starch acetylation was found (Nazarian *et al.*, 2007b). These results suggests that the MAT could transfer acetyl groups to the starch granules while at the same time competing acetyl-CoA with enzymes in lipid biosynthesis pathway and suppressing lipid biosynthesis. Furthermore, the limited acetyl-CoA pool in potato amyloplasts could be the reason that only a low degree acetylated starches were obtained. For producing high acetylated starches, enhancing the supply of acetyl-CoA in the potato amyloplast is one option. Moreover, the expression of MAT in other plants which have higher lipid content in starch granules such as maize and wheat is worth to try.

Table 1. Number of differentially expressed genes in different transformants studied in this thesis.

Transformant	Up-regulated	Down-regulated	Number of differentially expressed genes
SA-KD	147	36	183
MS-KD	55	174	229
GS-KD	229	78	307
SA-*amf*	19	6	25
MS-*amf*	21	5	26
GS-*amf*	82	16	98
SA-KD / SA-*amf*			6
MS-KD / MS-*amf*			3
GS-KD / GS-*amf*			18
SA/MS/GS in KD			25
SA/MS/GS in *amf*			8

SA, MS, and GS reprent SBD-Amylosucrase, MAT-SBD, and GlgB-SBD respectively.
KD and *amf* mean Kardal and amylose-free background.

Starch physico-chemical properties

In this thesis the GlgB and the amylosucrase fused with the SBD were introduced into potato amyloplasts to produce starches with altered amylose/amylopectin ratio and higher braching degree of amylopectin. It is well known that the chemical composition of starch such as amylose, amylopectin, lipids, phosphate ester groups, play important roles in the physico-chemical properties of starch, but the functional properties of starches are

not simply determined by the chemical composition. Many other factors, for instance, the granule size (especially the uniformity of starch granule size), the branching degree of amylopectin, and the branch chain length, also play important roles in the determination of the characteristics of starch granules (Ellis *et al.*, 1998; Hoover, 2001; Singh *et al.*, 2007).

The SBD fusion proteins accumulated in starch granules during starch biosynthesis but did not show the same activity *in planta* as *in vitro* resulting in no changes of the branching degree of amylopectin in GlgB/SBD transformants and no changes of the amylose/amylopetin ratio in amyloscurase/SBD transformants. Many factors could have contributed to this result. Firstly, the activity of an enzyme is dependent on temperature, pH value, enzyme and substrate concentration, and other factors. As the conditions in the potato amyloplast are not known, it is not known whether this environment is optimal for these heterologous enzymes in potato amyloplast. Secondly, the SBD fusion enzyme can bind the α-glucan chains during the starch biosynthesis and might be trapped in the matrix of amylopectin and amylose in starch granules, hindering its enzyme activity. Thirdly, it is well known that the double helices structure of the amylopectin in the starch granules is organized in a highly ordered way. The α-glucan branch chains of the amylopectin are not distributed randomly suggesting there are enzymes such as isoamylases which are responsible for correcting the misplaced branches during starch granule formation *in planta* (Zeeman *et al.*, 2010). These enzymes could keep the starch granule formation process in a normal way and diminish the effect of introduced enzymes resulting in no apparent changes in starch structure (Streb *et al.*, 2008). However, all these possibilities need to be confirmed by further investigations. The clearer starch biosynthetic pathway and how heterologous enzymes would work in the amyloplast, the higher the possibility for producing novel starches by these enzymes.

In this thesis the starch granules in amylosucrase/SBD transformants did not show changes in amylose/amylopetin ratio, amylopectin chain length, and branching degree of amylopectin, but the size of starch granules increased which gave rise to altered starch viscosity behavior. However, the mechanism of how larger starch granules obtain the increased end viscosity is not clear.

CBM25 is a novel tool for anchoring proteins in starch granules

Nine CBM families have affinity for starch granules (Christiansen *et al.*, 2009), but they interact with α-glucans in different ways and have distinct affinities (Boraston *et al.*, 2006). The great advantage of using CBM25 for introducing proteins in starch granules is that CBM25 binds single α-glucan chain of both amylose and amylopectin during starch biosynthesis rather than interacting with the termini of the amylopectin helix chains like the CBM20 (Morris *et al.*, 2005; Boraston *et al.*, 2006). Therefore, the CBM25 can be present in both crystalline and non-crystalline regions of starch during starch biosynthesis which could bring effector proteins in all the regions of the starch granule to maximize the potential of the proteins activity. For instance, the CBM25 might bring the *E. coli* glycogen branching enzyme (GlgB) distributing in all the regions in the starch granules and might have a bigger possibility than GlgB fused with CBM20 which should attached at the termini of double helix chains of amylopectin for producing higher branching degree of amylopectin in starch granules. The other advantage of using CBM25 is that the expression of the tandem CBM25 does not lead to severe starch granules morphological alteration as the double SBD. This study gives a new option for using starch binding domains as a tool to anchor effector proteins into starch granules for starch modification *in planta*.

Previous studies showed that multiple CBM20s have a higher affinity for starch granules than a single CBM20 domain (Ji *et al.*, 2004b). Since the CBM25 domain only has one binding site to α-glucan chains (Boraston *et al.*, 2006), the multiple CBM25 domains might have higher affinity for starch granules as the multiple CBM20s did. It would be worth to investigate the incorporation level of multiple repeats of the CBM25 domain in starch granules to optimize the using efficiency of CBM25 as the tool for anchoring proteins in starch granules. Furthermore, it would be complementary to experiments already conducted with the CBM20 protein to introduce enzymes, which have specific functions on modifying starch structure during biosynthesis, into potato by fusing with the CBM25.

The future of starch modification *in planta*

Producing novel starches *in planta* to enhance or diversify its functionality in food and non-food industries by modifying starch biosynthesis has a tremendous potential for reducing or eliminating the need for post-harvest chemical, physical, and enzymatic modifications which are costly and environmentally unfriendly. Many efforts have been made to modify starch metabolism to produce novel starches such as, to increase starch quantity; to alter amylose/amylopectin ratio; to change chain lengths and branching patterns of amylopectin. However, starch biosynthesis is a very complicated process and has not been completely characterized by the fact that many different enzymes are involved and most of them have several different isoforms. Furthermore, starch granules are organized by different starch chemical components in specific ways, the organization and interaction of these various components within the granule play key roles in determining the starch structure, morphology, and physico-chemical properties. However, so far, the relationships between the functional properties of starches and the starch composition, structure and morphology are not well defined. In the last two decades, significant progress in the understanding of starch biosynthesis has been made. With the development of new techniques such as molecular biological techniques, the starch biosynthesis and the starch granule formation process can be understood in more detail. Moreover, with the advancement of exploring the relationship between the starch physico-chemical properties and the given starch structure and composition, it will be a more thorough understanding of the mechanism of the starch functionality.

In this study, a small number of genes which were differentially expressed in different transformants seem to be accompanied with the starch granule morphological alteration. Further studies on the function of these genes might give better insight on the genetic mechanism of starch morphology. Moreover, we have generated starch granules with large size in the Amylosucrase/SBD in Kardal transformants. The starch granules with large size might have advantages not only in the potato starch industry because of less starch losses during the starch isolation process, but also in other applications such as in diet food because of its higher end viscosity than normal sized granules. The specific applications of this novel starch need to be further investigated. Furthermore, the SBD of

Carbohydrate Binding Module family 25 as a novel tool for anchoring proteins into starch granules has been explored in this study. Compared to SBD of CBM20, the CBM25 also can deposit in starch granules and interact with glucan chains in starch in a different way. It has a great advantage to maximize the potential of starch modification *in planta* by anchoring proteins in all regions of the granules.

In the future, producing "tailored" starches for specific applications could be efficiently achieved by better understanding the mechanism of starch biosynthesis and the advancement of unraveling the association of the starch composition and structure with the physico-chemical properties of the starch.

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Summary

Starch is mainly composed of two polymers of glucose, amylose and amylopectin. Producing starches with altered composition, structure and novel physico-chemical properties *in planta* by manipulating the enzymes which are involved in starch metabolism or (over)expressing heterologous enzymes has huge advantages such as broadening the range of starch applications and reducing the costs of the post-harvest starch modification. The starch binding domain (SBD) technology has been extensively explored in our lab for modifying starch *in planta* and producing so-called "tailored starches". The *E. coli* maltose acetyltransferase (MAT) fused with SBD acetylates amylose-containing starch granules during starch biosynthesis and makes this a successful example of using SBD with other enzymes.

The aim of this thesis was to modulate starch *in planta* by expressing different bacterial proteins fused to an SBD from CBM family 20 in potato, to explore the possibility of using SBDs from other CBM families for applications in starch bioengineering, and to better understand how the expression of heterologous proteins interferes with starch biosynthesis. In this thesis, two different microbial enzymes, *E. coli* glycogen branching enzyme (GlgB) and *Neisseria polysaccharea* amylosucrase, were chosen and expressed in potato to modify starch composition or structure by fusing with an SBD. Furthermore, earlier produced MAT/SBD transformants were analyzed at the transcriptional level using the POCI array to investigate whether and how the expression of these heterologous genes affect the expression levels of genes involved in starch biosynthesis. Next to these experiments, a tandem CBM25 domain of amylase from *Microbacterium aurum* was introduced in potato to assess whether it is a suitable tool for applications in starch bioengineering.

Previous studies showed that amalgamated starch granules could be observed in transformants expressing an *E. coli* maltose acetyltransferase (MAT) fused with a starch binding domain (SBD). To elucidate when and how the deposition of MAT/SBD fusion protein interferes with starch biosynthesis, two representative transformants KDMS-30 and *amf*MS-15 which showed a high MAT/SBD protein accumulation level in starch granules were chosen for further studies (Chapter 2). The amalgamated starch granules

appeared in the MAT/SBD transformant tubers when the tuber had a diameter of more than 1cm. However, the expression of MAT/SBD fusion protein in Kardal and *amf* potato tubers did not affect the transcript level of other genes in the starch biosynthetic pathway. Interestingly, many genes involved in lipid metabolism were down-regulated in amylose-containing transformant (KDMS-30) tubers, but not in amylose-free (*amf*MS-15) tubers. This result was consistent with earlier findings that only in the wild type Kardal background (not in *amf* background) a low degree of acetylation was found. Further, it suggests that the expression of the MAT in wild type potato suppresses the endogenous acyltransferases in lipid metabolism pathway probably because of the competition for the substrate acetyl-CoA in potato amylosplasts. It might be possible to produce higher acetylated starch by enhancing the acetyl-CoA content in the amyloplast of MAT/SBD transformants or expressing MAT in other plants which have a natural higher lipid content in starch granules.

Branching degree of amylopectin plays an important role in the physico-chemical behavior of starch. The *E. coli* glycogen branching enzyme (*glgB*) was fused to either the C- or N-terminus of a starch binding domain (SBD) and was expressed in the amylose-free mutant (*amf*) and an amylose-containing (Kardal) potato genetic background in order to increase the branching degree of potato tuber starch (Chapter 3). A large amount of GlgB/SBD fusion protein was accumulated inside the starch granules regardless of background or construct used. The presence of GlgB/SBD fusion proteins did result in altered morphology of the starch granules in both genetic backgrounds, however an increase of branching degree was not observed. The starch granules showed both amalgamated granules and porous starch granules in the *amf* genetic background and showed an irregular rough surface in Kardal background. The altered starch granules in both *amf* and Kardal backgrounds were visible from the initial stage of potato tuber development. Furthermore, the expression of GlgB/SBD fusion protein in potato tubers did not affect the expression level of most genes directly involved in the starch biosynthesis except for the up-regulation of a beta-amylase gene hinting at the possibility that the alteration of starch granule morphology is unlikely caused by the activity of the GlgB enzyme but should be the result of the deposition of GlgB/SBD fusion protein in starch granules which interferes with the normal assembly of amylopectin during

amylopectin biosynthesis.

To modify the ratio of amylose and amylopectin ratio which plays a key role in starch physico-chemical properties, an amylosucrase from *Neisseria polysaccharea* fused to a starch binding domain (SBD) was introduced in two potato genetic backgrounds (Kardal and *amf*) (Chapter 4). Expression of SBD-amylosucrase fusion protein in the amylose-containing potato resulted in starch granules with a rough surface, with a 2-fold increase in median granule size, and altered physico-chemical properties including higher end viscosity and higher enzymatic digestibility. Furthermore, the expression of SBD-amylosucrase in potato tubers resulted in increased expression of a glucose-6-phosphate/phosphate translocator 2 (GPT2) gene which might play an important role in transporting more glucose-6-phosphate into amyloplasts and thus causing the bigger size granules. Moreover, the starches from Amylosucrase/SBD transformants in Kardal not only show bigger granule size, but also have a different viscosity behavior which can find specific applications in industrial uses of starch.

The SBD from CBM family 20 has been used as a tool for starch engineering in our lab. Besides the CBM20, there are eight CBM families that have affinity for starch granules. A tandem CBM25 domain of amylase from *Microbacterium aurum* was assessed whether it is suitable as a novel tool for starch bioengineering *in planta* (Chapter 5). Two tandem CBM25 domain (2CBM25) and the tandem CBM25 with multiple fibronectin type III (FN3) domains of the α-amylase were expressed in Kardal and *amf*. The starches of the different transformants did not show significant differences in starch size distribution, apparent amylose content, and physico-chemical properties in comparison with that of untransformed controls which suggests that the tandem CBM25 can be used as a novel tool for targeting proteins to granules during starch biosynthesis without causing side-effects.

The expressing of heterologous enzymes to modify starch *in planta* using the SBD technology was described in this thesis. The results presented in this thesis demonstrate that SBD can be successfully used as a tool to introduce functional enzymes into starch granules, whose expression often results in starch morphological alterations. The understanding of how the effects of enzyme activities *in vitro* can be observed in the amyloplast will be a step forward to the construction of tailor-made starches.

Samenvatting

Zetmeel in planten bestaat uit twee polymeren van glucose; amylose en amylopectine. Het in de plant kunnen produceren van zetmelen met veranderde eigenschappen, structuur of fysisch chemische eigenschappen heeft potentieel grote voordelen vanwege de bijna ongelimiteerde mogelijke nieuwe producten die gemaakt kunnen worden alsmede de kostenbesparende aspecten. Binnen Wageningen UR Plant Breeding is de zogenaamde zetmeelbindende domeinen technologie (Starch Binding Domain; SBD) ontwikkeld om in de plant zetmeel te kunnen modificeren teneinde speciale zetmelen te kunnen produceren. Het *E. coli* enzym maltose acetyltransferase (MAT) gefuseerd aan het SBD is in staat om aardappelzetmeel te acetyleren en is een succesvol voorbeeld van deze technologie.

Het doel van het onderzoek beschreven in dit proefschrift was om de zetmeeleigenschappen te beïnvloeden door verschillende SBD van de CBM 20 familie te koppelen aan enzymen afkomstig uit bacteriën met als doel uit te vinden of diverse SBD ook verschillend binden in de zetmeelkorrel; zowel wat betreft affiniteit als wat betreft positie in de zetmeelkorrel. Hiertoe werd onder andere een tandem SBD domein van het amylase van *Microbacterium aurum* in aardappel tot expressie gebracht.

Twee microbiële enzymen namelijk het uit *E. coli* afkomstige vertakkingenzym en het uit *Neisseria polysacchareae* afkomstige amylosucrase werden gekoppeld aan SBD in aardappel zetmeel tot expressie gebracht en hun effect op het zetmeel bestudeerd. De al eerder genoemde MAT getransformeerde aardappelen werden via de aardappel micro-array (POCI array) intensief bestudeerd op transcriptie niveau om te achterhalen wat de transformatie voor effect heeft op andere genen betrokken bij de zetmeel biosynthese en bij de aardappelknol vorming in algemene zin.

Uit eerdere studies was naar voren gekomen dat in transformanten waarin het *E. coli* maltose acetyltransferase gefuseerd aan SBD tot expressie kwam dit leidde tot samengestelde zetmeel korrels. Om te onderzoeken wanneer en of de fusie-eiwitten interfereerden met de zetmeel biosynthese werden twee representatieve transformanten (KDMS-30 en *amf*MS-15) met een hoog fusie-eiwit gehalte verder bestudeerd (Hoofdstuk 2). Uit dit onderzoek bleek dat de samengestelde zetmeelkorrels verschenen wanneer de aardappel knollen een diameter van meer dan 1 centimeter hadden bereikt.

De expressie van het fusie-eiwit in welke genetische achtergrond dan ook had geen effect op de expressie van andere genen coderend voor enzymen betrokken bij de zetmeelbiosynthese. Opmerkelijk genoeg vertoonden wel veel genen coderend voor enzymen betrokken bij de vetzuur biosynthese een lagere expressie in de amylose bevattende knollen maar niet in de amylose-vrije knollen. Deze resultaten kwamen overeen met eerdere resultaten die lieten zien dat in amylose bevattende knollen, maar niet in amylose-vrije knollen, een lage graad van acetylatie van het zetmeel was gemeten. Een mogelijke verklaring voor deze resultaten kan liggen in een onderdrukking van de aardappeleigen acetyltransferases als gevolg van competitie voor het substraat acetyl co-A. Een verhoging van de acetyl co-A concentratie in de amyloplasten zou kunnen leiden tot een hogere acetylatie graad van het zetmeel. Een alternatief zou kunnen zijn het transformeren van planten die van nature al een hogere vetzuur gehalte in hun zetmeel hebben zoals maïs of tarwe.

De vertakkingsgraad van amylopectine speelt een belangrijke rol bij het fysisch chemisch gedrag van zetmeel. Het *E. coli* vertakkingsenzym (*glgB*) gefuseerd aan het SBD werd tot expressie gebracht in zowel de amylose bevattende als de amylose-vrije aardappel met als doel de vertakkingsgraad van het zetmeel en meer specifiek het amylopectine te verhogen (Hoofdstuk 3). Alhoewel een grote hoeveelheid fusie-eiwit kon worden aangetoond in zowel amylose bevattend als amylose-vrij zetmeel had dit geen verhoging van de vertakkingsgraad tot gevolg. Wel bleek dat de zetmeelkorrel morfologie drastisch veranderd was als gevolg van de aanwezigheid van het fusie eiwit in beide genetische achtergronden. In de amylose-vrije zetmeel achtergrond bleken zowel samengestelde als poreuze zetmeelkorrels voor te komen. In de amylose bevattende achtergrond hadden de zetmeelkorrels een onregelmatige en ruwe structuur in plaats van hun normale ovale ronde vorm. Deze afwijkingen van de normale zetmeel korrel vorm waren al waarneembaar vanaf de meest vroege stadia van zetmeelkorrel vorming. Ook hier bleek de expressie van de fusie-eiwitten nauwelijks enige invloed te hebben op de expressie van andere genen betrokken bij de zetmeelbiosynthese met uitzondering van de hogere expressie van een beta amylase gen.

In Hoofdstuk 4 zijn experimenten beschreven waarbij een amylosucrase van de bacterie *Neisseria polysaccharea* gefuseerd aan SBD tot expressie werd gebracht met als

doel de amylose/amylopectine ratio te verhogen. Expressie van dit fusie eiwit gaf geen verhoging van de hoeveelheid amylose te zien. Wel bleek in de amylose bevattende genetische achtergrond de morfologie van de zetmeelkorrels veranderd. De zetmeelkorrels bleken een zeer ruw uiterlijk te hebben en de gemiddelde grootte bleek met een factor 2 te zijn toegenomen. Daarnaast bleken ook een aantal fysische chemische eigenschappen zoals vriesdooi stabiliteit, enzymatische afbreekbaarheid en de eindviscositeit van gels van dit zetmeel veranderd te zijn ten opzichte van het controle zetmeel. Op gen expressie niveau bleek dat de glucose-6-phosphate/phosphate translocator 2 (GPT2), een gen dat codeert voor een enzym dat belangrijk is voor de import van de hoeveelheid glucose-6-fosfaat in de amyloplasten een hogere expressie vertoonde wat mogelijk een verklaring kan zijn voor de grotere zetmeelkorrels.

Er zijn ca 8 enzymfamilies die zetmeelbindende domeinen bevatten en tot dusver had het onderzoek zich gericht op SBDs van de familie CBM20. In Hoofdstuk 5 werd een dubbele SBD van de CBM25 familie getest om te zien of deze wellicht andere regio's bind in de zetmeelkorrel en dus andere effecten zou kunnen veroorzaken. De verkregen transformanten lieten geen verschillen zien voor wat betreft morfologie, vorm of andere eigenschappen en zijn dus mogelijk een alternatief voor de CBM20 SBD's.

De expressie van verschillende fusie-eiwitten in aardappel zetmeel lieten zien dat de zetmeelvorm, morfologie en fysisch-chemische eigenschappen veranderd kunnen worden. De beschikbaarheid van dit soort transformanten zal onze kennis van zetmeelbiosynthese en mogelijkheden om zetmeel te veranderen volgens vooraf gedefinieerde parameters vergroten .

论文摘要

淀粉主要由直链淀粉和支链淀粉组成。通过在植物体内调控淀粉合成酶和/或表达异源酶来改变淀粉的成分和结构，从而在植物体内制造具有新的理化特性的淀粉具有巨大的经济和环境效益：它既可扩大淀粉的应用范围，也可以减少淀粉收获后的改性费用。我们实验室已经对应用淀粉结合域（Starch Binding Domain）技术在植物体内制造所谓的“人工设计淀粉”进行了深入的探索研究。其中一个例子就是成功在马铃薯体内表达通过 SBD 引导的大肠杆菌麦芽糖乙酰转移酶（Maltose acetyltransferase）制造乙酰化淀粉。

本论文研究目的如下：其一, 在马铃薯体内表达通过 SBD 引导的几个不同的酶制造新型淀粉；其次, 探索使用其它家族的 SBD 在淀粉改良领域的应用；再次, 揭示这些异源蛋白的表达对马铃薯内源淀粉合成通路的影响。本论文的具体研究内容包括，在马铃薯中表达通过 SBD 引导的大肠杆菌糖原分支酶（GlgB）和多糖奈瑟菌淀粉蔗糖酶（Amylosucrase）两个微生物酶来合成具有改变的结构和成分的淀粉；使用马铃薯基因芯片（POCI microarray）来对麦芽糖乙酰转移酶/SBD 融合蛋白(MAT/SBD)的马铃薯转化体的转录水平进行检测，从而揭示 MAT/SBD 融合蛋白的表达对马铃薯体内淀粉合成通路的影响；最后对应用另一个家族的淀粉结合域（一个串联重复的 CBM25）进行在马铃薯体内淀粉改性进行探索研究。

先前的研究表明在马铃薯体内表达 MAT/SBD 融合蛋白不仅可以收获到低程度的乙酰化淀粉，同时还可以引起淀粉颗粒聚合形成新的淀粉颗粒形态。为了阐明 MAT/SBD 融合蛋白的表达对马铃薯淀粉合成通路的影响机制，本研究选择了两个具有高 MAT/SBD 融合蛋白表达水平的转化体（KDMS-30 和 *amf*MS-15）进行进一步研究（第二章）。研究发现当 MAT/SBD 转化体的块茎直径超过 1cm 以上时, 聚合淀粉颗粒开始出现在转化块茎里。而且 MAT/SBD 融合蛋白在 Kardal 和 *amf* 马铃薯块茎里的表达并没有影响淀粉生物合成途径中的其他基因的转录水平。有趣的是，在含直链淀粉的转化块茎（KDMS-30）里许多参与脂质代谢基因的表达水平被抑制，但在无直链淀粉的转化块茎（*amf*MS-15）里没有相似的发现。这一发现与先前的研究结果相一致，先前的研究发现只在野生型 Kardal 转化体里发现有低程度乙酰化淀

粉。此外，它表明了在野生型马铃薯里表达 MAT 抑制脂质代谢途径里的内源性乙酰转移酶（acyltransferases）的表达水平，可能是因为在马铃薯造粉体（amylosplasts）里竞争底物乙酰辅酶 A 造成内源性乙酰转移酶的抑制表达。本研究表明,通过提高 MAT/SBD 转化体的造粉体内乙酰辅酶 A 含量或在有较高脂肪含量的天然淀粉颗粒的其他植物里表达 MAT/SBD 融合蛋白有可能制造出高乙酰化淀粉。

支链淀粉分支程度很大程度上影响淀粉的物理化学特性。为了提高马铃薯淀粉的支链淀粉分支度，大肠杆菌的糖原分支酶（*glgB*）分别结合在 SBD 的 C -端或 N -端，并在无直链淀粉的突变体（*amf*）和含直链淀粉的马铃薯（Kardal）里表达（第三章）。无论是 GlgB-SBD, 还是 SBD-GlgB, 在 *amf* 和 Kardal 的淀粉颗粒内都积累着大量的 GlgB/SBD 融合蛋白。虽然支链淀粉分支度在 *amf* 和 Kardal 中都没有明显增加, 但是 GlgB/SBD 融合蛋白的存在引起了淀粉颗粒形态的改变。在 *amf* 的转化体中淀粉颗粒显示出两种新的形态：聚合淀粉颗粒和表面多孔淀粉颗粒；在 Kardal 的转化体中淀粉颗粒具有不规则的粗糙表面。在 *amf* 和 Kardal 的转化体中新形态的淀粉颗粒开始出现于马铃薯块茎发育早期。此外，除了一个 β -淀粉酶的表达量增加外，GlgB/SBD 融合蛋白在马铃薯块茎中的表达并没有影响绝大部分直接在淀粉合成通路上的基因表达水平。这个结果显示淀粉颗粒形态的改变可能不是由 GlgB 酶的直接作用造成的，而可能是由于 GlgB/SBD 融合蛋白在淀粉颗粒中的沉积影响了正常的支链淀粉在淀粉颗粒中的分布状态造成的。

直链淀粉和支链淀粉的比例决定着淀粉的物理化学特性。为了调控直链淀粉和支链淀粉的比例，一个多糖奈瑟菌的淀粉蔗糖酶（Amylosucrase）与淀粉结合域（SBD）融合并转化到两个马铃薯品种中（Kardal和*amf*）（第四章）。SBD-amylosucrase融合蛋白在含直链淀粉的马铃薯中表达不仅导致马铃薯淀粉颗粒表面粗糙，颗粒大小比未转化的淀粉大2倍；而且转化的大颗粒淀粉具有改变的物理化学性质包括高的终粘度和高的酶解力。此外，SBD-amylosucrase在马铃薯块茎中的表达导致了一个基因（GPT2）表达量的增加。GPT2表达量的增加能够运送更多的葡萄糖-6-磷酸进入造粉体，从而形成大颗粒淀粉。这种新型的大颗粒淀粉可以在淀粉工业中具有特定的用途。

在我们实验室，CBM 20 家族中的 SBD 已被成功用来作为淀粉生物改性的工具。除了 CBM20 外，另外还有八个 CBM 家族具有淀粉颗粒的亲和力。本论文对一个从 *Microbacterium aurum* 菌中的淀粉酶中的串联重复 CBM25 域进行了探索研究，评估它是否可以作为一个在植物淀粉生物工程中的新工具（第五章）。在 Kardal 和 *amf* 马铃薯中表达了两个基因片段：一个串联的 CBM25 域（2CBM25）片段和一个α-淀粉酶的纤维连接蛋白Ⅲ型（FN3）与串联 CBM25 区域片段。所有的马铃薯转化体中的淀粉与未转化对照中的淀粉在淀粉的颗粒度分布，直链淀粉含量，淀粉的物理化学性质上都没有显著差异。该研究结果表明这个串联的 CBM25 可以作为一个新的工具用于在淀粉合成过程中锚定其它的酶在淀粉颗粒上，而且对淀粉合成没有任何副作用。

本论文描述了利用 SBD 技术来在植物体内表达外源酶，从而进行淀粉改性。本论文的研究结果表明 SBD 可以成功地用来作为一种锚定工具来在淀粉合成过程中引导其它有特定功能的酶进入淀粉颗粒。SBD 和其它酶的融合体在植物体中的表达往往引起淀粉颗粒的形态学改变。如何使转入造粉体内的酶保持其特定的功能活性是为在植物体内成功进行淀粉改性的一个重要环节。

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Thank you!

Xingfeng Huang

Wageningen, the Netherlands
October, 2010

About the author

Xingfeng Huang was born on September 21st 1978 in Hexian, Anhui Province, China. He obtained his BSc in Horticulture in 2000 at Anhui Agriculture University. He continued his study at the same university and received his MSc with major in Plant Genetics and Breeding in 2004. From 2004 to 2006, he worked as a researcher in the Academy of State Administration of Grain, Beijing, China. He started his PhD research at Wageningen UR - Plant Breeding in October 2006. This thesis presents the outcome of his PhD research from 2006 to 2010.

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Xingfeng Huang
Date: 29 November 2010
Group: Laboratory of Plant Breeding, Wageningen University

1) Start-up phase	date
► **First presentation of your project**	
Analysis of starch granule morphology at different stages of potato tuber development	Jul 2007
► **Writing or rewriting a project proposal**	
Biogenesis of potato starch graunules	Feb 2007
► **Writing a review or book chapter**	
Production of heterologous storage polysaccharides in potato plants, Annual Plant Reviews 41 (2011), 387-406	May 2008
► **MSc courses**	
► **Laboratory use of isotopes**	
Safe handling with radioactive materials and sources	May 13-15, 2008
Subtotal Start-up Phase	*15.0 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day	Sep 13,2007
EPS PhD student day	Feb 26, 2009
EPS PhD student day	Jun 01, 2010
► **EPS theme symposia**	
EPS theme symposia 3 "Metabolism and Adaptation", University of Amsterdam	Nov 10, 2006
EPS theme symposia 3 "Metabolism and Adaptation", University of Wageningen	Nov 06, 2007
EPS theme symposia 3 "Metabolism and Adaptation" University of Amsterdam	Feb 18, 2009
► **NWO Lunteren days and other National Platforms**	
ALW meeting Lunteren Experiment Plant Sciences	Apr 02-03, 2007
ALW meeting Lunteren Experiment Plant Sciences	Apr 07-08, 2008
ALW meeting Lunteren Experiment Plant Sciences	Apr 06-07, 2009
ALW meeting Lunteren Experiment Plant Sciences	Apr 19-20, 2010
► **Seminars (series), workshops and symposia**	
Plant Breeding seminar series	Nov 2006-Oct 2010
Plant Breeding Research Day(WICC, Wageningen)	Sep 27, 2007
Plant Breeding Research Day(WICC, Wageningen)	Jun 17, 2008
Genstat user day 2008	Jun 18, 2008
Flying Seminar "DNA demethylation in Arabidopsis" by Prof. Jian-Kang Zhu	Nov 03, 2008
Plant Breeding Research Day(WICC, Wageningen)	Mar 03, 2009
Symposium "Photosynthesis: from femto to Peta and from nano to Global"	Nov 05, 2009
► **Seminar plus**	
► **International symposia and congresses**	
Starch 2008 Structure & Functionality (Universtity of Nottingham)	Mar 17-19, 2008
3rd Intern. Conference on Plant Molecular Biology (ICPMB), Beijing, China	Sep 05-09, 2010
► **Presentations**	
Poster presentation at Starch 2008 Structure & Functionality (University of Nottingham)	Mar 17-19, 2008
Poster presentation at ALW meeting Lunteren	Apr 07-08 2008
Poster presentation at Plant Breeding Research Day	Mar 03, 2009
Oral presentation at ALW meeting Lunteren	Apr 20, 2010
Poster presentation at ICPMB 3rd	Sep 05-09, 2010
► **IAB interview**	Dec 05, 2008
► **Excursions**	
Visit McCain company	Sep 15, 2008
Subtotal Scientific Exposure	*15.2 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Gateway to Gateway Technology	Nov. 17-21, 2008
Systems Biology:"Statistical analysis of ~omics data"	Dec 08-11, 2008
Introduction to R	Oct 08-09, 2009
Bioinformatics - A User's Approach	Mar 15-19, 2010
► **Journal club**	
Biweekly literature discussion at plant breeding	2006-2010
► **Individual research training**	
Using AFM to image the internal structure of starch granules (IFR, UK)	Mar 20-Apr 04, 2008
Subtotal In-Depth Studies	*10.7 credits**

4) Personal development	date
► **Skill training courses**	
Academic Writing II	Mar-Jun 2010
Career Perspectives	Mar-Apr 2010
Techniques for Writing and Presenting a Scientific Paper	Apr 13-16, 2010
► **Organisation of PhD students day, course or conference**	
Plant Breeding Lab-trip 2008	Jun 26, 2008
Plant Breeding Growth, Development and Quality Group bi-weekly colloquium series	2008-2009
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*7.0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**47.9**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study*

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