

***In Planta***

Modification of Potato Starch Granule Biogenesis

by

Different Granule-Bound Fusion Proteins

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***In planta* modification of potato starch granule biogenesis  
by different granule-bound fusion proteins**

**Farhad Nazarian Firouzabadi**

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## Abstract

**S**tarch is composed of amylose and amylopectin and it is deposited in amyloplasts/choloroplasts as semi-crystalline granules. Many biosynthetic enzymes are involved in starch degradation and biosynthesis. Some microbial starch degrading enzymes have a Starch Binding Domain (SBD) which has affinity for the starch granules on its own. In our laboratory, expression of SBD alone or fused to other effector proteins has been demonstrated. In industry, starch is modified after harvesting by chemical, enzymatic or physical treatments. In this thesis, the *in planta* modification of starch composition, structure and consequently its properties are demonstrated. Expression of multiple repeat SBDs (SBD<sub>2</sub>-SBD<sub>5</sub>) resulted in starch granule cluster formation. However despite the high intrinsic affinity for starch granules, they could not displace GBSSI from the starch granules. Acetylation of starch at low degree of substitution was obtained when *E. coli* maltose acetyltransferase (MAT) was expressed in potato amyloplasts. Expression of truncated form of a mutansucrase from *Streptococcus downei* fused to SBD led to mutan production in transgenic potato plants. Moreover starches with irregular surfaces and porous appearance were obtained in amylose-containing (Kardal) and amylose-free (*amf*) potato genetic background, respectively. Punctured and irregular starch granules were obtained when fusion proteins of SBD fused to an *E. coli* branching enzyme were expressed in potato amyloplasts. All these different approaches led readily to altered phenotypes of starches but without large consequences for their physical properties.

**To my loving wife *Akram* and our lovely kids, *Fatemeh* and *Amir Hossein***  
**Voor mijn lieve vrouw *Akram* en geweldige Kinderen, *Fatemeh* and *Amir Hossein***

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# ***Chapter 1***

**General introduction  
and outline of thesis**



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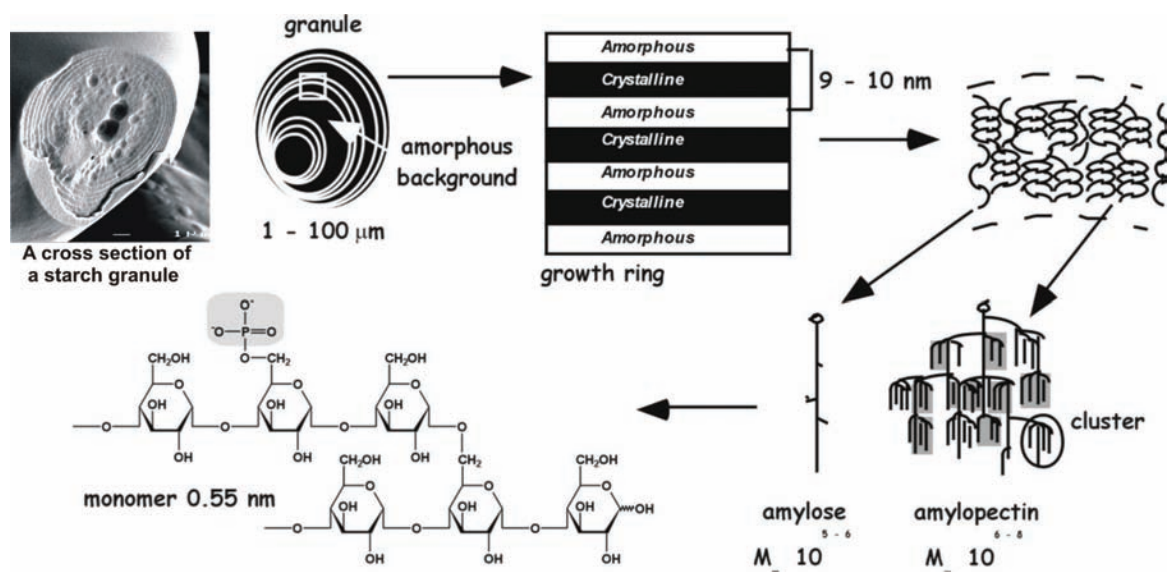
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Chemically, carbohydrate polymers such as starch and glycogen are polysaccharides which consist of several thousand monosaccharide units. In general, both polysaccharides have got glucose as their monosaccharide unit in common. They are synthesized by humans, animals, plants and microorganisms either to be metabolized to deliver energy or to serve as storage carbohydrates. Most plants store sugars in the form of starch which is unique among the different polysaccharides synthesized in nature because it occurs in discrete granules. Moreover, starch is not only an important human and animal food ingredient, it is also widely used as a raw material for many industrial applications such as glues, coatings, sizing, flocculating agents and building materials because it is abundant, renewable and safe. Starch can be found in cereal grains (wheat, rice, corn, oat and barley), roots (cassava and sweet potato), tubers (potato), fruits (banana), stems, leaves and even pollen grains.

### **Fine structure and starch granule organization**

Starch granules are composed of a mixture of two polymers, namely amylose and amylopectin and deposited as crystalline granules in chloroplasts of the green leaves (transitory starch) and storage organ amyloplasts (storage starch). Each starch granule consists of an amorphous and a semi-crystalline region (Fig. 1) (Myers *et al.*, 2000). The  $\alpha(1\rightarrow4)$  linked glucose residues, amylose makes up a fraction which can vary over a broad range among different species (Detherage *et al.*, 1955), from 11 to about 36%, but typically 20–25 % (w/w) of total potato starch is amylose (Orford *et al.*, 1987) and it is located in the amorphous part of the granules. Amylopectin having the same  $\alpha(1\rightarrow4)$  backbone but highly branched via  $\alpha(1\rightarrow6)$  linked glucosyl residues accounts for the rest of the starch and is located in the semi-crystalline part of the starch granules (Fig. 1). The  $\alpha(1\rightarrow6)$  linked glucosyl residues (branch points) in amylopectin are not randomly distributed, enabling this large molecule to be organized into clusters (Thompson, 2000). One of the interesting capabilities of amylopectin molecules is that despite their high molecular weight (average  $10^6$ – $10^8$ ), they can form double helices when they are packed together (Smith *et al.*, 1997; Ball *et al.*, 1998). This phenomenon brings about the starch semi-crystalline structure (Myers *et al.*, 2000). The ratio of these two polysaccharides influences the physical properties of starch such as viscosity, solubility, gel formation, gelatinisation temperature and brittleness of

formed films. There are also naturally occurring genotypes such as waxy mutants in cereal crops (maize, barley, wheat and rice) (Morrison *et al.*, 1984) and amf in potato (Hovenkamp-Hermelink *et al.*, 1987) containing little or no amylose at all. High-amylose mutants of maize and barley have also been found with up to 70% amylose (Morrison and Laignelet, 1983). Furthermore, there are other components such as lipids and phosphorus (Fig. 1) which are associated with starch granules.

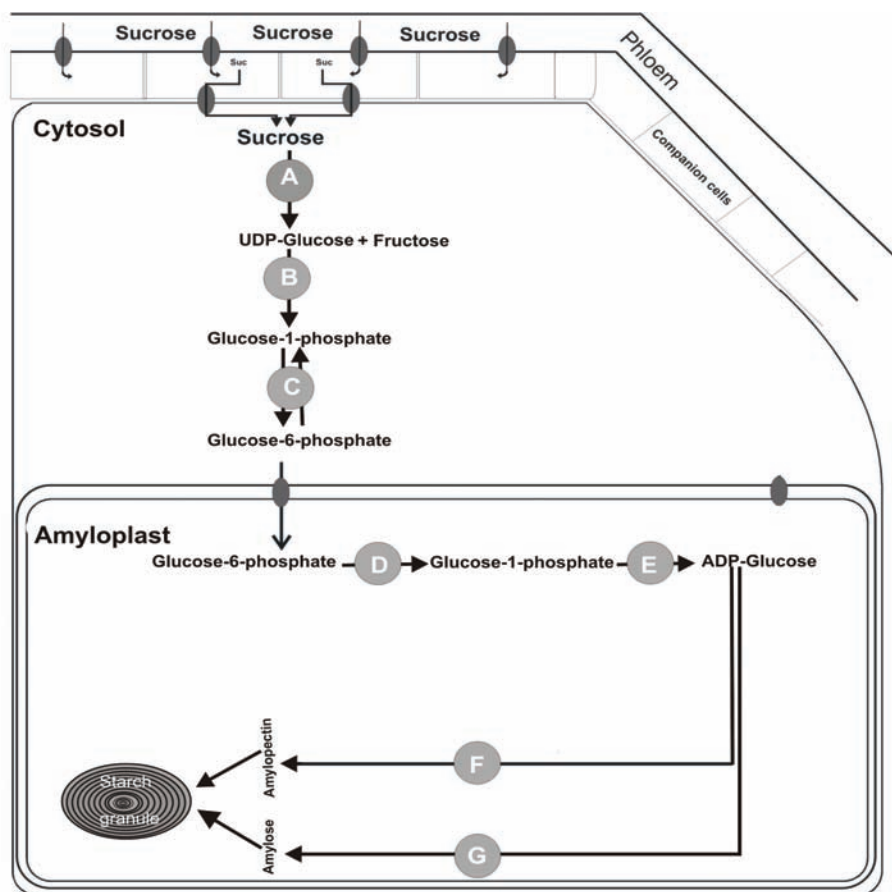


**Fig 1.** Schematic representation of the structure of a starch granule, with alternating amorphous and semi-crystalline regions constituting the growth rings. In potato starch one out of 200-300 glucose units of amylopectin is phosphorylated.

Depending on the source, the starch granule shape and size may vary widely. For instance potato starch granules are oval or spherical in shape and up to 100 μm in diameter (Ellis *et al.*, 1998) while taro starch granules are very tiny and about 0.5-2 μm. Most cereal starch granules have a bimodal granule size distribution, while native potato starch exhibits a normal granule size distribution.

**The pathway of starch biosynthesis**

Mutants in different starch biosynthetic enzymes have made it possible to allow the pathway of starch synthesis to be worked out in more detail. Many enzymes are involved in starch biosynthesis, among these enzymes some are directly involved in the process of amylose and amylopectin biosynthesis (Table 1).



**Fig 2.** Illustration of starch biosynthesis pathway in potato tubers.  represents putative transporters. Suc: Sucrose, A: Sucrose synthase, B: UGPase, C: Cytosolic phosphoglucomutase, D: Plastidic phosphoglucomutase, E: AGPase, F: Many enzymes such as Starch Synthases (SSI, SSII, and SSIII), Starch Branching Enzymes (SBEI and SBEII), Starch Debranching enzymes (SDEI, SDEII and SDEII), D-enzyme, etc and G: Granule-bound starch synthase I (GBSS I).

The synthesis of  $\alpha(1\rightarrow4)$  linked glucans (amylose and amylopectin) begins with sucrose conversion and consists of three major steps in the chloroplasts/amyloplasts of higher plants (Fig. 2) by which starch is being made. Briefly these three major steps are; (i) supply of Glc-6P into the plastids, the synthesis of ADP-glucose (ADP-Glc) from Glc-1P, and finally the synthesis of starch from ADP-Glc. At first, Glc-6P is transported to the amyloplast (Kammerer *et al.*, 1998) in order to be converted to ADP-Glc (Tauberger *et al.*, 2000; Muller-Rober *et al.*, 1992; Stark *et al.*, 1992).

**Table 1.** Summary of different genes involved in biosynthesis of potato tuber starch

| Enzyme                                           | place                             | gene   | Reaction catalyzed                                                                                                           |
|--------------------------------------------------|-----------------------------------|--------|------------------------------------------------------------------------------------------------------------------------------|
| Phosphoglucomutases<br>(EC 2.7.5.1)              | Cytosol and amyloplast            | Pgm    | Catalyzes Glc-6P ↔ Glc-1P                                                                                                    |
| ADP-glucose Pyrophosphorylase<br>(EC 2.7.7.27)   | Cytosol and amyloplast            | Agp    | Catalyzes formation of ADP-Glc from Glc-1P and ATP                                                                           |
| Granule-Bound Starch Synthase I<br>(EC 2.4.1.21) | Amyloplast (granule-bound)        | GbssI  | Synthesises amylose                                                                                                          |
| Granule-Bound Starch Synthase II<br>(EC 2.4.21)  | Amyloplast (mainly granule-bound) | GbssII | Synthesises amylopectin                                                                                                      |
| Soluble Starch Synthase I<br>(EC 2.4.1.21)       | Amyloplast (soluble fraction)     | SssI   | Synthesises amylopectin                                                                                                      |
| Soluble Starch Synthase II<br>(EC 2.4.1.21)      | Amyloplast (soluble fraction)     | SssII  | Synthesises amylopectin                                                                                                      |
| Soluble Starch Synthase III<br>(EC 2.4.1.21)     | Amyloplast (soluble fraction)     | SssIII | Synthesises amylopectin                                                                                                      |
| Starch-Branching Enzyme I<br>(EC 2.4.1.18)       | Amyloplast (partly granule bound) | SbeI   | Transfers a segment of a α(1,4) glucan chain to a primary hydroxyl group in a similar glucan chain                           |
| Starch-Branching Enzyme II<br>(EC 2.4.1.18)      | Amyloplast                        | SbeII  | Transfers a segment of a α(1,4) glucan chain to a primary hydroxyl group in a similar glucan chain                           |
| Disproportionating Enzyme<br>(EC 2.4.1.25)       | Amyloplast                        | Dpe    | Transfers a segment of a α(1,4) glucan chain to a new position in an acceptor, which may be glucose or another α(1,4) glucan |
| De-Branching Enzyme<br>(EC 3.2.1.41)             | Amyloplast                        | Dbe    | Hydrolyses the α(1,6)-glucosidic linkages in amylopectin                                                                     |
| α-Glucan Water dikinase<br>(EC 2.7.9.4)          | Amyloplast                        | Wdk    | Adds the β-phosphate group of ATP to either the C-3 or the C-6 of a glucosyl residue of amylopectin.                         |

The first committed and critical step in starch biosynthesis in potato amyloplasts involves the synthesis of ADP-Glc from Glc-1P and ATP, catalysed by ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27). The nucleotide sugar (ADP-Glc) serves as major substrate for different starch synthase isoforms (SS; EC 2.4.1.21). Starch synthase enzymes transfer glucose from ADP-Glc to the non-reducing end of an α(1→4) glucan resulting in the linear α(1→4) glucans. For instance in potato amyloplasts, the α(1→4) linked glucans, amylose, is synthesised by granule-bound starch synthase I (GBSSI; EC 2.4.1.21) (Kuipers, *et al.*, 1994). Following this, the linear α(1→4) glucans are used as substrates by starch branching enzymes (SBE; EC 2.4.1.18) to introduce α(1→6) linked side chains into linear α(1→4) glucan chains. Subsequently, amylopectin is crystallized into starch by the 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; Denyer *et al.*, 1997). Although the pathway of starch synthesis appears relatively simple, it is complicated by the fact that all the enzymes involved come in different isoforms, which differ in their behaviour and in the parts of a plant in which they are expressed.

### **Starch modification**

Since native starches do not have most of the desired characteristics demanded by the industry, they are often modified. To this end, starches from various sources undergo profound molecular, structural and enzymatic changes to suit various applications and provide a product to meet the specific needs of the end users (e.g. thickeners, binders, adhesives, gelling agents and texturisers; Table 2). To impart certain properties in starch, there are three main industrially available approaches. The most common approach is addition of chemical reagents to produce specific derivatives. Industry utilizes chemically modified starches for a large number of end uses. Such chemical reagents can be used to alter the arrangement of glucan chains in the starch granules (glucosidic bond cleavage through acid modification to dextrin) or to introduce additional chemical groups (carboxyl group formation through oxidation), or substitution of free hydroxyl groups (through etherification/esterification) or bridging of different chains (by cross linking reactions) to the native starch, making the starch more suitable for a particular industrial use. Another approach is the physical processing, such as heat and moisture treatment which can also alter the characteristics of the starch. The third method uses enzymes which biologically catalyses certain molecular reorganizations in the starch. All in all, the disadvantages of these methods are that they are time-consuming, expensive and in case of chemical derivatization often make use of hazardous chemicals. In addition, they may also generate undesirable and environmentally pollutant waste-products, which require disposal. There is nowadays a lot of interest in generating plants which produce so-called "designer" starches that are directly suitable for a particular industrial use. This would mean that the "raw" starch can be used immediately, without having to be modified industrially.

**Table 2.** An overview of some of different starch modifications

|   | Product               | Modification                       | Main objective                                                                 | Application(s)                                                                       |
|---|-----------------------|------------------------------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| 1 | Acetylated starch     | Acetic anhydride/<br>Vinyl acetate | Providing starches with less retrogradation and improved viscosity stabilities | Stabilizer, thickener, binder and biodegradable films                                |
| 2 | Anionic substitution  | Carboxymethyl-phosphate            | Freeze-thaw stability                                                          | Applicable in frozen food material                                                   |
| 3 | Cationic substitution | Quaternary ammonium                | Relatively lower gelatinization temperature                                    | Applicable in pigment retention for paper, sizing, coating and texture manufacturing |
| 4 | Hydroxypropylation    | Propylene oxide                    | Reduction of gelatinization temperature and increase in granule swelling       | Applicable in low temperature stable bakery products and processed food and candies  |
| 5 | Oxidation             | Hydrogen peroxide                  | Reduction of starch viscosity                                                  | Applicable in water resistancy and leather tanning                                   |
| 6 | Cross linking         | Phosphorus oxychloride             | Resistance to retrogradation                                                   | Applicable in soups, puddings and bakery products                                    |

Prospects for manipulating starch composition using modern molecular tools have given an encouragement to research on starch metabolism. Transgenic plants and starch mutants harbouring altered starch content, composition and morphology have been identified and/or produced in several plants such as barley, maize, pea, rice, oat, wheat and potato. It is now possible to tailor the characteristics of the native starch in planta (genetically) using manipulated genes. This will reduce the need for costly chemical or physical modifications and most important will get rid of the environmentally challenging effluents from such modifications. By inhibiting native genes (antisense or co-suppression approaches) involved in starch biosynthesis, the alteration of amylose/amylopectin ratio and modification of the degree of branching of amylopectin have been explored in potato. On the other hand adding characteristics is another very promising approach to modify starches. To this end, microbial enzymes are of great interest. By using these enzymes via molecular and genetic engineering methods, one may incorporate a gene of interest which encodes for a desired enzyme into amyloplasts. For instance, expression of *E. coli* branching enzyme (*glgB*) in tubers of amylose-free transgenic potatoes led to an increased branching degree of amylopectin (Kortstee *et al.*, 1996). Introduction of the *E. coli* glycogen synthase gene, *glgA*, into potato resulted in more amylopectin branch points as well as more amylose and starch yield penalty

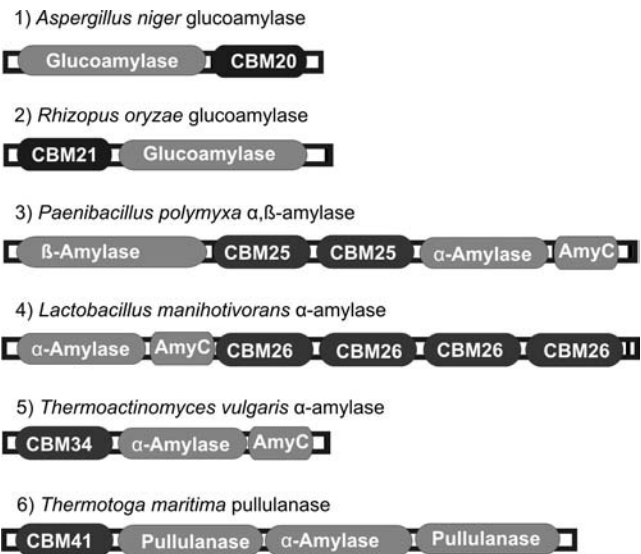
(Shewmaker *et al.*, 1994). Simultaneous antisense inhibition of two starch-synthase isoforms in potato tubers led to the accumulation of grossly modified amylopectin (Lloyd *et al.*, 1999). Transgenic plants with inhibition of starch branching enzyme by expressing antisense constructs containing cDNAs for potato SBE was reported by Safford *et al.* (1998). The suppression of SBE in potato (Jobling *et al.*, 1999; Schwall *et al.*, 2000) had severe effects on the starch granule morphology resulting in granules with irregular, elongated, multilobed or fissured shapes. Ji *et al.* (2003) were able to develop a technique called Starch Binding Domain (SBD) technology to anchor any protein of interest inside potato starch granules. Interestingly, expression of two copies of SBD (SBD2) in tandem in an amylose-free genetic background resulted in smaller starch granules (Ji *et al.*, 2004). Production of novel polymers (carbohydrates which are not normally produced in plants) having different linkages rather than  $\alpha$  (1 $\rightarrow$ 4) and  $\alpha$  (1 $\rightarrow$ 6) have also been successful in potato starch. For instance, Gerrits *et al.* (2001) introduced a *B. subtilis* levansucrase (an enzyme which uses sucrose as substrate and produces high molecular weight fructan, a polymer of fructosyl units) equipped with a chloroplastic targeting sequence into the potato tuber amyloplasts. Despite the starch yield penalty, production of fructan in potato amyloplasts was successful. Expression of a mature dextransucrase gene (*Dsrs*) from *Leuconostoc mesenteroides* led to production of dextran (polymers with mainly  $\alpha$ (1 $\rightarrow$ 6) linked glucosyl residues) in potato tubers. Although copolymerization of dextran was not evidenced with starch granules, starch granule phenotype in transformants was changed (Kok-Jacon *et al.*, 2005a). Alternan (a polymer with alternating  $\alpha$ (1 $\rightarrow$ 3)/ $\alpha$ (1 $\rightarrow$ 6) linked glucosyl residues) production was demonstrated when the alternansucrase gene (*Asr*) from *Leuconostoc mesenteroides* was expressed in potato amyloplasts. However, presence of alternan in potato tubers did not significantly change the morphology and physiochemical properties of such transgenic starches compared with starches from control plants (Kok-Jacon, 2005). Expression of full-length mutansucrase (*Gtfl*) from *Streptococcus downei* resulted in co-deposition of mutan (a polymer with  $\alpha$ (1 $\rightarrow$ 3) linked glucosyl residues) with starch granules (Kok-Jacon *et al.*, 2005c). A truncated form of the mutansucrase (*GtflCAT*) was also targeted to the potato amyloplasts. It was shown that mutan production with the truncated form of the enzyme was superior compared with that of the full-length enzyme, despite the fact that the truncated form retains 70% activity of the full-length counterpart. Mutan production in potato starch tubers by *GtflCAT* brought about profound morphological and physiological alterations at the tuber and starch granules levels. However, the presence of mutan inside the starch granules was not evidenced (Kok-Jacon *et al.*, 2005c).



The aforementioned examples show that biotechnology methods are able to tailor potato starch and have paved the way for starch manipulation *in planta*.

**Microbial Starch Binding Domain (SBD) and its biotechnological applications in starch modification**

Almost 10% of all amylolytic enzymes can bind to their substrate mediated by a distinct domain, the Carbohydrate Binding Module (CBM) (Janecek *et al.*, 2003; Machovic *et al.*, 2005). Based on their amino acid similarities, all CBMs have so far been classified into 47 different families ([http://afmb.cnrs-mrs.fr/CAZY/fam/acc\\_CBM.html](http://afmb.cnrs-mrs.fr/CAZY/fam/acc_CBM.html)). In the case of enzymes degrading starch, this domain is called Starch Binding Domain (SBD). Nevertheless, there are also some amylolytic enzymes such as barley  $\alpha$ -amylases which have no such functional domain (SBD) and are still capable of binding to the raw starch surface on their own (Hostinova *et al.*, 2003; Robert *et al.*, 2003; Bozonnet *et al.*, 2005). In the CAZy database and based on their sequence similarities SBDs have also been classified into six different families named, CBM20, CBM21, CBM25, CBM26, CBM34, CBM41 (Machovic *et al.*, 2005). They are about approximately 100 amino acids long and their amino acid sequences are very well conserved among different enzymes families (Svensson, *et al.*, 1989; Janecek and Sevcik, 1999). The domain organization of different enzymes comprising SBDs is diverse. For instance, SBDs can be found at C- or N- terminus of catalytic domains in single or multiple repeats (Fig. 3).



**Fig. 3.** An illustration of various starch-binding domains (SBDs) architecture. The six SBD families are represented as CBM20, CBM21, CBM25, CBM26, CBM34 and CBM41. It can be seen that SBDs are found at the N- or C-termini of the catalytic domains and are in single or multiple repeats.

As far as literature is concerned an enzyme with multiple SBDs at both C- and N-terminus has not been described yet. It has been well documented that SBDs play three different important roles; (i) they help the amylolytic enzymes to catch the substrate, (ii) they allow the interaction between insoluble substrate and the enzyme in the solution and (iii) they might scratch and disrupt the raw starch granule surface, facilitating the catalytic activity of the enzyme (Belshaw *et al.*, 1990; Sorimachi *et al.*, 1997; Southall *et al.*, 1999). It has been shown that enzymes that do not normally bind to starch granules can obtain affinity for the starch granules when they are fused to SBD (Dalmia *et al.* 1995; Ohdan *et al.* 2000). This offers opportunities for SBDs in starch bioengineering or so called “designer starches”. For instance, Ji *et al.* (2003) showed that any protein of interest can be anchored in potato starch during starch biosynthesis. They showed that SBD can be accumulated in starch granules either by itself or as a part of a SBD fusion protein. For Instance an active Luciferase-SBD fusion protein could be accumulated inside starch granules, whereas luciferase alone could not. These results obviously demonstrate that it is possible to target effector proteins (with no affinity for starch on their own) to the starch granule during starch biosynthesis. In other words, SBDs can be used as universal tools in order to alter starch structure and properties (Ji *et al.*, 2003). Based on results which were obtained by implication of SBDs, one might consider SBDs as potential tools to achieve certain applications such as *in planta* modification of starch, post-harvest starch modifications and production of various starch based products (Kok-Jacon *et al.*, 2003).

### Scope of this thesis

One of starch most important drawbacks which prevents some of its applications in industry is retrogradation. Retrogradation of starch pastes occurs by both intermolecular and intramolecular association of linear glucans, and to a lesser extent between amylopectin and amylose molecules. This phenomenon results in firmness, viscosity increase, opacity, turbidity and water syneresis (weeping) of starch based products. There are conditions that tend to prevent or inhibit retrogradation such as (i) lower amylose content (higher amylose/amylopectin ratio), (ii) higher number of amylopectin branching, and (iii) derivatization of the macromolecule, e.g. with acetyl, hydroxypropyl, cationic, phosphate, or

carboxyl groups. Therefore, this study is aiming at obtaining starches with different structures, properties and functionalities to be used by a wide range of end users through molecular approaches. To this end and to achieve *in planta* modification of starch, molecular biology has so far aided tremendously in the understanding of starch biosynthesis. Although it is now realistic to say that specific functions of starch biosynthetic enzymes in manufacturing of starch polymers can be assigned, one of the big questions is: how different enzymes alone or in combination control starch granule formation. Starch binding domain (SBD) technique has been developed in our laboratory (Ji *et al.*, 2003) by which the incorporation of foreign proteins in starch granules can be achieved during starch biosynthesis. The use of microbial SBDs is the key feature of this technology. Therefore, in this thesis a number of different microbial enzymes were chosen to confer certain characteristics to the starch granules. To facilitate and increase the impact of enzymes, they were fused to SBD either at the C- or N-terminus. In most cases the enzymes alone were also targeted to the potato tuber amyloplasts. We assume that the impact of particular enzymes on starch biosynthesis can be increased by fusing them to SBD, because in this way enzyme and substrate can be brought in closer proximity of each other. During this thesis, attempts to address a number of questions were made: (i) can multiple repeat SBDs challenge native granule-bound starch synthase I (GBSSI)?; (ii) is it possible to achieve *in planta* starch acetylation?; (iii) is it possible to promote the so called acceptor reaction of GtfiCAT and therefore co-polymerization of mutan with starch?; (iv) would it be possible to modulate the amylopectin branch points?

Amylose is an important component of starch. Presence/absence or a change in the ratio of this fraction determines some of the most important starch properties. Chapter 2 describes the attempts to out compete granule-bound starch synthase I (GBSSI), the amylose synthesizing enzyme from the starch granules during starch biosynthesis. To this end, an array of high-affinity multiple repeat family 20 starch binding domain constructs were employed. The influence of the SBDs on starch structure, functionalities and the interaction of SBDs with starch polymers are demonstrated. Acetylation of starch is an ongoing process in starch industry. Every year thousands of tons of starch are chemically acetylated. Acetylated starch has less retrogradation problems and therefore it is used in a variety of different applications. Chapter 3 of this thesis describes attempts to acetylate potato tuber starch by expressing an *E. coli* maltose-acetyltransferase (MAT) enzyme in potato tubers. Starch acetylation at low degree of substitution (DS), retention of MAT activity, its affinity towards starch granules as well as starch phenotype alterations are shown.

Introduction of novel polysaccharides in potato starch rather than amylose and amylopectin might broaden polymeric carbohydrate applications. Although we have shown in our lab the expression of different glucansucrases, in most cases the amount of final product in potato tubers was low. Chapter 4 of this thesis discusses production of mutan by expressing a catalytic domain of a mutansucrase from *Streptococcus downei* fused to SBD. The experiments described in this chapter were designed to show that it might be possible to increase the yield of mutan produced in potato tubers if substrate and acceptor molecules are brought together by SBD. In this chapter, mutan production, starch granule morphology alterations and effects of fusion proteins on starch properties in potato tuber starch are discussed with respect to the potato genetic background. Alteration of amylopectin branch points has been a challenging task of many labs worldwide because many starch properties depend on the amylopectin branching pattern. In chapter 5, introduction of *E. coli* branching enzyme (glgB) in potato tubers when it is fused to SBD, is investigated. In this chapter, it is investigated whether these fusion proteins are able to change the branching pattern of the starch polymers. Presence of glgB fused to SBD and affinity of glgB on its own for the starch granules were documented. Finally chapter 6 of this thesis tries to discuss all the results from the different chapters with respect to starch modifications. In this chapter the impact of each set of experiments either alone or together on starch structure and properties are discussed. Furthermore, future studies, drawbacks and opportunities are mentioned.

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# Chapter 2

## **Multiple-repeat starch-binding domains (SBD2-SBD5) does not reduce amylose content of potato starch granules**

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## Abstract

**T**his study investigates whether it is possible to produce an amylose-free potato starch by displacing the amylose enzyme, granule-bound starch synthase I (GBSSI), from the starch granule by engineered, high-affinity, multiple-repeat family 20 starch-binding domains (SBD2, SBD3, SBD4, and SBD5). The constructs were introduced in the amylose-containing potato cultivar (cv. Kardal), and the starches of the resulting transformants were compared with those of SBD2-expressing amylose-free (amf) potato clones. It is shown that a correctly sized protein accumulated in the starch granules of the various transformants. The amount of SBD accumulated in starch increased progressively from SBD to SBD3; however, it seemed as if less SBD4 and SBD5 was accumulated. A reduction in amylose content was not achieved in any of the transformants. However, it is shown that SBDn expression can affect physical processes underlying granule assembly, in both genetic potato backgrounds, without altering the primary structure of the constituent starch polymers and the granule melting temperature. Granule size distribution of the starches obtained from transgenic Kardal plants were similar to those from untransformed controls, irrespective of the amount of SBDn accumulated. In the amf background, granule size is severely affected. In both the Kardal and amf background, apparently normal oval-shaped starch granules were composed of multiple smaller ones, as evidenced from the many “Maltese crosses” within these granules. The results are discussed in terms of different binding modes of SBD.

**Key words:** Amylose, Granule-bound starch synthase, Starch-binding domain, Starch biosynthesis, Transgenic potato plant

Potato starch granules are composed of two polysaccharides, the more or less unbranched amylose (approximately 20%) and the highly branched amylopectin (approximately 80%) (Kossmann and Lloyd 2000). From an industrial viewpoint, the presence or absence of amylose is important, because it greatly determines the suitability of starch for different applications (Visser *et al.*, 1997a; 1997b). Properties as retrogradation of starch pastes (Visser *et al.*, 1997b), poor transparency of starch gels and low adhesiveness (Visser *et al.*, 1997a), poor freeze-thaw stability (Jobling *et al.*, 2002), and high granule melting temperatures (Schwall *et al.*, 2000) are all correlated with a high (apparent) amylose content of potato starch. Therefore, the *in planta* modulation of the amount of amylose of potato starch granules has been an important objective for the starch industry.

Various transgenic approaches have been explored for modifying the amylose content of potato starch. Granules with a very high apparent amylose content (~70%) were obtained by the simultaneous antisense inhibition of both potato starch-branching enzyme isoforms (Schwall *et al.*, 2000). An essentially amylose-free starch could be obtained by down-regulation of the activity of granule-bound starch synthase I (GBSSI), the amylose enzyme (Kuipers *et al.*, 1994), demonstrating that GBSSI is the only synthase involved in amylose synthesis. Interestingly, the amount of amylose of potato starch granules could also be reduced (to ~13%) by decreasing the ADP-glucose pool size (antisense AGPase; Lloyd *et al.*, 1999b). The fact that GBSSI has a lower affinity (higher  $K_m$ ) for ADP-glucose in comparison with (at least some) other starch synthases may explain this observation (Edwards *et al.*, 1999a). Thus, it seems as if GBSSI is the first synthase to suffer from a low ADP-Glc concentration, leading to the production of less amylose.

In a previous paper we have shown that microbial starch-binding domains (SBDs) can be accumulated in starch granules during the starch biosynthesis process, without affecting the amount of amylose (Ji *et al.*, 2003; Kok-Jacon *et al.*, 2003). It was also shown that more SBD could be incorporated in the granules of the amylose-free (*amf*) potato mutant. One explanation for that observation could be that GBSSI and SBD bind similar sites in the granule, and that GBSSI has the highest affinity for starch of the two proteins. Therefore, we engineered a high-affinity starch-binding domain by fusing two similar SBDs via a Pro-Thr rich linker peptide (SBD2). It was shown that SBD2 had an approximately ten-fold higher affinity for starch, and that much higher amounts of this protein could be accumulated in *amf* starch granules than of SBD (Ji *et al.*, 2004). Moreover, expression of SBD2 in the *amf* potato background resulted in an approximately two-fold reduction of the starch granule size. In

contrast, others have shown that expression of a tandem SBD in *Arabidopsis* increased starch granule size, particularly in the *sex 1-1* mutant, which has a mutation in the R1 gene involved in the phosphorylation of starch (Howitt *et al.*, 2006). In this study we investigated whether it is possible to obtain an amylose-free starch by introducing SBD2 in a wild-type potato background. Additionally, we have engineered a SBD3, SBD4, and SBD5, consisting of three, four, and five SBD repeats, respectively, separated by Pro-Thr rich linkers. Our hypothesis was that multiple appended SBDs might be strong enough to displace GBSSI from the starch granule.

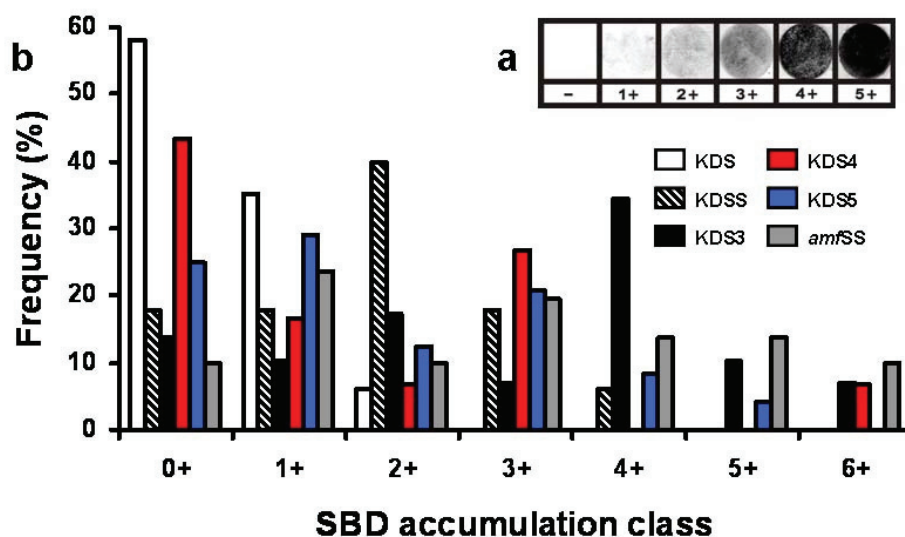
## Results

### Characterization of SBDn transformants

SBD2 was introduced into the KD background, yielding the KDSS#xx transformants, where xx represents the clone number. Untransformed control plants are referred to as KD-UT. Plant and tuber morphology, and tuber yield of all transgenic plants were comparable to that of control plants (data not shown).

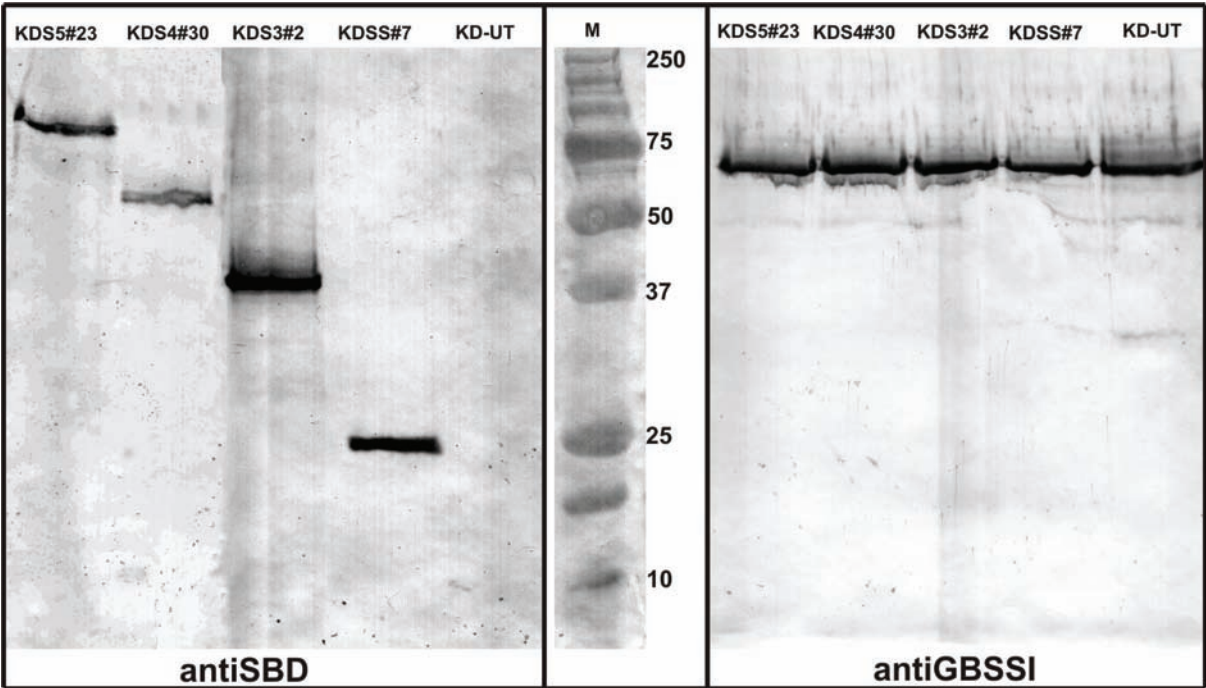
The levels of SBD2 protein accumulation in transgenic granules of the KDSS series were investigated by Western dot blot analysis. The SBD2 accumulating lines were divided into 6 classes (ranging from 0+ to 5+; see Fig. 1a) based on the amount of SBD2 protein associated with the starch granules, similar to the class-definition for the *amf*SS series described previously (Ji *et al.*, 2004). The SBD2 accumulation in the KDSS series is summarized in Figure 1b. For comparison, single SBD accumulation in the KDS series (Ji *et al.*, 2003) and SBD2 accumulation in the *amf*SS series (Ji *et al.*, 2004) are also indicated in Figure 1b. It can be seen that larger amounts of SBD2 than SBD can be accumulated in the WT background. This is consistent with our previous observations in the *amf* background (Ji *et al.*, 2004). Further, it is clear that much higher levels of SBD2 can be accumulated in *amf* granules than in amylose-containing ones, which is also in accordance with earlier observations for single SBD (Ji *et al.*, 2003).

SBD3, SBD4, and SBD5 were introduced into the KD background, yielding the KDS3#xx, KDS4#xx, and KDS5#xx transformants, respectively. Also for the plants of these series, plant and tuber morphology, and tuber yield were not consistently different from that of control plants.



**Fig. 1a,b** Accumulation levels of SBD in potato starch granules isolated from the various series of transformants of the Kardal background (KDSS, KDS3, KDS4, and KDS5 series). **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. The 6+ class 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 the Kardal background. SBD accumulation in the Kardal background (KDS series) and SBD2 accumulation in the *amf* background (*amfSS* series) are indicated for comparison.

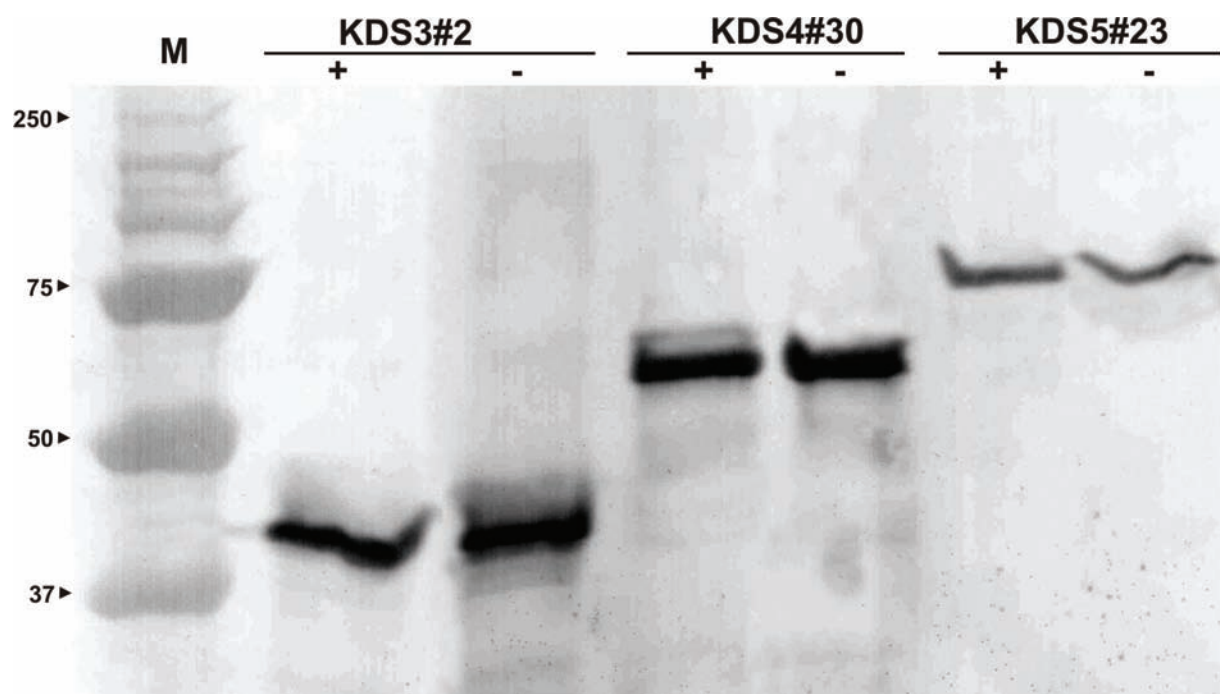
The granules of the KDS3, KDS4 and KDS5 series were subjected to Western dot blot analysis to determine the amount of SBD accumulated (Fig. 1b). We hereby assume that the appended SBDs act as individual epitopes for the antibody, i.e. a protein consisting of 5 appended SBDs binds 5 times more antibody than a protein consisting of one SBD. It can be seen that more SBD3 than SBD2 can be accumulated in the starch granules, consistent with our expectation that SBD3 is a higher-affinity starch-binding protein than SBD2. Many transformants with granules belonging to the 4+ to 6+ class were obtained with the KDS3 series, similar to the *amfSS* series. Our data do not indicate that SBD4 and SBD5 have a higher affinity for starch than SBD3, because only very few transformants of the KDS4 and KDS5 series had starch belonging to the higher SBD accumulation classes. SBD2, SBD3, SBD4, and SBD5 are correctly processed, as judged from the SDS polyacrylamide gel electrophoretic (SDS-PAGE) analysis combined with Western blot analysis using antiSBD antibodies (Fig. 2a, left panel). Starch from the best KDSS transformant showed a band of approximately 26 kDa, whereas that of the best KDS3, KDS4, and KDS5 transformants had a single band of approximately 40 kDa, 55 kDa, and 75 kDa, respectively; this corresponded well to the predicted molecular masses of 25,371 Da, 39,315 Da, 53,157 Da, and 66,999 Da for SBD2, SBD3, SBD4, and SBD5, respectively.



**Fig. 2** Western blot analysis of the highest expresser of each multiple repeat SBD series. The untransformed control (KD-UT) is shown for comparison. After SDS polyacryl amide gel electrophoresis, the proteins were blotted onto a membrane, and probed with anti-SBD (left panel) and anti-GBSSI (right panel) antibodies. The molecular mass marker (M) is indicated as well, with the protein masses in kDa.

The location (inside the granule or at its surface) of SBD-containing proteins was determined by treating the starch of the best transformant of each series with trypsin, and comparing the amount of SBD associated with the granules, with and without treatment, by SDS-PAGE/Western blot analysis. No differences were observed between the treated and untreated samples (Fig. 3), demonstrating that the various SBD-containing proteins are mainly inside the starch granule, and that they do not (re)deposit on the granular surface during starch isolation. From previous research it is known that trypsin can degrade the SBD protein (Ji *et al.*, 2003). The activity of the protease was verified by treatment of bovine serum albumine with trypsin, followed by SDS-PAGE analysis of the protein digest (data not shown).





**Fig. 3** Western blot analysis of the highest expresser of each multiple repeat SBD series, with and without trypsin treatment. Lanes marked + and – represent the granule-bound proteins in starch granules with and without trypsin treatment, respectively. Similar intensity of the bands in the + and – lanes indicates that the SBDn proteins are not accessible for the protease and are incorporated in the granule; a different intensity indicates that the SBDn protein is present at the surface of the granules. The molecular mass marker (M) is indicated as well, with the protein masses in kDa.

**Table 1** Accumulation levels of SBDn protein in juices of selected (transgenic) potato tubers of the KDSS, KDS3, KDS4, and KDS5 series. The amount of SBD2 in selected juices of the *amfSS* series are indicated for comparison. The number representation (1+, etc.) is according to the dot intensities in Fig. 1a

| Amount of SBD<br>in granules | Amount of SBD in potato juice |             |             |             |              |
|------------------------------|-------------------------------|-------------|-------------|-------------|--------------|
|                              | <i>KDSS</i>                   | <i>KDS3</i> | <i>KDS4</i> | <i>KDS5</i> | <i>amfSS</i> |
| 0+                           | traces                        | traces      | n.d.        | n.d.        | n.d.         |
| 1+                           | 1+                            | traces      | n.d.        | n.d.        | n.d.         |
| 2+                           | 1+                            | traces      | n.d.        | 1+          | n.d.         |
| 3+                           | 2+                            | traces      | traces      | 1+          | n.d.         |
| 4+                           | 2+                            | traces      | n.a.        | 1+          | n.d.         |
| 5+                           | n.a. <sup>b</sup>             | traces      | n.a.        | 1+          | n.d.         |
| 6+                           | n.a.                          | traces      | traces      | n.a.        | 2+           |

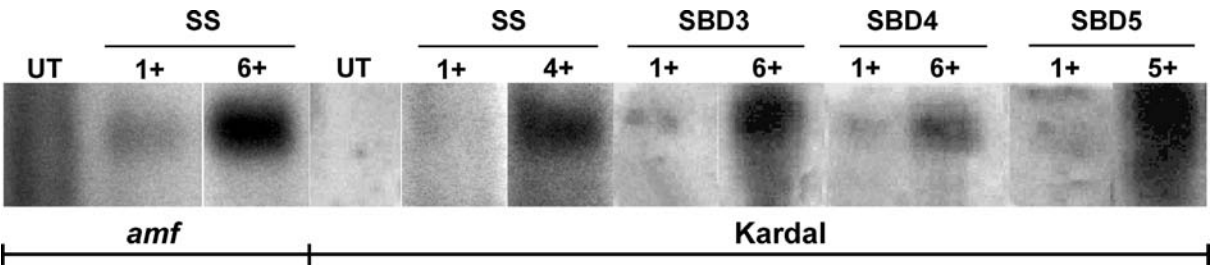
<sup>a</sup> not detected.

<sup>b</sup> not applicable.

The amount of SBDn protein was also determined (Western dot blot analysis) in the potato juice of one representative of each class from the various series. The results are summarized in Table 1. For comparison, the SBD2 concentration in the soluble fraction of representative *amfSS* potato tubers of each class (Ji *et al.*, 2004) are also indicated. The



amount of SBD2 found in class 1+ and 2+ KDSS tuber juice corresponded to the dot with an intensity of 1+ (see Fig. 1a), and that in class 3+ and 4+ KDSS tuber juice to 2+. Only trace amounts of SBD were present in the juices derived from the tubers of the various KDS3, KDS4 transformants. Typically, in the *amf*SS series, SBD2 can only be detected in the tuber juice derived of the 6+ class transformants, which suggests that the interaction of SBD2 with amylose-containing starch is of a different kind as with *amf* starch. Based on the Western dot blot results, one transgenic clone of a low (1+) and one of the highest accumulation class of the *amf*SS, KDSS, KDS3, KDS4 and KDS5 series were selected for Northern blot analysis, together with their respective controls (Fig. 4). The transcript levels of these transformants correlated well with the results obtained by the Western dot blot analysis, *i.e.* the transformants with starch granules of the 1+ class had low amounts of the transcript, whereas those with starch granules of the higher classes had much higher amounts. SBD transcripts were absent in the untransformed controls.



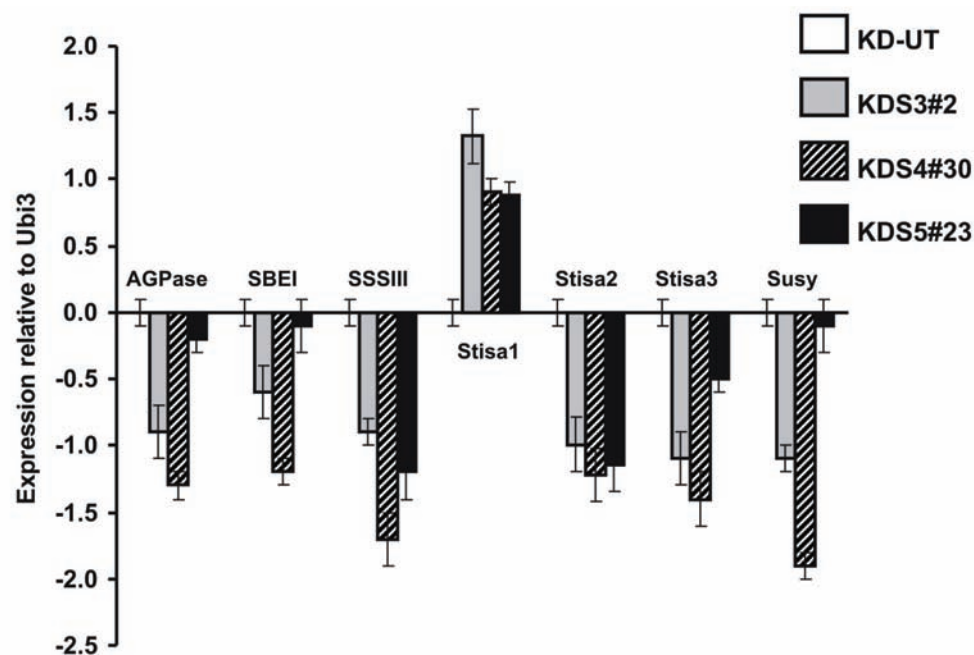
**Fig. 4** Northern blot analysis of high and low expressers of different multiple SBDs, together with their respective controls. Each lane contained 40 µg of total RNA

**Does expression of SBDn influence expression of key genes involved in starch biosynthesis?**

In order to assess whether expression of multiple appended SBDs influenced the gene expression pattern of key genes in starch biosynthesis, quantitative real time RT-PCR was performed with the most severe transformants from each series. No significant differences in the expression level of AGPase SBEI, Susy, SSSIII, Stisa1, Stisa2, and Stisa3 were found in comparison to that of control plants (Fig. 5), demonstrating that the expression of the various SBD-containing constructs does not affect the expression of starch biosynthetic genes.

Amylose content of starch granules of SBDn transformants

The starch granules of each transformed clone of the various series of transformants were stained with a Lugol solution, and subsequently the granules were investigated for the presence of amylose by light microscopy. No decrease in the amount of amylose was apparent in the granules from any of the clones (data not shown). In order to detect smaller differences in amylose content, also a colorimetric amylose determination was performed with starch samples of each SBD accumulation class of the various series of transformants. In all samples, an amylose content (approximately 20%) similar to the untransformed control was found. These data demonstrate that neither the introduction of SBD2 nor that of multiple-repeated SBDs (SBD3, SBD4, SBD5) in an amylose-containing background could reduce the amylose content.



**Fig. 5** Real-time quantitative RT-PCR analysis of RNA from the highest expresser of each multiple repeat SBD series and KD-UT. Primers specific for important genes in potato starch biosynthesis were used: ADP-glucose pyrophosphorylase (AGPase), starch branching enzyme I (SBEI), soluble starch synthase III (SSSIII), three isoforms of isoamylase (Stisa1, Stisa2, and Stisa3), and sucrose synthase (Susy). RNA levels for each gene were expressed relative to the amount of *Ubi3* RNA.

GBSSI content of starch granules of SBDn transformants

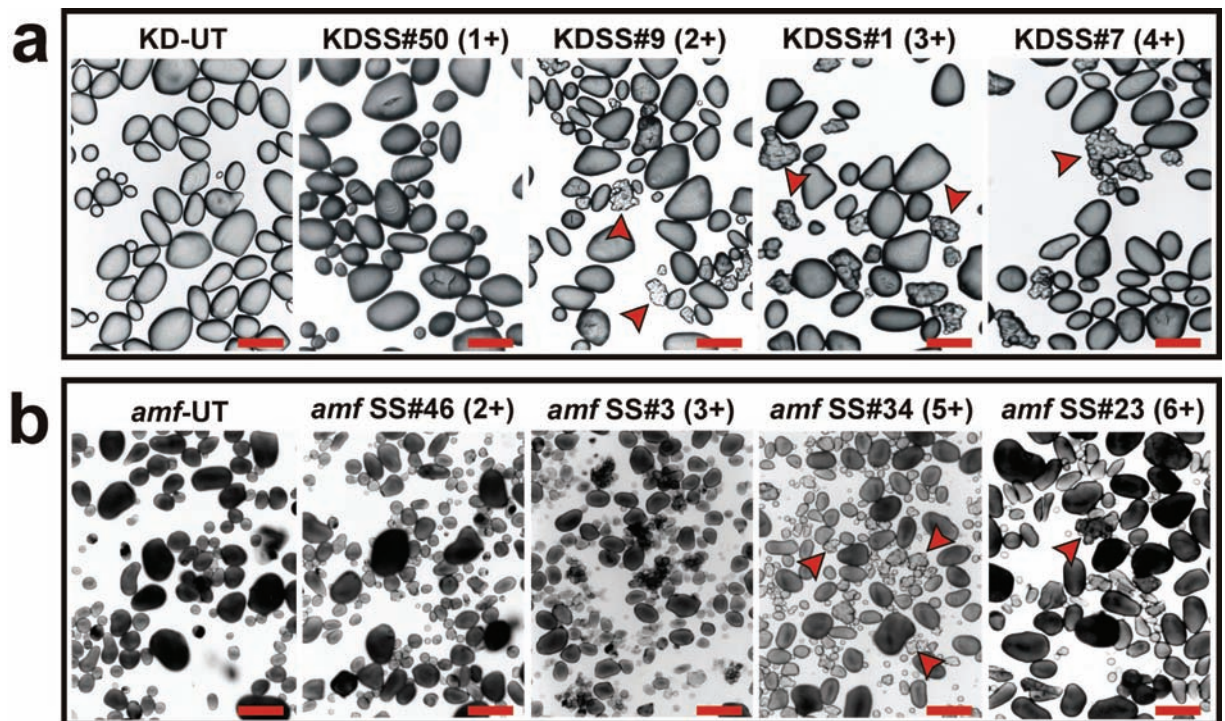
It has been shown previously that the GBSSI protein is present in surplus in potato starch granules, and that a reduction in the GBSSI content of the granules does not necessarily

correlate with less amylose (Flipse *et al.*, 1996). Therefore, we also determined the GBSSI content of the starch granules of the best KDSS, KDS3, KDS4, and KDS5 transformants by SDS polyacrylamide gel electrophoresis, followed by Western blotting using antiGBSSI. The analysis was repeated 3 times. No consistent differences in the amount of GBSSI were found in comparison to starch from KD-UT, with any of the transgenic starch samples (Fig. 2, right panel). These results suggest that all the multiple-repeat SBDs are unable to displace GBSSI from the starch granule during the biosynthesis process, or that they bind at a different location within the starch granule.

### Starch granule morphology

Starch granule morphology of each transformed clone from the KDSS, KDS3, KDS4, KDS5 and *amf*SS series were investigated by light microscopy. The selected micrographs of various KDSS and *amf*SS starch granules and their corresponding controls are shown in Figure 6. It can be seen that KD-UT and KDSS#50 (1+) starch granules are phenotypically similar, except for the presence of apparent cracks in the granules. Further analysis of these granules by SEM showed that their surface was smooth (data not shown), indicating that the pronounced staining (cracks) does not represent a groove in the surface, but rather a slightly altered internal organization. Such apparent cracks were also observed with single SBD, although they seem to be less pronounced. Higher SBD2 accumulation levels in the KDSS series showed altered granule morphology in both light and scanning electron micrographs. Granules were sometimes organized in amalgamated clusters. It seemed that this phenomenon was most pronounced in KDSS#1 (3+) and KDSS#7 (4+), although it could also be observed in KDSS#9 (2+). Starch from the various classes of KDS3, KDS4, and KDS5 transformants gave similar results, and there seems to be a tendency that, within one series of transformants, starches belonging to class 2+ and upwards show the amalgamated clusters at higher frequency than those of the lower accumulation classes (data not shown). The *amf*SS#3 starch contained loosely associated clusters. In *amf*SS#34 and *amf*SS#23 with higher levels of SBD2 than *amf*SS#3, these loosely associated clusters were not observed, but amalgamated clusters of very small granules were encountered. Thus, the amalgamated clusters are observed in both the Kardal and the *amf* backgrounds, but in Kardal they appear at lower levels of SBD2 accumulation.

Analysis of KDSS, KDS3, KDS4, KDS5 and *amf*SS granules by SEM revealed the clustered appearance of starch granules in more detail. Fig. 7a shows a magnification of amalgamated clusters of very small granules in the Kardal background, whereas the loosely associated clusters of the *amf*SS#3 starch are shown in Fig. 7b; the granules of the latter, but not of the former, could be cut loose by  $\alpha$ -amylase treatment, indicating that the small granules were connected through  $\alpha$ -glucans (data not shown).



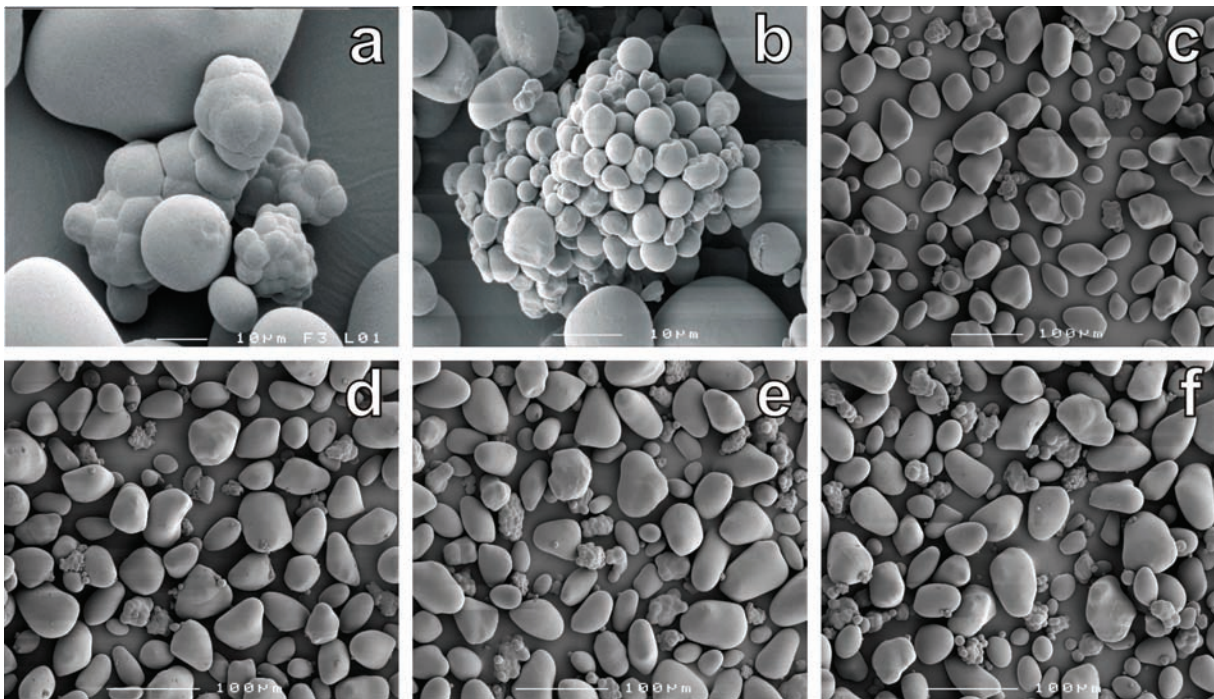
**Fig. 6a,b** Light micrographs showing the morphology of control and transgenic starch granules. The granules were stained with a 20× diluted Lugol solution. **a** Representative granule preparations of selected plants of KD-UT and the KDSS series. **b** Representative granule preparations of selected plants of *amf*-UT and the *amf*SS series. *Arrow heads* indicate amalgamated clusters. Scale bar represents 40  $\mu$ m.

The micrographs of KDS3#2, KDS4#30, and KDS5#23 also showed the amalgamated clusters (Fig. 7d-f), similar to starches derived from the KDSS series (Fig. 7a,c). The size and frequency of appearance of the amalgamated clusters did not seem to be correlated to the number of appended SBDs.



### Starch granules with SBD2 can assemble differently

In the starch granules from a number of transformants of the KDSS series, we observed the contour lines of amalgamated clusters of small granules inside larger starch granules (Fig. 8a,b). The normal, concentric pattern of growth rings was absent in these granules. Furthermore, it can be seen that normal-looking granules and granules with internalized amalgamated clusters can both occur in the starch of KDSS transformants. Similar observations were made for starches from the KDS3, KDS4, and KDS5 series. This phenomenon was not observed in the starch granules from any of the *amf*SS transformants. These data suggest that the irregular amalgamated clusters can grow out to mature granules.



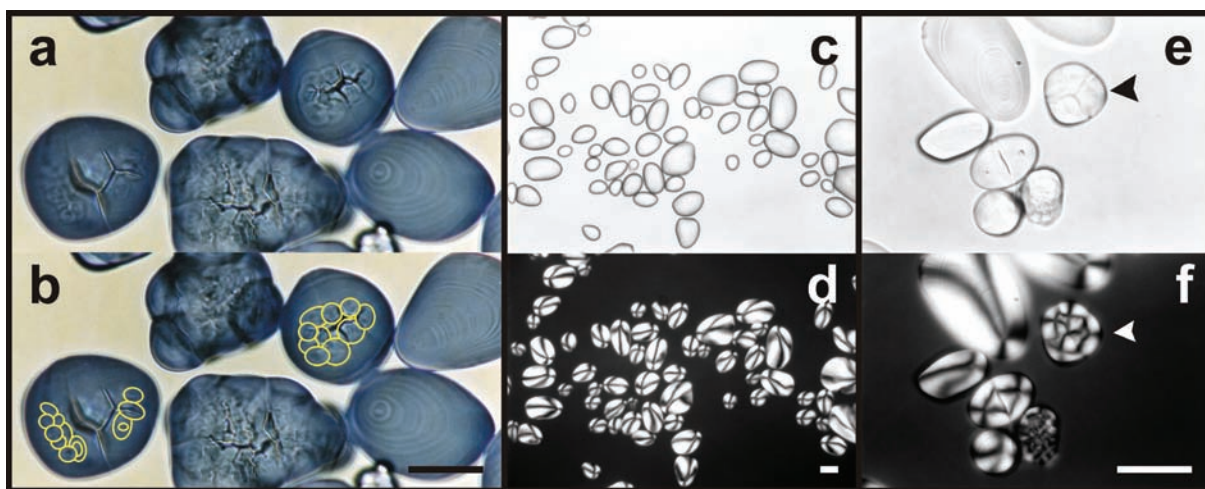
**Fig. 7a-f** SEM analysis of transgenic starch granules from the KDSS, KDS3, KDS4, KDS5, and *amf*SS series. **a** KDSS#1 (3+): close-up of amalgamated clusters. **b** *amf*SS#3 (3+): close-up loosely associated cluster. **c** KDSS#7 (4+). **d** KDS3#2 (6+). **e** KDS4#30 (6+). **f** KDS5#23 (4+). The granule size is indicated by the scale bar.

Since the morphology of the granules appeared very different, a number of the starches were investigated by microscopy under polarized light. The untransformed controls, KD-UT and *amf*-UT, clearly showed the characteristic “Maltese cross” in the starch granules (Fig. 8d). The starches from the KDSS series (Fig. 8f), as well as those from the *amf*SS and other multiple SBD series (data not shown), showed an altered birefringence pattern. It appeared as

if a large portion of the granules were built from many small ones, because often one granule contained many “Maltese crosses”. This indicates that the radial molecular ordering within these granules is different from the starches that do not contain SBD2 (or multiple SBD).

### Granule size distribution

The granule size distributions of all transgenic Kardal starch clones were determined. Additionally, the starches from transformants expressing SBD2 in the *amf* background were analyzed. For SBD2 transformants (both the Kardal and *amf* background), all granule size distributions recorded within one class of transformants were averaged, the result of which is summarized in Fig. 9a. The averaged granule size distributions of the starch of the various classes of the KDSS series were not deviated from the profile of the control, indicating that the granule size of the KDSS starches is not affected by SBD2 accumulation in the granules.



**Fig. 8a-f** Light micrograph of KDSS#2 (2+) starch granules stained with a 10× diluted Lugol solution. **a** Original image of the starch granules. **b** Same image, highlighting in yellow the contour lines of amalgamated clusters of small granules, which seem to be incorporated in larger starch granules. **c-f** Light micrographs of starch granules of KD-UT (**c,d**) and KDSS#9 (**e,f**) viewed under normal (**c,e**) and polarized light (**d,f**). Arrow heads indicate granules with multiple Maltese crosses. Scale bar represents 25 μm.

This was not observed with the *amf*SS series; there, the granule size distribution became bimodal with increasing SBD2 accumulation. Furthermore, it seemed as if the smaller granules in the bimodal distribution become smaller with increasing SBD2 accumulation, and the larger ones larger. The granule size distributions of the KDS3, KDS4, and KDS5

transformants gave similar results as found with the KDSS series; the granule size distribution was unaltered, irrespective of the amount of SBDn accumulated.

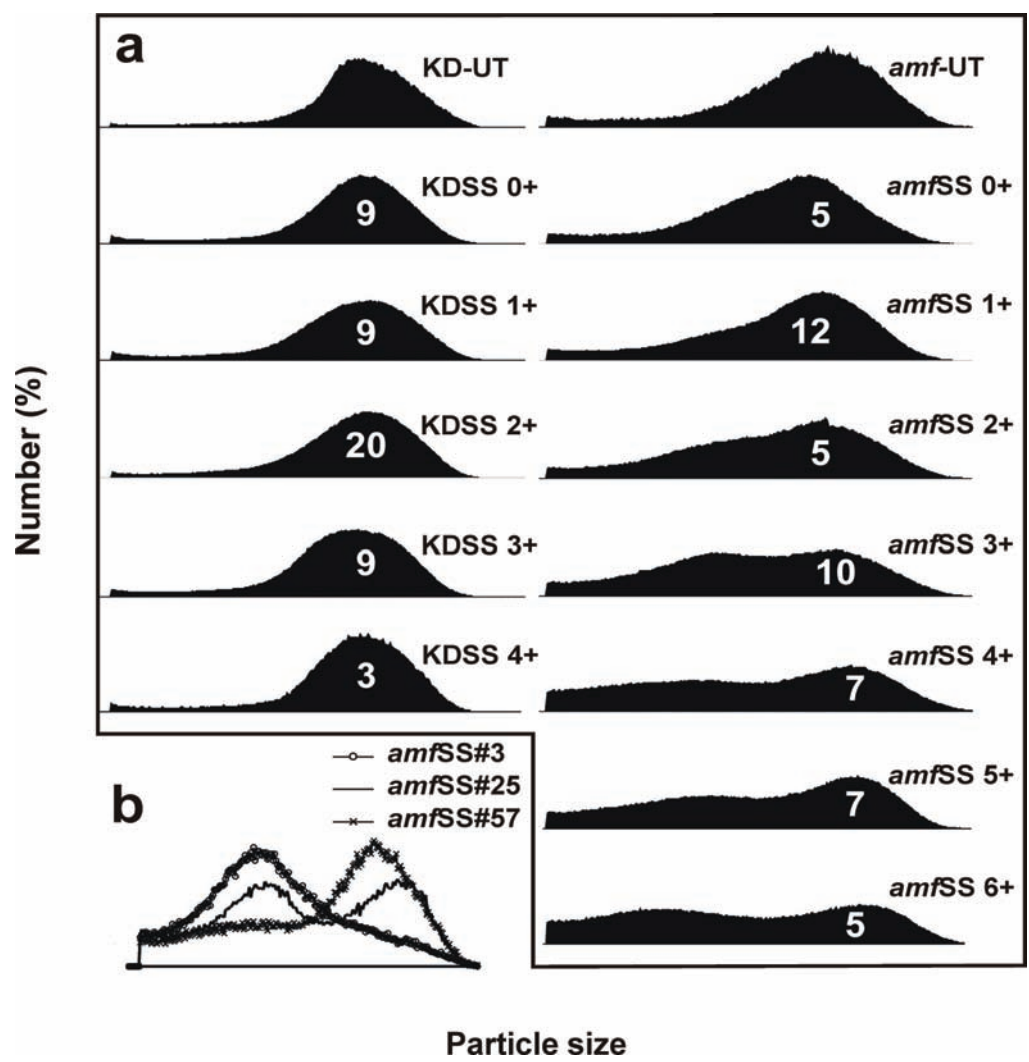
Although the triplicate measurements with the Coulter Multisizer appeared to be very reproducible for each (transgenic) starch sample, we found a large variation in granule size distributions within one SBD2 accumulation class, particularly in the 3+ class of the *amf* background. An example of this is provided in Figure 9b, which shows three different granule size distributions of starches belonging to the 3+ class: *amf*SS#3 has very small starch granules (mean granule size 7.8  $\mu\text{m}$ ), whereas *amf*SS#57 has relatively large ones (mean granule size 13.3  $\mu\text{m}$ ). *Amf*SS#25 had a bimodal distribution with peaks at 7.0 and 27.2  $\mu\text{m}$ .

### Fractionation of starch granules

In order to investigate the relationship between granule size and the level of SBD2 accumulation in the granule, the *amf*SS#22 starch (6+ class) with a bimodal granule size distribution was fractionated into granules larger and smaller than 20  $\mu\text{m}$ . Microscopic examination revealed that the size of the starch granules in the two fractions was more uniform than in the starting material (data not shown), which was further substantiated by measuring the granule size distributions of the two starch fractions (Fig. 10a). Subsequently, the amount of SBD2 was determined in the two granule fractions by Western dot blot analysis. It appeared that the small granules contained more SBD2 protein than the larger ones (Fig. 10b).

### Starch content and physico-chemical properties of the starches

The impact of SBDn accumulation in granules on their physico-chemical properties and on the starch content of the tubers was investigated for the KDSS series. For this, one transgenic clone from each SBD accumulation-class of each series and their respective controls were selected. The starch content of the selected transformants and their controls were measured. Granule-melting behaviour ( $T_0$  and  $\Delta H$ ) of the selected transgenic clones and their controls were investigated by differential scanning calorimetry (DSC). The results showed that there were no consistent differences in the various parameters between the transgenic starches and their controls (data not shown), similar to the results for the *amf*SS series reported before (Ji *et al.*, 2004).

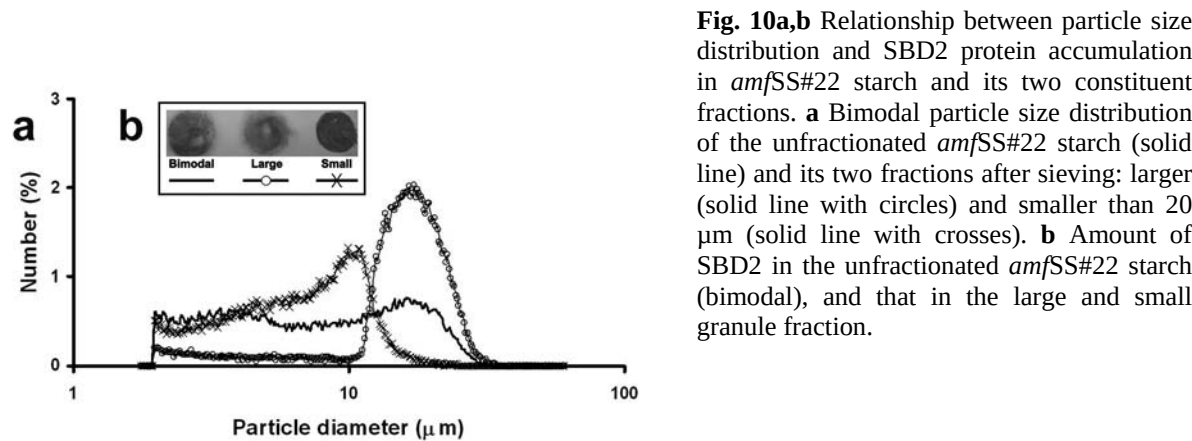


**Fig. 9a,b** Relationship between particle size distributions of the various starches and their SBD accumulation levels. **a** Averaged particle size distributions of the transgenic starches of the various SBD2 classes of the KDSS and *amfSS* series. Each starch sample was analyzed in triplicate, and the granule size distribution profiles of all starches belonging to the same class were averaged. The number inside the distribution indicates the number of transformants belonging to that class. **b** Averaged particle size distributions of *amfSS*#3, *amfSS*#25, and *amfSS*#57 transgenic starches from the 3+ class.

Schwall *et al.*, (2000) inhibited the expression of both starch branching enzymes in potato tubers, and observed that the birefringence patterns of these transgenic starches were different from untransformed controls. Because there seems to be a relationship between birefringence and branching enzyme activity, the same transgenic starches as which were used for polarized light microscopy were investigated for differences in their chain-length distribution. With both high-pH anion-exchange chromatography (HPAEC; good separation of maltodextrins up to a degree of polymerization of approximately 45) and high-performance size-exclusion chromatography (HPSEC; a broader range separation than HPAEC, but with



lower resolution), no differences were found between transgenic starches, and between transgenic starches and the respective untransformed controls (data not shown).



Since the granule packing appeared to be affected by the presence of SBD2, three *amfSS* and two KDSS starches were selected for X-ray diffraction analysis. The untransformed and transgenic starch granules all consisted of B-type crystallites, demonstrating that SBD2 expression did not alter the crystal type. Small, but consistent, differences in the crystallinity of the granules were found. The *amf*-UT starch had a crystallinity of 42%, that of *amfSS#3* (3+) 45%, that of *amfSS#22* (6+) 44% (the small and large granule fraction of *amfSS#22* were also measured, and gave crystallinity values of 42% and 46%, respectively), and that of *amfSS#23* (6+) 47%. The KD-UT starch had a crystallinity of 42%, that of KDSS#9 (2+) 39%, and that of KDSS#7 (4+) 42%. This suggests that there is no apparent correlation between the amount of SBD2 in the granule and crystallinity. Thus, the altered packing of the starch granule does not seem to lead to a different crystallinity of the granules.

### Discussion

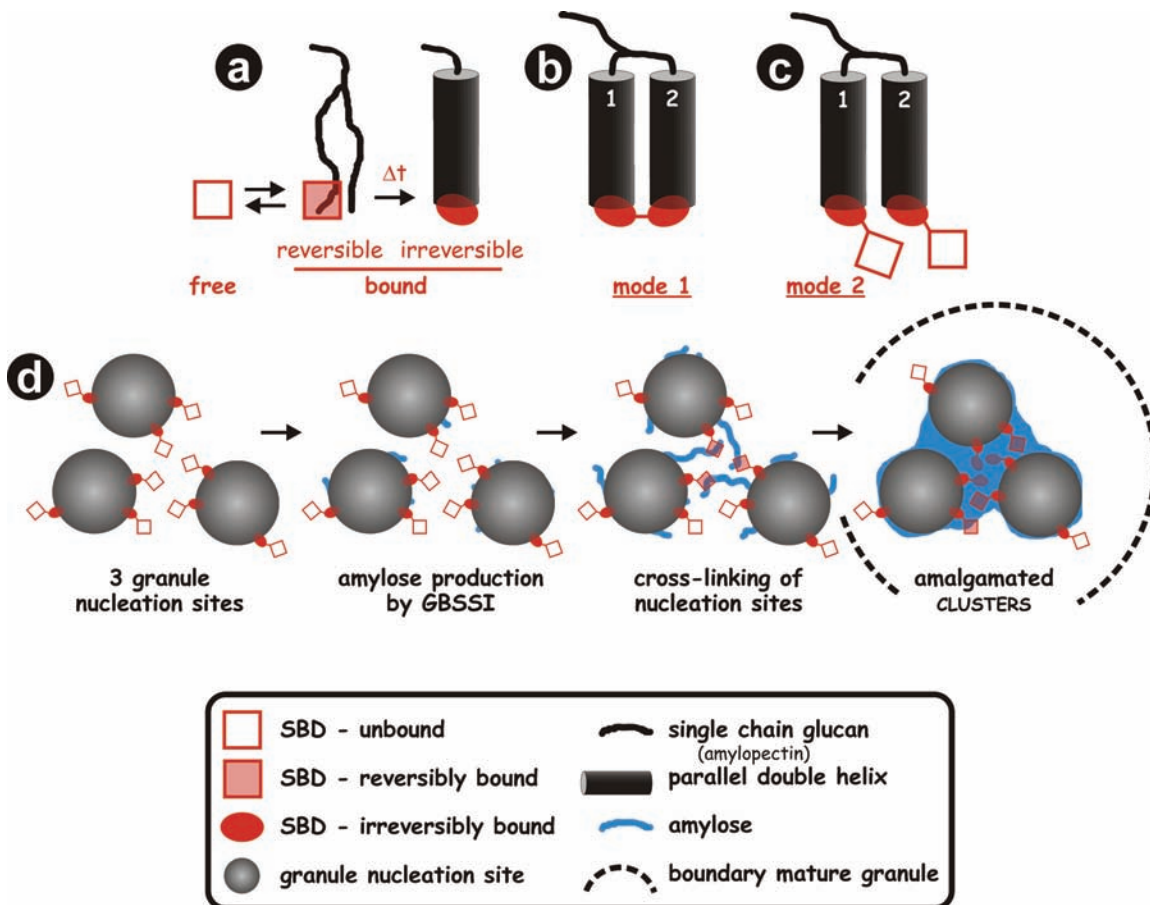
In this study, engineered high-affinity multiple-repeat SBDs were introduced into the amylose-containing potato genotype Kardal to investigate whether they could displace GBSSI from the starch granule during biosynthesis. Higher amounts of SBD2 than of SBD could be accumulated, which was similar to our previous observations in the *amf* background (Ji *et al.*, 2004). SBD3 seemed to have a higher affinity for starch than SBD2 (Fig. 1), but the data on the affinity for starch of SBD4 and SBD5 are not conclusive. None of the multiple appended

SBD proteins had the ability to reduce the GBSSI content of the starch granule, and therewith the amylose content. As it has been shown that the affinity for the target ligand of a protein increases with the number of carbohydrate-binding module repeats (Boraston *et al.*, 2002; Ji *et al.*, 2004), it was anticipated that particularly SBD4 and SBD5 would prevent GBSSI from binding the growing granule. As this does not occur, it seems most straightforward to assume that GBSSI and SBD bind different sites inside the starch granule. However, it can not be excluded that they bind at similar sites. For this, it is important to consider the mechanism by which SBD binds  $\alpha$ -glucans. This mechanism has been most extensively studied for the SBD of *Aspergillus niger* glucoamylase (Southall *et al.*, 1999; Giardina *et al.*, 2001; Paldi *et al.*, 2003; Morris *et al.*, 2005); the SBD of *Bacillus circulans* cyclodextrin glycosyltransferase, used in this study, is believed to display similar features (Kok-Jacon *et al.*, 2003). SBD has two binding sites with distinct affinities for maltooligosaccharides. It is believed that the small and accessible site 1 is responsible for the initial recognition, whereas the longer site 2 is involved in tighter binding and undergoes conformational changes upon binding (Giardina *et al.*, 2001). Paldi *et al.* (2003) observed that binding of SBD to starch granules is not instantaneous (unlike the binding of certain cellulose-binding domains), but requires approximately one hour, which seems to be consistent with the fact that site 2 needs to be moulded onto the  $\alpha$ -glucan. After one hour, binding of SBD to starch was irreversible (see Fig. 11a). Furthermore, SBD with its two binding sites has been shown to induce conformational changes in linear  $\alpha$ -glucan (Giardina *et al.*, 2001), and it has been suggested that they can bind (and unwind) the non-reducing termini of two parallel side chains of amylopectin engaged in a double helix (Morris *et al.*, 2005). When SBD and GBSSI are simultaneously present, as during starch biosynthesis, it might be that GBSSI displays an instantaneous, higher initial affinity for  $\alpha$ -glucan than SBD, and that the conformational change of SBD's binding site 2 simply takes too much time for making SBD a true competitor of GBSSI. By increasing the number of SBDs in the fusion protein, the initial affinity for  $\alpha$ -glucan might be enhanced; as it is possible that the appended SBDs interact with different  $\alpha$ -glucan chains on the growing granule, this does not necessarily mean that SBD<sub>n</sub> has more competitive power than SBD alone, and therefore displacement of GBSSI is not self-evident. The above is consistent with our observations: (i) SBD and SBD2 can be accumulated in larger amounts in *amf* than in Kardal starch granules; and (ii) more SBD2 was found in the tuber juices of all KDSS transformants than in those of the *amf*SS series, which hints at a more reversible binding in the Kardal background (in the *amf* background, SBD2 was only

found in transformants with 6+ class granules, suggesting that the binding is of a more high-affinity type, and that the granule surface was only saturated in these high expressers).

Although SBD2 (Ji *et al.*, 2004), and likely also SBD3, has more affinity for the starch granule than a single SBD, our data for SBD4 and SBD5 are not conclusive in this respect; the lower frequency of SBD4 and SBD5 transformants in the higher SBD accumulation classes compared to that of SBD3 transformants was unexpected (Fig. 1). Given the large amount of transcript found in the KDS5 (5+) transformant (Fig. 4; compare with SBD3 (6+)), it seems unlikely that an altered transcription of these multiple-repeat genes underlies this phenomenon. It might be that translation or import into the amyloplast becomes less efficient with proteins of more than 3 SBD repeats, but this was not further investigated, and needs to be substantiated. It can also not be excluded that the SBD antibody can not bind all domains in proteins with more than 3 appended SBDs, leading to an underestimation of the amount of SBD-containing protein. However, in that case, we would have expected less high SBD accumulators (4+ to 6+) in the KDS5 series in comparison with the KDS4 series.

SBD2 seems to interfere with various aspects of the starch biosynthesis process, *i.e.* granule packing and granule morphology (Kardal and *amf*), and granule size (*amf*). For this, it is important to realize that SBD2 can bind to starch granules in two modes. In mode 1 (Fig. 11b), both SBDs of SBD2 are attached to the same granule surface. This mode presumably occurs at lower SBD2 concentration, when not all granule surface is covered with SBD. In mode 2 (Fig. 11c), one SBD binds the granule surface, whereas the other one is exposed to the granule surrounding. This mode might occur at higher SBD2 concentration, when the granule surface is saturated with SBD. We speculate that at low SBD2 concentration in the *amf* background mode 1 predominates, whereas at higher SBD2 levels in the *amf* background, and at any SBD2 concentration in the Kardal background, mode 2 becomes more important (see also Ji *et al.*, 2004). The exposed SBD of SBD2 in mode 2 (or the “cross-linking” mode) might capture soluble glucans from the stroma or protruding amylose (Fig. 11d), and cross-link different granule nucleation sites, eventually leading to the clusters: the amalgamated ones (Kardal, *amf* high SBD2 expressers), and the loosely associated ones (*amf* intermediate expressers). The amalgamated clusters are observed in Kardal at lower levels of SBD2 accumulation than in the *amf* background. This might be due to the presence of amylose protruding from the granule surface, which may facilitate cross-linking of the small granules.



**Fig. 11a-d.** Schematic representation of possible binding mechanisms and modes of SBD2. **a** SBD can bind the non-reducing termini of the double-helical side chains of amylopectin with high affinity, as suggested by Morris *et al.* (2005). Initially, binding site 1 of SBD is suggested to be involved in the initial, instantaneous recognition of  $\alpha$ -glucan (reversible binding). Subsequently, SBD undergoes a conformational change to facilitate the stronger interaction of binding site 2 with the parallel, double helical amylopectin side chains (Giardina *et al.*, 2001). This process requires time and is thought to be irreversible (Paldi *et al.*, 2003). These double helices can be bound by SBD at high-affinity (irreversible). **b** At low SBD2 concentrations, both domains are expected to interact with the same granule surface (mode 1). **c** At higher SBD2 concentrations, the surface area may become limiting, and both domains can not be accommodated on the same surface anymore. Consequently, the SBD2 proteins may have one SBD attached to a double helix, whereas the other one is available for interaction with soluble glucans or amylose protruding from the granule surface (mode 2). **d** SBD2 binds in mode 2 to granule nucleation sites (note that only a few SBD2 proteins are shown). The exposed SBD of SBD2 is available for interaction with amylose-like molecules produced by GBSSI. If the exposed SBD of SBD2 binds amylose-like molecules from a different nucleation site, then the amalgamated clusters may be formed, which eventually develop into a starch granule.

The larger granules seem to have over-grown the clustered appearance, and develop into normal-looking ones (although multiple “Maltese crosses” occur within one granule; see Fig. 8f). It seems as if SBD2 is present in non-limiting amounts at the onset of starch biosynthesis, and becomes limiting at a certain time point in starch biosynthesis, after which the clustering stops, and the granules develop normally.

We have reported previously that SBD2 expression can reduce granule size in the *amf* background, depending on the amount of SBD2 accumulated (Ji *et al.*, 2004). In the intermediate

to high SBD2 accumulators (3+ to 6+ classes) of the *amfSS* series, bimodal granule size distributions were observed, a phenomenon which is common for wheat starch (Peng *et al.*, 2000). A thorough study in which granule size is monitored during tuber development might shed more light on this. Preliminary studies have indicated that small and large granules occur in one tuber cell, but also this needs further investigation. In the Kardal background, the granule size distributions are not affected by the presence of multiple appended SBDs, irrespective of the amount accumulated. This is consistent with SBDn binding mainly in mode 2 (see above) and the production of amylose close to the granule surface, the combination of which facilitates cross-linking of small granules. Typically, larger granules than those present in tubers of control plants are not formed. However, expression of SBD2 in the *Arabidopsis* *sex1* mutant leads to larger starch granules, and interestingly the starch granules from this mutant have a higher amylose content than those from wild-type *Arabidopsis* (Howitt *et al.*, 2006). Some caution is required with this extrapolation, as one compares transitory starch (*Arabidopsis*) to storage starch (potato), and a mutant in starch degradation (*Arabidopsis*) to a mutant in starch biosynthesis (potato). At moderate to high SBDn accumulation in the Kardal background, it was observed that the assembly of oval-shaped granules was impaired (Fig. 7a,c-f). A kind of clustered appearance of starch granules was also observed by Howitt *et al.*, (2006). Altered granule morphology has also been evidenced in other transgenic potato tubers, but in all these cases an enzyme activity involved in starch biosynthesis had been down-regulated (Edwards *et al.*, 1999b; Lloyd *et al.*, 1999a; Schwall *et al.*, 2000; Fulton *et al.*, 2003; Bustos *et al.*, 2004). In these examples, the ratio of  $\alpha$ -1,4 to  $\alpha$ -1,4,6 linked glucose was altered in comparison to starch from untransformed potato plants, as evidenced from clear differences in chain-length distributions. In our study, the chain-length distribution of the various transgenic starches was unaltered, irrespective the amount of SBDn accumulated, and so were the expression levels of major starch biosynthetic genes. Therefore, it seems unlikely that the activity of starch synthase, branching enzyme and/or isoamylase is affected to any large extent. Our results and those from Howitt *et al.*, (2006) suggest that differences in granule morphology can be mediated by protein binding only, a process by which the physical interactions between the constituent  $\alpha$ -glucans can be modified; alterations in enzyme activities do not seem necessarily required. We believe that by using mutant (e.g. in the residues mediating binding of glucan) SBD2 (or SBDn), and fusing various partner proteins to SBD2, the physical interactions at play during starch biosynthesis can now be probed further.

## Materials and methods

### Constructs for transformation

The pBIN19/SBD2 plasmid was used for the expression of SBD2 protein in potato plants; for the preparation of this construct we refer to Ji *et al.*, (2004). The gene was expressed in potato plants under the control of the tuber-specific potato GBSSI promoter. Amyloplast entry of SBD2 was mediated by the potato GBSSI transit peptide. The construct for expression of SBD3 (pBIN19/SBD3) in potato plants was made as follows. The pBIN19/SBD3 construct was assembled from the pUC19/SBD2 (Ji *et al.*, 2004). A SBD-encoding sequence was amplified by polymerase chain reaction proof-reading *Pfu* turbo DNA polymerase (Stratagene, Cambridge, UK) with the primers 5'-ATAGCAACCTCGAGTAGTACCATGGCCGGGGATCAG-3' and 5'-CGCCTGGTGTCTAGAAATTCGGTCGACGGGGT-3', which contained an *Xho*I and *Sal*I at their 5'-ends, respectively. The pUC19/SBD (Ji *et al.*, 2003) plasmid was used as a template. The amplified fragment was inserted into pUC19/SBD2, which was opened with *Sal*I, to give the pUC19/SBD3 plasmid (*Xho*I and *Sal*I have compatible overhangs). The orientation of the inserted fragment was checked by digestion with *Bgl*II. The gene sequence contains two *Bgl*II sites, one at the beginning of the first linker, and one at the beginning of the second linker. The length of the *Bgl*II-*Bgl*II fragment in the correct orientation should be 387 nucleotides. In order to verify its correctness, the construct was sequenced (long runs). In a similar way, the pUC19/SBD4 plasmid was obtained. Briefly, the *Xho*I-*Sal*I fragment (see above) was cloned in pUC19/SBD3, after opening this plasmid with *Sal*I. The orientation of the insert was checked by digestion with *Hind*III, and by sequencing. Similar procedures were followed for generating pUC19/SBD5. After digestion of the plasmids pUC19/SBD3, pUC19/SBD4, and pUC19/SBD5 with *Hpa*I and *Kpn*I, the *Hpa*I-*Kpn*I fragment was inserted into the corresponding sites of the pBIN19/SBD2 to generate the pBIN19/SBD3, pBIN19/SBD4, and pBIN19/SBD5, respectively. The predicted molecular masses of the SBD3, SBD4, and SBD5 produced in plants is 39,315 Da, 53,157 Da, and 66,999 Da, respectively, excluding the transit peptide.



## Plant transformation and regeneration

The pBIN19/SBD2, pBIN19/SBD3, pBIN19/SBD4, and pBIN19/SBD5 plasmids were introduced into the amylose-containing potato (*Solanum tuberosum*) cultivar (cv. Kardal; Laboratory of Plant Breeding, Wageningen University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands) via *Agrobacterium*-mediated transformation described by Visser (1991). More than 50 independent shoots were harvested for each genotype. Shoots were tested for root growth on a kanamycin-containing (100 mg/L) MS30 medium (Murashige and Skoog 1962). For each genotype, fifty transgenic, root-forming, shoots were multiplied and five plants of each transgenic clone were transferred to the greenhouse for tuber development. In addition, 10 untransformed controls were grown in the greenhouse.

## Determination of SBDn transcript levels

Total RNA extraction was performed according to Kuipers *et al.* (1994). Total RNA was extracted from 5 g (fresh weight) of potato tuber material. The amount of RNA was determined spectrophotometrically in each sample. The RNA concentration was verified by running an appropriate volume corresponding to 40 µg on a 0.8% w/w agarose gel. Based on a spectrophotometric RNA determination, similar amounts of total RNA were fractionated on a 1.5% (w/v) agarose-formaldehyde gel, and transferred to a Hybond N nylon membrane (Amersham, Little Chalfont, UK). The membrane was hybridized with a [<sup>32</sup>P]-labeled *Nco*I-*Bam*HI DNA fragment of SBD2 as a probe; labelling was performed using a *rediprime*<sup>TM</sup> II kit (Amersham) according to the instructions of the manufacturer.

## Isolation of tuber starch

All tubers derived from the five plants of each greenhouse-grown clone were combined, and their peels were removed in an IMC VC7T Peeler (Spangenberg, Waardenburg, 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 in 0.01% (w/w)  $\text{N}_2\text{S}_2\text{O}_5$  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).

### **Determination of SBD content of starches by Western dot blot analysis**

A 12.5% sodium dodecyl sulfate-polyacrylamide gel (50 mm × 50 mm × 3 mm) with nine, equally-spaced, holes ( $\varnothing$  = 9 mm) was placed in contact with a similarly-sized Hybond ECL nitrocellulose membrane (Amersham). For determining the SBD2, SBD3, SBD4, and SBD5 content, 20 mg of (transgenic) starch was boiled for 5 min with 200  $\mu\text{L}$  of 2× SDS sample buffer (Laemmli 1970). After cooling to room temperature, the starch gel was transferred into one of the holes. The proteins in the transgenic starch gels were blotted to the membrane using a PhastSystem (Pharmacia, Uppsala, Sweden; 20 V, 25 mA, 15 °C, 45 min) (Ji *et al.*, 2003). The protein was identified with anti-SBD antibody according to the method described by Ji *et al.* (2003).

### **Determination of the expression of major starch biosynthetic genes**

Gene expression of major starch biosynthetic enzymes was measured using quantitative real time PCR (qRT-PCR) analysis. The PCR reaction consisted of 25  $\mu\text{L}$  of a mixture containing: 1x PCR buffer, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM dNTPs, 0.2  $\mu\text{M}$  of each sequence-specific primers 3  $\mu\text{L}$  SYBR Green I (1:15,000 diluted), 3  $\mu\text{L}$  of transcribed cDNA, and 0.5 U of Taq polymerase. Specific primers for starch branching enzyme I (SBEI), sucrose synthase (Susy), ADP-glucose pyrophosphorylase (AGPase), starch synthase III (SSSIII) (according to Kok-Jacon *et al.*, 2005), for three isoamylase isoforms (Stisa1, Stisa2 and Stisa3; Bustos *et al.*, 2004), and for the internal control gene ubiquitin (ubi3; 5'-TTCCGACACCATCGACAATGT-3' and 5'-CGACCATCCTCAAGCTGCTT-3') were designed by the primer express software (version 1.5, PE Applied Biosystems, CA, USA). In qRT-PCR analysis, quantification is based on  $C_t$  values. The  $C_t$  (cycle threshold) is a measurement taken during the exponential phase of amplification when limiting reagents and small differences in starting amount have not yet influenced the PCR efficiency.  $C_t$  is defined as the cycle at which fluorescence is first detectable above background, and is inversely



proportional to the log of the initial copy number. Each reaction was performed in triplicate and the corresponding  $C_t$  values were determined. The  $C_t$  values of each qRT-PCR reaction were normalized in relation to the  $C_t$  value corresponding to the Ubi3 gene. These values were then used to determine the changes in gene expression among the different transformants and their corresponding control plants.

### **Electrophoretic separation of granule proteins SBDn and GBSSI**

Fifty mg of starch was boiled for 2 min in 1 mL of SDS sample buffer, containing 5% (v/v)  $\beta$ -mercaptoethanol. The gelatinized samples were centrifuged for 10 min at 14,000 *g*. Twenty five  $\mu$ L of supernatant were loaded onto a 12% polyacrylamide gel (145 mm x 95 mm x 3 mm), and the proteins were separated by electrophoresis. Subsequently, the proteins were transferred onto a Hybond ECL membrane (Amersham), and detected using anti-SBD as described in the previous section, or anti-GBSSI polyclonal antibody (Vos-Scheperkeuter *et al.*, 1986) in a 1:250 dilution.

### **Determination of SBDn content of potato juice**

SBD2, SBD3, SBD4 and SBD5 proteins in the soluble fraction of potato tubers were determined as follows. 500  $\mu$ L of tuber juice was freeze-dried. The dried material was dissolved in 200  $\mu$ L of SDS sample buffer, containing 5% (v/v)  $\beta$ -mercaptoethanol. In order to make the sample suitable for Western dot blot procedure, the mixture was boiled for 5 min in the presence of 20 mg starch from the control samples. The resulting starch gel was applied to one of the holes in the sodium dodecyl sulfate-polyacrylamide gel. The rest of the procedure was conducted in the same way as described for granule-bound SBD2 (Ji *et al.*, 2004).

### **Analysis of physico-chemical properties of starch granules**

The average granule size and granule size distribution of the (transgenic) starches were determined in triplicate with a Coulter Multisizer II, equipped with an orifice tube of 200  $\mu$ m (Beckman-Coulter, High Wycombe, UK). Approximately 10 mg of starch was dispersed in

160 mL of Isoton II. The granule size distributions were recorded by counting approximately 50,500 ( $\pm 500$ ) 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 and birefringence were investigated by light microscopy (LM, Axiophot, Oberkochen, Germany). Starch granules were stained with a 20 $\times$  diluted Lugol solution (1% I<sub>2</sub>/KI). For determining the birefringence of the granules the polarized light device was used. For scanning electron microscopy (SEM, JEOL 6300F, Japanese Electron Optics Laboratory, Tokyo, Japan), dried starch samples spread on silver tape and mounted on a brass disc were coated with a 20 nm platinum layer. Samples were then examined with a scanning electron microscope operating at an accelerating voltage of 1.5-3.5 keV. The working distance was 9 mm.

The apparent amylose content was determined according to the method described by Hovenkamp-Hermelink *et al.* (1989). The temperature at which starch granules start to gelatinize was determined by differential scanning calorimetry (DSC) using a Perkin-Elmer Pyris 1 (Perkin-Elmer, Vlaardingen, The Netherlands), equipped with a Neslab RTE-140 glyco-cooler (Ji *et al.*, 2003).

Prior to X-ray diffraction, the water content of the starch was equilibrated at 90% of relative humidity (RH) under partial vacuum in presence of a saturated barium chloride solution. The samples (20 mg) were then sealed between two tape foils to prevent any significant change in water content during the measurement. Diffraction diagrams were recorded using an INEL spectrometer (Artenay, France) working at 40 kV and 30 mA, operating in the Debye-Scherrer transmission mode. The X-ray radiation CuK $\alpha_1$  ( $\lambda=0.15405$  nm) was selected with a quartz monochromator. Diffraction diagrams were recorded during 2 h exposure periods, with a curve position sensitive detector (INEL CPS 120). Relative crystallinity was determined after normalization of all recorded diagrams at the same integrated scattering between 3 and 30° (2 $\theta$ ). B-type recrystallized amylose was used as crystalline standard, after scaled subtraction of an experimental amorphous curve in order to get nul intensity in the regions without diffraction peaks. Dry extruded potato starch was used as the amorphous standard. The degree of crystallinity of samples having a pure polymorphic type was determined using the method initially developed for cellulose (Wakelin *et al.*, 1959). The percentage of crystallinity was taken as the slope of the line  $(I_{\text{sample}} - I_{\text{amor}})_{2\theta} = f(I_{\text{crys}} - I_{\text{amor}})_{2\theta}$  where  $I_{\text{sample}}$ ,  $I_{\text{amor}}$  and  $I_{\text{crys}}$  are the diffracted intensity of the sample, the amorphous and the crystalline standards, respectively.

### Determination of chain-length distribution

For chromatography, five mg of (transgenic) starch was suspended in 250  $\mu$ L of DMSO, and the starch was gelatinized by keeping this suspension for 15 min in a boiling water bath. Subsequently, the solution was cooled down to 40 °C, and 700  $\mu$ L of 50 mM phosphate buffer (pH 4.0), containing sufficient isoamylase (Hayashibara Biochemical, Okayama, Japan) to debranch the starch polymers completely, was added. After 2 h of incubation at 40 °C, the enzyme was inactivated in a boiling water bath for 10 min. To each sample, 1 mL of 25% DMSO was added, and the samples were centrifuged (7,500 *g*, 2 min). For HPAEC, the supernatant was diluted 5 times with a 25% DMSO solution; for HPSEC, the samples were used as such.

HPSEC was performed on a P680 HPLC pump system (Dionex, Sunnyvale, CA, USA) equipped with three TSKgel SWXL columns in series (a G3000 and two G2000; 300  $\times$  7.5 mm; Montgomeryville, USA) in combination with a TSKgel SWXL guard column (40  $\times$  6 mm) at 35 °C. Aliquots of 100  $\mu$ L were injected using a Dionex ASI-100 Automated Sample Injector, and subsequently eluted with 10 mM NaOAc buffer (pH 5.0) at a flow rate of 0.35 mL/min (3 h 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). Dionex Chromeleon software version 6.50 SP4 Build 1000 was used for controlling the HPLC system and data processing.

HPAEC was performed on a GP40 gradient pump system (Dionex) equipped with a CarboPac PA 100 column (4  $\times$  250 mm; Dionex) at 35 °C. The flow rate was 1.0 mL/min and 20  $\mu$ L sample was injected with a Dionex AS3500 automated sampler. Two eluents were used, eluent A (100 mM NaOH) and eluent B (1 M NaOAc in 100 mM NaOH), for mixing the following gradient: 0 $\rightarrow$ 5 min, 100% eluent B (rinsing phase); 5 $\rightarrow$ 20 min, 100% eluent A (conditioning phase); 20 $\rightarrow$ 25 min, linear gradient from 0 $\rightarrow$ 20% eluent B (100 $\rightarrow$ 80% eluent A); 25 $\rightarrow$ 50 min, linear gradient from 20 $\rightarrow$ 35% eluent B (80 $\rightarrow$ 65% eluent A); 50 $\rightarrow$ 55 min, linear gradient from 35 $\rightarrow$ 50% eluent B (65 $\rightarrow$ 50% eluent A); 55 $\rightarrow$ 60 min, 50% eluent B (50% eluent A). The sample was injected at 20 min. The eluent was monitored by an ED40 electrochemical detector in the pulsed amperometric mode (Dionex).

### Determination of starch content

Approximately 50 mg of potato tuber material was 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 was determined enzymatically using a test kit (Boehringer, Mannheim, Germany), according to the instructions of the manufacturer. The values are an average of three independent measurements.

### $\alpha$ -Amylase treatment of starch granule preparations

Twenty milligrams of *amfSS#3* or *KDSS#7*, as well as their respective control starches, was suspended in 1 mL of 50 mM NaOAc buffer (pH 4.8), and treated with 5 U of porcine pancreas  $\alpha$ -amylase (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) for 4 h at 25 °C. Samples without  $\alpha$ -amylase addition served as controls. After incubation, the samples were centrifuged (7,500 *g*, 10 min). The starch pellets were washed three times with water, and air-dried. The dried starch granules were used for light microscopic analysis and granule size distribution determination.

### Fractionation of starch granules

Approximately 5 g of transgenic starch from clone *amfSS#22* was fractionated by sieving (20 µm DIN-ISO 3310/1, Retsch, Haan, Germany), using a continuous flow of distilled water. Two fractions were obtained (with granules smaller and larger than 20 µm), which were air-dried at room temperature.

### Trypsin treatment of starch granules

In order to test whether the various multiple SBD proteins are present inside starch granules or at the granular surface, 20 mg of starch was treated with a non-limiting amount of trypsin (Sigma-Aldrich Chemie B.V.) as described by Ji *et al.* (2003). The result of trypsin digestion was evaluated by Western blot analysis. Digestion of bovine serum albumine with trypsin was performed to verify that the protease was active.

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# Chapter 3

## Expression of an engineered granule-bound *E. coli* maltose acetyl transferase in wild-type and *amf* potato plants

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## Abstract

**S**tarch is used in many industrial applications, but often it requires chemical derivatization to enhance its properties before use. More in particular, the stability of starch polymers in solution is improved by acetylation. A drawback of this treatment is the use of pollutant chemicals. A biological alternative to chemical derivatization was investigated by expressing an amyloplast-targeted *E. coli* maltose acetyltransferase gene (*MAT*) in tubers of wild type (Kardal) and mutant amylose-free (*amf*) potato plants. *MAT* was expressed as such, or fused to the N- or C-terminus of a non-catalytic starch-binding domain (SBD) for targeting the starch granule. Starch granules derived from transgenic plants were found to contain acetyl groups, although their content was low, opening an avenue to move away from post-harvest chemical derivatization of starch. *MAT* inside starch granules was found active post-harvest, when supplied with acetyl-CoA and glucose or maltose, but it did not acetylate starch polymers *in vitro*. Starch granules from transformants in which *MAT* alone was expressed also showed *MAT* activity, indicating that *MAT* is accumulated in the starch granules, and has affinity for starch by itself. Furthermore, starch granule morphology was altered, and fusion proteins with *MAT* and SBD seem to have a higher affinity for starch granules than two appended SBDs. These results are discussed against the background of the quaternary structure of *MAT*.

**Key words:** Starch-binding domain, SBD, biosynthesis, transgenic potato, acetylation, amylose-free potato

**S**tarch, the main storage carbohydrate *in planta*, is abundant in almost all major agricultural crops. Although native starches have their own food and non-food applications, more often industry requires the functionality of chemically modified starch because some native characteristics of starch like shear-stress, thermal resistance and high degree of retrogradation are drawbacks (Singh *et al.*, 2004). Among different chemical modifications of starch, acetylation and hydroxypropylation have been applied for decades to improve the functional properties and storage stability of starch-based foods. Both acetylation and hydroxypropylation have been particularly effective in reducing retrogradation, increasing water holding capacity, and improving freeze-thaw stability (Perera and Hoover, 1999).

Industrial acetylation is achieved with acetic anhydride or vinylacetate. Upon acetylation of starch the hydrophilic hydroxyl groups (at C-2, C-3 and C-6) are substituted with hydrophobic acetyl esters (Perera and Hoover, 1999; Lawal, 2004). The degree of substitution (DS) is used as a measure to indicate the number of acetyl groups per glucosyl unit; theoretically, the maximum acetylation that can be achieved is close to three. In food applications, starch acetates with a DS = 0.01-0.2 are used to improve, in particular, the stability of starch pastes (Xu *et al.*, 2004). Acetyl groups can disrupt the interaction among linear  $\alpha$ -glucan chains and, consequently, acetylated starches have a lower gelatinization temperature, higher peak viscosity, reduced retrogradation on cooling, and better freeze-thaw stability (Singh *et al.*, 2004).

The disadvantage of chemical derivatization procedures is that they often rely on hazardous chemicals, which must be separated from the derivatized polymers at the end of the process. With more strict discharge regulations for industrial waste, a biological alternative for derivatization is becoming increasingly desirable. Therefore, we aimed to introduce an enzyme with the capability of attaching acetyl groups to  $\alpha$ -glucan into potato plants, in order to synthesize starch acetate *in planta*. An enzyme that might be suitable for this purpose is the *E. coli* maltose acetyl transferase (MAT) (EMBL accession number, AJ223173). This enzyme cleaves acetyl-coenzymeA (acetyl-CoA) (donor substrate), and transfers the acetyl group to the C6 position of a glucosyl residue of an acceptor molecule (Boos *et al.*, 1981; Leggio *et al.*, 2003). The acceptor can be glucose or malto-oligosaccharides, but it seems that the rate of acetylation decreases with increasing length of the acceptor (Leggio *et al.*, 2003).

In this study, *E. coli* MAT and MAT with an appended N- or C-terminal starch-binding domain (SBD) (Ji *et al.*, 2003; Kok-Jacon *et al.*, 2003) were introduced into potato plants. The

SBD serves two purposes, (i) to bring MAT and acceptor substrate in more intimate contact, and (ii) to incorporate (active) MAT in starch granules, which may enable laboratory-scale post-harvest starch acetylation experiments (Kok-Jacon *et al.*, 2003). The various gene constructs were transformed into two potato backgrounds: the amylose-containing (Kardal) and the mutant amylose-free (*amf*) genotype. It is realized that introduction of the *MAT* gene constructs in the *amf* background is commercially irrelevant, because the starch from the *amf* genotype is much less sensitive to retrogradation than amylose-containing potato starch (Visser *et al.*, 1997). However, it should be noted that more SBD-containing fusion proteins can be accumulated in *amf* starch granules (Ji *et al.*, 2003, 2004), and therefore *amf* starch may be better suited a priori for studying MAT activity in starch granules.

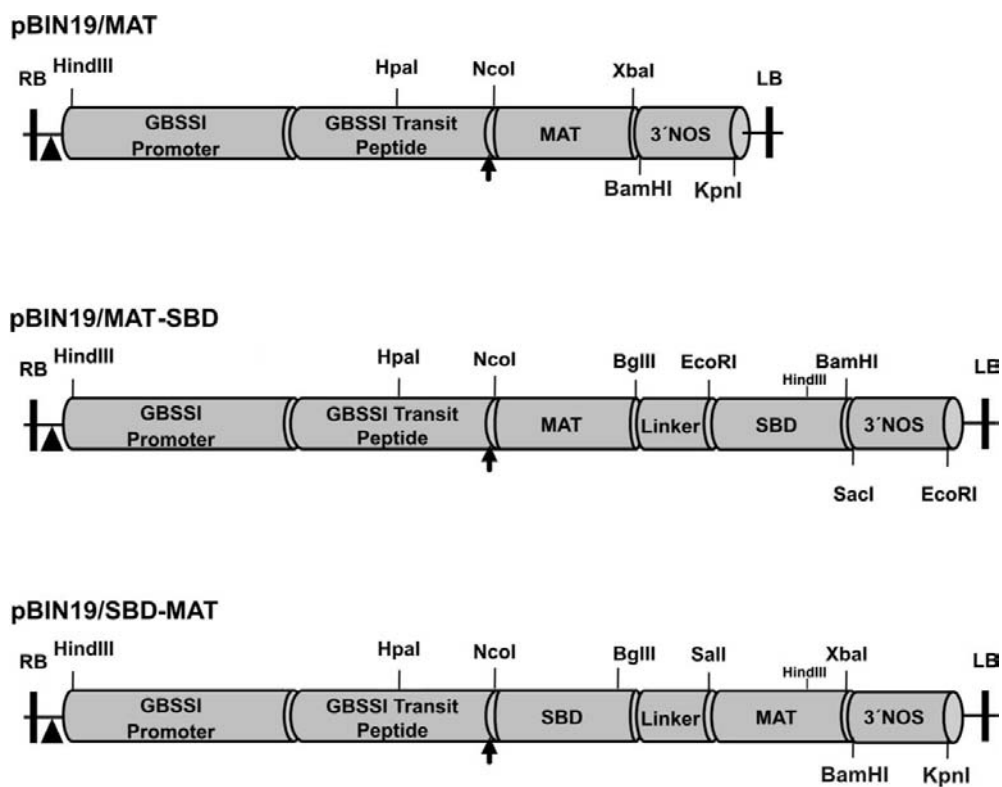
## Results

### Screening of the transformants by Western and Northern blot analyses

Three different constructs (Fig.1) were introduced into two different backgrounds, i.e. the amylose-free (*amf*) and amylose-containing (Kardal) genotype, using *Agrobacterium*-mediated plant transformation. Around 30 independent transformants, five of each clone, were harvested and transferred to the greenhouse to produce tubers. The resulting transformants are referred to as *amf*Mxx, *amf*MSxx and *amf*SMxx for the *amf* background, and KDMxx, KDMSxx and KDSMxx for the Kardal background, in which M, MS and SM represent MAT, MAT-SBD and SBD-MAT, respectively, and xx designates the clone number. During the experiments, no consistent difference(s) in the morphology and growth characteristics potato plant's, or in their tuber yield, size and morphology, were observed, when compared with control plants.

The starch of all transformants expressing the SBD-containing gene fusions was subjected to Western dot blot analysis. The accumulation of the fusion protein was quantified, using the number representation from 0+ - 6+ (Fig. 2A), according to Ji *et al.* (2004). Figure 2B shows how the transformants are distributed over the various classes. The figure shows that, in both backgrounds, the transformants with a large amount of fusion protein (3+ - 6+) are most predominant. For the *amf* background, 82% and 89% of the SBD-MAT and MAT-SBD transformants, respectively, belong to the classes 3+ - 6+, whereas the corresponding values

are 73% and 77% for the Kardal background. With respect to the affinity for starch, the position (N- or C-terminal) of SBD in the fusion protein did not seem to matter much.



**Fig. 1.** Schematic representation of the different constructs used in this study. Maltose acetyltransferase (MAT) is expressed as such, and with starch-binding domain (SBD) fused to its N- or C-terminus. The three genes are driven by the potato granule-bound starch synthase I (GBSS I) promoter. Amyloplast entry was mediated by the GBSSI transit peptide. The entire expression cassettes are flanked by right border (RB) and left border (LB) respectively. ▲ and ↑ represent the kanamycin resistance gene and transit peptide cleavage site, respectively.

The amount of fusion protein in the potato juices of selected transformants was also quantified by Western dot blot analysis. For the *amf* series, only the transformants with granules in the 4+ class and higher contained the fusion protein in the juice; for the amylose-containing background, the fusion protein was only detected in transformants with starch granules in the 5+ and 6+ classes (Table 1). Apparently, the fusion proteins are present in the juice when they occur in large quantity in the granules. All transformants from the KDM and *amfM* series were classified into four different categories by Northern blot analysis (Fig.3). The figure shows that 22%, 39%, 22% and 18% of the KDM series, and 22%, 18%, 39% and 22% of the *amfM* series, can be classified as none (N), low (L), intermediate (M) and high (H) expressers, respectively.

**Table 1.** Overview of various parameters determined for the transgenic starches in both backgrounds.

| Clone           | Classification by |       | DS×10000          | AM (%)     | T <sub>onset</sub> (° C) | Mean (μm)   |
|-----------------|-------------------|-------|-------------------|------------|--------------------------|-------------|
|                 | Western dot blot  |       |                   |            |                          |             |
|                 | Granule           | Juice |                   |            |                          |             |
| KDUT            | Nd                | Nt    | 5.0               | 20.8±(0.1) | 63.7±(0.2)               | 18.7±(0.3)  |
| KDM4            | Nt                | Nt    | 5.6 <sup>ns</sup> | 20.8±(0.1) | 63.3±(0.1)               | 17.8±(0.1)  |
| KDM9            | Nt                | Nt    | 6.0 <sup>ns</sup> | 21.2±(0.1) | 62.8±(0.6)               | 19.0±(0.1)  |
| KDM27           | Nt                | Nt    | 7.2 <sup>ns</sup> | 21.3±(0.1) | 63.9±(0.1)               | 18.6±(0.2)  |
| KDM19           | Nt                | Nt    | 14.5**            | 19.3±(0.0) | 64.6±(0.0)               | 15.8±(0.4)  |
| KDMS23          | 3+                | Nd    | 4.3 <sup>ns</sup> | 21.0±(0.3) | 63.0±(0.1)               | 21.3±(0.3)  |
| KDMS2           | 5+                | 1+    | 7.5 <sup>ns</sup> | 20.8±(0.0) | 63.0±(0.2)               | 15.1±(0.2)  |
| KDMS29          | 6+                | 2+    | 10.7*             | 20.7±(0.4) | 62.5±(0.1)               | 15.6±(0.5)  |
| KDSM12          | 1+                | Nd    | 13.6**            | 21.0±(0.4) | 63.4±(0.0)               | 15.8±(1.1)  |
| KDSM8           | 2+                | Nd    | 19.0**            | 21.0±(0.1) | 64.0±(0.1)               | 14.9±(0.7)  |
| KDSM18          | 6+                | 2+    | 12.1**            | 20.7±(0.4) | 64.0±(0.1)               | 13.9±(0. 9) |
| <i>amf</i> UT   | Nd                | Nt    | 3.8               | 3.4±(0.1)  | 69.7±(0.0)               | 7.5±(0.2)   |
| <i>amf</i> M9   | Nt                | Nt    | 5.5 <sup>ns</sup> | 3.6±(0.2)  | 70.1±(0.1)               | 8.3±(0.2)   |
| <i>amf</i> M15  | Nt                | Nt    | 3.9 <sup>ns</sup> | 3.6±(0.3)  | 69.2±(0.2)               | 8.2±(0.2)   |
| <i>amf</i> M21  | Nt                | Nt    | 5.0 <sup>ns</sup> | 3.7±(0.2)  | 69.7±(0.1)               | 7.6±(0.1)   |
| <i>amf</i> MS21 | 4+                | 2+    | 2.4 <sup>ns</sup> | 3.7±(0.2)  | 71.0±(0.0)               | 4.5±(0.1)   |
| <i>amf</i> MS19 | 6+                | 2+    | 4.8 <sup>ns</sup> | 3.6±(0.1)  | 71.4±(0.1)               | 4.5±(0.1)   |
| <i>amf</i> SM18 | 5+                | 2+    | 2.5 <sup>ns</sup> | 3.8±(0.2)  | 70.3±(0.1)               | 6.8±(0.0)   |
| <i>amf</i> SM6  | 6+                | 2+    | 4.6 <sup>ns</sup> | 3.5±(0.1)  | 70.0±(0.2)               | 5.3±(0.3)   |

AM, apparent amylose content; DS, degree of substitution; Mean, mean granule size; Nd, not detected; Ns, not significant; T<sub>onset</sub>, temperature of onset of starch gelatinization. \* and \*\* significantly different from control at α=0.05 and α=0.01, respectively, using the analysis of variance (ANOVA) test for statistical analysis.

Selected transformants from the *amf*MS, *amf*SM, KDMS, and KDSM series were also analysed in this way. A comparison of results of the Western and Northern blot analyses for fusion proteins showed that the level of mRNA transcripts of fusion proteins did not always correlate with the amount of the accumulated protein in Western blot (data not shown).

**Are the MAT fusion proteins of the right size?**

To verify that the fusion proteins in transformants were of the correct size, protein extracts from the KDMS29 (6+) and KDSM18 (+6) transformants were subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Western blot analysis. Figure 4A shows the presence of a band of about 37 kDa, which corresponds

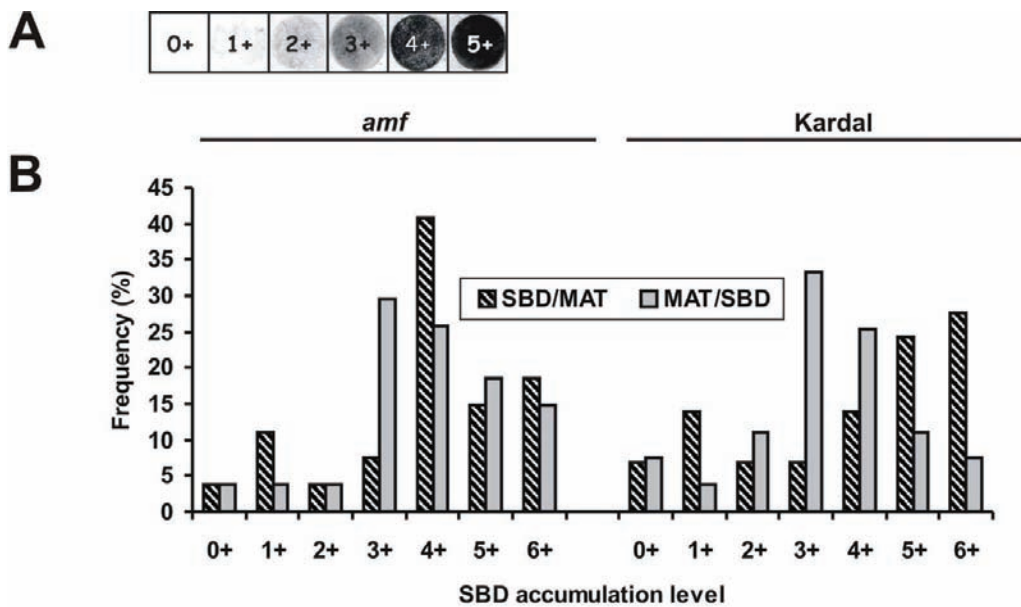
well with the predicted molecular mass of the MAT fusion proteins. MAT accumulation in the granules was also visualized by Coomassie staining. A band with a molecular mass of about 20 kDa was found, which corresponded well with the predicted molecular mass of 20,166 Da of MAT alone (Fig. 4B).

### **Is the starch derived from transgenic plants acetylated?**

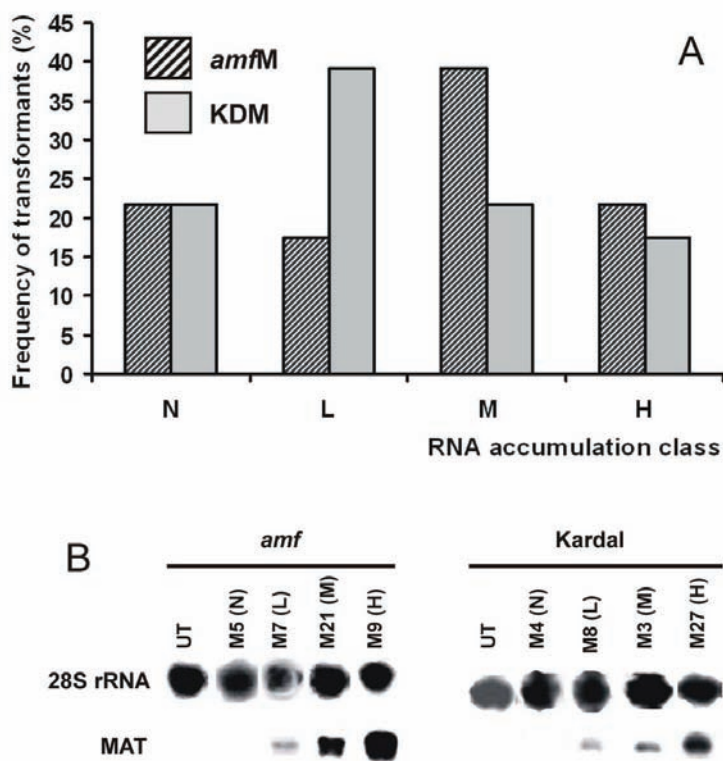
A calibration curve for the range  $2 \times 10^{-5}$  -  $2 \times 10^{-4}$  was made by dilution of an industrial, chemically acetylated sample with a DS = 0.02. An analysis of the acetyl content of starches from different transformants showed that the DS values of starches from all series of transformants were low, regardless of the type of construct. However, in all selected transgenic starches of the Kardal series, except one, the DS values were higher than those of the untransformed control starches (Table 1). Moreover, the DS values in the amylose-containing background was almost two-fold higher than that in the *amf* background. Furthermore, in some of the best transformants a three –four fold increase in acetylation was observed, although there did not seem to be a consistent correlation between DS, protein accumulation and RNA level (Table 1). The DS value of the transgenic starch with the highest acetylation was 10 times lower than that of the industrial sample (DS=0.02). It is unknown where in the starch granule the acetyl groups are attached; they may be attached to amylopectin, amylose, and/or maltooligosaccharides, which are entrapped in the granule. Given the low amount of acetylation, it is impossible to obtain a fraction enriched in acetylated  $\alpha$ -glucan to determine this unambiguously.

### **Is MAT incorporated in starch granules active?**

Our experiments so far have demonstrated that a large amount of MAT protein is present in starch granules, but this had not resulted in a high degree of *in planta* acetylation of the glucan polymers. Therefore, an important question was whether MAT retained its activity inside starch granules.



**Fig. 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.



**Fig. 3.** Gene expression levels of maltose-acetyltransferase (MAT) in transgenic potato plants of the amylose free (*amf*) and Kardal backgrounds. (A) Distribution of individual transformants over different classes of transgenic mRNA expression. The panel defines four different mRNA expression levels in transformants: none (N), low (L), medium (M), and high (H). (B) Northern blot analyses for selected KDM and *amfM* transformants. For all transformants 40 µg of total RNA was separated by gel electrophoresis, after transfer onto a nylon membrane and hybridized with a <sup>32</sup>P-labelled 551 bp MAT fragment. 28S rRNA was used as a loading control for classification.



It has previously been reported that glucose is the best acceptor molecule for MAT (Brand and Boos, 1991; Leggio *et al.*, 2003). Hence MAT activity was determined using acetyl-CoA as donor substrate and glucose as acceptor substrate. Based on Denyer *et al* (2001), it can be assumed that these substrates are sufficiently small to diffuse into the granule. Starches from different transformants belonging to the various protein accumulation classes were tested (Fig.5). In comparison with the untransformed controls, MAT activity was found in all transgenic starches tested. The *amf* starches showed higher MAT activity than those of the Kardal background (Fig.5). Moreover, the level of activity correlated with the amount of fusion protein in the granules. Within starches from each series of transformants, the high accumulators of the fusion protein always showed higher MAT activity than the lower accumulators, at least when transformants of the same genetic background (the ease with which substrates diffuse into the granule might be background-dependent), and plants transformed with the same construct (the position of SBD in the fusion protein may affect its activity), were compared. Surprisingly, a high MAT activity was found in the granules from the high expressers of the KDM and *amfM* series (KDM27 and *amfM9*), in which MAT did not have an appended SBD. This activity was comparable to that observed with 6+ class starches, in the respective backgrounds, containing the fusion protein. The acetylation activity in the KDM27 and *amfM9* starch granules suggests that MAT has affinity for the starch granules by itself.

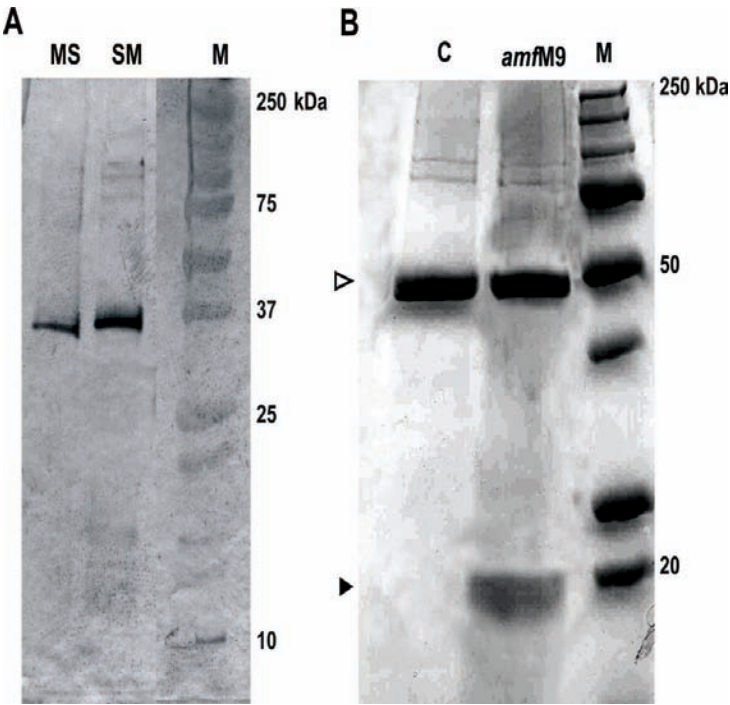
### **MAT activity on different acceptor substrates**

Because the MAT activity assay suffered from relatively high background readings for absorbance (partially because of the reagents themselves and, to a lesser extent, because the reagent can react with SH-groups present in granule-bound proteins), the validity of our results was verified by incubating the transgenic starch granules with different compounds. Some of these were known to be acceptor molecules for MAT (glucose and maltose), whereas others were known not to be acceptor molecules [glucose-1-phosphate (Glc-1P), glucose-6-phosphate (Glc-6P), ADP-glucose, fucose, and *myo*-inositol] (Leggio, *et al.* 2003). Glc-1P, Glc-6P, and ADP-glucose were of particular interest to us, because they are part of the starch biosynthetic pathway. Comparison of granule-bound MAT activity on different acceptor substrates showed that glucose was the best substrate of all substrates tested (Table 2). This was in accordance with observations of others (Brand and Boos, 1991; Leggio *et al.*, 2003).

**Table 2.** Activity of granule bound maltose acetyltransferase (MAT) ( $\mu\text{U}/\text{mg}$  starch) with different acceptor substrates. All reactions were performed with  $40\text{ }\mu\text{M}$  acetyl-CoA as the acetyl donor, and started with addition of acceptor substrate.

| Clone               | Acceptor substrate*   |               |               |        |        |     |
|---------------------|-----------------------|---------------|---------------|--------|--------|-----|
|                     | Glucose               | Maltose       | ADP-Glc       | Glc-1P | Glc-6P | NS‡ |
| <i>amfM9</i> (H)    | $4.5\pm(0.6)^\dagger$ | $3.7\pm(0.0)$ | $2.8\pm(0.3)$ | 0      | 0      | 0   |
| <i>amfMS19</i> (6+) | $2.5\pm(0.2)$         | $3.1\pm(0.4)$ | $1.5\pm(0.4)$ | 0      | 0      | 0   |
| <i>amfSM6</i> (6+)  | $4.9\pm(0.2)$         | $3.1\pm(0.2)$ | $1.2\pm(0.2)$ | 0      | 0      | 0   |
| KDM27(H)            | $2.6\pm(0.2)$         | $0.3\pm(0.2)$ | 0             | 0      | 0      | 0   |
| KDMS30(6+)          | $2.1\pm(0.4)$         | $0.1\pm(0.1)$ | 0             | 0      | 0      | 0   |
| KDSM18(6+)          | $2.5\pm(0.4)$         | $0.5\pm(0.2)$ | 0             | 0      | 0      | 0   |

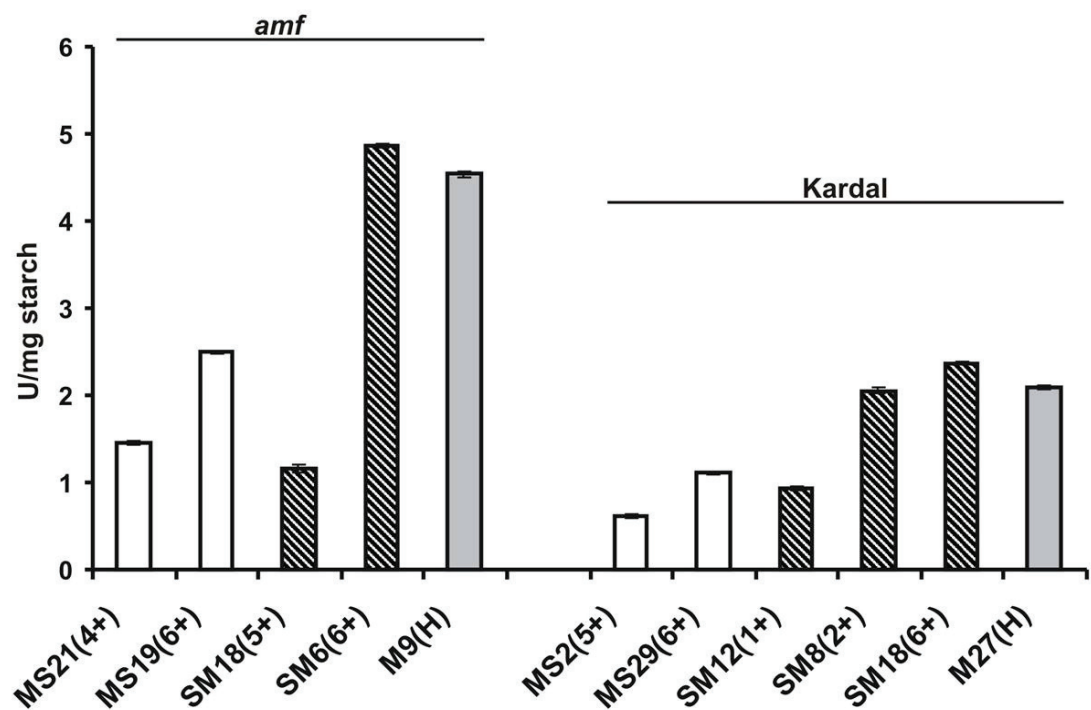
\* At an acceptor concentration of  $100\text{ mM}$ .  $^\dagger$ Values ( $\pm\text{SD}$ ) of at least two independent measurements were averaged; the values were corrected for the background absorbance of the corresponding untransformed control.  $^\ddagger$ Incubation was carried out without supply of an acceptor substrate.



**Fig. 4.** (A) Polyacrylamide (12%) gel electrophoresis of starch granule-bound fusion proteins of *amfMS19* (6+) and *amfSM6* (6+). Lane M shows the marker. MS and SM represent MAT-SBD and SBD-MAT fusion proteins, respectively. (B) Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis followed by Coomassie blue staining of a starch granule extract from the *amfM9* transformant. filled triangle indicates MAT, whereas the open triangle indicates the porcine pancreas  $\alpha$ -amylase. Lane c represents the untransformed *amf* control.

Typically, the activity with maltose was much lower with the starches from the Kardal background than with those from the *amf* background, whereas the activity with glucose was similar. Furthermore, granule bound MAT showed a low activity towards ADP-glucose in the *amf* background (in contrast with the results obtained by Leggio *et al.* (2003)), but not in the Kardal background. In the absence of an acceptor substrate, or in the presence of the non-acceptors Glc-1P, Glc-6P (Table 2), fucose and *myo*-inositol (data not shown), no activity was

found, providing further evidence that our assay truly detects MAT activity, despite the high background reading of the assay.



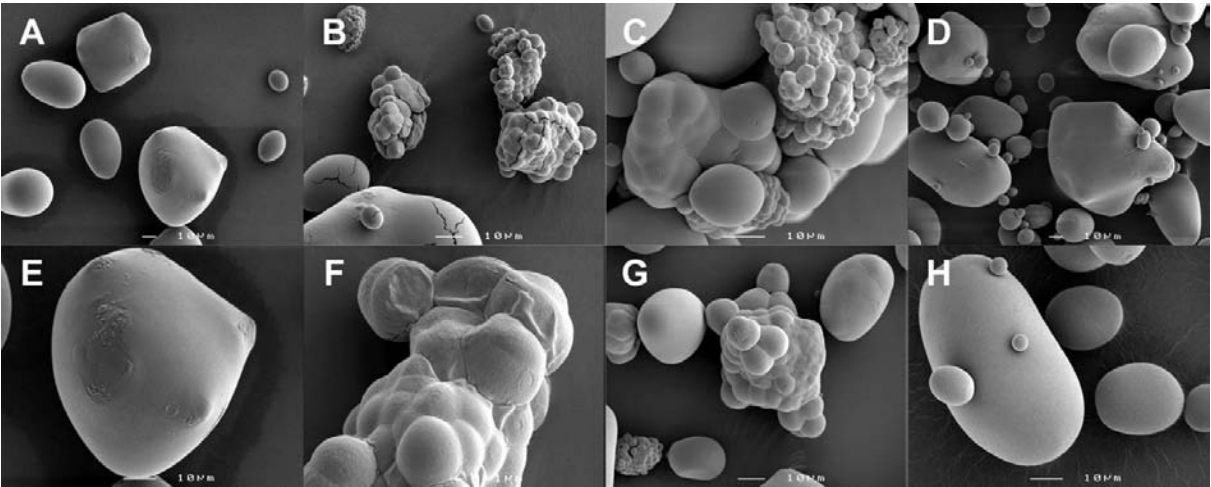
**Fig. 5.** Maltose-acetyltransferase (MAT) activity ( $\mu\text{U}/\text{mg}$  starch) with glucose as acceptor substrate. The white, hatched, and gray bars represent the granules from the MAT-SBD, SBD-MAT, and MAT series, respectively. SBD, starch-binding domain.

### MAT-SBD fusion protein affects granule morphology

Our experiments have shown that active MAT can be accumulated in large amounts in starch granules. We have previously observed that accumulation of foreign proteins in starch granules during their biosynthesis can affect starch granule morphology (Ji *et al.*, 2003; 2004). To investigate possible effects of MAT on granule morphology in the various transformants, light microscopy (LM) and scanning electron microscopy (SEM) were carried out. The transformants of the *amf*MS and KDMS series showed altered starch granule morphology (Fig. 6 and 7). Granules with cracks (KDMS23 and *amf*MS19) and amalgamated granules (KDMS23 and KDMS2) were abundant. The phenotypes of starches with approximately the same amount of fusion protein, but from different backgrounds, showed different morphologies (compare KDMS2 and *amf*MS19 in Fig. 6C,G and Fig. 7B,E,

respectively). It appears as though the amalgamated cluster phenotype is less pronounced in the *amf* background. The percentage of altered granules in the *MAT-SBD* transformants was correlated to the amount of fusion protein accumulated in the granules (Fig. 8).

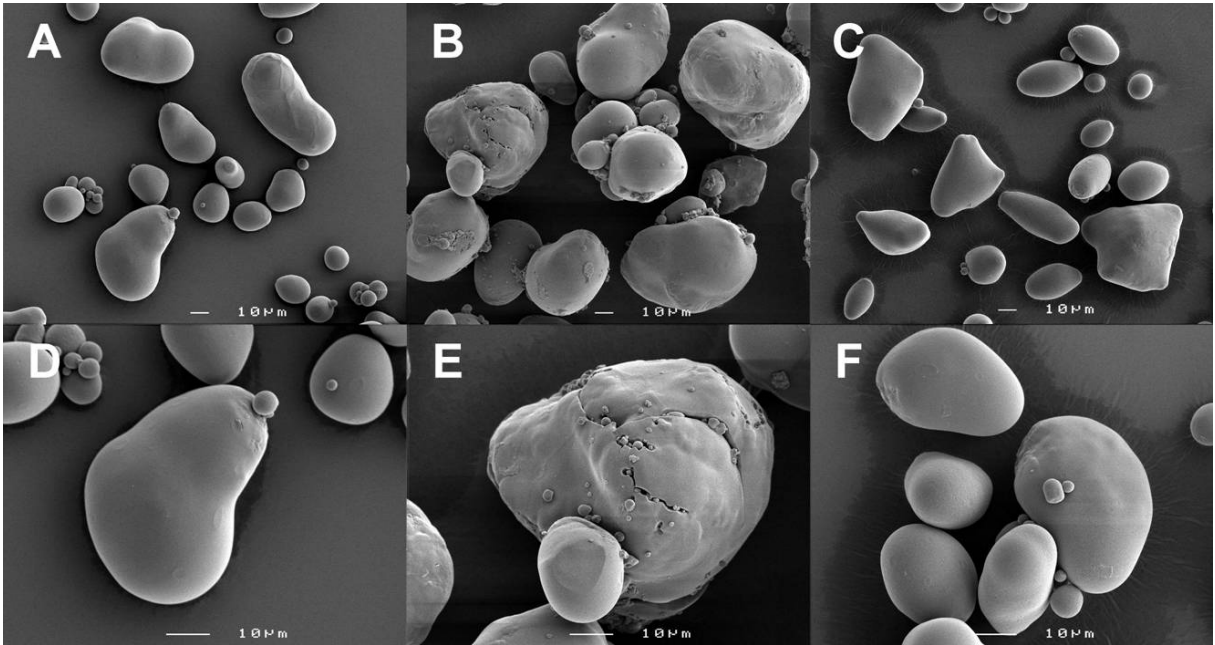
Irrespective of protein level, altered phenotypes were observed for almost every KDMS transformant; however, in the *amf* background, the alteration was only seen in the 3+ class and higher (Fig. 8). In other words, 21 transformants of the 27 counted (78%) in *amf*MS showed altered starch phenotypes. The granule morphology of starch containing SBD-MAT or MAT alone, even the high expressers (KDSM18 and *amf*M9, respectively), was similar to that of control plants, indicating that not only is the simultaneous presence of SBD and MAT important for altering granule morphology, but also their order in the fusion protein.



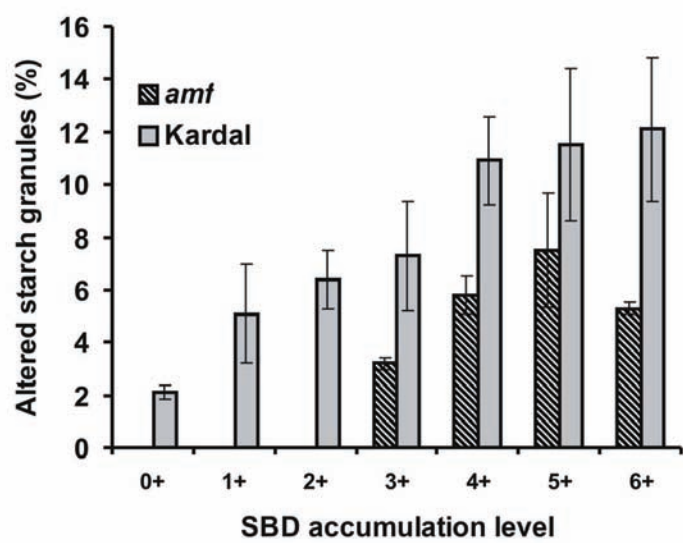
**Fig. 6.** Scanning electron microscopy (SEM) analyses of starch granules at different magnifications in the Kardal background. (A-D) (×1000) and (E-H) (×4000) for KD-UT (A , E), KDMS23 (3+; B , F), KDMS2 (5+; C, G) and KDSM18 (6+; D, H).

**Starch granule size, gelatinization properties and amylose content**

Possible effects of MAT expression, as well as expression of fusion proteins, on physicochemical properties of starch such as gelatinization characteristics [onset temperature of starch granule melting ( $T_{onset}$ ), enthalpy released ( $\Delta H$ ) (data not shown)], apparent amylose content and mean granule size were measured for selected transformants. These results are summarized in Table 1. No consistent differences in these parameters were found for the selected samples compared with the control starches.



**Fig. 7.** Scanning electron microscopy (SEM) analyses of starch granules at different magnifications in the *amf* background. (A-C) ( $\times 1000$ ), (D-F) ( $\times 4000$ ) for *amf*-UT (A, D), *amf*MS19 (6+; B,E) and *amf*M9 (RNA class H; C, F).



**Fig. 8.** Percentage of granules with altered morphology for the various classes of starch-binding domain (SBD)-containing fusion proteins in both backgrounds. A population of 100 starch granules in triplicate was counted. Bars indicate mean  $\pm$ SD. *amf*, amylose-free.

### Discussion

In this study, the *MAT* gene from *E. coli*, alone or with an appended N- or C-terminal SBD, was expressed in normal and *amf* potato plants to test the possibilities for *in planta* acetylation of starch as an alternative to chemical derivatization. In this study we found evidence for *in planta* starch acetylation, albeit at a low level. Furthermore, it was shown that



MAT is active inside starch granules, and also that starch granule morphology is affected. These observations are discussed in more detail below.

### **MAT is active in granules and acetylates starch *in planta* to a limited extent**

MAT was found to be active inside starch granules (Fig. 5), even without an appended SBD. However, this did not result in extensive acetylation of the starch polymers, particularly in the *amf* background, although the starch granules of some of the Kardal transformants seemed to contain acetyl groups. There may be several reasons for the poor acetylation *in planta*. First, it is possible that the acetyl-CoA pool is limiting in potato amyloplasts, and, consequently, MAT has a limited supply of donor substrate. The fact that potato starch has one of the lowest lipid contents of the known starch sources corroborates this (Vasanthan and Hoover, 1992), as acetyl-CoA serves as the starting point of fatty acid biosynthesis (Sasaki and Nagano, 2004). Second, this might be related to the fact that MAT activity decreases with increasing length of the  $\alpha$ -glucan acceptors (Leggio *et al.*, 2003). Maltotriose is acetylated 20 times more slowly than maltose, and maltotetraose about 200 times more slowly. From this, it might be expected that longer maltodextrins, amylose, and amylopectin will be acetylated even more slowly. This is corroborated by our finding that incubation of MAT-containing starch granules with acetyl-CoA, but without an appropriate acceptor, did not lead to an increased acetylation of the starch within 48 hours.

The activity of MAT appeared to be dependent on the potato background (Table 2). The activity with the acceptor substrate glucose was similar for both backgrounds. However, the activity with maltose and ADP-glucose was negligible in the Kardal background, whereas in the *amf* background these molecules were used as acceptor. This might be explained by the fact that both ADP-glucose and maltose are substrates (donor and acceptor, respectively) for granule-bound starch synthase I (GBSSI), whereas glucose is not (Denyer *et al.*, 1996). GBSSI is present in starch granules from the Kardal background, whereas it is absent in the mutant *amf* starch (Van der Leij *et al.*, 1991). By binding to ADP-glucose or maltose, GBSSI might decrease the availability of these substrates for MAT, leading to a much lower rate of acetylation. Another possibility might be that the diffusion of ADP-glucose and maltose into amylose-containing starch granules is slower than that into amylose-free granules.

Although the Kardal starch particular was found to contain acetyl groups, the starch properties did not deviate from that of the control plants (Table 1). It is possible that the acetyl

groups found are not part of amylopectin, but are present on small malto-oligosaccharides (MOS), which are subsequently elongated by GBSSI, after which they can not escape from the granule. This is consistent with MAT's preference for smaller acceptor molecules (Leggio *et al.*, 2003) and our post-harvest acetylation experiments. Furthermore, it may explain why a low degree of acetylation was found in the granules from the Kardal but not from the *amf* background: in the latter, acetylated malto-oligosaccharides are not elongated and can escape from the granule during starch biosynthesis or starch isolation.

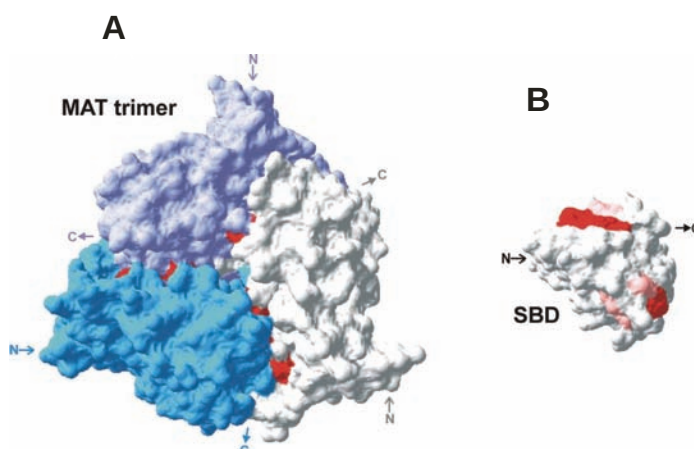
Taken together, our results show that an active MAT can successfully be targeted to the starch granules; therefore, there may be opportunities for *in planta* starch acetylation. The possibly limiting supply of acetyl-CoA may be solved by introducing MAT-containing constructs in plants such as maize or wheat, which are known to contain larger amounts of fatty acids in their starch. Furthermore, it might be possible to improve MAT's capabilities for utilizing longer acceptor substrates. The finding that NodL, an acetyltransferase with high sequence similarity to MAT (and likely to have a similar three-dimensional structure), prefers oligosaccharides over mono- to trisaccharides as an acceptor substrate (Leggio *et al.*, 2003), may offer opportunities in this direction. Future studies on the *in planta* acetylation of starch should consider both acetyl-CoA availability and improvement of MAT's activity towards longer acceptors. The importance of the former needs to be established experimentally, whereas there seems to be sufficient evidence for the importance of the latter. It is probable that success involving *in planta* starch acetylation can only be achieved by combining these two aspects, which is probably best served by expressing an optimized MAT gene (when available) in a cereal background. In such an investigation, it should be re-evaluated whether appending an SBD to MAT can increase acetylation of starch.

### **MAT has affinity for starch granules, and can alter starch morphology**

The morphology of the granules in both backgrounds was severely altered when MAT-SBD was accumulated in starch granules. Neither SBD-MAT, nor MAT alone, had a similar effect. It seems unlikely that MAT activity is responsible for the differences in the starch granule morphology. We have observed previously that the incorporation of non-catalytic proteins, SBD2, into starch granules can affect granule morphology (Ji *et al.*, 2004; Nazarian Firouzabadi *et al.*, 2006). Interestingly, our results indicate that MAT-SBD (and also SBD-MAT) has a higher affinity for starch granules than SBD2, because a larger percentage of the

KDMS and *amf*MS transformants belonged to the higher accumulation classes (3+ - 6+) in comparison with KDSS and *amf*SS (two series of SBD2 transformants) (Nazarian Firouzabadi *et al.*, 2006), despite the fact that MAT-SBD has only one copy of SBD, and SBD2 has two. In the *amf* background, about 60% of the transformants belonged to the higher classes, whereas this was about 85% for MAT-SBD; in the Kardal background, these values were 27% and 75%, respectively.

The unexpected high content of MAT-SBD in starch granules may originate from the quaternary structure of MAT. It has been shown that MAT spontaneously forms a trimer (Fig. 9) in aqueous solution (Leggio *et al.*, 2003), which is a common structural feature of the enzymes belonging to the hexapeptide acyltransferase (HexAT) superfamily (Wenzel *et al.*, 2005). At the interface of two monomeric units, a tunnel is formed in which the acceptor substrate maltose can be accommodated. Thus, the trimeric MAT can bind three maltose molecules simultaneously, with their non-reducing end pointing to the outside of the trimer (to the catalytic residues His113, the outermost residues indicated in red in each subunit).



**Fig. 9.** Typical trimeric organization of maltose acetyltransferase (MAT) (A) and three-dimensional structure of starch binding domain (SBD) (B). For MAT, the residues highlighted in red are involved in substrate-binding (N83 and F84; Wenzel *et al.*, 2005), and catalysis (H113; Leggio *et al.*, 2003). The three monomeric units are represented by different colours. For SBD, aromatic amino acids playing a pivotal role in sugar-binding (W616, Y633, W662) are indicated in bright red. Less important amino acids in sugar-binding (A599, G601, N627, K651, N667) are indicated in light red. Numbering of the amino acid residues of SBD refers to the amino acid sequences of the *Bacillus circulans* cyclodextrin glycosyltransferase (Lawson *et al.*, 1994). N and C indicate the N- and C-termini of the proteins.

Because the activity of MAT decreases rapidly with acceptor substrates longer than two glucosyl residues and maltose is acetylated at the C6 position of the non-reducing glucosyl residue (Leggio *et al.*, 2003), this indicates that glucan chains will not enter the tunnel at one side and exit at the other, but rather will interact at their non-reducing chain ends with an



interaction ranging no more than two glucosyl residues. Such a 3×2 interaction is apparently sufficient for MAT to bind glucans and be incorporated in starch granules, because we have demonstrated MAT activity with transgenic starch granules. We can not exclude the possibility that MAT binds as a separate unit. With an appended SBD an even higher-affinity trimer will be obtained, although our data do not allow us to compare the amount of protein present in starch granules of the highest expressers of the MAT and MAT-SBD series. We hypothesize, that after entry into the amyloplast MAT-SBD will assemble into a trimer (the N- and C-terminus of the monomeric subunits are far enough apart from the interface at which they interact; see Fig. 9), in which the core of the trimer binds glucans as described above, and the three exposed SBDs may bind the non-reducing termini of double helical amylopectin side chains, suggested by Morris *et al.* (2005). In principle, the protein complex may be regarded as SBD3<sup>PLUS</sup>, which may explain that it has a higher affinity for starch granules than SBD2 and SBD3 (Nazarian Firouzabadi *et al.*, 2006). Despite its high affinity for starch granule, the trimer does not appear to influence amylose biosynthesis by GBSSI, e.g. by competing for binding sites on the granular surface. This might suggest that GBSSI binds a different domain of the starch granule from MAT, which most likely interacts with the non-reducing ends of amylopectin side chains, or that GBSSI binds α-glucan in a different way.

## Experimental procedures

### Preparation of constructs

Three different constructs were made in this study. The pBIN19/MAT-SBD and pBIN19/SBD-MAT plasmids were used for the expression of the MAT-SBD and SBD-MAT fusion proteins in potato plants (Kardal and *amf*), respectively. The pBIN19/MAT plasmid was used as a control in which MAT was directed to the amyloplast without granule-targeting sequence. To obtain the pBIN19/MAT-SBD plasmid (Fig. 1), a MAT-encoding fragment was obtained by PCR amplification with the primers 5'-CCATGGGCACAGAAAAAG-3' and 5'-AGATCTTTTTTTAATTATACTGGC-3', which contained *NcoI* and *BglII* sites at their 5' ends, respectively. *E. coli* genomic DNA was used as a template for PCR. The amplified fragment was used to replace the first SBD fragment (also *NcoI*-*BglII*) in the pUC19/SBD2 (Ji *et al.*, 2004), giving the pUC19/MAT-SBD plasmid. After digestion of this plasmid, the *HpaI*-*SacI* fragment was inserted into the corresponding sites of the pBIN19/SBD2 to generate the

pBIN19/MAT-SBD. The predicted molecular mass of the MAT-SBD produced in plants is 33,986 Da, excluding transit peptide. The pBIN19/SBD-MAT plasmid (Fig. 1) was assembled from the following fragments: (1) GBSSI promoter, GBSSI transit peptide and SBD-linker fragment (*HindIII-SalI*) obtained from the pUC19/SBD2 plasmid, (2) a MAT fragment (*SalI-XbaI*), and (3) a NOS terminator (*BamHI-KpnI*). The MAT-encoding fragment was amplified by PCR using the genomic DNA as the template with the primers 5'-TTGAGTCGACGATGAGCACAGAAAAAGAAAAC-3' and 5'-GAAGTCTCTAGAGTTACGATTTTTTGATTATTATGGCTGG-3', which contained *SalI* and *XbaI* sites at their 5' ends, respectively. The predicted molecular mass of the SBD-MAT produced in plants is 33,864 Da, excluding transit peptide. For making the pBIN19/MAT plasmid (Fig. 1), a MAT-encoding fragment was obtained by PCR amplification with the primers 5'-CCATGGGCACAGAAAAAG-3' and 5'-GAAGTCTCTAGAGTTACGATTTTTTGATTATTATGGCTGG-3', which contained *NcoI* and *XbaI* sites at their 5' ends, respectively. This amplified fragment was introduced into the plasmid pUC19/SBD-MAT, which was opened by *NcoI* and *XbaI* restriction enzymes, giving the pUC19/MAT plasmid. After digestion of this plasmid, the *HpaI-SacI* fragment was inserted into the corresponding sites of the pBIN19/SBD-MAT to generate the pBIN19/MAT. The predicted molecular mass of the MAT produced in plants is 20,166 Da, excluding transit peptide. All constructs were sequenced to verify their correctness.

### Potato transformation

The pBIN19 constructs were transformed into electro-competent cells of *Agrobacterium tumefaciens* strain LBA4404 by electroporation (Takken *et al.*, 2000). A wild type potato cultivar (Kardal) and an amylose-free (*amf*) potato, lacking a functional GBSSI, were used for *Agrobacterium*-mediated transformation. Plantlets grown on MS30 selection medium (Murashige and Skoog, 1962), containing kanamycin (100 mg/L), were harvested and multiplied; five plantlets of each clone were subsequently taken to the greenhouse to generate tubers.

### Starch isolation

All tubers derived from the five plants of each greenhouse-grown clone were combined,

and their skins were removed in an *IMC Peeler* (Spangenberg, The Netherlands). Starch from different transformants was isolated with a Sanamat rotor (Spangenberg, The Netherlands) according to Ji *et al.* (2003), after addition of Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> (0.01% w/w). Starch in the collected juices was allowed to settle at 4° C for at least 3 h. A portion of the juice was stored at –20 °C. The settled starch was washed at least 3 times and passed through Whatman filter paper (Whatman international Ltd, Maidstone, kent, UK) placed in a Buchner funnel. All starch samples were air dried and kept at room temperature.

### **Determination of the acetyl content of starch**

Twenty milligrams of starch was saponified with 400 µL of 50 mM sodium hydroxide for 6 h at 40 °C. Saponified samples were neutralized with 50 mM citric acid (Chen *et al.*, 2003), and centrifuged at 10,000 *g* for 5 min. The released acetic acid was determined in duplicate, using an acetic acid UV-kit (Boehringer, Mannheim, Germany), according to the specifications of the manufacturer. A calibration curve was obtained, using chemically acetylated samples from AVEBE (The Netherlands) with a known degree of substitution of 0.02.

### **Determination of the content of fusion protein in starch granules**

The amount of SBD-containing fusion protein accumulated in transgenic starch granules was quantified by Western dot blot analysis as described by Ji *et al.* (2003). The potato tuber juice analyzed for the presence of SBD as described by Ji *et al.* (2003).

### **Electrophoretic separation of granule proteins**

Fifty mg of starch from two selected transformants, KDMS29 (6+) and KDSM18 (6+), was boiled for 2 min in a mixture of 1 mL of SDS and 5% β-mercaptoethanol (v/v). The gelatinized samples were centrifuged for 10 min at 14,000 × *g*. 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-SBD as described by Ji *et al.* (2003). The same amount of *amf*M9 (H, high expresser) starch was treated with α-amylase and concentrated

with a Microcon column YM-10 according to the instructions of the manufacturer (Millipore, USA). The concentrated sample (25  $\mu$ L) was analyzed as described above.

### **Northern blot analysis**

Relative expression of mRNA was determined by using Northern blot analysis as described by Kuipers *et al.* (1994). Total RNA from 5 g of ground tubers was isolated with 3 mL RNA extraction buffer (2% SDS, 10 mM EDTA, 50 mM Tris, pH 9.0, and 3 ml phenol). After centrifugation ( $1,300 \times g$ , 10 min), the supernatant was extracted with 3 mL phenol/ $\text{CHCl}_3$ /isopropanol alcohol (25:24:1). Subsequently, RNA was precipitated with 3 mL isopropanol. The pellet was re-suspended in 1125  $\mu$ L water. RNA was precipitated by the addition of 375  $\mu$ L 8 M LiCl (0  $^{\circ}\text{C}$ , overnight). After centrifugation ( $7,500 \times g$ , 10 min), the pellet was dissolved in 400  $\mu$ L of water and re-precipitated with 40  $\mu$ L of 3 M sodium acetate (pH=5.0) and 1 mL ice-cold ethanol. The pellet was dissolved in 50  $\mu$ L of water. Based on spectrophotometric RNA determination, similar amounts of total RNA were fractionated on a 1.5% (w/v) agarose-formaldehyde gel, and transferred onto a Hybond N+ nylon membrane (Amersham, UK). The membrane was hybridized with a [ $^{32}\text{P}$ ]-labeled MAT gene fragment as a probe; labeling was performed with a rediprime II kit (Amersham), according to the instructions of the manufacturer.

### **Analysis of physiochemical properties of starches from different transformants**

Granule morphology was investigated by both light microscopy (LM; Axiophot, Germany) and scanning electron microscopy (SEM; JEOL 6300F, Japan). A solution of a 20x diluted Lugol's solution (1%  $\text{I}_2/\text{KI}$ ) was used to stain starch granules. For SEM, 1 mg of dried starch was mounted onto brass holders with double-sided sticky carbon tape (EMS, Washington, USA), sputter coated with gold, (Edwards S150B, Crawley, England), and subsequently transferred into a FESEM (JEOL JSM-6300F, Tokio, 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.* (1998).

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 Ji *et al.* (2003). The Tonset and the enthalpy ( $\Delta H$ ) were calculated automatically. Granule size distributions were measured with a Coulter Counter Multisizer II, equipped with an orifice tubes of 100  $\mu\text{m}$  or 200  $\mu\text{m}$  (Beckman-Coulter, UK), as described by Ji *et al.* (2003). All samples were measured at least in triplicate.

### **Determination of MAT activity in transgenic starch granules**

An activity assay was carried out in a final volume of 250  $\mu\text{L}$ , consisting of 25  $\mu\text{L}$  of assay buffer (50mM potassium phosphate (pH 7.5) and 2 mM EDTA), 25  $\mu\text{L}$  of 1 M glucose, 10  $\mu\text{L}$  of 1 mM acetyl-CoA, 190  $\mu\text{L}$  of demineralized water, and 20 mg of the various (transgenic) starches. This mixture was incubated for 1 h at 25 °C with continuous shaking. Subsequently, 25  $\mu\text{L}$  of freshly made 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) was added. Acetyl-CoA consumption was immediately quantified spectrophotometrically (412 nm;  $\epsilon=13,600 \text{ M}^{-1} \text{ cm}^{-1}$ ; Ultraspec 2000, Pharmacia Biotech) as described by Leggio *et al.* (2003). Values for starches from untransformed plants served as a control, and their average was subtracted from the values obtained with the transgenic starch samples.

In a similar way, MAT activity was determined with different acceptor substrates; in these experiments, 1 M of maltose, ADP-glucose (ADP-Glc), glucose-1-phosphate (Glc-1P), glucose-6-phosphate (Glc-6P), fucose and *myo*-inositol were used instead of glucose. Furthermore, experiments were done without the addition of an acceptor substrate, to investigate whether the starch granules themselves could be used as an acceptor substrate.

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# Chapter 4

## **Fusion proteins comprising the catalytic domain of mutansucrase and a starch-binding domain can alter the morphology of amylose-free potato starch granules during biosynthesis**

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*Transgenic Research: In press*

## Abstract

**I**t has been shown previously that mutan can be co-synthesized with starch when a truncated mutansucrase (GtfICAT) is directed to potato tuber amyloplasts. The mutan seemed to adhere to the isolated starch granules, but it was not incorporated in the starch granules. In this study, *GtfICAT* was fused to the N- or C-terminus of a starch-binding domain (SBD). These constructs were introduced into two genetically different potato backgrounds (cv. Kardal and *amf*), in order to bring GtfICAT in more intimate contact with growing starch granules, and to facilitate the incorporation of mutan polymers in starch. Fusion proteins of the appropriate size were evidenced in starch granules, particularly in the *amf* background. The starches from the various GtfICAT/SBD transformants seemed to contain less mutan than those from transformants with GtfICAT alone, suggesting that the appended SBD might inhibit the activity of GtfICAT in the engineered fusion proteins. Scanning electron microscopy showed that expression of SBD-GtfICAT resulted in alterations of granule morphology in both genetic backgrounds. Surprisingly, the *amf* starches containing SBD-GtfICAT had a spongy appearance, *i.e.* the granule surface contained many small holes and grooves, suggesting that this fusion protein can interfere with the lateral interactions of amylopectin sidechains. No differences in physico-chemical properties of the transgenic starches were observed. Our results show that expression of granule-bound and “soluble” GtfICAT can affect starch biosynthesis differently.

**Key words:** mutan, glucansucrase, starch-binding domain, granule-boundness, transgenic potato, microscopy, granule morphology.

We have shown, in our previous study, that expression of the truncated mutansucrase gene *GtfICAT* (*Gtfl* without a glucan-binding domain) in potato amyloplasts led to a more efficient production of mutan in comparison with that of the full-length *Gtfl* (Kok-Jacon *et al.*, 2005a). Mutan production by *GtfICAT* was accompanied with pronounced morphological and physicochemical alterations at the tuber and starch levels. However, from this study, the presence of mutan inside starch granules was not evidenced. We hypothesized that even more dramatic effects on starch granule morphology and properties might be obtained, if mutan could be incorporated in the starch granule. This might be achieved by bringing *GtfICAT* and the growing starch granule in intimate contact with each other. For this purpose, we have engineered two genes in which a starch-binding domain (SBD) was fused to either the 5' or the 3' end of *GtfICAT*. It has been shown before that SBD is an efficient tool for targeting effector proteins to the growing starch granule (Ji *et al.*, 2003; 2004). The use of SBD technology may offer three advantages. (i) Mutan will be produced close to the granule surface, which will increase the chance that it is co-crystallized with starch during granule formation. (ii) The more intimate contact between *GtfICAT* and starch may facilitate the so-called acceptor reaction of the enzyme (Monchois *et al.*, 2000), which in turn may lead to covalent attachment of mutan to starch. (iii) Mutan may be produced post-harvest by supplying sucrose, a cheap and abundant substrate, to transgenic starch granules containing the fusion proteins (Kok-Jacon *et al.*, 2003).

In this study, we investigate if starch granule properties can be more severely affected with a granule-bound *GtfICAT* than with a "soluble" *GtfICAT*. For this, SBD was fused to the N- or C-terminus of *GtfICAT*, since our previous studies have pointed out that the position of the SBD in the fusion protein can influence the activity of the appended effectors. The *amf* background was used for transformation, because we have demonstrated before that accumulation of SBD was most efficient in the amylose-free (*amf*) potato background (Ji *et al.*, 2003; 2004; Nazarian *et al.*, 2006a). Moreover, the constructs were introduced into the wild type (Kardal) background, in order to facilitate a more direct comparison with the *GtfICAT* potato transformants that had already been made (Kok-Jacon *et al.*, 2005a).

## Results

The SBD-*GtfICAT* and *GtfICAT*-SBD fragments were cloned in frame to the plastidic protein targeting sequence (FD) (Gerrits *et al.*, 2001), and driven by the highly tuber-

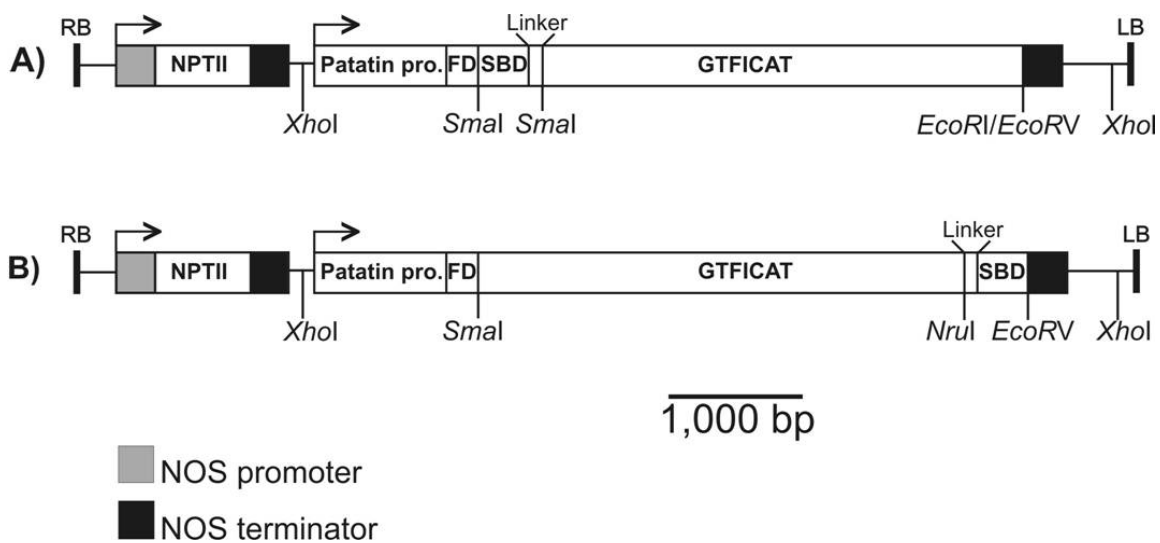
expressed patatin promoter (Wenzler *et al.*, 1989), resulting in the pPFSBDIC and pPFICSBD constructs (Fig. 1). These constructs were used for *Agrobacterium*-mediated transformation of amylose-containing [cv. Kardal (KD)] and amylose-free (*amf*) mutant potato plants; thirty independent transgenic potato clones, per background and construct, were obtained, which were named KDSICxx, KDICSxx, *amf*SICxx and *amf*ICSxx. SIC and ICS represent the SBD-*GtfICAT* and *GtfICAT*-SBD genes, respectively, and xx the clone number. The untransformed genotypes are referred to as KD-UT and *amf*-UT. During the growth period in the greenhouse, the transgenic plants were morphologically similar to the controls (data not shown).

### Classification of transformants by RT-PCR analysis

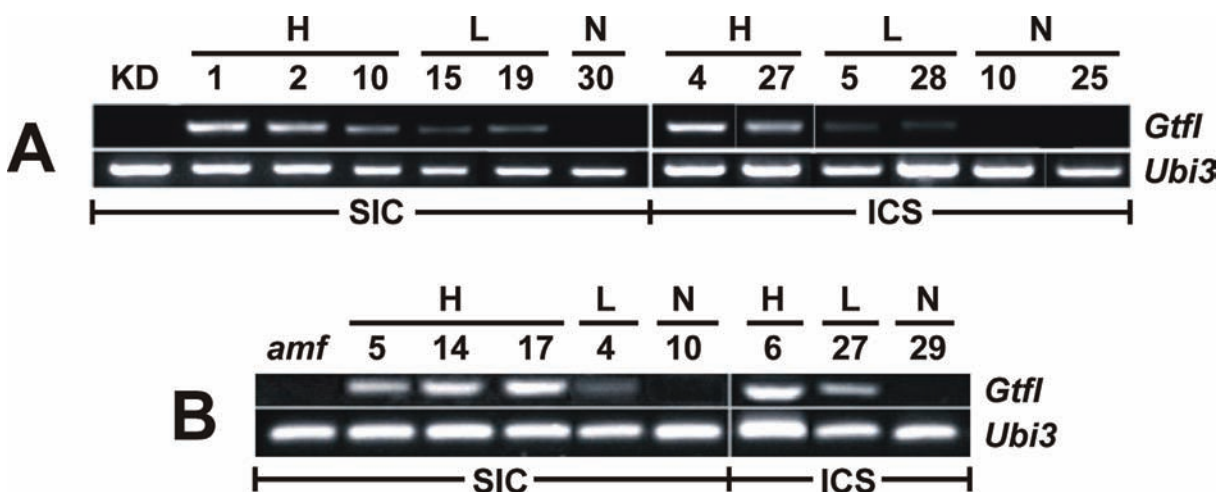
*GtfICAT* expression was determined by RT-PCR analysis. Based on the band intensity of the PCR products, the transformants were divided in different classes (Fig. 2). The ubiquitin-ribosomal gene expression (*Ubi3*), which is known as constitutive (Garbarino and Belknap, 1994), was used as an internal control. The (N), (L), and (H) classes represent plants with no, low, and high levels of mRNA, respectively. From the KDSIC series, the KDSIC30 (N), KDSIC15 (L), KDSIC19 (L), KDSIC1 (H), KDSIC2 (H), and KDSIC10 (H) transformants were selected for further analysis; from the *amf*SIC series, *amf*SIC10 (N), *amf*SIC4 (L), *amf*SIC5 (H), *amf*SIC14 (H), and *amf*SIC17 (H) (see Fig. 2A,B) were selected. From the KDICS series, the KDICS10 (N), KDICS25 (N), KDICS5 (L), KDICS28 (L), KDICS4 (H), and KDICS27 (H) transformants were subjected to further characterization; from the *amf*ICS series, *amf*ICS29 (N), *amf*ICS27 (L), and *amf*ICS6 (H) (see Fig. 2A,B) were selected. Given the intensity of the *Ubi3* PCR product, KDICS5 might also be assigned to the (H) class. As expected, no *GtfICAT* mRNA was detected in the KD-UT and the *amf*-UT control plants.

### Accumulation of SBD-containing proteins in the transformants

In the selected transformants, the amount of SBD-containing protein in the granule was analyzed by Western dot blot analysis. Quantification was performed by using starch from the (+), (++) and (+++) classes of the *amf*S series of transformants (Ji *et al.*, 2003) for calibration. Subsequently, the dot intensities obtained for the starches of the selected transformants were



**Fig. 1.** Schematic representation of pPFSBDIC (A) and pPFICSBD (B) binary vectors used for potato plant transformation.



**Fig. 2.** RT-PCR analysis of the selected transformants in Kardal (A) and *amf* (B) and their corresponding untransformed control plants. In each series, the upper panels show the PCR products using the primers designed on the *Gtfl* sequence. The lower panels show the PCR products using the primers designed on the *Ubi3* sequence, and served as an internal control.

compared with these positive controls. Starch from *amf* transformants not only showed higher SBD accumulation than those from Kardal, but also the number of plants with detectable SBD protein was higher, showing that more fusion protein could be incorporated into the *amf* starches compared with the Kardal starches. The *amf*SIC5, *amf*SIC14, *amf*SIC17, and *amf*ICS6 starches showed a dot intensity comparable with that of the (++) class, whereas the dot intensities of the KDSIC10 and *amf*SIC4 starch were comparable with that of the (+)

class. The starch of the other selected transformants showed a dot intensity comparable with that of either KD-UT or *amf*-UT, in which no SBD-containing protein was present. The results of the Western dot blot analysis correlated well to the results obtained by RT-PCR analysis. The tuber juices of the selected transformants were also analyzed for the presence of the fusion protein. None of the juices gave a positive response in the Western dot blot assay, indicating that the amount of SBD-containing protein was too low to be detected.

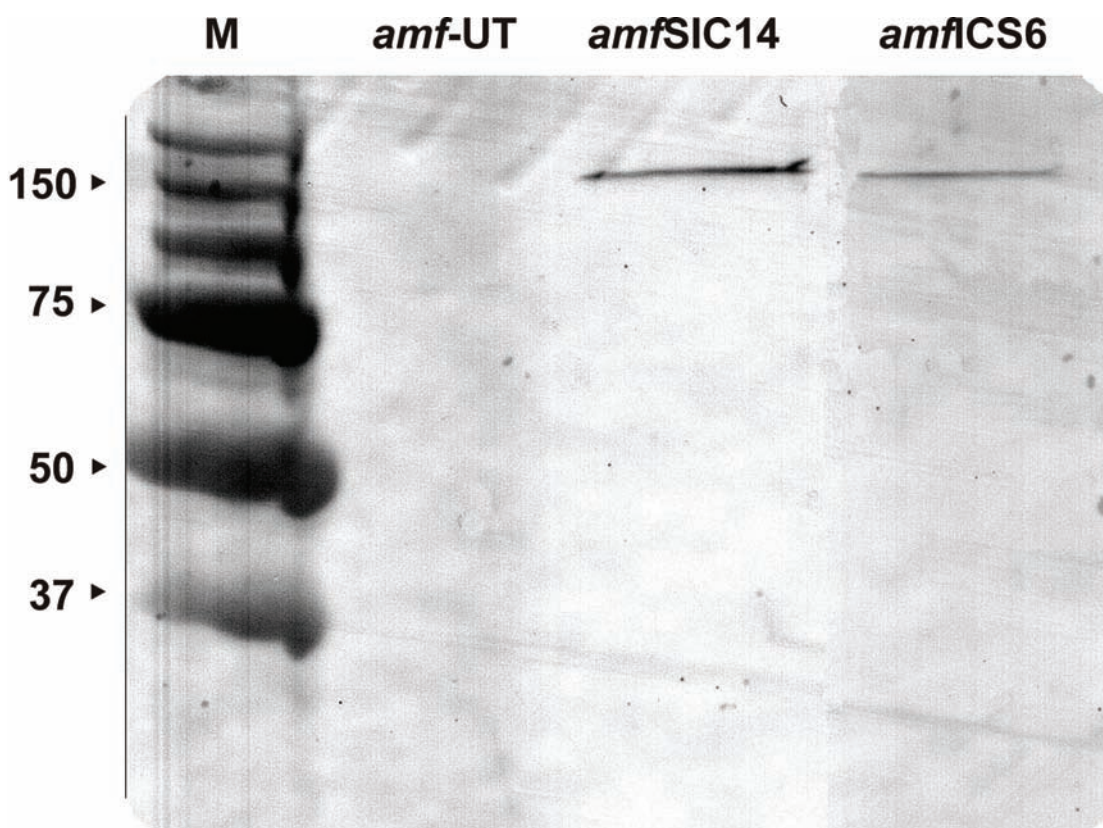
### **SBD-containing fusion proteins of expected size in starch granules**

In order to determine whether the fusion proteins in the starch granules had the correct size, the granule proteins of the *amf* starches with the highest SBD content (*amf*SIC14 and *amf*ICS6, representing both series) were separated by SDS polyacrylamide gel electrophoresis, followed by Western blotting using the SBD antibody (Fig. 3). For both starches, a band of almost 140 kDa was found, which corresponded well with the predicted molecular weight of 125 kDa for the fusion proteins (excluding the transit peptide). This showed that the fusion protein was correctly imported into the amyloplast, and incorporated into the starch granules.

### **Mutan is detected in the (H) and (L) classes of KDSIC transformants**

As described previously (Kok-Jacon *et al.*, 2005a), mutan can be visualized using an erythrosine red dye (Fig. 4). A purified mutan (Wiater *et al.*, 1999) was used as a positive control (Fig. 4B). A number of surfaces of starch granules from the (H) class (Fig. 4C), and from (L) classes (data not shown), of the KDSIC series coloured red, indicating the presence of mutan. Starch granules from the (N) class transformants did not stain with the erythrosine dye (data not shown), similar to those from the *amf*-UT and KD-UT controls (Fig. 4A). For none of the *amf*ICS, *amf*SIC, and KDICS starch granules, a red colouration was observed upon erythrosine treatment (data not shown). KDSIC10 starch granules were exhaustively treated with exo-mutanase in order to detach the mutan from the granule surface. From previous results (Kok-Jacon *et al.*, 2005a), it was shown that the 48 h incubation time was sufficient for detaching mutan from the granule surfaces (Fig. 4E). From Figure 4D, it can be





**Fig. 3.** Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of granule-proteins of the starches from *amfICS6* and *amfSIC14* transformants, followed by antibody detection. Lane M shows the migration of the marker proteins. Molecular mass of the proteins is indicated in kDa.

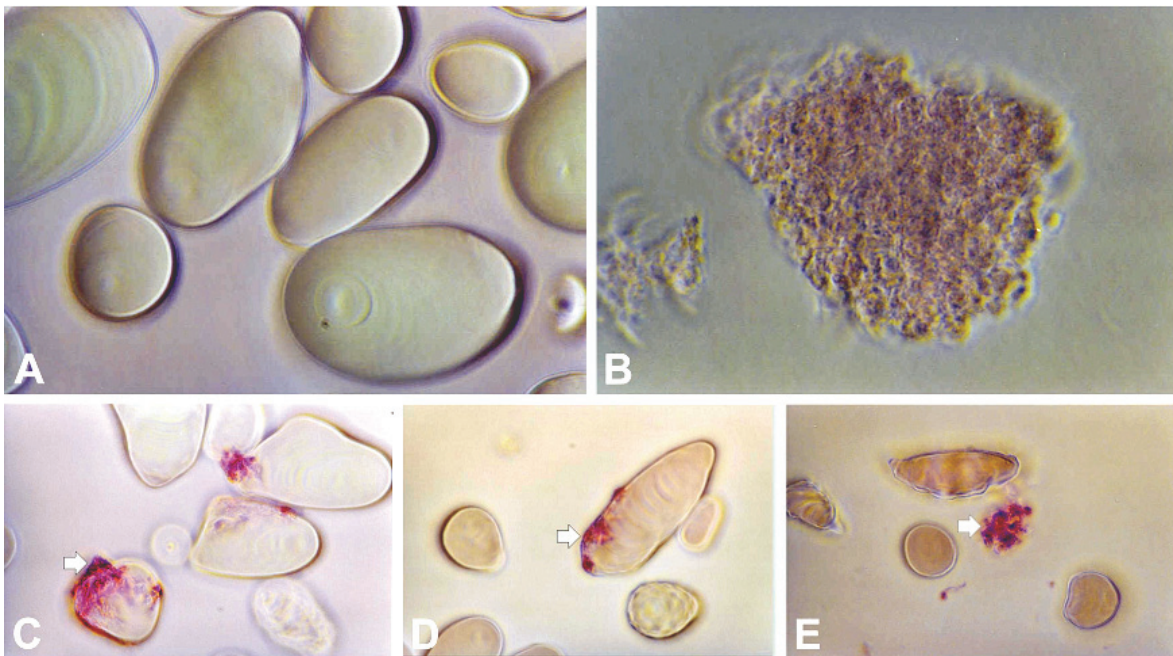
seen that most of the mutan remained attached to the granule, which suggests that a proportion of the mutan might be present inside the starch granules, and therefore inaccessible to the exo-mutanase.

### **Accumulation of SIC and ICS fusion proteins interferes with granule packing**

SEM analysis was performed on the selected transgenic starches. The presence of altered starch granule surfaces was visualized in the (H) class of the KDSIC series (Fig. 5B,F,G,H) in contrast to the (N) class, which was comparable with that of KD-UT (Fig. 5A). It can be seen that surfaces of the starch granules were irregular (Fig. 5F,G,H). In addition, small protrusions on the granular surface were present. Such granule phenotypes were not observed in the (H) class of the KDIC transformants (Fig. 5D,I). For the (H) class of the KDICS series (Fig. 5C), starch granules were not altered, and similar to those of KD-UT. In the *amf* background, granules with altered morphology were found in both the SIC and ICS

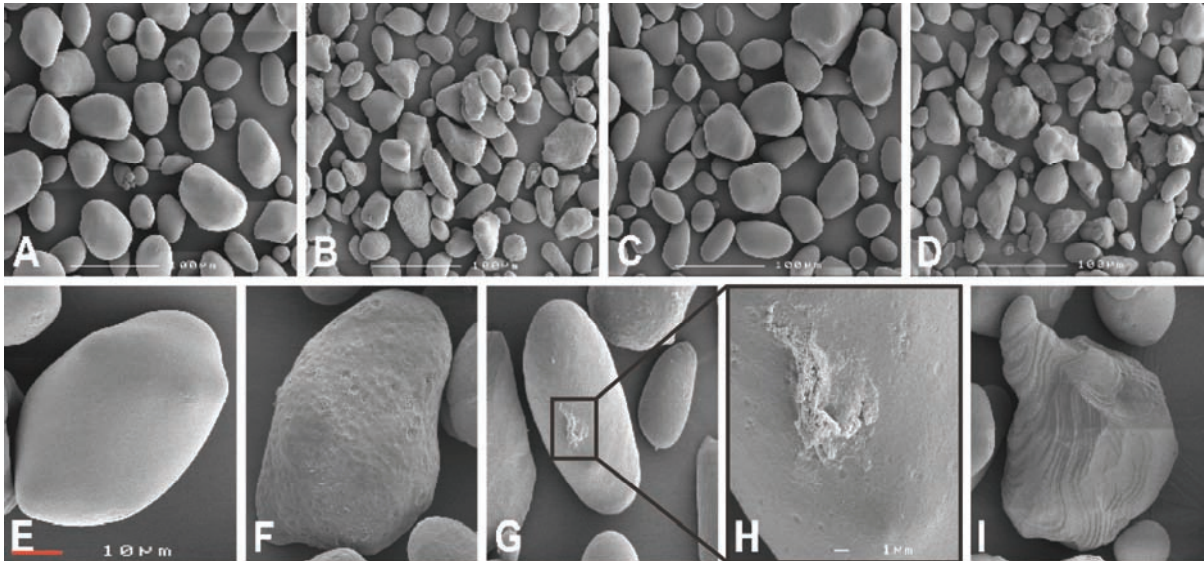
series (Fig. 6). Also, the position of SBD in the fusion protein seems to matter with respect to morphological changes. For instance, the surface of *amfSIC* granules (Fig. 6B,E) appeared spongy or punctured, just as though a crust of material was deposited on top of the surface. The surface of *amfICS* granules [with (++) level of SBD accumulation] showed a bumpy and rough appearance (Fig. 6F). The different phenotypes observed for the two potato backgrounds might find their origin in the presence of amylose, or in the fact that in the *amf* background more of the fusion protein is accumulated in the starch granules.

From the SEM pictures, the number of altered granules was scored by counting a population of 100 starch granules in triplicate for one transformant of the (N) and two transformants of the (H) class of each series. This quantification is shown in Figure 7 for the selected transformants. The highest numbers of altered starch granules were found in the (H) class of the *amfSIC* and *KDSIC* series,  $25.5\% \pm 3.5$  and  $24.7\% \pm 3.8$  for *amfSIC17* and *KDSIC10*, respectively. In the (H) class of the *amfICS* and *KDICS* series, the number of altered starch granules was similar, ranging from  $12.0\% \pm 2.8$  in *amfICS6* to  $10.7\% \pm 2.3$  in *KDICS27*. For the (N) class of transformants, the frequency of altered starch granules was less than 7%. In general, it seemed as though SBD-GtfICAT affected starch granule morphology more than GtfICAT-SBD.

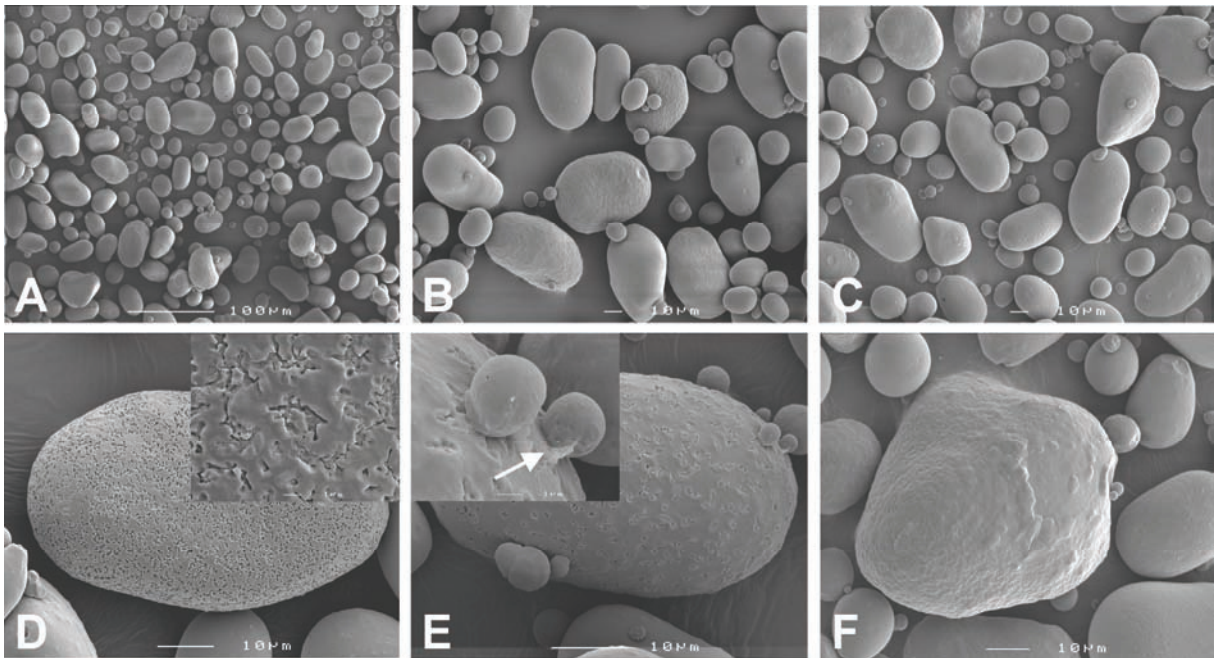


**Fig. 4.** LM analysis of starch granules (x 800) from KD-UT (A), purified mutan (B), and *KDSIC10* (+) (C), stained with erythrosine. The granules of *KDSIC10* (D) and *KDIC15* (E) are shown after treatment with 25 mU of exo-mutanase.



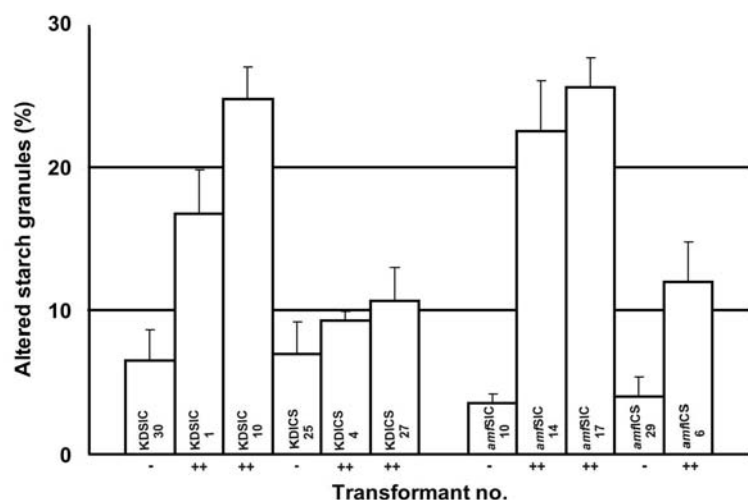


**Fig. 5.** SEM analysis of starch granules from KD-UT (A), KDSIC10 (B), KDICS27 (C), KDIC15 (D), and close-ups of the granule surface of KD-UT (E), KDSIC10 (F), KDSIC10 (G), KDSIC10 (H), and KDIC15 (I).



**Fig. 6.** SEM analysis of starch granules from *amfSIC4* (+) (A,D), *amfSIC14* (++) (B,E), and *amfICS6* (++) (C,F). Arrow (E) might indicate mutan, which is co-deposited with starch.

However, in the Kardal background, the granule morphology of the best SBD-GtfICAT transformants was less affected than that of the best KDIC transformants, in which  $31.3 \pm 2.3$  % of the starch granules exhibited altered morphologies (Kok-Jacon *et al.*, 2005a).



**Fig. 7.** Percentage of starch granules with altered morphology of various transformants. A population of 100 granules was counted in triplicates. Bars indicate the mean of three independent counts, error bars represent the standard deviation. Classification of transformants based on RNA (N, H) is indicated underneath bars.

**GtfICAT/SBD does neither influence starch properties nor content**

Starch content and various starch characteristics were determined for the different transformants in comparison with KD-UT and *amf*-UT. Table 1 shows that, there are no consistent differences, particle size distribution, granule melting temperature, and amylose content, indicating GtfICAT-SBD and SBD-GtfICAT do not influence these parameters in neither the Kardal, nor the *amf* background. Starch content does not seem to be affected in the transformants of the *amf* background; for the Kardal background it seems as though the starch content of the transgenic tubers is reduced, but this still needs to be further established.

**GtfICAT with appended SBD does not seem to alter starch fine structure**

The chain length distribution was determined in order to detect deviations in starch structure, which may indicate the presence of  $\alpha(1\rightarrow3)$ -linked glucosyl residues. After complete debranching of starch with isoamylase, no consistent changes were detected with HPSEC and HPAEC in comparison with the control starches (data not shown). In addition, isoamylase debranched starches were further incubated with  $\alpha$ -amylase, and analyzed with HPAEC in order to detect the presence of possible  $\alpha(1\rightarrow3)$  linkages. No consistent changes in amylopectin fine structure were detected in comparison with control starches (data not shown). These results suggest that mutan is not covalently attached to the starch polymers, but rather is present as a separate carbohydrate.

**Table 1.** Summary of transformant classification, granule size ( $d_{50}$ , median value of the granule size distribution), onset temperature of starch granule melting ( $T_o$ ), amylose content, and starch content of (starches from) selected transformants, KDIC15 (H; Kok-Jacon *et al.*, 2005a), KD-UT, and *amf*-UT. Data (mean  $\pm$  SD) are the average of two or three independent measurements.

| Transformant     | RNA <sup>1</sup> | SBD <sup>2</sup> | $d_{50}$ ( $\mu$ m) | $T_o$ ( $^{\circ}$ C) | Amylose Content (%) | Starch content (mg/g FW) |
|------------------|------------------|------------------|---------------------|-----------------------|---------------------|--------------------------|
| KD-UT            | N                | -                | 28.8 ( $\pm$ 0.5)   | 66.7 ( $\pm$ 0.1)     | 20.1 ( $\pm$ 0.5)   | 242.9 ( $\pm$ 93.5)      |
| KDSIC10          | H                | +                | 25.1 ( $\pm$ 0.8)   | 69.5 ( $\pm$ 0.1)     | 18.0 ( $\pm$ 0.2)   | 179.7 ( $\pm$ 38.3)      |
| KDICS27          | H                | -                | 28.6 ( $\pm$ 0.2)   | 67.4 ( $\pm$ 0.1)     | 20.0 ( $\pm$ 0.2)   | 191.6 ( $\pm$ 29.4)      |
| KDIC15           | H                | -                | 20.2 ( $\pm$ 0.4)   | 67.2 ( $\pm$ 0.1)     | 19.7 ( $\pm$ 0.2)   | 81.3 ( $\pm$ 37.3)       |
| <i>amf</i> -UT   | N                | -                | 13.1 ( $\pm$ 0.2)   | 70.1 ( $\pm$ 0.1)     | 3.3 ( $\pm$ 0.1)    | 140.8 ( $\pm$ 24.2)      |
| <i>amf</i> SIC4  | L                | +                | 13.9 ( $\pm$ 0.1)   | 69.4 ( $\pm$ 0.1)     | 3.0 ( $\pm$ 0.1)    | 161.0 ( $\pm$ 17.5)      |
| <i>amf</i> SIC5  | H                | ++               | 14.9 ( $\pm$ 0.1)   | 71.4 ( $\pm$ 0.2)     | 3.0 ( $\pm$ 0.0)    | 152.0 ( $\pm$ 17.5)      |
| <i>amf</i> SIC14 | H                | ++               | 15.1 ( $\pm$ 0.4)   | 72.3 ( $\pm$ 0.1)     | 3.5 ( $\pm$ 0.0)    | 133.8 ( $\pm$ 21.3)      |
| <i>amf</i> SIC17 | H                | ++               | 11.7 ( $\pm$ 0.2)   | 70.9 ( $\pm$ 0.1)     | 3.6 ( $\pm$ 0.0)    | 109.6 ( $\pm$ 8.6)       |
| <i>amf</i> ICS27 | L                | -                | 12.3 ( $\pm$ 0.1)   | 70.6 ( $\pm$ 0.1)     | 3.6 ( $\pm$ 0.1)    | 96.5 ( $\pm$ 21.9)       |
| <i>amf</i> ICS6  | H                | ++               | 11.8 ( $\pm$ 0.1)   | 70.5 ( $\pm$ 0.1)     | 3.2 ( $\pm$ 0.1)    | 125.2 ( $\pm$ 9.1)       |

<sup>1</sup>Based on RT-PCR analysis.  
<sup>2</sup>Based on Western dot blot analysis.

### Post-harvest incubation of GtfICAT-containing starch granules with sucrose

In an effort to produce more mutan, starch granules of the best transformants were incubated with an excess of sucrose at 37° C, which is the optimal temperature for GtfICAT, and at 45°C. The starch from the high expresser KDIC15 (Kok-Jacon *et al.*, 2005a) was selected as a control, representing GtfICAT without an appended SBD. After 66 h of incubation, the production of fructose and glucose was determined by HPAEC. The release of fructose is indicative for the amount of GtfFICAT activity, whereas the release of glucose is not; glucose can either be released as such (hydrolytic activity of the enzyme) or as part of the mutan that is formed (polymerizing activity of the enzyme). GtfICAT was found active, but at a low level (data not shown). After 66 h, fructose was released in a higher amount for the best transformant (33  $\mu$ g/ml at 45° C), in contrast to KD-UT for which no fructose was released at all. The glucose concentration was similar to that of fructose, suggesting that the enzyme inside starch granules mainly catalyzes a hydrolysis reaction and no polymerization. This is in line with our observations that no increased amounts of mutan were visualized upon light microscopic analysis of transgenic starch granules, which were stained with erythrosine after

66 h of incubation with sucrose. The post-harvest experiments demonstrated that granules from KDIC (H) were more active in presence of excess of sucrose than those from KDSIC (H) and *amf*SIC (H) (data not shown). This suggested that GtfICAT with appended SBD is less active than GtfICAT alone.

## Discussion

In this study, a microbial SBD was fused to the N- or C-terminal end of GtfICAT in order to bring this effector enzyme in more intimate contact with starch granules. We have obtained indications that it is possible to co-deposit starch and mutan in this way, but we have not obtained evidence for covalent attachment of mutan to starch. Surprisingly, it was found that the morphology of *amf* starch was severely altered in the highest expressers of GtfICAT with an appended SBD. These results will be elaborated below.

### Granule-targeting of GtfICAT without covalent attachment of mutan

By light microscopy, combined with erythrosine staining, qualitative indications were obtained that more mutan was produced in the Kardal than in the *amf* background (data not shown), despite the fact that more GtfICAT with appended SBD was present in *amf* starch. Similar observations were made for Kardal and *amf* potato transformants expressing the *Leuconostoc mesenteroides* DSRS gene (Kok-Jacon *et al.*, 2005b). With this study, we provide a second example that the *amf* background might be less suitable for the production of carbohydrate polymers or oligomers from sucrose.

Staining of granules from SIC, and particularly from ICS, transformants with erythrosine was less pronounced than that of starch from IC transformants. This might suggest that the fusion proteins are less active. Furthermore, the post-harvest activity of GtfICAT with appended SBD in starch granules was very low, even lower than that of GtfICAT alone. Taken together, our results suggest that the presence of an appended SBD compromised the activity of GtfICAT. Our data indicate that the activity of the enzyme decreases in the order of GtfICAT alone, SBD-GtfICAT, and GtfICAT-SBD. Possibly, the appended SBD might induce conformational changes in the enzyme, making it less active, or it might decrease the accessibility of the enzyme's active site to substrates.

Interestingly, the starch from the KDSIC10 transformant had mutan attached, which seemed more difficult to degrade or detach from the granule than that of transformants in which the mutan was synthesized by GtfICAT without an appended SBD. Our experiments in which starch polymers were treated with isoamylase and  $\alpha$ -amylase only gave the common maltooligosaccharide degradation products, without indications for the presence of more peculiar oligosaccharides having  $\alpha(1\rightarrow3)$  linkages. These results lend some support to the idea that, with an appended SBD, it is possible to co-deposit novel polymers with starch in granules, although the more intimate contact between the growing starch granule and mutansucrase do not seem to lead to covalent connections between the polymers.

### **GtfICAT with an appended SBD alters granular morphology in *amf* background**

Consistent with previous results, it was found that more GtfICAT with appended SBD accumulated in the *amf* than in the Kardal background (Ji *et al.*, 2004; Nazarian *et al.*, 2006a); a protein of the expected size was evidenced in the starch granules of transgenic *amf* plants (Fig. 3). Surprisingly, the starches of the high expressers of the *amf*SIC and *amf*ICS displayed an altered granule morphology, the appearance of which was different for the two series: a spongeous and a bumpy surface, respectively. It is unlikely that these different phenotypes can be explained by the presence of mutan (no indications for the presence of mutan was found in the *amf* transformants) or activity of GtfICAT (only little evidence for active fusion proteins was found). Therefore, it is thought that the different morphologies are related to GtfICAT's ability to bind maltooligosaccharides (MOS). From a number of studies (Fu and Robyt, 1990; 1991; Kralj *et al.*, 2005; Monchois *et al.*, 1999b; Monchois *et al.*, 2000), a view has emerged in which GtfI seems to have several glucosyl-binding pockets (likely three or more), which can accommodate MOS acceptor molecules, after which they receive a glucosyl residue derived from the donor substrate sucrose (the so-called acceptor reaction). The presence of this MOS-binding site, together with the observation by others that deletion of the C-terminal one third of GtfI, as in our GtfICAT, did not seem to affect the acceptor reaction (Monchois *et al.*, 1999a), lead us to believe that GtfICAT can bind MOS. Our observation that GtfICAT displays little activity does not exclude this possibility, because it is thought that donor and MOS acceptors can bind independently (Kralj *et al.*, 2005; Monchois *et al.*, 2000). It is hypothesized that GtfICAT requires an appended SBD for prolonged contact with starch granules. When present in the proximity of the granule surface, it may interact with  $\alpha$ -



glucan side chains of the nascent amylopectin, which are in the process of assembling into double-helical structures, which subsequently pack laterally into a crystalline lamella. By binding to amylopectin side chains, GtfICAT might influence their lateral interaction, which in turn could lead to perturbations at the granule surface, and eventually the spongy or bumpy appearance of the granule surface. It is unclear why the phenotype of the granules in the high expressers of the *amfSIC* and *amfICS* series are different. Possibly, this is related to how GtfICAT and SBD are oriented with respect to each other.

In the Kardal background, only the SIC transformants showed altered granule morphology, although the spongy appearance was not observed. Possibly the small holes in the granule surface are filled with amylose (it is considered very unlikely that the different ploidy level and alleles of starch biosynthetic enzymes of Kardal and the *amf* potato mutant are responsible for the observed altered granular morphology). The alterations are less severe and at lower frequency than in the *amf* background, which is consistent with the lower accumulation of SBD-containing fusion proteins in Kardal. It has been shown that GtfICAT alone also gives an altered granular phenotype (Fig. 5I, and Kok-Jacon *et al.*, 2005a), which is different from that of GtfICAT with appended SBD. It is believed that, in case of GtfICAT alone, the morphology of the granule is determined by the presence of mutant, whereas, in case of GtfICAT with appended SBD, the phenotype is determined by the presence of the fusion protein interfering with lateral interactions of amylopectin side chains.

In previous investigations in which SBD was fused to an effector protein (either other SBDs (Ji *et al.*, 2004; Nazarian *et al.*, 2006a), or maltose acetyl transferase (Nazarian *et al.*, 2006b)), it was observed that the different domains constituting the fusion proteins showed cooperativity in binding, i.e. more of the fusion protein was accumulated in the granule than of SBD alone. This was not observed with GtfICAT/SBD. Interestingly, much more of maltose acetyl transferase (MAT) with appended SBD was incorporated in starch granules than of GtfICAT/SBD (Nazarian *et al.*, 2006b). Despite this, the morphology of the starch granules of the former was less affected than that of the latter. This might be related to the fact that MAT has at the most two glucosyl-binding pockets, whereas GtfICAT has at least three. The ability of GtfICAT to bind more glucosyl residues simultaneously is expected to have a larger impact on lateral interactions of amylopectin side chains, and consequently on granule morphology. The starch from GtfICAT/SBD transformants does not show multiple Maltese crosses upon analysis by microscopy with polarized light (data not shown), as was observed in starches with multiple SBDs (Nazarian *et al.*, 2006a). This suggests that GtfICAT and SBD

bind different parts of the granule, or that SBD binds  $\alpha$ -glucans with higher affinity than GtfICAT.

## Materials and methods

### Preparation of constructs containing GtfICAT and SBD

pPF and pPFGtfICAT (Kok-Jacon *et al.*, 2005a) were used as starting material for cloning the SBD-linker and linker-SBD fragments, resulting in pPFSBDGtfICAT and pPFGtfICATSBD, respectively. The SBD-linker and linker-SBD fragments were obtained from the pTrcHisB/SBD2 plasmid (Ji *et al.*, 2004) that was used as a template for PCR amplification. The SBD fragment originated from the *CGTase* gene of *Bacillus circulans* strain 251 (Lawson *et al.*, 1994), and the linker fragment is similar to the Pro-Thr rich linker of the *Cellulomonas fimi* exoglucanase (*Cex*) (Gilkes *et al.*, 1991). For the construction of pPFSBDGtfICAT, the SBD-linker fragment was obtained by PCR amplification with a forward primer, containing a *ScaI* restriction site (5'- AGTACTATGGCCGGAGATCAGGTC- 3'), and a reverse primer, containing a *NruI* restriction site (5'- TCGCGACGACGGGGTC- 3'), and cloned into the *SmaI* restriction site of pPFGtfICAT. For the construction of pPFGtfICATSBD, the linker-SBD fragment was obtained by PCR amplification with a forward primer, containing an *EcoRV* restriction site (5'- GATATCTCCGACGCCGACGC- 3'), and a reverse primer, containing a *SmaI* restriction site (5'- CCCCGGGATCCACCAAAAC- 3'), and cloned into the *EcoRV* restriction site of pPF, resulting in pPFSBD. In order to remove the stop codon in the original *GtfICAT* sequence, the *GtfICAT* fragment was amplified with a forward primer, containing a *SmaI* restriction site (5'- CCCCGGGACTGAAAAGTGTAG- 3'), and a reverse primer, containing a *NruI* restriction site (5'- TCGCGAACATTGAGGTACTTG- 3'), and cloned into the *SmaI* restriction site of pPFSBD, resulting in pPFGtfICATSBD. pPFSBDGtfICAT and pPFGtfICATSBD were completely sequenced in one direction by Baseclear (The Netherlands) to verify the correctness of the construct. pPFSBDGtfICAT and pPFGtfICATSBD were digested with *XhoI* and ligated into a pBIN20 binary vector (Hennegan and Danna, 1998), resulting in pPFSBDIC and pPFICSBD (Fig. 1). The predicted molecular mass of the fusion proteins excluding the signal peptide is 125,870 Da and 125,390 Da for SBD-GtfICAT and GtfICAT-SBD, respectively.

## Transformation and regeneration of potato plants

pPFSBDIC and pPFICSBD were transformed into *Agrobacterium tumefaciens* strain LBA4404 using electroporation (Takken *et al.*, 2000). Internodal stem segments from the tetraploid [cv. Kardal (KD)] and diploid mutant (*amf*) potato genotypes were used for *Agrobacterium*-mediated transformation, which was performed as described by Kok-Jacon *et al.* (2005b).

## Starch isolation

Starch isolation was performed as described by Kok-Jacon *et al.* (2005a).

## Expression analysis of *GtfICAT*, using semi-quantitative RT-PCR analysis

Total RNA was isolated from 3 g (fresh weight) of potato tuber material from selected transgenic lines according to Kuipers *et al.* (1994). Semi-quantitative RT-PCR was performed as described by Kok-Jacon *et al.* (2005b). *GtfI* RT primers, 5'-CCGTGCTTACAGTACCTCAGC-3' and 5'-GGTCGTTAGCATTGTAGGTGAAA-3' (Tm=59°C, 35 cycles) were based on the *GtfI* gene sequence (Ferretti *et al.*, 1987). The primers, 5'-GTCAGGCCCAATTACGAAGA-3' and 5'-AAGTTCCAGCACCGCACTC-3' were used to amplify the ubiquitin gene (L22576), which was used as an internal control (Garbarino and Belknap, 1994).

## Determination of morphological and physicochemical properties of starch granules

Analysis of starch granule morphology was performed by light microscopy (LM) and scanning electron microscopy (SEM) as described by Kok-Jacon *et al.* (2005b). Mutan polymers were visualized with LM, and the exo-mutanase treatment was performed as described by Kok-Jacon *et al.* (2005a). Median values of the granule size distribution ( $d_{50}$ ), gelatinization behaviour, amylose content, starch content, and chain length distributions (HPSEC, HPAEC) were determined as described by Kok-Jacon *et al.* (2005b).

## Quantification of SBD transgenic starches and tuber juices

Western dot blot analysis was performed according to the method described by Ji *et al.* (2003). A 12.5 % sodium dodecylsulphate-polyacrylamide gel (50 mm × 50 mm × 3 mm), with nine equally spaced holes (9 mm diameter), was placed in contact with a similarly sized Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Amersham, UK). Twenty milligrams of (transgenic) starch was boiled for 5 min with 200 µl of 2 × SDS sample buffer, containing 5% (v/v) β-mercaptoethanol. After cooling to room temperature, the starch gel was transferred into one of the holes. SBD proteins from transgenic starch gels were blotted onto the membrane with PhastSystem (Pharmacia, Uppsala, Sweden; 20 V, 25 mA, 15°C, 45 min). The blot was incubated overnight in a 1 % blocking solution (10 ml 10 × western blocking reagent; Roche, Germany) in 90 ml TBS (20 mM Tris, 500 mM NaCl pH 7.5) at room temperature. Subsequently, the blot was washed in TTBS (0.05 % Tween-20 in TBS) for 5 min, and incubated for 2 h at room temperature with a 1:500 dilution of the primary antibody (anti-SBD) in a 0.96 % blocking solution in TTBS. After this, the blot was washed twice in TTBS for 5 min, and incubated for 2 h at room temperature with a 1:2000 dilution of goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate (BioRad, Hercules, CA, US) in a 0.64 % blocking solution in TTBS. The blot was washed twice in TTBS, and once in TBS for 5 min. Finally, the blot was stained for 15 min in dark with a 0.1 M NaHCO<sub>3</sub> solution of pH 9.8, containing 1 % NBT/BCIP (Roche Molecular Biochemicals, Mannheim, Germany), and 0.01 M MgCl<sub>2</sub>. The amount of SBD-containing protein in potato tuber juice was also determined as described by Nazarian *et al.* (2006a).

## Electrophoretic separation of granule-bound proteins

Fifty mg of *amf*SIC14, *amf*ICS6 or *amf*-UT starch were boiled for 2 min in 1 mL of SDS sample buffer, containing 5% (v/v) β-mercaptoethanol. The gelatinized samples were centrifuged for 10 min at 14,000 *g*. The supernatant was subjected to a microconcentrator column (Millipore, Bedford, MA, US). Twenty five µl of the concentrated samples were analyzed using a 12% SDS-polyacrylamide gel (BioRad, Hercules, CA, US), and detection of the fusion proteins was carried out as described by Nazarian *et al.* (2006a). The Precision Plus Protein all blue standards (BioRad, Hercules, CA, US) was used for calibration.

### Post-harvest determination of mutansucrase activity

Post-harvest experiments were performed using 10 mg of starch, suspended in 1 ml of 50 mM Tris/HCl pH 7.0, containing 1 M sucrose; incubation was done for 66 h at 37 °C and 45 °C. After centrifugation (1 min; 10,000 *g*), the supernatant was submitted to HPAEC analysis.

HPAEC was performed as described previously except that the column temperature was 28°C and a different gradient was used. Three eluents were used, eluent A (100 mM NaOH), eluent B (1 M NaAc in 100 mM NaOH) and eluent C (H<sub>2</sub>O) with the following gradient: 0→12 min, 25→85% eluent A and 75→15% eluent C (linear gradient); 12→25 min, 0→10% eluent B and 15→5% eluent C (linear gradient); 25→25.1 min, 85→0% eluent A, 10→100% eluent B, and 5→0% eluent C (linear gradient); 25.1→30 min, 100% eluent B (rinsing phase); 30→45 min, 0→25% eluent A, 100→0% eluent B, and 0→75% eluent C (equilibration phase). The eluents were monitored by an ED40 electrochemical detector in the pulsed amperometric mode (Dionex).

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# Chapter 5

## **Introduction of an engineered granule-bound *E. coli* glycogen branching enzyme results in irregular and punctured starch granules in wildtype and amylose-free potatoes**

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**To be submitted**

## Abstract

**I**n order to affect the degree of branching in potato tuber starch, the coding region of the *E. coli* glycogen branching enzyme (*glgB*) was fused to either the C- or N-terminus of a starch binding domain (SBD). Two chimeric gene cassettes (SBD-*glgB* and *glgB*-SBD) under control of the potato granule-bound starch synthase I (GBSSI) promoter were expressed in the mutant amylose-free (*amf*) and an amylose-containing (Kardal) potato genetic background. Regardless of background or construct used, a large amount of fusion protein was accumulated inside the starch granules, suggesting that *glgB* also has affinity for the starch granules on its own. Furthermore, Western blot analysis with both SBD and *glgB* antibodies showed that correctly-sized fusion proteins were targeted to the starch granules. A good correlation was found between *glgB* protein levels and the level of corresponding mRNA transcripts. Presence of *E. coli* granule-bound branching enzyme seemed to result in punctured starch granules with relatively large holes in the *amf* genetic background, whereas those of the amylose-containing showed irregular surfaces, suggesting that holes might be filled with amylose which seems to protrude from the starch granules. Despite these starch granule alterations, the physicochemical properties of such starches were unaltered.

**Key words:** SBD, starch, *E. coli* glycogen branching enzyme, potato, amylose-free

The  $\alpha$  (1→4) glucan polymers, starch and glycogen, are the main energy suppliers for a wide range of organisms (Ball *et al.*, 1996). Starch as semi-crystalline granules, consists of two types of macromolecules, amylose and amylopectin (Smith, 2001). The proportion between these two varies depending on the starch source, but the majority of starches contain between 20% to 30% amylose. Amylose is an essentially linear molecule of  $\alpha$  (1→4) linked glucosyl units with few branches, while amylopectin is a much larger molecule than amylose and consists of shorter chains of  $\alpha$  (1→4) linked glucosyl residues that are connected by  $\alpha$  (1→6) glucosidic linkages resulting in a branched structure. Interestingly, glycogen, in bacteria and animal tissues, consists of a similarly branched molecule like amylopectin, however it has a shorter average length of the side chains and it is water-soluble (Myers *et al.*, 2000). For both molecules, branching enzymes (BE) (EC 2.4.1.18) catalyze the formation of  $\alpha$  (1→6) branch points, in plants, starch branching enzymes (SBEs) and in *E. coli*, glycogen branching enzyme (Nakamura, 2002; Thompson, 2000). However, in contrast to the plant SBEs, *E. coli* glycogen branching enzyme effectively introduces a much narrower size of side chains (Guan *et al.*, 1997).

Many starch characteristics including freeze-thaw stability, are defined by the ratio of amylose/amylopectin and degree of amylopectin branching. A much desired property for starch is freeze-thaw stability, i.e., starch pastes can maintain their integrity without syneresis when subjected to a number of repeated thermal cycles between ambient and freezing temperatures. From an industrial viewpoint starches with higher freeze-thaw stability are desirable and they are therefore subjected to chemical starch modifications such as acetylation and hydroxypropylation. In these ways, added chemical groups prevent reassociation of the glucan chains, and therefore improve starch freeze-thaw stability (Jobling, 2004). Although these types of chemical modifications are feasible, from environmental perspective such chemicals are not desirable so that it is important to look for biological alternatives which are not polluting.

It is known that the long and unbranched  $\alpha$ -glucans such as amylose have higher tendency to retrograde than highly branched and much shorter chain molecules of 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 principal, it

may be possible to create a freeze-thaw stable starch 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, antisense inhibition of three isoamylase isoforms in potato tubers led to deposition of phytoglycogen, a highly branched  $\alpha$ -glucan molecule that does not have the capability at all to form semi-crystalline structures (Bustos *et al.*, 2004). Antisense down regulation of three starch synthase genes (GBSSI, SSII and SSIII) in potato led to reduction in the chain length of the amylopectin branches and 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). Expression of *E. coli* branching enzyme (*glgB*) in tubers of amylose-containing background did not result in any significant changes in starch branching (Krohn *et al.*, 1994). However *glgB* expression in amylose-free potatoes led to an increased branching degree of amylopectin. Despite the fact that a 25% increase in branching points of amylopectin was reported, neither the physiochemical behavior of starch nor starch granule morphology was altered (Kortstee *et al.*, 1996). Therefore, one may speculate that a 25% increase in amylopectin branch points may not have been sufficient to see a significant change in starch properties.

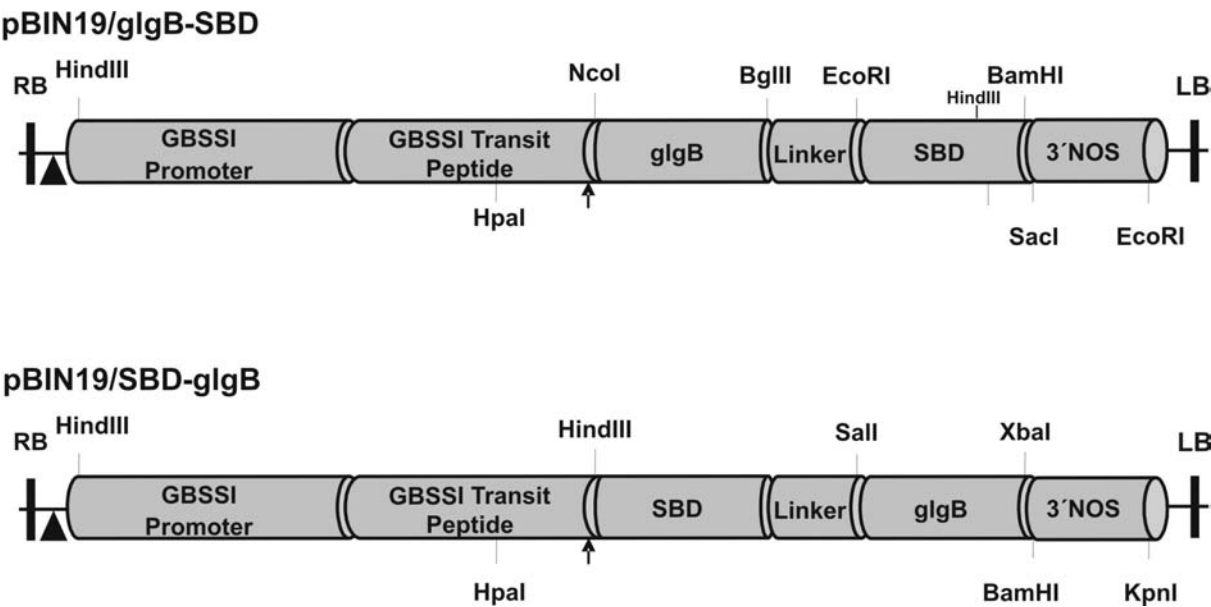
We have shown previously that effector proteins which are fused to a microbial Starch Binding Domain (SBD), remain functional despite the fact that they are locked in the highly crystalline starch material. For instance a granule-bound *luciferase* (Ji *et al.*, 2003) and a granule-bound *E. coli* maltose acetyltransferase (MAT) (Nazarian Firouzabadi *et al.*, 2006c) were found active in the transgenic starches.

In an attempt to increase amylopectin branching more than 25% (Kortstee *et al.*, 1996) an *E. coli* *glgB* with different minimum chain length requirement and specificity than potato SBEs was fused to either carboxyl or amino terminus of SBD (Ji *et al.*, 2003). This will bring *glgB* in more intimate contact with its substrate and acceptor molecules. In this study, the influence of a granule-bound *glgB* on structure and properties of potato starch is discussed.

## Results

In order to manipulate starch branching pattern during starch biosynthesis, 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. An 800 bp potato GBSSI promoter segment and its transit peptide (TP) sequence were used for tuber-specific expression and amyloplast entry of the proteins, respectively (Fig. 1). Thirty individual transformant clones were taken to the greenhouse for tuberization. Transformed potato plants in this study are referred to as *amf*GSxx, *amf*SGxx, KDGSxx and KDSGxx, in which GS and SG stand for the glgB-SBD and SBD-glgB fusion proteins, respectively and xx represents the clone number in each series of transformants. Besides, untransformed genotypes are referred to as *amf*-UT and KD-UT.



**Fig 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 is incorporated into starch granules

Expression of fusion proteins in tubers was quantified in both genetic backgrounds by Western dot blot analysis according to Ji *etal.* (2003) in which numbers from 0+ to 6+ were allocated, with 0+ meaning that no trace of fusion protein, and 6+ the highest amount of fusion protein, was found in the starch granules. 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)

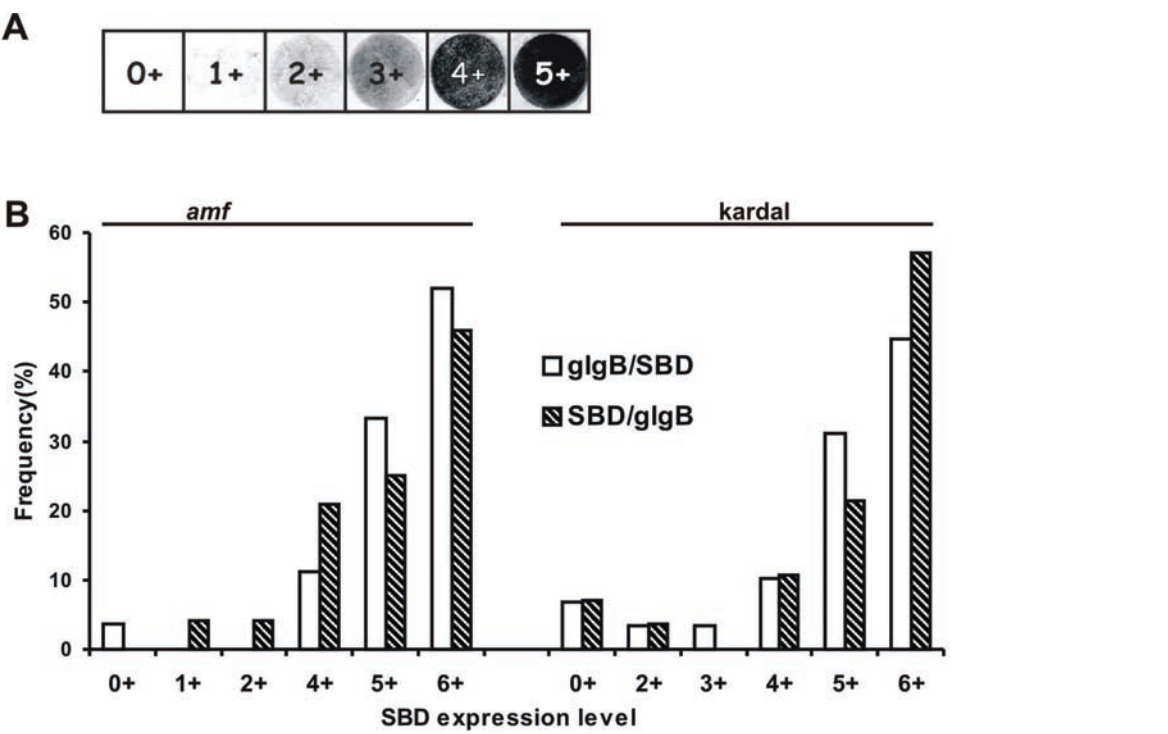
were classified from 4+ through 6+ classes (Fig. 2). Thus regardless of either construct or background used, almost half of the transformants accumulated the highest amount of the fusion proteins (6+). As can be seen from Table 1, a relatively low (2+) amount of protein was found in the juices of 6+ transformants; no fusion protein was found in the juice fractions of any of the other classes.

### **Fusion proteins of the correct size are present in the granules**

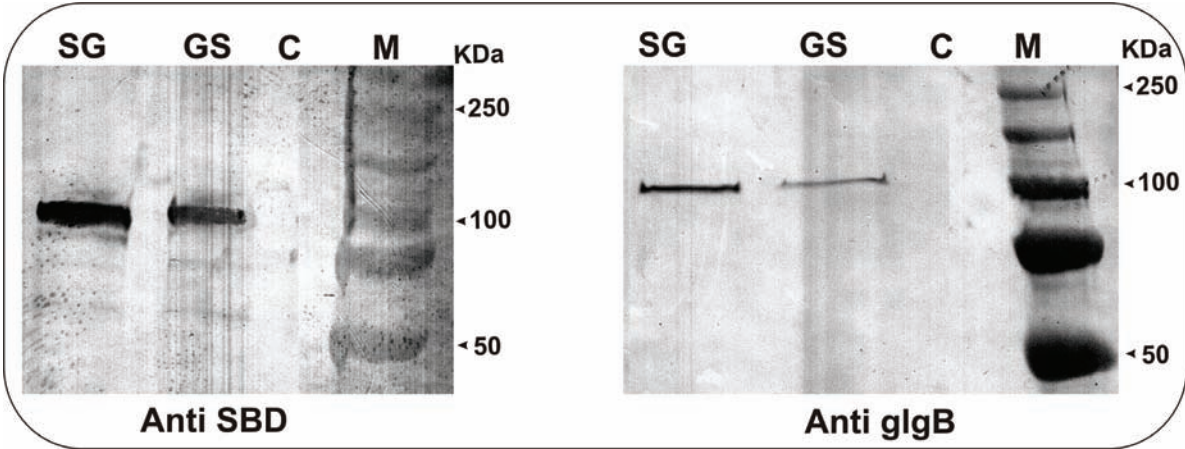
Engineered granule-bound fusion proteins were analyzed, using SDS-PAGE/immunoblotting with both anti-SBD and anti-*glgB* antibodies. Electrophoretic analysis of the granule-bound proteins extracted from potato starch showed the presence of fusion proteins in starch granules. The observed mass of about 100 kDa corresponded well with the predicted molecular mass of the fusion proteins which were detected with both anti-SBD (Fig. 3 left) and anti-*glgB* (Fig. 3 right), indicating that fusion proteins in starch granules are composed of both domains. Starches from control plants did not contain the fusion proteins. These results show that correctly processed fusion proteins have been successfully targeted to the starch granules. In general, it can be concluded that the amount of fusion protein for the *glgB*-SBD transformants was lower than that of the SBD-*glgB* transformants (Fig. 2)

### **mRNA transcript levels correlate with Western blot analysis**

To study the level of mRNA transcripts of fusion proteins, total RNA from tubers was isolated and subjected to real time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) using the *glgB* gene as a template. The constitutively expressed potato *Ubi3* gene (Garbarino and Belknap, 1994) was used as an internal control in both genetic backgrounds. Results from qRT-PCR showed that in both backgrounds within each series, the level of fusion protein in the granules (Western dot blot analyses) correlated well with corresponding mRNA transcripts in the transformants.



**Fig 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.



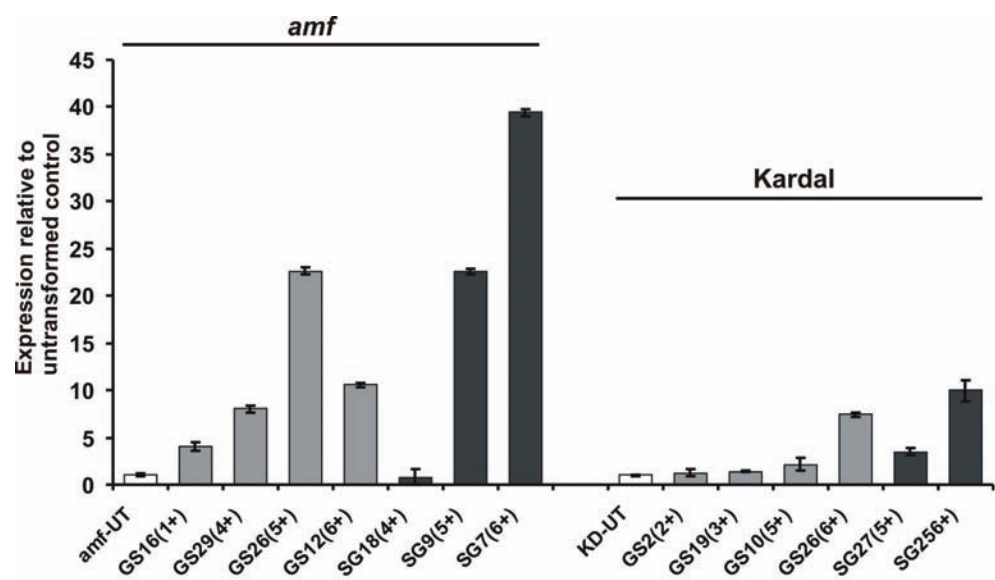
**Fig 3.** SDS-PAGE/Immunoblot analyses of starch granule-bound proteins of *amfGS26* and *amfSG7*. Migration of marker proteins (M) is shown to the right in each panel. SG and GS represent SBD-glgB and glgB-SBD fusion proteins respectively. Lanes designated with “C” represent starches from untransformed 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 |                    |                  |                    |
| <i>amf</i> -UT  | NP      | NP    | 3.0 ( $\pm 0.4$ )  | 69.4 $\pm$ (0.0) | 19.3 ( $\pm 0.2$ ) |
| <i>amf</i> GS26 | 6+      | 2+    | 3.0 ( $\pm 0.3$ )  | 70.7 $\pm$ (0.2) | 10.7 ( $\pm 0.4$ ) |
| <i>amf</i> SG12 | 6+      | 2+    | 3.4 ( $\pm 0.1$ )  | 69.2 $\pm$ (0.1) | 18.9 ( $\pm 0.3$ ) |
| KDUT            | NP      | NP    | 18.6 ( $\pm 0.4$ ) | 63.1 $\pm$ (0.1) | 25.2 ( $\pm 0.1$ ) |
| KDGS26          | 6+      | 2+    | 17.9 ( $\pm 0.5$ ) | 61.8 $\pm$ (0.1) | 22.8 ( $\pm 0.3$ ) |
| KDSG25          | 6+      | 2+    | 18.3 ( $\pm 0.3$ ) | 61.9 $\pm$ (0.1) | 14.2 ( $\pm 0.2$ ) |

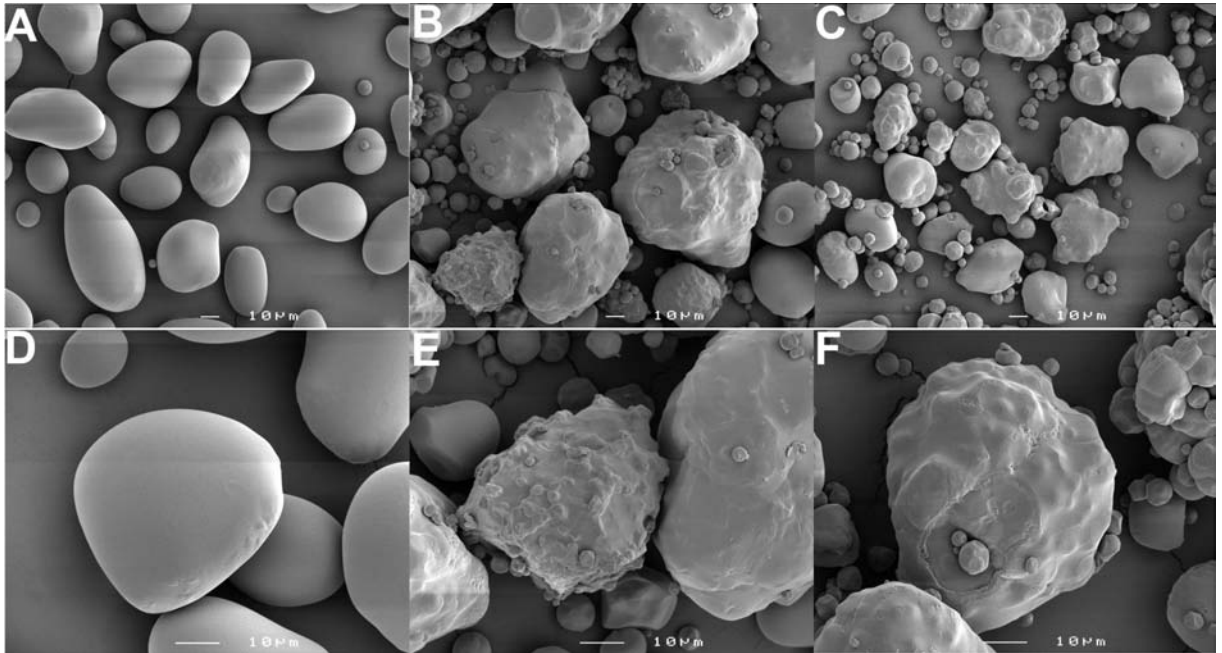
W.B= Western dot blot, NP= Not present



**Fig 4.** Quantitative RT-PCR analysis of the selected transformants in both backgrounds relative to the untransformed controls. Data (mean $\pm$ SD) are the average of three independent replications. Expression quantification of *glgB* gene is based on  $C_t$  values as described in Materials and methods section. Each QRT-PCR reaction was normalized using the  $C_t$  values corresponding to a potato *Ubiquitin* gene as a constitutively expressed control.

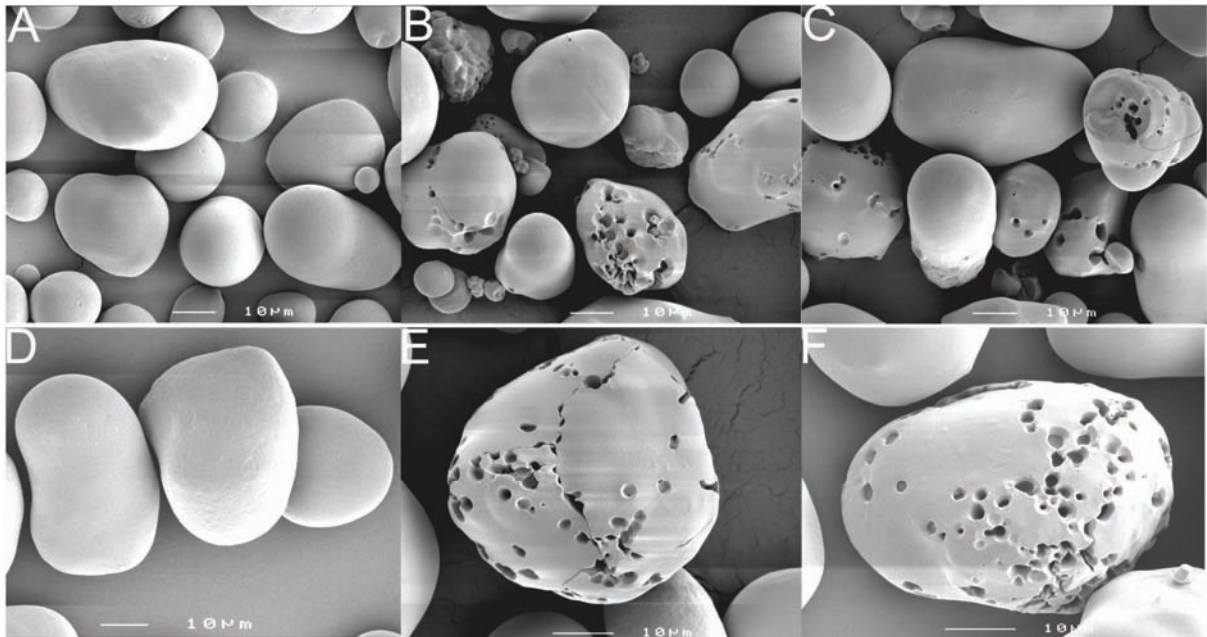
Granule morphology is affected by glgB with appended SBD

Investigation of starch granule morphology of transgenic starches in both genetic backgrounds, using light microscopy (LM), revealed that transgenic starches showed a different phenotype in comparison with untransformed control starches.



**Fig 5.** SEM analyses of starch granules from Karda-UT (A and D) in comparison with that of the different selected transformants at different magnifications. KDSG26 (6+) (B and E) and KDSG25 (6+) (C and F). Magnifications are; A ( $\times 250$ ), B and C ( $\times 500$ ) D, E and F ( $\times 2000$ ).

Punctured and irregular starch granules can be seen in starches from amylose-free and amylose-containing transformants, respectively (data not shown). In depth inspection of granule morphology from selected transformants by scanning electron microscopy (SEM) showed that starch morphology was severely affected as a result of the expression of the fusion proteins in both genetic backgrounds. Transgenic starches from amylose-containing plants exhibit irregular bumpy surfaces (Fig. 5 B,C,E and F). This phenomenon can be seen in different transformants at all levels of SBD accumulation (data not shown). Surprisingly, transgenic amylose-free starches exhibit starch granules with many deep holes (Fig. 6 B,C,E, and F) in such a way that sometimes starch granules show a crack connecting holes (Fig. 6E). 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.

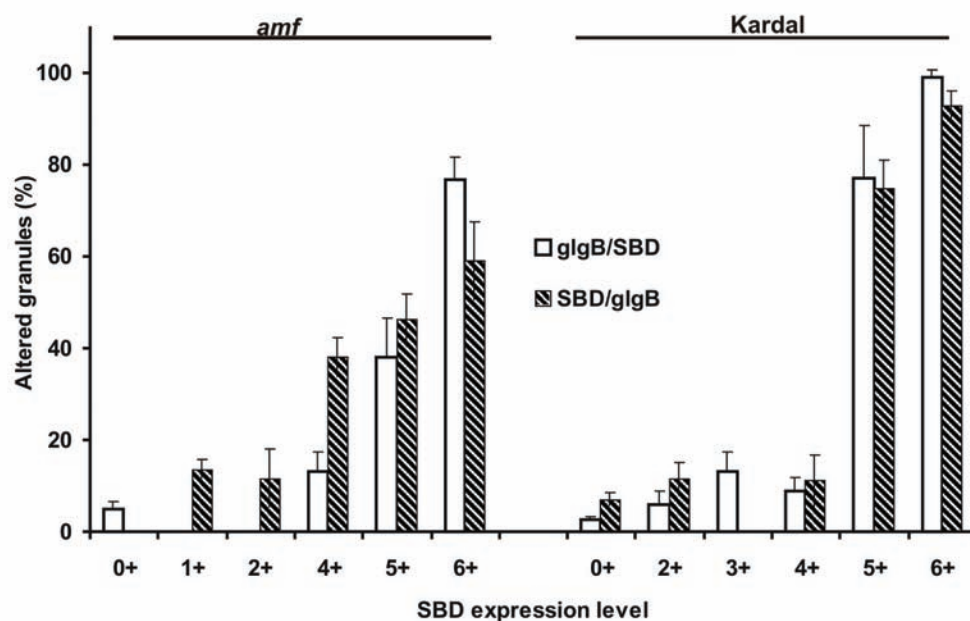


**Fig 6.** SEM analyses of starch granules from *amf*-UT (A and D) in comparison with that of the different selected transformants at different magnifications. *amf*GS26 (6+) (B and E) and *amf*SG7 (6+) (C and F). Magnifications are; A (x250), B and C (x500), D (x200), E and F(x4500).

Despite severe alterations in starch granule morphology in both genetic backgrounds, altered starch granules showed a normal “Maltese cross” under polarized light in the microscope, indicating that the overall crystallinity of the altered starch granules was not changed (data not shown). Quantification of such altered starch granule phenotypes as shown in Figure 5 and 6 revealed that the higher the amount of granule-bound fusion proteins, the more altered starch granules (Fig. 7).

### Starch characteristics remain unaltered

Starch characteristics such as gelatinization behavior and apparent amylose content (%AM) as well as starch content (data not shown) were also determined. No significant differences were found in these analyses in comparison with the control plants (Table 1).



**Fig 7.** 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  $\pm$ SD.

Although the starch median granule size ( $d_{50}$ ) is not significantly changed, scanning electron micrographs (Fig 5 and 6) showed a broader granule size distribution. To this end the half peak height of all starches from transgenic plants were calculated and plotted against median granule size. No consistent conclusion could be drawn with respect to the amount of accumulated fusion proteins (data not shown).

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

Dextrose equivalent numbers (which is the percentage of reducing sugar calculated as dextrose on starch dry weight basis) of the various transformant starches was also measured. Results of these types of analyses did also not deviate from those of control plants (data not shown).

### Granule-bound *glgB* in transgenic starches does not show activity

Since accumulation of *glgB* in starch granules during starch biosynthesis did not seem to change the pattern of the amylopectin branching, it was investigated whether granule-bound *glgB* was active in the starch granules. Our attempts to measure *glgB* activity directly were not successful because the activator molecule (glycogen) could not penetrate into starch granules. Therefore, given that *glgB* is granule-bound, transgenic starch granules were incubated in proper buffer, at different temperatures and different time points during post harvest experiments. Since the donor and acceptor molecules are in close proximity of the fusion proteins comprising *glgB* in transgenic starches, it can be expected that an active *glgB* may translocate amylopectin side chains leading to an altered chain length distribution in amylopectin/amylose structure. After treatment of such transgenic starches with isoamylase followed by HPSEC analysis, no detectable differences between transgenic and control starches were observed (data not shown).

### Discussion

We have demonstrated that *E. coli glgB* can be targeted to the starch granules during starch biosynthesis when it is fused to SBD either at the C- or N-terminal position. As a result of these transgenes, a large amount of fusion proteins was accumulated in the transgenic starch granules. Moreover, starch granule morphology was severely altered resulting in punctured and irregular bumpy phenotypes in amylose-free and amylose-containing genetic backgrounds, respectively. Surprisingly, regardless of the background and construct used, more than 85% of the transformants comprising *glgB* in their fusion proteins, accumulated the highest amount of the fusion proteins (4+ and higher) (Fig. 2). This was unexpected and even higher in comparison with SBD2 (KDSS and *amfSS*) transformants (Nazarian Firouzabadi *et al.*, 2006b). Furthermore, regardless of the construct used both amylose-free and amylose-containing transformants accumulated the same amount of fusion proteins. The unexpected high accumulation may indicate that *glgB* has affinity for the starch granules on its own. The reason why we assume that *glgB* has affinity for the starch granules may be explained by the domain structure of *glgB*. It seems that the N-terminal region of *glgB* looks like a SBD, and although it has not yet been experimentally proven that this domain truly is an SBD, there seems to be sufficient evidence that this domain along with the catalytic domain plays an

important role in glucan binding by the enzyme. The affinity of glgB for starch granules is corroborated by the observation that glgB was visualized as a granule-bound protein with Western blot analysis in transgenic potato starches (Kortstee *et al.*, 1998; Kortstee *et al.*, 1996).

Accumulation of fusion proteins in this study resulted in altered phenotypes (Fig. 5 and 6), despite the fact that expression of the *glgB* gene alone in potato amyloplasts of an amylose-free genotype (Kortstee *et al.*, 1998), wild type potato (Krohn *et al.*, 1994) and rice caryopses (Kim *et al.*, 2005) did not lead to any starch morphology alterations. The type of altered granules varies from what had been observed in multiple appended SBDs (Nazarian Firouzabadi *et al.*, 2006b) and GtfiCAT fusion proteins with SBD (Nazarian Firouzabadi *et al.*, 2006a). In the *amf* genetic background altered starch granules are punctured (Fig. 6). On the contrary, starches from Kardal transformants showed irregular bumpy surfaces with protrusions appearing (Fig. 5). Interestingly expression of a truncated mutansucrase (GtfiCAT) aimed at starch granules with SBD in potato tubers resulted in a kind of punctured/irregular bumpy starches depending on orientation of SBD as well as the genetic background used (Nazarian Firouzabadi *et al.*, 2006a). Since on the one hand the physicochemical properties of altered potato starches were not changed and on the other hand glgB was not found active after post harvest experiments, it is unlikely that glgB activity may be responsible for these differences in starch phenotypes. Therefore glgB/SBD may display a similar manner of interacting with starch as GtfiCAT/SBD, albeit in a strange way. Furthermore, we assume that the holes in *amf* are being filled with amylose later on in the amylose-containing genetic background such that amylose is protruding from these holes and some of it is deposited on the granule surface which eventually gives rise to bumpy surfaces as can be seen in Figure 5. However, the reason why such holes are being formed in the starch granules of *amf* starches needs to be investigated further and remains elusive.

Chain length profiles of transgenic starches were comparable to the untransformed control plants, indicating that glgB fused to SBD either do not introduce new  $\alpha$  (1→6) linkages or the number of newly introduced side chains is too little to be detected. Our finding however confirmed a previous finding in amylose-containing genetic background (Krohn *et al.*, 1994). Based on the trimming model suggested to explain the role of isoamylases in starch biosynthesis (Ball *et al.*, 1996; Myers *et al.*, 2000), it is thought that the size of amylopectin clusters (9 nm repeat) and crystalline lamella (4 to 6 nm) (Jenkins *et al.*, 1993) are mandatory values for crystallization of starch granules. It is thought that dp 10 is the minimum chain



length requirement for double helix formation by linear maltooligosaccharides (MOS) and crystalline lamella of 4 to 6 nm corresponds to a dp 12-18 by linear  $\alpha$ -glucans. Interestingly it seems that these values are in agreement with the minimum chains length required by most starch branching enzymes (Guan *et al.*, 1997; Rydberg *et al.*, 2001). In contrast, glgB needs a minimum chain length of 12 for branching (Guan *et al.*, 1997) while producing irregularly branched molecules. Furthermore, it seems that starch debranching enzymes (isoamylases) work on irregularly branched glucan molecules (like glycogen) by preferably removing small chains (Burton *et al.*, 2002; Fujita *et al.*, 2003) and cleave them in such a way to generate a regularly branched glucan structure (competent to crystallize). Production of phytoglycogen which resembles glycogen in antisense plants lacking functional isoamylases further corroborate this (Bustos *et al.*, 2004). Taken together, these findings may suggest that glgB may actively be involved in branching during starch biosynthesis, however such randomly branched molecules may serve as substrate for the native isoamylases to become unbranched. Therefore it is possible that a minimum chain length required, differences in substrate specificity of glgB from potato SBEs, crystallinity requirement obligation of the starch granule formation and trimming mode of isoamylases hampers glgB from further branching starch under *in planta* conditions.

In this study we have shown that it is possible to obtain punctured starch granules in amylose-free potato starch. In comparison with native starch, punctured starch may in principle absorb a larger amount of liquid and gaseous additives; this enables punctured starches to retain the absorbed material for a longer period than native starches (WeiRong and HuiYuan, 2002). Industrially, punctured starch is achieved either by enzymatic digestion or chemical treatments. As a biological alternative, we think our *amf* transgenic starches may find application in industry.

## Materials and methods

### Preparation of constructs

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 *NcoI* and *BglII* sites at their 5' ends, respectively. This amplified *glgB* fragment was used to replace the first SBD fragment (also *NcoI*-*BglII*) in the pUC19/SBD2 (Ji *et al.*, 2003), giving the pUC19/glgB-SBD plasmid. After digestion of this plasmid with *HpaI* and *SacI*, the *HpaI*-*SacI* 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 pBIN19/SBD-glgB plasmid was assembled from below fragments: (1) GBSSI promoter, GBSSI transit peptide and SBD-linker fragment (*HindIII*-*SalI*) obtained from pUC19/SBD2 plasmid, (2) a *glgB* fragment (*SalI*-*XbaI*), and (3) a NOS terminator (*BamHI*-*KpnI*). The *glgB* fragment was amplified by PCR with 5'-GTGTCGACCATGGCCGATC-3' and 5'-TCTAGAGTCATTCAGCCTCCCG-3' as primers, which contained *SalI* and *XbaI* 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 *Agrobacterium tumefaciens* strain LBA4404 by electroporation (Takken *et al.*, 2000). 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.

### Starch Isolation

All tubers from each individual transformant ( five clones) were peeled, mixed and ground using a Sanamat rotor (Spangenberg, The Netherlands) to which 0.01% of sodium

metabisulphite ( $\text{Na}_2\text{S}_2\text{O}_5$ ) 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).

### **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). To visualize the fusion proteins, a portion of 1:500 dilution of anti-SBD (Ji *et al.*, 2003) was used as primary antibody. 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.* (2006a). 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)  $\beta$ -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  $\mu$ l 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 immunoblotted using either anti-SBD or anti-glgB antibodies as described by Nazarian *et al.* (2006a).

### **Expression analyses of the *glgB* gene using quantitative RT-PCR**

The gene expression pattern of the *glgB* gene in tubers of transgenic plants was measured using quantitative real time PCR (qRT-PCR). One microgram of total RNA from transgenic tubers was treated with DNase I (Boehringer Mannheim) and standard cDNA template was obtained using random hexamers as primers and Multiscribe™ Reverse

Transcriptase (Applied Biosystems). Specific primers for the *glgB* gene, (5'-GGGCTTTACCCACCTCGAAC-3' and 5'-CCATCGAAGGGATGCTCGT-3') and for the potato *ubi3* gene (Garbarino and Belknap, 1994) as an internal control (5'-TTCCGACACCATCGACAATGT-3' and 5'-CGACCATCCTCAAGCTGCTT-3') were designed by the primer express software (version 1.5, PE Applied Biosystems, CA, USA). Aliquots of 50 ng of cDNA for each transgenic potato plant were amplified with gene specific primers on the Perkin Elmer ABI Prism 7700 sequence detector in conjunction with the SYBR green PCR master mix kit (Applied Biosystems). The PCR thermal conditions were 10 min at 50 ° C, 5 min at 95° C followed by 40 cycles (15s at 95° C, 1 min at 60° C). The relative quantification of the target RNA (*glgB*) expression level was calculated based on  $C_t$  (Cycle threshold) values, using the User Bulletin #2 (ABI PRISM 7700 sequence detection system, December 1997, Applied Biosystems).

### **Analysis of physico-chemical properties of different transformants**

Granule morphology and shape were investigated by both light microscopy (LM; Axiophot, Oberkochen, Germany) equipped with a Sony color video camera (CCD-Iris/RGB) and Scanning Electron Microscopy (SEM; JEOL 6300F, Tokyo, Japan). A solution of 20X dilution of Lugol's solution (1% I<sub>2</sub>/KI) was used to stain starch granules. For SEM, 1 mg of dried starch samples was spread on silver tape and mounted on a brass disc coated with a 20 nm platinum layer. Samples were then examined with a scanning electron microscope operating at an accelerating voltage of 1.5–3.5 keV.

Parameters such as enthalpy, heat capacity and thermal behaviour were measured by Differential Scanning Calorimetry (DSC), using a Perkin-Elmer pyris 6 machine (Perkin-Elmer, Vlaardingen, The Netherlands). The onset temperature of melting and differences in enthalpy ( $\Delta H$ ) were measured automatically by its software. The rest of the experiment was done as described by Ji *et al.* (2003).

The particle size of transformants as well as control starches were measured using a Coulter Counter Multisizer II (Beckman- Coulter, High Wycombe, UK) using 100  $\mu$ m and 200  $\mu$ m orifice tube for *amf* and wild type starches, respectively. Approximately 10 mg of starch was suspended in about 160 ml of isotone solution, each sample was measured at least twice and granule size distribution was recorded for at least 50,000 particles.

To test the chain length distribution, 5 mg of starch from transgenic potatoes was suspended in 250  $\mu$ l of DMSO and gelatinized for 10 min at boiling temperature. After cooling down to the ambient temperature, 700  $\mu$ l 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 40 °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 35 °C. Aliquots of 100  $\mu$ l 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 35 °C. The flow rate was 1.0 ml/min and 20  $\mu$ l 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).

The amount of reducing sugars was determined with the *Nelson-Somogyi* method (Nelson, 1944) and expressed as dextrose equivalents (DE).

## Post harvest experiments

Five mg of starch from selected transformants (Fig. 4) was incubated in two different ways. Assay (a): Starches were incubated in 100 mM phosphate buffer pH=7.5 (Hilden *et al.*, 2000) and incubated for 5 days at 40° C and 55° 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 250 µl DMSO, 800 µl of 10mM phosphate buffer was added and the mixture was incubated for 48 hours at 30° 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, 550 µl distilled water and 250 µl DMSO were added. The HPSEC and HPAEC analyses were performed as mentioned earlier.

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# *Chapter 6*

**General discussion**

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Successful incorporation of an active granule-bound luciferase protein into potato starch granules during starch biosynthesis, using starch binding domain (SBD) (Ji *et al.*, 2003) and *in planta* modification of starch towards so-called “designer starches” were the main driving forces to equip different candidate proteins with a SBD from *B. circulans* CGTase. Given SBDs high affinity for raw starch, it is possible to bring starch granules in close contact with effector enzymes during starch biosynthesis. By doing this we intended to alter the starch granule structure, its components and functionalities.

One of the drawbacks which limits native starch applications in food and non-food industries is retrogradation of starch pastes, i.e. when starch is heated and then is cooled down, the continuous cooling makes the starch solution cloudy. In industry, this phenomenon is called “retrogradation of starch” caused by recrystallization/realignment of amylose chains through cooling. To this end, in the different chapters of this thesis, we have tried to improve this property of potato starch through implementing SBD technology fused to different effector proteins. We therefore fused a number of interesting candidate proteins to both the carboxyl and amino termini of SBD as an anchor. Different microbial enzymes, each performing a desired function on oligosaccharides and/or starch were chosen from different enzyme families; (i) an *E. coli* maltose acetyltransferase (MAT), to acetylate starch (Chapter 3), (ii) a truncated mutansucrase to achieve introduction of novel  $\alpha(1\rightarrow3)$  side chains in potato starch (Chapter 4) (iii) and finally a glycogen branching enzyme from *E. coli* to introduce extra  $\alpha(1\rightarrow6)$  branch points (Chapter 5) were investigated in this thesis. Besides, since it was found in our previous study that SBD2 had an almost 10 fold higher affinity for starch granules than single SBD (Ji *et al.*, 2004) multiple repeats of SBDs (SBD2-SBD5) linked via a Pro-Thr rich linker were also expressed in wild type potato genetic background to displace Granule-Bound Starch Synthase I (GBSSI) (Chapter 2), hoping to get amylose-free starch in this way. In all these aforementioned cases diminishing of starch retrogradation behavior was the objective. For instance, acetyl groups reduce reassociation of glucan chains and consequently retrogradation. Introduction of new types of side chains by mutansucrase interfere with the interaction between amylose and amylopectin chains and therefore less retrogradation is expected. If extra short chains are introduced by *glgB*, starch gels are less susceptible to retrogradation. Finally, elimination of all amylose as the main source of retrogradation might be achieved if SBDs could displace GBSSI from the granules. An overview of different achievements for each experimental chapter is discussed here.

## Expression of fusion proteins affects starch granule morphology

Depending on the enzyme fused, position of the SBD and sometimes the genetic background used, starch granule structure was severely altered. Dramatic alterations occurred in all cases whenever enzymes had affinity towards  $\alpha$ -glucans.

Expression of glycogen branching enzyme (*glgB*) and a catalytic domain of mutansucrase while these enzymes were fused to SBD changed the starch granule morphology severely. It is thought that starch components (amylose and amylopectin) can interact not only with each other but also with the heterologous fusion proteins during starch biosynthesis (Chapter 4 and 5). Strikingly, from a 3D point of view of these two enzymes, there seem to be similarities which might help to decipher the way they interact with  $\alpha$ -glucans. Mutansucrase and *glgB* belong to GH70 and GH13 ( $\alpha$ -amylase) families, respectively. Although the 3D structure of glucansucrases (GTFs) is not yet available, comparative sequence and secondary structure analysis of GTFs with members of GH13 ( $\alpha$ -amylase) family have shown that the catalytic core of these enzymes possess a  $(\beta/\alpha)_8$ -barrel structure similar to  $(\beta/\alpha)_8$ -barrel central core of members of GH13 family but it seems to be circularly permuted (MacGregor *et al.*, 1996). *GlgB* is a member of  $\alpha$ -amylase family and possesses a long catalytic cavity in which  $\alpha$ -glucans and protein interact. This long cleft of *glgB* is thought to be large enough to accommodate relatively long maltooligosaccharide (MOS) (dp, 7-30) molecules (Binderup *et al.*, 2000) in the course of starch biosynthesis. On top of that, the N-terminal region of *glgB* seems to look like a SBD and determines the length of the transferring chains as well as is involved in MOS binding and helping the enzyme to cut and introduce new side chains (Devillers *et al.*, 2003). Therefore, taken together fusion proteins comprising *glgB* are capable of binding the  $\alpha$ -glucans during starch biosynthesis. Likewise mutansucrase (*GtfI*) is capable of accommodating MOS and therefore the so-called acceptor reaction of the enzyme is facilitated (Fu and Robyt, 1990; Fu and Robyt, 1991; Kralj *et al.*, 2005; Monchois *et al.*, 1999; Monchois *et al.*, 2000). Fusion of *GtfICAT* to SBD with two maltose binding sites, increases the acceptor reaction of *GtfICAT* and bringing the enzymes to the proximity of the granule surface may facilitate the interaction of enzymes with  $\alpha$ -glucan side chains of the nascent amylopectin molecules. Upon such a binding to amylopectin side chains, *GtfICAT* might influence the lateral interactions, which in turn could lead to perturbations at the granule surface, and eventually the spongy or bumpy appearance of the granule surfaces. Therefore, it is thought that the different morphological alterations of the starch granules are

related to GtfICAT's ability to bind maltooligosaccharides (MOS) on the one hand and affinity of the SBD for the starch granules on the other hand. In these fusion proteins, the individual granules were affected while expression of *E. coli* Maltose acetyltransferase (MAT) resulted in amalgamated granules, i.e. many granules are assembled in a structure which looks like a cluster. This apparent difference between the MAT protein and the other fusion proteins is striking and intriguing. MAT does not have a catalytic domain similar to GH13 and GH70 enzymes, it belongs to the hexapeptide acyltransferase (HexAT) superfamily (Wenzel *et al.*, 2005). It forms a trimer (Lo Leggio *et al.*, 2003) and at the interface of two monomeric units a tunnel is formed in which the acceptor substrate, maltose, can be accommodated (Wenzel *et al.*, 2005). This interesting feature of MAT presumably enables the enzyme to cross-link different growing starch granules at the same time and forms thereby maybe the amalgamated granules. A similar phenomenon was observed with multiple repeat SBDs in which, depending on SBDs concentration the way how SBDs binds to starch is thought to be responsible for the so called cluster formation (Chapter 2). In other words, multiple SBDs are able to cross-link different granule nucleation sites, eventually leading to the clusters. We however found it difficult to draw conclusions on why different positions of SBD within fusion proteins or different genetic backgrounds might result in different starch phenotypes.

### **Effect of potato genetic background on starch granule morphology alteration**

Although Ji *et al.* (2003; 2004) found a genetic background dependency in term of SBD accumulation, in this study there was not such a strong relationship. The reason why such phenomenon was not so obvious could be that the effector proteins had affinity for the starch granules on their own. Therefore, fusion proteins regardless of construct used, showed an overall affinity for starch granules. Altered starch granules showed different phenotypes with respect to the potato genetic background. The only difference between amylose-free and amylose-containing genetic backgrounds is the presence of the granule-bound starch synthase I (GBSS I), the amylose enzyme, and therefore amylose. It is likely that amylose plays an important role in the modifying type of the altered starch granules. Nevertheless, we believe that either the interaction between GBSS I and heterologous proteins or between amylose and heterologous proteins may play a pivotal role in this regard. To shed light on the exact

mechanism underlying these altered starch granule phenotypes, expression of the same constructs in different genetic backgrounds or crops would be worth trying.

### **Multiple repeat SBDs seem not to be a good competitor of GBSSI**

A double SBD (SBD2) is a better tool with a higher affinity for anchoring foreign proteins in starch granules than a single SBD (Ji *et al.*, 2004). Furthermore, it was demonstrated that the two SBDs act in synergy which might theoretically mean that increasing the number of appended SBDs (resulting in 3, 4 and 5 appended SBDs) will probably behave the same and will eventually have a much higher affinity for starch granules. Therefore, it might be possible to displace the GBSSI protein from the granules during starch biosynthesis. In the course of this study, reduction of GBSSI protein and consequently amylose production were not evidenced. We assume that SBD might not be a good competitor to challenge GBSSI because binding of SBD to the starch granules is time consuming and depends on SBD conformational changes. Furthermore, it seems that there is no correlation between the concentration of amylose in starch and the GBSSI concentration in potato amyloplasts in such way that at a certain level of GBSSI activity, a maximum amount of amylose is formed, but a further increase in GBSSI activity does not result in an increase in amylose concentration (Flipse *et al.*, 1996a). This however does not exclude the possibility that GBSSI and SBD might occupy the same locations in starch granules.

### **Incorporation of novel $\alpha(1\rightarrow3)$ glucan side chains in the starch granules is possible**

*In planta* production of foreign polymers have engaged many scientists world-wide, (Cairns, 2003; Gerrits *et al.*, 2001; Kok-Jacon, 2005; Kok-Jacon *et al.*, 2005a; Kok-Jacon *et al.*, 2005b;; Neumann *et al.*, 2005). The idea to turn plants into green factories can in principle be achieved in such a way that plants produce compounds which normally are found in animals or bacteria. Despite some negative effects on growth and development of transgenic plants producing such foreign compounds, in all cases the amounts of such compounds are low and sometimes might be below the detection limit of available methods (Bohmert *et al.*, 2000; Bohmert *et al.*, 2002). Although the pioneering experiments have opened up possibilities to produce foreign polymers and compounds, these types of molecular farming requires much more research before being introduced into agricultural production. One reason



could be the fact that at least 15% dry weight production of a foreign polymer is required to commercialize its production, which so far seems impossible to be achieved. In this thesis the introduction of novel  $\alpha(1\rightarrow3)$  glucosyl linkages to the starch polymers by a mutansucrase from *Streptococcus downei* Mfe28 is described (Chapter 4). Kok-Jacon et al. (2005a) showed that a truncated form of a mutansucrase had a higher efficiency for mutan production in potato amyloplasts than the full-length enzyme, although in principle the truncated one had a lower activity (Monchois *et al.*, 1999). Presence of mutan in wild type potato background led to some physico-chemical alterations such as 1.7 fold higher viscosity and starch granule alterations in comparison with both the control plants and GtfI transformants (Kok-Jacon *et al.*, 2005a). The wish to bring GtfICAT and acceptor molecules (starch polymers) to closer contact with each other and the idea to produce mutan inside the starch granules led us to equip GtfICAT with SBD. Although GtfICAT was successfully targeted to the starch granules, to our surprise this did not lead to introduction of  $\alpha(1\rightarrow3)$  glucosyl linkages as expected. We speculate that the acceptor reaction was not particularly favored inside the starch granules because we found no evidence(s) for altered chain length distribution. The donor substrate (sucrose) seems to be less available in potato amyloplasts than other subcellular organelles (Farre *et al.*, 2001). This can be another reason why neither GtfI nor GtfICAT expression in potato tubers could efficiently result in a lot of mutan production (Kok-Jacon *et al.*, 2005a). On top of that we postulate that nascent starch polymers might not be good acceptors for GtfICAT or SBD might somehow inhibit GtfICAT catalytic activity. Nevertheless it seems possible to co-produce mutan along with potato starch but at very low amount.

### **Acetylation of starch requires more than MAT expression alone**

Although the *in planta* acetylation of starch was possible, the degree of substitution (DS) was low. We assume that in order to be able to acetylate starch *in planta* efficiently, a high sink of acetyl-CoA is necessary in potato amyloplasts. Indications that acetyl-CoA is not present in high amounts in potato amyloplasts were obtained by Romano *et al.* (2005) who tried to produce polyhydroxybutyrate (PHB) (a final product which substrate is acetyl-CoA) in potato tubers but only a very small amount of PHB was produced. Granule-bound MAT was found active inside the starch granules in post harvest experiments. Despite retention of MAT activity even in a crystalline matrix, this did not result in starch acetylation during post

harvest experiments. Nevertheless, glucose, maltose and some of the starch biosynthetic enzyme substrates were found acetylated. This is probably due to the fact that MAT is mainly active towards smaller maltooligosaccharides (MOS) (Lo Leggio *et al.*, 2003) and not towards large polysaccharides such as starch. Perhaps also the limited acetyl-CoA pool in potato amyloplasts might be a second reason why MAT could not acetylate starch efficiently (Chapter 3). Three alternative approaches can be considered to obtain a high degree of acetylation in potato. The first option could be to rationally design MAT in such a way that its activity increases as long as the length of MOS increases. Second, if possible, the acetyl-CoA supply should be increased in the potato amyloplasts by expression of an acetyl-CoA transporter. Finally, expression of the NodL enzyme, an enzyme with similar sequence and as functionality MAT (Bloemberg *et al.*, 1994), but capable of acetylating longer MOS can be considered.

### ***E. coli* glycogen branching enzyme (*glgB*) fused to SBD does not lead to an increased branching degree of starch**

Although *E. coli* glycogen branching enzyme seemed to be able to increase the number of branch points of the starch (Kortstee *et al.*, 1996), to see significant changes in starch properties, a much higher increase rate than 25% in branching points is required. It is possible that *glgB* branches amylose/amylopectin in starch at a higher level but such differently branched molecules are likely to be accepted by endogenous enzymes (starch synthases) because glycogen-like molecules can not form double helix structures which are a prerequisite for starch granule formation. Therefore we think that these highly branched molecules are good substrates for isoamylases. Another explanation for a low degree of branching might be the *glgB* affinity for starch granules which might prevent the enzyme to make branch points more efficiently. In fact our finding of accumulation of large amounts of fusion proteins comprising *glgB* in starch granules corroborates on the high binding affinity of this enzyme for starch granules.

Interestingly expression of *glgB* fused to SBD generated starches with perforated and irregular phenotypes in amylose-free and amylose-containing genetic backgrounds, respectively (Chapter 5). It is difficult at this point to pinpoint why starch granules in both backgrounds show different phenotypes. However, it is tempting to assume that amylose plays an important role in this respect.

## Concluding remarks

For three reasons, the potato tuber is a suitable model system for the study of starch metabolism: (i) the conversion of sucrose to starch is the dominant metabolic flux in potato tubers, (ii) almost all the major genes involved in potato starch biosynthesis are known, making it possible to study effect(s) of individual genes on starch biosynthesis, and (iii) *Agrobacterium*-mediated transformation of potato is easy. However, the mechanism by which starch granules are made in the form of dense granules largely remains unknown. Depending on the protein of interest fused to SBD, starch granule morphology was changed. The phenotype of these altered granules has something to do with either affinity of heterologous proteins for the starch or interaction of fusion proteins with starch biosynthesis enzymes. One of the intriguing questions about starch biosynthesis is how starch granules are being made. Although most of the enzymes involved in starch biosynthesis are known, the analysis of starch biosynthesis is complicated by the fact that starch granule formation is not in a linear pathway. That means that alteration of starch granule morphology due to inhibition of a particular gene involved in starch biosynthesis can not be directly correlated to the reduction of an enzyme. There are many reports showing that antisense down regulation of enzymes involved in starch biosynthesis alone or simultaneously has resulted in starch granule alterations (Bustos *et al.*, 2004; Edwards *et al.*, 1999; Flipse *et al.*, 1996b; Jobling *et al.*, 2002; Lloyd *et al.*, 1999). Interestingly introduction of foreign genes into potato amyloplasts has also resulted in starch granule morphology alterations (Ji *et al.*, 2003; Ji *et al.*, 2004; Kok-Jacon *et al.*, 2005a; Kok-Jacon *et al.*, 2005b; Kok-Jacon *et al.*, 2005c; this thesis chapters 2,3,4 and 5). Surprisingly, in all cases the shape of the altered starch granules is different.

Despite a lot of progress on starch biosynthesis, there remains uncertainty and controversy regarding the starch granule initiation. In glycogen synthesis, there seems to be some sort of priming called glycogenin for the enzymes involved in glycogen biosynthesis. Since the mechanism of starch granule initiation is not yet fully understood, we assumed that generation of different altered starch granules in this thesis might shed light on this mysterious story and help researchers to decipher starch granule formation.

The principle of *in planta* starch acetylation in this thesis was shown and has paved the path for further attempts to obtain starches with a high degree of acetylation. This will be durable if new techniques such as site-directed mutagenesis are employed to generate a new

generation of MAT to accept longer maltooligosaccharides as acceptor molecules. Enrichment of acetyl-CoA in potato amyloplasts through genetic engineering approaches might be another necessary step in this.

Co-deposition of mutan polymers with starch might result in new generations of polymer compounds with numerous industrial applications such as adhesives for paper coating industry (Haag *et al.*, 2004). Although the suitability of the punctured starches produced with *GtfICAT* and *glgB* have not yet been tested for industrial applications, these types of starches might be interesting for starch based derivatives.

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## Note

# ***Appendixes***

**Summary in English**

**Summary in Dutch (Samenvatting)**

**Acknowledgment**

**List of Publications**

**Training and Supervision Plan (TSP)**

**About The Author**

**Sponsorship**

**Summary in Persian (خلاصه فارسی)**

**Persian Appendixes**

**S**tarch, a complex carbohydrate, is a polymer of glucose residues. It occurs in two main forms: amylose, consisting of predominantly linear chains of glucose units linked by  $\alpha(1,4)$  glycosidic bonds, and amylopectin, in which the chains are highly branched by the addition of  $\alpha(1,6)$  glycosidic bonds. Depending upon the plant species and the site of storage, the proportion between these two components varies. In most plant species, amylose comprises about 20% of the starch and the rest is amylopectin.

Starch is one of the cheapest renewable resources of carbohydrates. Although in its native form it has some applications in food and non-food industries, the properties of currently available starches (native starch) do not comply with most industrial standard and enhanced commercial applications. To obtain starches with particular properties such as starches with lower retrogradation and more freeze-thaw stability, starch is often chemically modified. Manipulation of the starch structure with chemical reactions or additives will eventually impart certain properties which are desired for industrial uses. Techniques including cross-linking (to strengthen against shear) or acetylation (to reduce the retrogradation) are the most common starch modifications. The use of chemicals, however, may not only cause concern over health and safety, but there is also a cost involved with the chemical modification.

Over the last decade many enzymes have been identified that can influence the amount or type of starch that a plant produces. Interestingly, these findings have provided useful targets for starch bioengineering. Alterations in the level of the enzymes of the starch biosynthesis pathway have been shown to improve the quantity of starch produced by plants such as potatoes. Genetic manipulation of starch synthase genes allowed the proportions of amylose and amylopectin in starch to be altered, yielding starches tailored for different industrial purposes.

The aim of the research described in this Ph.D thesis was to investigate possibilities to alter starch functionalities through implementing starch binding domain (SBD) technology. In order to produce starches with different properties such as lower retrogradation and higher freeze-thaw stability, a number of interesting microbial enzymes were used either alone or fused to the non-catalytic, E domain (SBD domain) of cyclodextrin glycosyltransferase (*CGTase*) of *Bacillus circulans*. SBD as a tool enables effector proteins to be targeted to starch granules during starch biosynthesis. In this way some of the starch precursors or components (maltooligosaccharides, amylose and amylopectin) are brought in intimate

contact with the microbial proteins. Therefore, given SBD affinity for the starch granules, we think that effector proteins fused to SBD have more impact on starch properties than the effector proteins alone, provided the substrate for the effector proteins is available.

From an industrial viewpoint, the presence or absence of amylose greatly determines the suitability of starch for different applications. Starch properties such as retrogradation of starch pastes, poor transparency of starch gels, low adhesiveness, poor freeze-thaw stability and high granule melting temperatures are all correlated with a high (apparent) amylose content of starch. Therefore, *in planta* modulation of the amount of amylose of potato starch granules has been an important objective for the starch industry. Various transgenic approaches have been explored for modifying the amylose content of potato starch. In earlier research, we were able to show that a tandem repeat of two SBDs had a 10 fold higher affinity towards starch granules than a single SBD domain. Hence we hypothesized that engineering of a series of multiple high-affinity repeats of family 20 starch binding domain might be able to out compete native granule-bound starch synthase I (GBSSI) from the starch granules. Therefore, a series of constructs of SBDs (SBD<sub>2</sub>-SBD<sub>5</sub>) were introduced in an amylose-containing genetic background. Our results showed that none of the multiple repeat SBDs was able to displace GBSSI from the starch granules. Neither the amount of GBSSI protein nor the apparent amylose content of any of the transformants was changed (**Chapter 2**). However, it was shown that SBD<sub>n</sub> expression can affect physical processes underlying granule assembly, in both amylose-containing and amylose-free potato genetic backgrounds, without altering the primary structure of the constituent starch polymers and the granule melting temperature. The amount of SBD accumulated in starch granules increased progressively from SBD to SBD<sub>3</sub>; however, it seemed as if less SBD<sub>4</sub> and SBD<sub>5</sub> were accumulated inside the starch granules.

Starch acetylation is required because native starch does not have the desired characteristics demanded by industry. Improved stability against retrogradation, lower gelatinization temperature, more transparency and improved elasticity are some of the desired properties of acetylated starches. Starch acetylation is achieved through chemical reactions using some environmentally pollutant procedures. In an attempt to acetylate potato tuber starch *in planta*, *E. coli* maltose-acetyltransferase (MAT) was targeted to the starch granules either alone or fused to SBD. A significant higher degree of acetylation was obtained in an amylose-containing genetic background. However, this did not seem to be enough to see altered physicochemical properties of starch. Interestingly, MAT maintained its activity inside

the starch granules when provided with acetyl-CoA and different acceptor substrates. Our results evidenced for the first time that MAT has affinity for starch granules on its own. In comparison to the SBD2 transformants, fusion proteins comprising MAT were accumulated in a larger amount inside the starch granules. MAT trimer formation on the one hand and fusion to SBD on the other hand might explain its affinity for starch granules as well as its high accumulation in the starch granules (**Chapter 3**).

Starch biosynthetic enzymes are only able to make  $\alpha(1,4)$  and  $\alpha(1,6)$  linkages. However, some bacterial enzymes are able to join glucose units through  $\alpha(1,2)$  and  $\alpha(1,3)$  linkages. These types of extracellular polysaccharides possess unique properties/functionalities and have made them industrially important. In an attempt to diversify the nature of glucosyl linkages in the potato starch structure, a truncated but active form of a mutansucrase gene (*GtfiCAT*) from *Streptococcus downei* Mfe28 bacteria was fused in frame with SBD. Our experiments showed that production of such a predominantly  $\alpha(1,3)$  polymer (mutan) was achieved. Specific erythrosine staining, exo-mutanase treatments of transgenic starches and some differences in physicochemical properties of transgenic starches corroborated the mutan production. Starch granule morphology was severely altered in an amylose-free genetic background, resulting in starch granules with many small holes in the granules. These alterations were however less pronounced and led to a different phenotype in an amylose-containing genetic background (**Chapter 4**).

Despite the fact that glycogen and starch share the same type of  $\alpha(1,4)/\alpha(1,6)$  glucosidic linkages, the properties of these two are totally different. From a structural viewpoint, the main differences between starch and glycogen are the number of the  $\alpha(1,6)$  glucosidic bonds and the length of the side chains. Thus, modification of the number of these  $\alpha(1,6)$  branch points might change starch properties for new applications. Although expression of *E. coli* branching enzyme (*glgB*) in potato tubers had shown a 25% increase in overall amylopectin branching, no obvious physicochemical alteration had been reported. In an attempt to manipulate the number and length of amylopectin branch points further, *E. coli* branching enzyme (*glgB*) was equipped with SBD either at the C- or N- terminus and expressed in two potato genetic backgrounds. In comparison to other transgenic plants generated in this thesis, starches from transgenic plants comprising *glgB* showed a surplus of fusion proteins inside the starch granules. Despite the fact that starch main characteristics did not deviate from control plants, presence of fusion proteins brought about severe granule

morphology alterations. Moreover, the potato genetic background in which *glgB* was expressed mattered. Porous starch granules in an amylose-free genetic background were observed, whereas those of the Kardal background had irregular surfaces. The chain length distribution in the starch of transformants was not different from that in the control plants. This might be due to the fact that potato de-branching enzymes (isoamylases) play a proofreading role, i.e. isoamylases will eventually cleave randomly made chains by *glgB* from the amylopectin chains and make them competent for crystallization (**Chapter 5**).

The work in this thesis describes the applicability of SBD technology during starch biosynthesis. It shows the great potential to alter starch structure. Although there are many candidate genes to be fused to SBD and targeted to the starch granules, care must be taken to consider all possible ins and outs of the enzymes and their substrate specificity. To achieve further alteration of starch polymers, a rational design of enzymes is required.



Zetmeel een complex koolhydraat is een polymeer van glucose. Het komt voor in twee vormen namelijk als amylose (bijna lineaire ketens met alpha 1,4 bindingen) en als amylopectine (vertakte ketens met alpha 1,4 en alpha 1,6 bindingen). In de meeste plantensoorten is de ratio amylose/amylopectine ca 20/80 alhoewel er mutanten bekend zijn waar deze ratio kan variëren. Zetmeel is een van de meest voorkomende hernieuwbare grondstoffen. Natief zetmeel kent beperkt toepassingen in de voedingsindustrie en voor industriële doeleinden. Voor de meeste toepassingen dient het zetmeel gederiviseerd te worden: chemisch, enzymatisch of fysisch. De meest gewenste eigenschappen van zetmeel zijn lage retrogradatie en een hoge vriesdooi stabiliteit. Manipulatie van de zetmeel structuur via derivatisering is een belangrijke handeling om producten te krijgen die geschikt zijn voor industriële toepassingen. Meest gebruikte technieken daarbij zijn cross-linking en acetylering waarbij niet zelden chemicalien gebruikt worden die niet alleen een kostenfactor van belang kunnen zijn, maar daarnaast ook problemen voor milieu en gezondheid kunnen opleveren.

Gedurende het laatste decennium zijn de meeste enzymen die een rol spelen bij de biosynthese van zetmeel geïdentificeerd en geïsoleerd. Genetische modificatie met deze enzymen hetzij alleen hetzij in combinatie heeft ‘tailor-made’ zetmelen opgeleverd met verschillende nieuwe eigenschappen en verschillende ratio’s van amylose en amylopectine alsmede andere zetmeelkorrel structuren. In een aantal gevallen heeft dat geleid tot zetmelen die zonder derivatisering al eigenschappen bezitten die voordien alleen door middel van chemische derivatisering bereikt konden worden.

Het doel van het onderzoek beschreven in deze dissertatie was om de mogelijkheden te onderzoeken om de functionaliteit van zetmeel te moduleren door gebruik te maken van de zetmeel bindings domein (Starch Binding Domain: SBD) technologie. De functionaliteiten die daarbij speciaal werden aangepakt betroffen de verlaging van de retrogradatie en verhoging van de vriesdooi stabiliteit. Hiertoe werden een aantal microbiële enzymen alleen of na fusie aan het zetmeel bindings domein van het cyclodextrine glycosyltransferase (*CGTase*) van *Bacillus circulans* gefuseerd. Dit domein zorgt ervoor dat de fusie-eiwitten in de zetmeelkorrel worden afgezet tijdens zetmeel biosynthese. Door op die manier de enzymen in direct contact te brengen met hun substraat verwachten we een groter effect op de uiteindelijk gevormde zetmeelkorrel en zetmeel samenstelling.

De hoeveelheid amylose in zetmeel bepaalt in grote mate de geschiktheid van het zetmeel voor zijn uiteindelijke toepassingen. Eigenschappen zoals retrogradatie van zetmeel

gels, slechte transparantie van gels, lage adhesie, slechte vriesdooi stabiliteit van gels en hoge smelt temperaturen van zetmeel zijn alle gecorreleerd met een relatief hoog amylose gehalte. Daarom is een van de meest gewenste modificaties de verlaging of complete verwijdering van de amylose fractie uit zetmeel geweest. De meest bekende is de uitschakeling van het gen coderend voor korrelgebonden zetmeel synthase (KGZ) hetgeen aanleiding geeft tot amylose-vrij zetmeel. In eerder onderzoek hadden we aangetoond dat SBD mogelijk op de zelfde plaats in zetmeel zou terecht komen als waar het enzym KGZ terecht komt. Derhalve was een van de werkhypothesen dat met meerdere SBD's in tandem verdringing van KGZ zou kunnen optreden hetgeen als resultaat amylose-vrij zetmeel zou kunnen opleveren. Een serie constructen werd gemaakt met meerdere SBD's (SBD<sub>2</sub>-SBD<sub>5</sub>), en deze werden geïntroduceerd in wildtype amylose-bevattende aardappels. De resultaten van dit onderzoek lieten zien dat geen van de constructen in staat was om het KGZ weg te concurreren uit de zetmeelkorrel. Noch de hoeveelheid KGZ eiwit, noch de hoeveelheid amylose was veranderd (Hoofdstuk 2). Wat wel kon worden aangetoond is dat expressie van SBDn eiwitten het zetmeelkorrel assemblage proces danig kan beïnvloeden zonder echter de fysisch-chemische eigenschappen van het zetmeel te veranderen. Niet alleen in een amylose-bevattende genetische aardappel achtergrond, maar ook in een amylose-vrije achtergrond. De hoeveelheid SBD eiwit nam constant toe in zetmeelkorrels van transformanten met één, twee of drie SBD's, maar minder SBD eiwit werd aangetoond in zetmeelkorrels van transformanten met vier of vijf SBD's.

Zetmeel acetylering is een veel toegepaste chemische modificatie van zetmeel omdat natief zetmeel niet de door de industrie gewenste eigenschappen bezit zoals hierboven reeds aangegeven. Naast lagere gelerings temperatuur zijn grotere transparantie en verhoogde elasticiteit van gels de voordelen van geacetyleerd zetmeel. In Hoofdstuk 3 worden experimenten beschreven die tot doel hadden geacetyleerd zetmeel in de plant te maken. Daartoe werd het *E. coli* maltose-acetyltransferase (MAT), alleen of gefuseerd aan SBD, tot expressie gebracht in aardappel. Acetylering van zetmeel kon worden vastgesteld in transformanten van amylose bevattende aardappel. Echter dit was niet genoeg om de fysisch-chemische eigenschappen van het zetmeel te veranderen. MAT was actief in de zetmeelkorrel zoals bleek uit zogenaamde 'na oogst' experimenten waarbij acetyl-CoA en verschillende substraten werden aangeboden aan zetmeelkorrels die het enzym bevatten.

Zetmeel biosynthese enzymen zijn enkel in staat om  $\alpha(1,4)$  en  $\alpha(1,6)$  bindingen te maken. Er zijn echter ook enzymen bekend, met name van bacteriële herkomst, die andere

verbindingen zoals  $\alpha(1,6)$  en  $\alpha(1,3)$  kunnen maken. De polymeren met dit soort bindingen hebben andere unieke eigenschappen ten opzichte van zetmeel. Teneinde een diversificatie te verkrijgen van de verschillende bindingstypen in zetmeel is een getrunceerde maar biologisch actieve vorm van het mutansucrase gen (*GtfiCAT*) van *Streptococcus downei* Mfe28 gefuseerd met het SBD. De resultaten lieten zien dat een polymeer met hoofdzakelijk  $\alpha(1,3)$  verbindingen (mutan genaamd) geproduceerd werd in de zetmeelkorrel. Het feit dat het zetmeel te kleuren was met een specifieke kleurstof (erythrosine) die mutan aantoont, het mutan na exo-mutanase behandeling verwijderd kon worden en dat het zetmeel van de meest extreme transformant verschillen liet zien in zijn fysisch-chemische eigenschappen, onderstreepten het feit dat hier een nieuw zetmeel geproduceerd was. Naast de verandering van fysisch-chemische eigenschappen was ook de zetmeelkorrel structuur in transformanten van met name de amylose-vrije genetische achtergrond dramatisch veranderd. De zetmeelkorrels vertoonden zeer veel kleine gaten. In transformanten van de amylose bevattende achtergrond waren de afwijkingen minder duidelijk en ook van een andere aard dan in de amylose-vrije transformanten (Hoofdstuk 4).

Ondanks het feit dat glycogeen en amylopectine dezelfde type bindingen hebben waarmee de glucose units aan elkaar gekoppeld zijn [ $\alpha(1,4)/\alpha(1,6)$ ], zijn de eigenschappen van de twee polymeren totaal verschillend. Vanuit structuur oogpunt zijn de enige verschillen het aantal  $\alpha(1,6)$  verbindingen en de lengte van de zijketens. Dus indien het aantal bindingen in amylopectine (of zetmeel) verhoogd kan worden zou dat kunnen leiden tot andere eigenschappen van dat zetmeel. In eerder onderzoek was al aangetoond dat het mogelijk was om het aantal bindingen te verhogen met ca 25% door expressie van het *E. coli* vertakkingsenzym (*glgB*) in aardappel. Dit leidde echter niet tot fysisch chemische veranderingen van het zetmeel. In Hoofdstuk 5 werd getracht dit eerder beschreven effect te vergroten door het vertakkingsenzym te koppelen aan een SBD. In vergelijking tot andere transformanten hadden de GLGB-SBD transformanten veel meer fusie-eiwit in hun zetmeelkorrels dan alle andere geteste combinaties. Ondanks het feit dat de aanwezigheid van veel fusie-eiwit aanleiding gaf tot zeer afwijkende zetmeelkorrels in zowel amylose bevattende als amylose-vrije genetische achtergrond waren de fysisch-chemische eigenschappen van het zetmeel van die transformanten vergelijkbaar met die van de controle planten. In de amylose-vrije achtergrond vertoonden de zetmeelkorrels gaten, terwijl in de amylose bevattende achtergrond de zetmeelkorrels zeer onregelmatig van vorm waren. Een verklaring voor deze verschillende fenotypes van de zetmeelkorrels is nog niet voorhanden.

Waarom de zetmelen geen hogere vertakingsgraad hebben is ook nog niet duidelijk, maar een mogelijke verklaring zou kunnen liggen in het feit dat andere enzymen (zogenaamde isoamylases) de zwaarder vertakte ketens van het zetmeel wegnippen teneinde de pakking van de zetmeelkorrel beter mogelijk te maken.

Het werk beschreven in dit proefschrift toont de grote potentie aan van de SBD technologie. Niet alleen zal in toekomstige experimenten getracht moeten worden andere enzymen te koppelen aan SBD, maar ook zullen de specifieke eigenschappen van de hier gepresenteerde en nieuwe enzymen aangepast moeten worden via specifieke mutagenese, teneinde substraat specificiteit en activiteit optimaal te moduleren. Deze zogenaamde rationele modulering van enzymen zal moeten leiden tot nieuwe zetmeelkorrels en zetmeelpolymeren.

## **In the name of Allah, the Most Beneficent, the Most Merciful**

**“Those who do not thank people, they do not thank Allah”**

*The Prophet of Islam (pbuh)*

**B**elieve me or not, I am getting emotional as I write this part of my thesis. Having said that, I will be failing in my tasks if I do not thank those people who have made a difference in my life and scientific career. Therefore, I would like to start my acknowledgment with a common sense of great feeling in my heart. Greetings to my friends and supporters and to those whose life is devoted to the humanity in the name of our almighty Allah. Oh my almighty god, words can not express my heart feeling at this moment, I am not able to express my feeling and to say how grateful I am. God, you have promised to all of us “as long as we put our trust to you, you will expand our chest, ease our tasks and remove implement from our speech”. Thank you so much for all your blessings through my thin and thick.

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My episode of acknowledgement would be definitely incomplete if I would forget to thank the others. A Persian saying bells in my mind which says, “If you wish your merit to be known, acknowledge that of other people” Who are these other people? Obviously, those who even may not have been involved in my research but still made a large contribution to both my personal and social life here among Dutch community. My tenure in Wageningen was enriched by so many people. They helped me grow both as a person and as a scientist. Therefore, it is my pleasure to express my gratitude to many people without whom these achievements would not have been possible in time. In my daily work in the labs, I have been blessed with a friendly and cheerful group of fellow students and colleagues. Bjorn Kloosterman, as well as Jaap Timmers assistances provided me good arguments about

molecular aspects of plant breeding and helped me regain some sort of fitness in science, healthy body and healthy mind. Therefore I would like to thank both of you for your kind helps and friendly chit chats. Jeroen Werij, often kept me entertained with his charming smiles. I would also like to thank Annelies, Marcel, Koen, Jan, Simon and Nicolas for their friendly supports. To Christian Bachem, Ralph, Dirkjan, Anne-marie and Bea, I praise Allah that I met you guys, your fruitful discussions and suggestions made this thesis through. Splendid and thank you so much for all you did to help me. I take this opportunity to thank Herman for our nice debates on religious matters and the way we could reach to the same destination. I learnt a lot from about Dutch's rich cultural inheritance as well as different topics while drinking coffee together. To my Chinese officemates, Shujun and Jianjun, I enjoyed being in the same office during my writing phase. What a fast and difficult language you are speaking, I mean Chinese!!!. I got a good impression about wonderful Chinese culture. I am also really grateful to you for the friendly and peaceful atmosphere that we had together.

In many ways, I have learnt much from our technician in molecular, cell biology and tissue culture labs. Irma, Elly, Fien, Petra, Marian, Isolde and Marjan have impressed me with their willingness to teach me how to use both the equipments and to set up experiments in such a way that I can now be considered to be in their capable hands. They always kept me on my theoretical toes with their tricky questions. I really appreciate what you have done to support me to fulfill my experiments and finish up this thesis.

Although far from our relatives and close friends, the Iranian ethnic minority in Wageningen is/was unbelievable from all the angles. We never felt lonely as long as Iranian friends were around and ready to offer helps and assistances. I hereby would like to thank all my Iranian friends and families in Wageningen. My special thanks go to Dr. Otroshy and Mahdizadeh families for their kindness. I was blessed to be in the same office for almost two and half year of my PhD period with Hossein Jafary. Dear Hossein, I want to thank you for all your helps and great moments in which we were together. I really enjoyed our tea (coffee) gathering with Hossein Jafary, Rahim Mehrabi, Reza Aghnoum, Mahmoud Tabib ghafari, Alireza babaie and Alireza Seifi inside or outside the PRI building. We never forget our family outings/parties with Talebi, Vazifiedoust, Khalesi and Mahdizadeh's families. Our family journeys and trips inside and outside the Netherlands will not be forgot. Apart from friends in Wageningen, I should appreciate my Iranian friend, Reza Shirzadian from Groningen University for all his mental supports during my PhD study. To my esteemed



friend Dr. Saeed Mohamadzadeh in Iran, I really appreciate your attention and hospitalities whenever we were in Iran. I wish you a very prosperous life and scientific career as well.

I also want to thank my parents, who taught me the value of hard work by their own example. I would like to share this moment of happiness with my wife's family and those in IRAN who rendered me enormous support during the whole tenure of my academic study since 2003.

Dear Fatemeh and Amir Hossein, I know that it was difficult for you to put up with a tired father when he got home late in the evening during last 4 years but I hope some day when you grew up you will justify me for that. I was always facing this question from you in the weekends "Won't you go to the Department?" but the answer was always disappointing (Yes, I will). Sorry, mates. It was my entire fault and I am now ready to compensate at any cost.

By the time, my wife gets here; she will definitely be asking herself why she has married to someone who does not even include her in his acknowledgements. One should remember that hidden between lines, figures and chapters of this thesis was many stories. Story of adventure, sacrifice, maturation, gratitude, friendship, and most importantly love. My dear wife (Akram), I kept you for last because you are the best part of my life. Every day, I am amazed by how much you have become a part of me. Besides, if I call myself a successful man for fulfillment of my thesis, then I should memorize another Persian saying, "Behind every successful man, there stands a woman". You keep saying that leaving behind everything to come with me is not a sacrifice for you. You readily agreed to give up all your dreams of higher education and a scientific career for the sake of family, and me. You are right, it is not a sacrifice, there are no sacrifices in the ties of love to the family future. I must confess that your support and understanding was enough to keep me running, day, night, weekends and holidays in the labs. Thank you for all you have done for kids and me. You have assured me that my life with you would definitely not be boring.

## List of publications

### Full papers

- (1) **Nazarian Firouzabadi, F.**, Kok-Jacon G,A., Vincken, J-P., Ji, Q., Suurs, L.C.J.M. and Visser, R.G.F. (2006) Fusion proteins comprising of the catalytic domain of mutansucrase and starch-binding domain can alter the morphology of amylose-free potato starch granules during biosynthesis. *Transgenic Res*, in press.
- (2) **Nazarian Firouzabadi, F.**, Vincken, J-P., Ji, Q., Suurs, L.C.J.M., Buléon, A. and Visser, R.G.F. (2006) Accumulation of multiple-repeat starch-binding domains (SBD2-SBD5) does not reduce amylose content of potato starch granules. *Planta*,in press.
- (3) **Nazarian Firouzabadi, F.**, Vincken, J-P., Ji, Q., Suurs, L.C.J.M. and Visser, R.G.F. (2006) Expression of an engineered granule-bound *E. coli* maltose acetyl transferase in wild-type and *amf* potato plants. *Plant Biotechnology Journal*, in press.
- (4) **Nazarian Firouzabadi, F.**, Vincken, J-P., Ji, Q., Suurs, L.C.J.M., Visser, R.G.F. (2006) Introduction of an engineered granule-bound *E. coli* glycogen branching enzyme results in irregular and punctured starch granules in wildtype and amylose-free potatoes. Submitted for publication.

### Abstracts

- (1) **Nazarian Firouzabadi, F.**, Vincken, J.P., Ji, Q., Suurs, L.C.J.M. and Visser, R.G.F. (2006) Expression of an engineered granule-bound *E. coli* maltose acetyl transferase in wild-type and *amf* potato plants. Page 238 in: Proc. The XXIIIrd International Carbohydrate Symposium, Whistler, Vancouver Canada.
- (2) **Nazarian Firouzabadi, F.**, Vincken, J.P., Ji Q., Suurs, L.C.J.M. and Visser R.G.F. (2006) Accumulation of multiple-repeat starch-binding domains (SBD2-SBD5) does not lead to a reduction of amylose in potato tuber starch. Page 94: Proc. The 8<sup>th</sup> International Congress of Plant Molecular Biology (ISPMB), Adelaide, South Australia.
- (3) Visser, R.G.F., **Nazarian Firouzabadi, F.**, Kok-Jacon, G., Ji, Q., Vincken, J.P., Suurs, L. (2006) Breeding for improved and novel starch characteristics in potato. Page 20 in: Proc. The 11<sup>th</sup> IAPTC&B Congress, Beijing, China.

### Patent

**Nazarian Firouzabadi F.**, Vincken, J.P., and Visser, R.G.F. (2006) Methods and means for producing starch having at least one altered characteristic. **EP 06076710.0**

### Award

The best student poster prizewinner from the 8<sup>th</sup> International Congress of Plant Molecular Biology (ISPMB), Adelaide, South Australia, 2006.

## Education Statement of the Graduate School

## Experimental Plant Sciences



|                                                                                                                |                     |
|----------------------------------------------------------------------------------------------------------------|---------------------|
| Issued to: Farhad Nazarian Firouzabadi                                                                         |                     |
| Date: 08 January 2007                                                                                          |                     |
| Group: Laboratory of Plant Breeding, Wageningen University                                                     |                     |
| 1) Start-up phase                                                                                              | <i>date</i>         |
| ► First presentation of your project                                                                           |                     |
| ► Modification of starch biosynthesis in potato tuber using starch binding domain                              | Mar 1, 2003         |
| ► Writing or rewriting a project proposal                                                                      |                     |
| ► <i>In planta</i> modification of potato starch granule biogenesis by different granule-bound fusion proteins | Feb-Jun 2003        |
| ► Writing a review or book chapter                                                                             |                     |
| ► MSc courses                                                                                                  |                     |
| ► Genetic variation and modification (Code F750-210)                                                           | Mar 2003            |
| ► General aspects of Plant Breeding (Code F750-209)                                                            | Mar 2003            |
| ► Laboratory use of isotopes                                                                                   |                     |
| ► safe handling with radioactive materials and sources                                                         | Apr 2004            |
| <b>Subtotal Start-up Phase</b>                                                                                 |                     |
| <b>15.0 credits*</b>                                                                                           |                     |
| 2) Scientific Exposure                                                                                         |                     |
| ► EPS PhD student days                                                                                         |                     |
| ► EPS PhD student day, Vrije Universiteit Amsterdam                                                            | Jun 3, 2004         |
| ► EPS PhD student day, Radboud University Nijmegen                                                             | Jun 2, 2005         |
| ► EPS PhD student day, Wageningen University                                                                   | Sep 19, 2006        |
| ► EPS theme symposia                                                                                           |                     |
| ► EPS theme symposia III 'Metabolism and Adaptation', University of Amsterdam                                  | Dec 11, 2003        |
| ► EPS theme symposia III 'Metabolism and Adaptation', Utrecht University                                       | Nov 24, 2005        |
| ► EPS theme symposia III 'Metabolism and Adaptation', University of Amsterdam                                  | Nov 10, 2006        |
| ► NWO Lunteren days and other National Platforms                                                               |                     |
| ► ALW meeting Lunteren Experimental Plant Sciences                                                             | Apr 7-8, 2003       |
| ► ALW meeting Lunteren Experimental Plant Sciences                                                             | Apr 5-6, 2004       |
| ► ALW meeting Lunteren Experimental Plant Sciences                                                             | Apr 3-4, 2006       |
| ► Seminars (series), workshops and symposia                                                                    |                     |
| ► Plant breeding seminar series (9x)                                                                           | 2003-2007           |
| ► Seminar plus                                                                                                 |                     |
| ► International symposia and congresses                                                                        |                     |
| ► 4th national Biotechnology Congress, Kerman (Iran)                                                           | Aug 15-17, 2005     |
| ► The XXIIIrd International carbohydrate symposium, Whistler (Canada)                                          | Jul 23-28, 2006     |
| ► The 8th international congress of Plant molecular biology, Adelaide (Australia)                              | Aug 20-25, 2006     |
| ► Presentations                                                                                                |                     |
| ► Oral presentation, 4 th national Biotechnology Congress, Kerman (Iran)                                       | Aug 15-17, 2005     |
| ► Oral presentatoin at the XXIIIrd International carbohydrate symposium, Whistler (Canada)                     | Jul 27, 2006        |
| ► Poster presentation at the 8th intern. congress of Plant molecular biology, Adelaide (Australia)             | Aug 20-25, 2006     |
| ► IAB interview                                                                                                | Jun 3, 2005         |
| ► Excursions                                                                                                   |                     |
| <b>Subtotal Scientific Exposure</b>                                                                            |                     |
| <b>11.2 credits*</b>                                                                                           |                     |
| 3) In-Depth Studies                                                                                            |                     |
| ► EPS courses or other PhD courses                                                                             |                     |
| ► Summer course glycoscience                                                                                   | Jun 28- Jul 1, 2003 |
| ► Summer course protein engineering                                                                            | Mar 8-10, 2004      |
| ► Journal club                                                                                                 |                     |
| ► Weekly literature discussion at plant breeding                                                               | 2003-2007           |
| ► Individual research training                                                                                 |                     |
| <b>Subtotal In-Depth Studies</b>                                                                               |                     |
| <b>5.4 credits*</b>                                                                                            |                     |
| 4) Personal development                                                                                        |                     |
| ► Skill training courses                                                                                       |                     |
| ► Upper intermediate English course                                                                            | Feb-Mar 2003        |
| ► Fluent English course                                                                                        | Mar-Apr 2003        |
| ► Scientific writing                                                                                           | Apr-May 2003        |
| ► Organisation of PhD students day, course or conference                                                       |                     |
| ► Membership of Board, Committee or PhD council                                                                |                     |
| <b>Subtotal Personal Development</b>                                                                           |                     |
| <b>4.6 credits*</b>                                                                                            |                     |
| <b>TOTAL NUMBER OF CREDIT POINTS*</b>                                                                          |                     |
| <b>36.2</b>                                                                                                    |                     |

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 credits

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

## About the Author

**F**arhad Nazarian Firouzabadi was born on Sep. 23, 1972 in Nahavand, Hamedan, Iran but raised in a small village some 100 kilometers north of Khorramabad in Lorestan province in a mountainous area with a fierce and beautiful climate. He finished primary school at his village. After finishing secondary and high school in Nahavand in biology sciences, he took part in the central university entrance exam and was accepted in 1991 to the University of Tabriz to study Agronomy and Plant Breeding. After 4 years, he received a B.Sc degree in the field of Agronomy and Plant Breeding. He took part in the central entrance exam for M.Sc degree in plant breeding upon finishing undergraduate studies and he was accepted at the University of Tarbiat Modares in Tehran in 1995. His research project was to study the genetic inheritance of physiological and morphological characters in sugar beet seedlings. Obtaining his M.Sc with distinguished degree led him to a job at the Department of Agronomy and Plant Breeding at the University of Lorestan as a lecturer. He taught the principles of Genetics, general plant breeding and advanced plant breeding while carried out some research projects. After five years at the University of Lorestan, he wanted to expand his knowledge in plant biotechnology and molecular biology. With a scholarship from the Iranian government, he had the opportunity to come to study in The Netherlands. Upon coming to The Netherlands, Farhad settled down in Wageningen along with his family. Farhad began working with Prof. Richard G.F. Visser and Dr. Jean-Paul Vincken in Feb 2003 on a project involving research on starch biosynthesis in potato. He will continue his research and scientific career as an assistant professor in Plant biotechnology and genetic engineering at agronomy and plant breeding group of Lorestan University in Iran.

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تحقیق ارائه شده در این پایان نامه پتانسیل کاربری تکنیک SBD در جهت تغییر ساختمان نشاسته و در جریان بیوسنتز آنرا نشان می دهد. هر چند آنزیم های زیادی را می توان بعنوان کاندید جهت اتصال به SBD و بیان آنها در سیب زمینی انتخاب نمود، لیکن می بایست آنزیم هایی را در نظر گرفت که شرایط و ویژگیهای مورد نظر آنها در سیب زمینی مهیا باشد. گاهی اوقات تغییر جهت دار<sup>1</sup> آنزیم ها در جهت بهره برداری از شرایط موجود در سیب زمینی می تواند مفید و موثر باشد.

آنزیم های دخیل در امر سنتز نشاسته تنها قادرند که واحد های گلو کز را به دو صورت  $\alpha(194)$  و  $\alpha(196)$  به هم متصل نمایند. این در حالی است که برخی آنزیم های موجود در تعدادی از میکروبها قادرند واحد های گلوکز را به صورت دیگری مانند  $\alpha(192)$  و  $\alpha(193)$  به هم متصل نمایند. چنین پلی ساکارید های خارج سلولی، حائز خصوصیات و توانایی های منحصر به فردی بوده و آنها را از نظر صنعتی پراهمیت نموده است. در پژوهشی دیگر به منظور تنوع بخشیدن به طبیعت پیوند های گلوکزیدی موجود در نشاسته سیب زمینی، آنزیم Mutansucrase (GtfiCAT) موجود در میکروب *Streptococcus downi* که فقط دارای قسمت فعال کاتالیتیکی بود، کلون و به SBD متصل گردید و سپس به سیب زمینی انتقال داده شد. نتایج این تحقیق نشان داد که تولید پلیمری با پیوند های غالب  $\alpha(193)$  (موتان)<sup>1</sup> در سیب زمینی امکان پذیر است. رنگ آمیزی مخصوص با Exo-mutanase و تفاوت های محرز در خصوصیات فیزیکی و شیمیایی نشاسته های تراریخته، بوجود موتان در نشاسته های تولید دلالت می کرد. ساختمان دانه های نشاسته در زمینه ژنتیکی فاقد آمیلوز (*amf*) به شدت تغییر نمود به طوریکه نشاسته های حاصل متخلخل<sup>2</sup> بوده و سطح آنها پوشیده از سوراخ های ریز متعددی بود. چنین تغییراتی در نشاسته های حاصل از زمینه دارای آمیلوز (Kardal) کمتر دیده شده بود (فصل چهارم).

علیرغم آنکه گلیکوژن<sup>3</sup> و نشاسته هردو از پیوندهای  $\alpha(194)$  و  $\alpha(196)$  سود می برند، خصوصیات فیزیکی و شیمیایی این دو کاملاً متفاوت می باشد. از نظر ساختمانی تفاوت عمده این دو در تعداد و طول شاخه های جانبی  $\alpha(196)$  می باشد. بنابراین تغییر در تعداد شاخه های  $\alpha(196)$  ممکن است خصوصیات و کاربردهای نشاسته را تغییر دهد. با وجود آنکه تظاهر آنزیم سازنده شاخه های جانبی *E. coli* (*glgB*) توانسته است مجموع شاخه های  $\alpha(196)$  را در آمیلوپکتین سیب زمینی 25% افزایش دهد، هیچگونه تغییر فیزیکی- شیمیایی قابل توجهی در خصوصیات نشاسته اتفاق نیفتاد. در پژوهشی دیگر به منظور تغییر هرچه بیشتر شاخه های جانبی  $\alpha(196)$  ساختمان آمیلوپکتین ژن *glgB* حاصل از *E. coli* هم از طرف انتهای کربوکسیلی (-C) و هم از طرف انتهای آمینی (-N) به SBD متصل گردید. در مقایسه با سایر نشاسته های تراریخته تولیدی در پژوهش های قبلی، نشاسته های تراریخته ای که دارای *glgB* بودند، به میزان بیشتری در دانه های نشاسته انباشته گردیدند. علیرغم اینکه خصوصیات نشاسته های تولیدی در مقایسه با نشاسته های شاهد تغییر چندانی ننموده بود، لیکن وجود چنین آنزیم های سبب تغییر شدید در مورفولوژی دانه های نشاسته حاصل گردید. نشاسته های متخلخل و با سطوح ناهموار، به ترتیب در زمینه ژنتیکی فاقد آمیلوز (*amf*) و دارای آمیلوز (Kardal) تولید گردیدند. توزیع<sup>4</sup> تعداد و طول شاخه های جانبی  $\alpha(196)$  در نشاسته های تراریخته در مقایسه با نشاسته های شاهدهی تغییر نکرده بود. علت این امر را می توان در خاصیت آنزیم های شاخه زد<sup>5</sup> موجود در سیب زمینی جستجو کرد که قادرند شاخه های نامتقارن موجود بر روی آمیلوپکتین را حذف نموده، و از این راه امکان کریستالیزه شدن آنها فراهم آورند (فصل پنجم).

<sup>1</sup>Mutan

<sup>2</sup>Porous

<sup>3</sup>Glycogen

<sup>4</sup>Distribution

<sup>5</sup>Debranching enzymes (Isoamylases)



از آنجایی که SBD دارای کشش ذاتی به سمت نشاسته می باشد، اثرات آنزیم های میکروبی متصل به آن می تواند افزایش یابد چونکه در این حالت گیرنده ها و پذیرنده ها همگی در دسترس پروتئین مورد نظر هستند.

از نظر صنعتی وجود و یا عدم وجود آمیلوز در نشاسته از اهمیت بالایی برخوردار بوده و تا حدود زیادی تعیین کننده مصارف صنعتی گوناگون آن است. خصوصیتی مانند نقص در ذوب شدن و یخ بستن مجدد، پسرفت ژل، شفافیت کم ژل، نقص در چسبندگی و نقطه بالای ذوب نشاسته همگی با مقدار بالای آمیلوز نشاسته ارتباط مستقیم دارند. از این رو تغییر میزان آمیلوز نشاسته به صورت بیولوژیکی و از طریق روشهای مهندسی ژنتیک و بیو تکنولوژی در سبب زمینی یکی از اهداف مهم صنعتی است. تا کنون روشهای بیولوژیکی متعددی در جهت این مهم ابداع شده اند.

در یکی از پژوهش های پیشین نشان دادیم که SBD2 ده برابر بیشتر از SBD برای دانه های نشاسته تمایل نشان می دهد. لذا با این فرض اگر تعداد SBD ها را افزایش دهیم، این احتمال وجود دارد که چندین (SBD2-SBD5) متصل به هم، قادر باشند که آنزیم سنتز کننده آمیلوز (GBSSI) را از گردونه خارج نموده و نشاسته ای را در سبب زمینی تولید نمایند که آمیلوز نداشته و یا مقدار آن ناچیز باشد. نتایج حاصل از این تحقیق نشان داد که هیچ کدام از این SBD ها قادر نبودند که GBSSI را از گردونه خارج نموده و خللی در میزان تولید آمیلوز ایجاد نمایند (فصل دوم) با وجود این SBD ها توانستند فرایند یا فرایندهای فیزیکی موجودی را که در سنتز دانه های نشاسته دخالت دارند، را در هر دو زمینه ژنتیکی سبب زمینی (amylose-free and amylose-containing) تحت تاثیر خود قرار دهند. اگرچه ساختار اولیه نشاسته و نقطه ذوب آن تغییر نکرد، میزان انباشته شدن SBD در نشاسته از SBD تا SBD3 قدری افزایش یافت در حالیکه میزان کمتری از SBD4 و SBD5 در دانه های نشاسته انباشته گردید.

همانطوریکه گفته شد نشاسته به صورت معمولی دارای خصوصیات مورد توجه صنعت نمی باشد لذا باید آن را با مواد شیمیایی تیمار نمود. یکی از این تیمارها تیمار نشاسته با مواد استیل کننده است. اصلاح پایداری نشاسته در مقابل پسرفت، کاهش درجه ژله ای شدن، افزایش شفافیت نشاسته و اصلاح ارتجاع پذیری ژل نشاسته از جمله خصوصیات مورد توجه نشاسته های استیل شده صنعتی می باشند. به دلیل آنکه چنین مواد شیمیایی برای سلامت انسان و محیط زیست مضر هستند، سعی گردید تا Maltose-Acetyltransferase (MAT) باکتری *E. coli* کلون شده و از طریق تکنیک SBD به داخل نشاسته سبب زمینی فرستاده شود. نتایج نشان داد که درجه استیل شدن به طور معنی داری در زمینه ژنتیکی فاقد آمیلوز (*amf*) بالاتر از کنترل بود. با این وجود به نظرمی رسد که چنین سطحی از استیل شدن برای تغییر اساسی خصوصیات فیزیکی و شیمیایی نشاسته کافی نباشد. جالب آنکه MAT توانسته بود که توانایی استیل نمودن گیرنده های مناسب خود را حتی در درون دانه های نشاسته حفظ نماید. این حقیقت با فراهم نمودن مولکولهای دهنده (Acetyl-CoA) گروه استیل و گیرنده های مختلف (گلوکز، مالتوز،...) با استفاده از نشاسته های تراریخته ثابت گردید. در این تحقیق همچنین برای اولین بار نشان داده شد که MAT تمایل دارد که به خودی خود در دانه های نشاسته جمع گردد. در مقایسه با نشاسته های تراریخته دارای SBD2، نشاسته های تراریخته حامل MAT متصل به SBD میزان بیشتری از SBD را در خود انباشته نموده بودند. تشکیل trimer توسط MAT از یک طرف و اتصال آن به SBD از طرف دیگر ممکن است علت تمایل زیاد چنین پروتئین های برای دانه های نشاسته باشند (فصل سوم).

## خلاصه فارسی:

نشاسته پلی ساکاریدی مرکب از واحدهای گلوکز بوده که از دو جزء آمیلوز<sup>1</sup> و آمیلوپکتین<sup>2</sup> تشکیل شده است. آمیلوز ترکیبی خطی از واحدهای گلوکز (4 و 1)  $\alpha$  بوده درحالی که آمیلوپکتین نسبتاً شاخه ای بوده و دارای شاخه های (6 و 1)  $\alpha$  زیادی است. میزان این دو جزء درنشاسته بسته به نوع گونه گیاهی و منبع ذخیره کننده آن متفاوت است اما بیشتر گونه های گیاهی دارای حدوداً 20% آمیلوز در ساختارنشاسته خود بوده و مابقی آن را آمیلوپکتین تشکیل می دهد. نشاسته یکی از منابع تجدید شونده و ارزان قیمت در بین کربوهیدرات ها است. با وجود این که نشاسته معمولی دارای کاربردهای متعددی در صنایع غذایی و غیر غذایی می باشد، اما استفاده از آن بدون اعمال هیچ گونه تیماری دارای محدودیت های فراوانی بوده و مناسب کاربردهای صنعتی نبوده، بدین منظور و برای تولید نشاسته ای با خصوصیات مطلوبی چون حد اقل پس رفت<sup>3</sup> و پایداری در چرخه های متعدد ذوب و انجماد مجدد<sup>4</sup>، می بایست در نهایت نسبت به تیمار نشاسته با مواد شیمیایی اقدام نمود، تا این ماده از خصوصیات مطلوب برای کاربردهای گوناگون صنعتی برخوردار گردد. از جمله این تیمارها می توان به تیمارهای در جهت Cross-linking و یا استیله نمودن (به منظور کاهش پسرقت) اشاره نمود. صرف نظر از هزینه بر بودن چنین تیمارهایی در صنعت، نگرانی در مورد سلامت بشرو آلودگی محیط زیست را می توان از مضرات دیگر این روش ها بر شمرد. طی دهه گذشته آنزیم های متعددی شناسایی شده اند که قادرند مقدار یا نوع نشاسته تولیدی یک گیاه را تحت تاثیر قرار دهند. چنین یافته هایی راه را برای تغییر بیولوژیکی نشاسته هموار نموده است. دست ورزی در سطح آنزیم های دخیل در بیوسنتز نشاسته امکان اصلاح کیفیت نشاسته تولیدی در گیاهانی مانند سیب زمینی را فراهم آورده است. برای نمونه دست ورزی در چنین ژنهایی سبب تغییراتی در نسبت آمیلوز به آمیلوپکتین شده و در نهایت نشاسته های با خصوصیات متنوعی برای کاربردهای صنعتی تولید شده است.

هدف تحقیق ارائه شده در رساله دکتری حاضر بررسی راهکارهای در جهت تغییر خصوصیات نشاسته سیب زمینی از طریق تکنیک<sup>5</sup> SBD می باشد. به منظور تولید نشاسته های با خصوصیات متفاوتی مانند پس رفت کمتری و دارای توانایی بیشتر در تحمل چندین سیکل ذوب شدن و یخ بستن، تعدادی از آنزیم های جالب توجه از منابع میکروبی متعدد به صورت مجزا و یا متصل به SBD حاصل از آنزیم Cyclodextrin Glycosyltransferase (CGTase) موجود در میکروب *Bacillus circulans* به سیب زمینی وارد گردیدند. در جریان بیوسنتز نشاسته، SBD به عنوان یک ابزار پروتئین های میکروبی مورد نظر را قادر می سازد که در دانه های نشاسته انباشته شوند. از این رو با این روش بسیاری از مواد اولیه ساخت نشاسته (الیگوساکاریدها، آمیلوز و آمیلوپکتین) به حد اقل فاصله ممکنه از چنین پروتئین های می رسند.

<sup>1</sup>Amylose

<sup>2</sup>Amylopectin

<sup>3</sup>Retrogradation

<sup>4</sup>Freeze-thaw stability

<sup>5</sup>Starch Binding Domain-technology

# دست ورزی در بیوژنز نشاسته سیب زمینی با استفاده از پروتئین های نو ترکیب

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گروه اصلاح نباتات  
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دانشگاه واگنینگن  
هلند

## تقدیم به:

همسر عزیز اکرم

و

گل های زندگیمان



امیر حسین

و

فاطمه

