Microbial starch-binding domains as a tool for modifying starch biosynthesis

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Chapter 1

General Introduction

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Starch structure

Starch is an important reserve carbohydrate found in many plant species. It is deposited as crystalline granules, which consist of two polysaccharides, amlyose and amylopectin. Amylose (20-30%) is an essentially linear molecule, which is composed of α-1,4-linked glucose residues with less than 1% of branch points. Amylopectin (70-80%) is a highly branched molecule, mainly composed of a collection of α-1,4-linked glucan chains, which are connected by α-1,6-linkages (the branch points). The distribution of the branch points is not *at random*, enabling the unique, cluster-based structure of amylopectin (Thompson, 2000). Clustering of the branch points in the amorphous lamellae enables the polymer chains to line up in a parallel fashion. The chains can associate with each other to form double helices (Smith *et al*., 1997; Ball *et al*., 1998). This chain organization forms the basis of the semi-crystalline structure of the starch granule (Myers *et al*., 2000) (Figure 1). It is generally believed that amylopectin plays a major role in establishing the semicrystalline organization of starch, while amylose is generally considered as an amorphous polymer with little or no impact on the overall crystalline organization (Wattebled *et al*., 2002). The crystalline granules can vary in size, shape, composition and the physicochemical properties between different species.

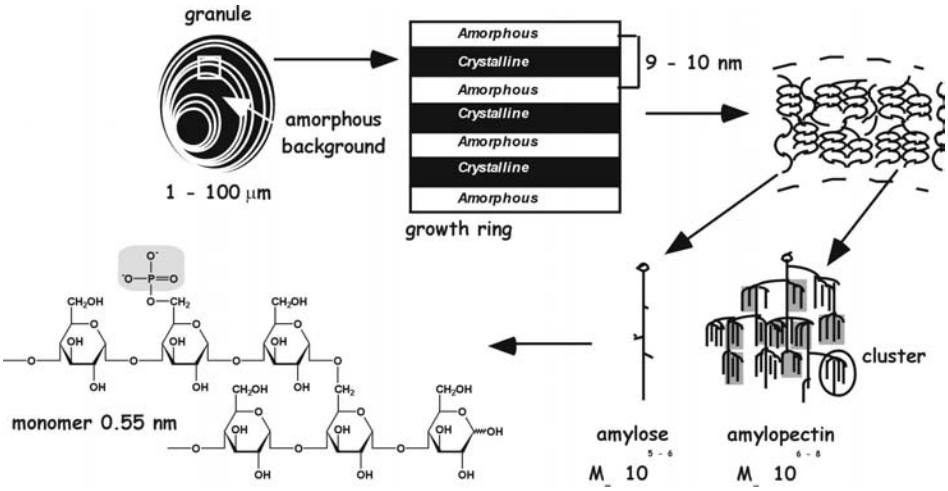

Figure 1. Overview of the various levels of polymer organization within the starch granule, which is composed of amylopectin and amylose molecules.

Starch biosynthesis

In plant storage organs, starch biosynthesis takes place within the amyloplast and is the result of different reactions such as synthesis (polymerization of glucosyl residues), rearrangement and degradation, in which various starch synthases (E.C.2.4.1.21), transferases (branching (E.C.2.4.1.18) and disproportionating enzyme (E.C.2.4.1.25)), and hydrolytic enzymes (debranching enzyme (E.C.3.2.1.41)), respectively, play key roles. Sucrose is the starting point of starch biosynthesis, which is converted into hexose-phosphate sugars in the cytoplasm. In potato, glucose-6-phosphate (Glc-6P) is transported into the amyloplast (Kammerer *et al*., 1998). It is first converted to glucose-1-phosphate (Glc-1P) by plastidial phosphoglucomutase (PGM) (E.C.5.4.2.2) and, subsequently, to ADP-glucose (ADP-Glc) by ADP-glucose pyrophosphorylase (AGPase, E.C.2.7.7.27) (Tauberger *et al*., 2000; Müller-Röber *et al*., 1992; Stark *et al*., 1992). ADP-Glc serves as a substrate for the different starch synthase isoforms, some of which are mainly present in the soluble phase or stroma (SS-isoforms), and others, which are associated with the granule. The exclusively granule-bound starch synthase (GBSSI) catalyzes the formation of amylose (Kuipers *et al*., 1994). Furthermore, it was shown that GBSSI could also contribute to amylopectin synthesis in potato, pea and the unicellular alga, *Chlamydomonas reinhardtii* (Baba *et al*., 1987; Denyer *et al*., 1996; van de Wal *et al*., 1998). All starch synthases elongate glucan chains by transferring the glucose moiety of ADP-Glc to the non-reducing end of α-1,4 linked glucans. The branching enzymes (BE) cleave α-1,4 linkages and form α-1,6 linkages. Schwall *et al*. (2000) showed that antisensing these enzymes in potato led to less branching of the starch. Additional enzymes such as the disproportionating enzymes (D-enzyme) cleave and rejoin α-1,4 linkages in starch polymers and the debranching enzymes (DBE) hydrolyze the α-1,6 linkage at branch points.

The starch biosynthesis process is summarized schematically in Figure 2. The model shows clearly that amylopectin is synthesized first, and that amylose is formed later. Two possible mechanisms for amylose biosynthesis have been proposed: (i) the amylopectin-primed pathway, and (ii) the malto-oligosaccharides (MOS)-primed pathway (Mouille *et al*., 1996; Ball *et al*., 1998; van de Wal *et al*., 1998, 2000; Denyer *et al*., 1999; 2001). First evidence for the first pathway was provided by van de Wal *et al*. (1998), who showed that amylose could be synthesized *in vitro* by cleavage of amylopectin molecules in mutant *Chlamydomonas* starches. It was postulated that amylopectin is cleaved by the action of hydrolytic enzymes as α-amylase (E.C.3.2.1.1). The *Arabidopsis* genome sequence indicates that these enzymes may be granule-bound, because they

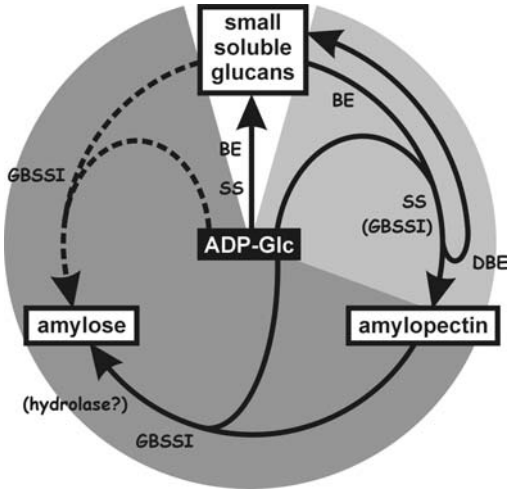

Figure 2. Schematic representation of the starch biosynthesis pathway. The timing of the biosynthesis process is as follows: the darker the background colour, the later the event takes place. The role of the various enzymes is described in detail in the text.

can be equipped with a specialized region for attachment to starch granules (Coutinho and Henrissat, 1999). An alternative explanation is that GBBSI has a dual activity, initially working as a polymerase, and at a particular time point as a hydrolase. Also in higher plants, the amylopectin-primed pathway seems to take place. However, in this case it is more difficult to demonstrate amylopectin-priming, due to the much lower GBSSI activity in the granules (van de Wal, 2000). For the second pathway, Denyer *et al*. (1999) showed that amylose could be synthesized *in vitro* in pea granules by the processive elongation of small soluble glucans or MOS, which can diffuse into the granule. It is estimated that the MOS concentration is sufficiently high in starch-producing organs of plants, thereby providing enough acceptor substrate for amylose synthesis (Denyer *et al*., 2001). The MOS pool may be replenished continuously by the action of DBE and other hydrolytic enzymes. Van de Wal *et al*. (2000) have suggested that the two pathways can occur side-by-side. Depending on the conditions in the plant, amylose biosynthesis may switch between the two mechanisms.

Partitioning of starch synthases during starch biosynthesis

It has been observed by many investigators that the biosynthetic enzymes are partitioned between the soluble phase or stroma and the growing crystalline granule during the biosynthesis process. This partitioning process is illustrated in Figure 3. For clarity, the number of enzymes is kept constant during granule growth, which is most probably a simplification of the reality. Three types of enzymes are distinguished: (i) exclusively soluble enzymes, (ii) partially granule-bound

enzymes, and (iii) exclusively granule-bound enzymes. The partially granule-bound enzymes are sometimes referred to as dual-location enzymes (Cao *et al*., 1999). To this end, it is unknown which factors determine granule-boundness. At the onset of granule formation, the surface area is small, and the number of binding sites for exclusively granule-bound enzymes may be so limited that not all the enzyme molecules can attach to the granule. This situation gradually changes as the granule grows larger, and eventually all exclusively granule-bound enzymes will be incorporated in the starch granule. It is possible that these enzymes possess a high-affinity starch-binding region, which locks them into place. The partially granule-bound enzymes may also operate at the granule surface, but with a lower affinity for the starch granule than exclusively granule-bound enzymes. Occasionally, they may be entrapped inside the granule, when starch polymers crystallize around them before they had a chance to dissociate from the granule. The exclusively soluble enzymes may be envisaged as a swarm of enzymes around a granule, displaying a "hit and run" strategy. The enzyme-substrate complex dissociates after each encounter of enzyme and substrate.

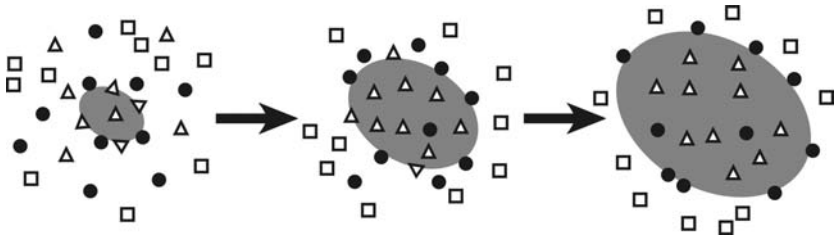

Figure 3. Schematic illustration of the partitioning of starch biosynthetic enzymes between the granule and the soluble phase (stroma) during the biosynthesis process. □: soluble enzymes; ●: partially granule-bound or entrapped enzymes; △: exclusively granule-bound enzymes. For details, see text.

Starch synthases are present within each of the classes described above. In potato, GBSSI and SSIII belong to the exclusively granule-bound enzymes and the exclusively soluble enzymes, respectively, whereas SSII is a representative of the partially granule-bound enzymes (Edwards *et al*., 1995). There is some evidence that starch synthase isoforms in other plants partition between stroma and granule in a similar fashion (Edwards *et al*., 1996; Cao *et al*., 1999). However, a good comparison is hampered by the fact that the various isoforms are not expressed in similar amounts in the starch-accumulating compartments of different plants. For instance, SSI is an abundant dual-location enzyme in maize kernels (Mu-Forster *et al*., 1996), whereas the potato orthologue of this enzyme is hardly expressed in tubers (Kossmann *et al*., 1999). Examples of other dual-location

enzymes that have been described to date are R1 (or starch excess 1 [SEX1]) (Ritte *et al*., 2000; Yu *et al*., 2001), and maize BEII (Mu-Forster *et al*., 1996). R1 is involved in controlling the phosphate content of starch, which seems to be important for starch mobilization (Yu *et al*., 2001). Recently, phosphoserine/threonine-binding 14-3-3 proteins were identified in the starch granules of *Arabidopsis* leaves and maize kernels (Sehnke *et al*., 2001). The 14-3-3 proteins seem to control the rate of synthesis of starch that is produced, since antisense suppression of these genes in *Arabidopsis* gives a two-fold increase in starch content. In addition, the polymers of the transgenic starch were more heavily branched. There is some evidence that 14-3-3 proteins can bind to SSIII. This suggests that the granule-bound 14-3-3 proteins determine the partitioning of the soluble SSIII between stroma and granule. Thus, granule-boundness may be imparted indirectly to proteins. Of particular interest is the granule-boundness of certain wheat BEs (Peng *et al*., 2000). These BEs are mainly found in A-type starch granules, and in much smaller amounts in B-type granules. Possibly, these enzymes determine the type of crystallite that is formed during biosynthesis.

Starch binding domain

The ability to bind to starch granules is not restricted to its biosynthetic machinery. Also, microbial starch-degrading enzymes can attach to starch granules. A large number of these enzymes have been studied extensively, and they are often composed of two or more domains (Svensson *et al*., 1989). Figuer 4A illustrates the modular structure of four starch-degrading enzymes. Attachment of these enzymes to (raw) starch granules is mediated by a so-called starch-binding domain (SBD) or domain E. Without a SBD, these enzymes show little activity on insoluble substrates (such as the crystalline granule), whereas the activity on soluble substrates is not affected substantially (Southall *et al*., 1999). It has also been suggested that SBD plays a more active (but non-hydrolytic) role in starch degradation by disrupting the granule surface (Southall *et al*., 1999). Besides their affinity for starch granules, SBDs can bind malto- and cyclodextrins (Svensson *et al*., 1989). In their natural setting, SBDs are combined with catalytic domains belonging to different protein families (domain A/B is a GH13 member, whereas domain J belongs to GH15). The function of domains C and D is still unclear. The SBD can be present on the N-terminus or on the C-terminus of a protein. SBD can be embedded in a multi-domain structure as in the cyclodextrin glycosyltransferase (CGTase) from *Bacillus circulans* (see Figure 4B; Lawson *et al*., 1994), or it can be spatially separated from the catalytic domain by a heavily glycosylated linker peptide (the glucoamylases of *Aspergillus niger* and *Rhizopus oryzae*).

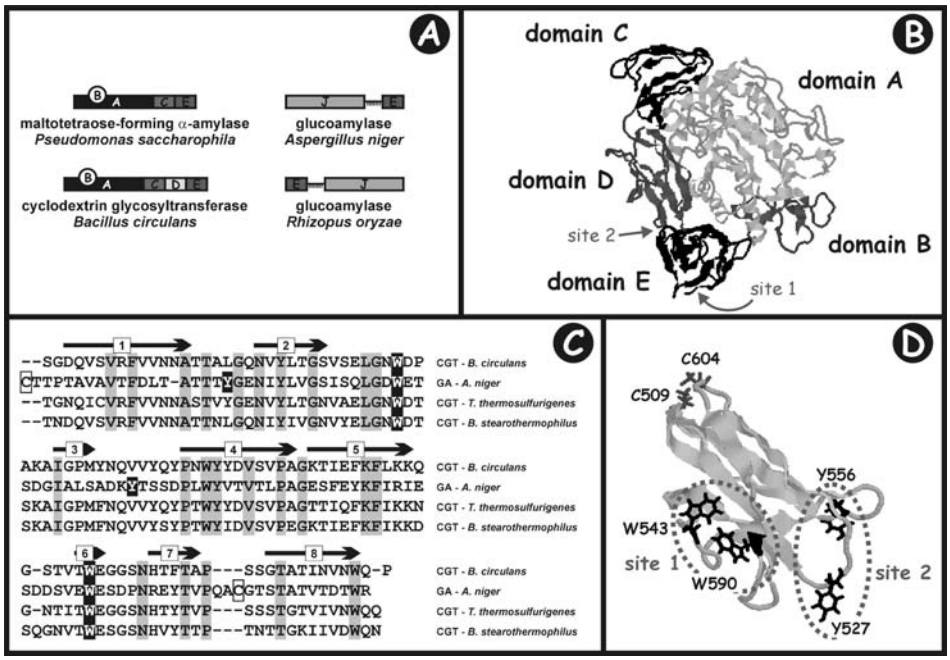

Figure 4. Overview of the domain structure of various microbial starch-degrading enzymes, and some structural features of starch-binding domains. **A**: Domain organization of selected starch-degrading enzymes. Domain A and J represent the catalytic domains belonging to two different enzyme families, GH13 and GH15, respectively. Domain E represents the starch-binding domain. The exact function of the domains B, C, and D is not known. In glucoamylases, SBD is connected to the catalytic domain by a glycosylated linker peptide. **B**: Ribbon diagram of the structure of the cyclodextrin glycosyltransferase from *Bacillus circulans*, showing that the SBD is an integral part of a rather compact 5-domain structure. Maltose-binding sites 1 and 2 of domain E are indicated by the arrows. **C**: Alignment of the amino acid sequences of 4 different SBDs. Numbered arrows illustrate the β-strand structural elements. **W** residues represent the highly conserved maltose-binding site 1. **Y** residues indicate the less conserved maltose-binding site 2. Boxed cysteine residues participate in the formation of a disulfide bridge. **D**: Ribbon diagram of the structure of the separate SBD of glucoamylase from *Aspergillus niger*. The exposed aromatic amino acids, belonging to maltose-binding sites 1 and 2, are indicated in black.

Compared to the catalytic domains (> 200 amino acids), the SBDs are usually relatively small (approximately 100 amino acids) (Svensson *et al*., 1989; Janeček and Ševčík, 1999). Their amino acid sequences seem very well conserved among different enzymes (α-amylase, β-amylase, glucoamylase, CGTase, *etc*.), as well as among different microbial species (*Aspergillus niger*, *Bacillus circulans*, *Streptomyces limosus*, *Clostridium thermosulfurogenes*, *Pseudomonas stutzeri*, *Klebsiella pneumoniae*, *etc*.) (Svensson *et al*., 1989; Janeček and Ševčík, 1999; Lin *et al*., 2003; Paldi *et al*., 2003). Figure 4C shows amino acid alignments of four SBDs. The overall identity of the domains is approximately 25%.

In particular, a number of aromatic amino acids (Trp and Tyr) are highly conserved. Some of these play a pivotal role in binding carbohydrate structures (see below; Penninga *et al*., 1996; Williamson *et al*., 1997). Crystallographic studies revealed that SBDs are rather rigid structures, which are predominantly composed of β-strands (Lawson *et al*., 1994; Sorimachi *et al*., 1996). Both the SBDs of *Bacillus circulans* CGTase and *Aspergillus niger* glucoamylase contain two separate sugar-binding sites (see Figure 4D), a finding confirming earlier observations by Belshaw and Williamson (1993). Each site contains two or three exposed aromatic amino acids. The structure of site 1 is better conserved among the different SBDs than that of site 2 (see also Figure 4C). It contains two easily accessible Trp residues, which more or less keep their orientation upon ligand binding (Sorimachi *et al*., 1996). Site 2 is much longer than site 1 and contains two or three Tyr residues, which are located on a rather flexible loop of the SBD. Upon binding, site 2 undergoes significant structural changes, which may allow the SBD to interact with starch in various orientations (Sorimachi *et al*., 1996). In CGTase, site 1 is on the outside of the protein, whereas site 2 is more buried in the protein structure forming part of a channel (between domain D and E) leading to the catalytic site of the enzyme (see Figure 4B). Site-directed mutagenesis of a number of aromatic amino acid residues belonging to both site 1 and 2 have shown that site 1 is the actual (raw) starch-binding site (Penninga *et al*., 1996; Williamson *et al*., 1997), whereas site 2 is involved in guiding linear starch chains to the active site of the CGTase (Penninga *et al*., 1996). To this end, it is unclear whether site 2 of the *Aspergillus niger* glucoamylase SBD has a particular function. Several studies show that SBDs retain their affinity for starch, even when they are separated from the other constituent domains of the natural proteins (Belshaw and Williamson, 1993; Dalmia *et al*., 1995; Williamson *et al*., 1997; Lin *et al*., 2003). Further, it has been shown that enzymes that normally do not bind to starch can acquire affinity for starch granules by fusion of an SBD to their catalytic domain (Dalmia *et al*., 1995; Ohdan *et al*., 2000). These results suggest that SBDs can be used as universal tools to target an attached biosynthetic enzyme to starch granules during the biosynthesis process.

Starch modification

Starch is an important raw material for industrial applications, such as in the paper, textile, plastics, food and pharmaceutical industry. It is currently being used in the production of biodegradable packing materials (Riaz, 1999) and the development of biodegradable plastics is becoming an increasingly attractive alternative to petroleum-based products (Davis *et al*., 2003). There is

considerable interest from the industry in the prospect of diversifying the structure of starch polymers. Improvement of starch properties for industrial uses can be achieved by chemical or physical modification after isolation, but also through the *in planta* modification of starch. Over the years, the recombinant DNA technology has provided many examples of this. A number of modified starches have been tailored *in planta*. In most cases certain endogenous genes were inhibited. For instance, an amylose-free potato starch was obtained by the introduction of an antisense GBSS I construct, showing an increase in gelatinisation temperature of granules, compared to the control, and absence of retrogradation in starch solution after gelatinisation (Kuipers *et al*., 1994). The amylose-free starches can be used in frozen foods to improve the freeze-thaw character of the products. In paper manufacturing, it is used to enhance paper strength and printing properties (Slattery *et al*., 2000). Another example is that a very-high-amylose potato starch (60-89%) was generated by inhibition of SBE A and B (Schwall *et al*., 2000). High amylose starches also have numerous industrial applications. They can be used in fried snack products to create crisp, and in the production of gum candies due to their rapid setting properties (Slattery *et al*., 2000). However, there are also examples in which heterologous genes were added to the biosynthetic machinery. For instance, introduction of *Escherichia coli* glycogen-branching enzyme (GLGB) in an amylose-free potato mutant background led to a more heavily branched amylopectin structure than the control (Kortstee *et al*., 1996). The examples mentioned above show that modification of the biosynthetic pathway have an enormous potential to produce novel or tailor-made starches.

Aim and outline of this thesis

The aim of this thesis research was to explore the possibility of engineering artificial granule-bound proteins by using microbial SBDs, which might be incorporated into granules during starch biosynthesis. In this way, starches with new or improved functionalities may be generated, which cannot be obtained by conventional breeding. It is expected that this new technology has a much wider range of applicability. A number of important aspects were studied in this thesis research: (i) can SBDs be accumulated in starch granules?; (ii) can active enzymes be targeted to starch granules by fusion to SBD?; (iii) can potato GBSSI be used as an alternative for SBD?; (iv) is it possible to engineer a higher affinity SBD with which more protein can be accumulated in granules, and (v) does the anchor's position (C- or N-terminal) in fusion proteins influence the activity of the effector

and binding affinity of the anchor? Normal amylose-containing wild-type (WT) potato plants, as well as an amylose-free (*amf*) mutant plant were chosen for these studies, because potato is one of the crop plants amenable to genetic transformation, and therefore very well suited for the production of modified starch polymers. In addition, the starch of potato has a number of characteristics, which make it very suitable for various industrial applications, such as high molecular weight, high phosphate content, and a low level of contaminants (such as protein and fat). It is expected that the genetically modified starches can combine the advantageous potato starch characteristics with the newly acquired functionalities, which will open new applications for these starches. For example, introduction of more charge, such as phosphate groups, to starch can alter the rheological properties of the transgenic starch, and introduction of reactive groups might reduce or eliminate the need for some chemical modifications, such as the addition of crosslinkers (Slattery *et al*., 2000).

A family 20 SBD derived from *Bacillus circulans* CGTase was used as an anchor for making granule-bound proteins. To investigate whether the microbial SBD can be targeted to granules during starch biosynthesis, a separated SBD was expressed in the tubers of two potato cultivars (cv. Kardal and cv. Karnico) and the *amf* potato mutant under the control of the potato GBSSI promoter. Amyloplast entry of SBD was mediated by the potato GBSSI transit peptide (Chapter 2). The possibility of incorporating active proteins (SBD/luciferase fusion) in starch granules using SBD technology was also investigated in Chapter 2.

The efficiency of two granule targeting sequences, SBD and GBSSI, as well as the effect of their position (C- or N-terminal) in luciferase-containing fusion proteins on the activity of luciferase and the binding affinity of the targeting sequences, are described in Chapter 3.

To investigate whether SBD2 (an artificial tandem-repeat of a family 20 starch-binding domain) is a higher-affinity anchor than SBD, a comparison of affinity of the SBD and SBD2 for starch (granules) *in vitro* and *in planta* is described in Chapter 4. Chapter 5 describes the introduction of SBD2 into an amylose-containing potato background. It was investigate whether SBD2 could displace GBSSI from the granules to generate an amylose-free starch.

The implications of this research are given in Chapter 6.

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Chapter 2

Microbial starch-binding domains as a tool for targeting proteins to granules during starch biosynthesis

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Summary

Modification of starch biosynthesis pathways holds an enormous potential for tailoring granules or polymers with new functionalities. In this study, we have explored the possibility of engineering artificial granule-bound proteins, which can be incorporated in the granule during biosynthesis. The starch-binding domain (SBD)-encoding region of cyclodextrin glycosyltransferase from *Bacillus circulans* was fused to the sequence encoding the transit peptide (amyloplast entry) of potato granule-bound starch synthase I (GBSS I). The synthetic gene was expressed in the tubers of two potato cultivars (cv. Kardal and cv. Karnico) and one amylose-free (*amf*) potato mutant. SBDs accumulated inside starch granules, not at the granule surface. Amylose-free granules contained 8 times more SBD (estimated at approximately 1.6% dry weight) than the amylose-containing ones. No consistent differences in physicochemical properties between transgenic SBD starches and their corresponding controls were found, suggesting that SBD can be used as an anchor for effector proteins without having side-effects. To test this, a construct harbouring the GBSS I transit peptide, the luciferase reporter gene, a PT-linker, and the SBD (in frame), and a similar construct without the linker and the SBD, were introduced in cv. Kardal. The fusion protein accumulated in starch granules (with retainment of luciferase activity), whereas the luciferase alone did not. Our results demonstrate that SBD technology can be developed into a true platform technology, in which SBDs can be fused to a large choice of effector proteins to generate potato starches with new or improved functionalities.

Key words: luciferase, starch-binding domain, starch biosynthesis, transgenic potato

Introduction

Starch is an important storage material in many plants, such as cassava, maize, rice, pea and potato. It is deposited as crystalline granules, which generally consist of two polysaccharides, amylose (20-30%) and amylopectin (70-80%). Amylose is an essentially linear molecule, which is composed of (1→ 4)-linked α-D-glucopyranosyl (α-D-Glc*p*) residues. Amylopectin is a highly branched molecule composed of a collection of α-(1→ 4)-glucan chains, which are connected by α-(1→ 6)-linkages (the branch points) (Kossmann and Lloyd, 2000). Many enzymes are involved in assembling the starch granule. During the biosynthesis of the starch granule, these enzymes are partitioned over the soluble phase (or stroma) and the crystalline granule. Most of the enzymes occur in the stroma, whereas one enzyme, the granule-bound starch synthase I (GBSS I), is found exclusively attached to the starch granule. The presence of GBSS I determines whether amylose is synthesized (Kuipers *et al*., 1994). Other examples of biosynthetic enzymes that associate with starch granules include particular starch synthases other than GBSS I (Cao *et al*., 1999), a protein involved in starch phosphorylation (Ritte *et al*., 2000), and particular wheat branching enzymes (Peng *et al*., 2000).

Granule-boundness is also encountered in starch-degrading enzymes. Many of these enzymes have a modular structure, *i.e.* they are composed of a number of domains (Svensson *et al.*, 1989). The composition and arrangement of domains varies greatly between enzymes. Besides a catalytic domain (domain A and B), and domains of unknown function (domains C and D), the cyclodextrin glycosyltransferase (CGTase) from *Bacillus circulans* (Lawson *et al*., 1994) contains a starch-binding domain (SBD; domain E). SBDs are approximately 100 amino acids long. Their amino acid sequences seem very well conserved among different enzymes (α-amylase, β-amylase, glucoamylase, CGTase, *etc*.), as well as among different species (*Aspergillus niger*, *Bacillus circulans*, *Streptomyces limosus*, *Clostridium thermosulfurogenes*, *Pseudomonas stutzeri*, *Klebsiella pneumoniae*, *etc.*) (Svensson *et al*., 1989; Janeček and Ševčík, 1999). One of the functions of SBD is to attach the amylolytic enzyme to the insoluble starch granule. Several studies have shown that these enzymes lose (most of) their activity towards raw starch granules upon removal of the SBDs, whereas their activity towards soluble substrates remains unaltered. It has also been suggested that (next to a passive attachment) SBDs play a more active (but non-hydrolytic) role in starch degradation by disrupting the granule surface (Southall *et al*., 1996). Besides their affinity for starch granules, SBDs can also bind maltodextrins and cyclodextrins (Svensson *et al*., 1989).

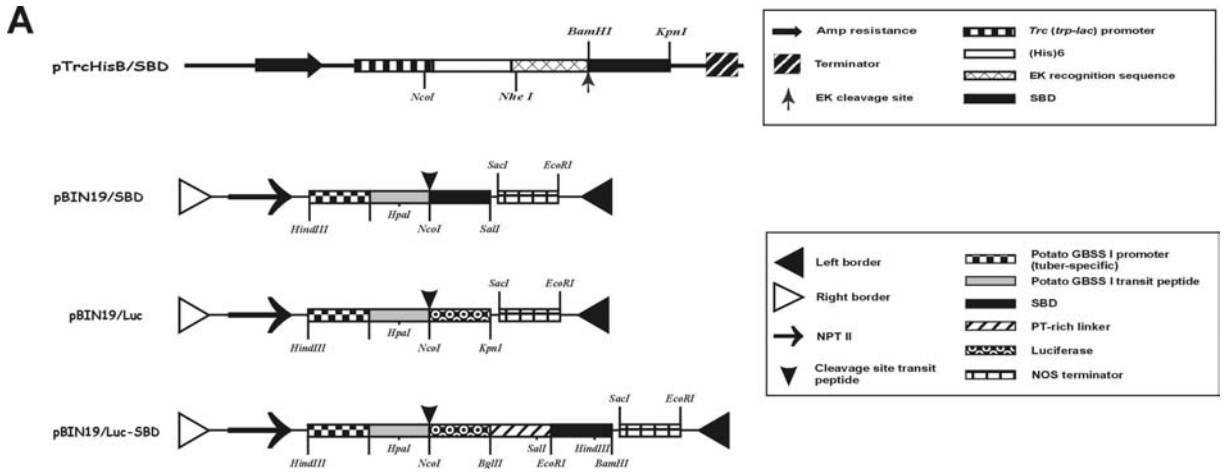
There is considerable interest from industry in the prospect of diversifying in the structure of starch polymers. On the one hand this is done with various chemical derivatization procedures, on the

other hand recombinant DNA technology offers many opportunities to do this. Over the years, a number of potato starches with new or improved functionalities have been tailored *in planta*. In most cases certain biosynthetic enzymes were inhibited. For instance, an amylose-free starch was obtained by the introduction of an antisense GBSS I construct (Kuipers *et al*., 1994). However, there are also examples in which heterologous enzymes were added to the biosynthetic machinery. For instance, introduction of *Escherichia coli* glycogen-branching enzyme (GLGB) in an amylose-free potato background led to a more heavily branched amylopectin structure (Kortstee *et al*., 1996). In this paper we describe a platform technology, which is widely applicable in starch bioengineering. The technology applies SBDs in a different context, *i.e.* in starch biosynthesis instead of degradation. By fusing a SBD to an enzyme of interest (the effector), the partitioning of an enzyme over the crystalline granule phase and the stroma can be determined. In principle, granule-boundness of any effector can be engineered.

Results

Preparation of constructs

The pTrcHisB/SBD vector was constructed by cloning the SBD gene into the pTrcHisB *E. coli* expression vector (Figure 1A). The purified SBD protein from *E. coli* lysates was used to raise polyclonal antibodies against SBD. The amino acid sequence of SBD and that preceding SBD in pTrcHisB is shown in Figure 1B. To investigate whether SBD can be targeted to starch granules in potato tubers, the pBIN19/SBD (Figure 1A) vector was made for SBD expression in potato plants. The SBD gene was cloned into the plant expression vector pBIN19 under control of tuber-specific potato GBSS I promoter. Amyloplast entry of SBD is mediated by the potato GBSS I transit peptide. The splice site for amyloplast entry is slightly different from **ATIVC↓GK** in the original potato GBSS I transit peptide (see Figure 1C). Also, a luciferase-SBD fusion protein was included in this study. The pBIN19/Luc-SBD (Figure 1A) vector for the expression of Luciferase-SBD fusion proteins in potato plants was used to investigate whether an enzyme connected to a SBD could be incorporated in starch granules with retention of the enzyme's activity. Because the SBD in its natural setting is present at C-terminus of CGTase, the SBD was fused to the C-terminus of luciferase. The fusion was made by an artificial PT-rich linker, normally found in the exoglucanase from *Cellulomonas fimi* (Gilkes *et al*., 1991; Tomme *et al*., 1995). The linker has a function in spatially separating the luciferase and the SBD, so that both domains can operate independently.



B pTrcHisB/SBD sequence

MGGSHHHHHHGMASMTGGQQMGRDLY*DDDDK*↑DPLSGDQVSVRFVVNNATTALGQNVYLTG SVSELGNWDPAKAIGPMYNQVVYQYPNWYYDVSVPAGKTIEFKFLKKQGSTVTWEGGSNHT FTAPSSGTATINVNWQRS

C pBIN19/SBD sequence

MASITASHHFVSRSQTSLDTKSTLSQIGLRNHTLTHNGLRAVNKLDGLQSRTNTKVTPKMA SRTETKRPGCSATIVC↓TMAGDQVSVRFVVNNATTALGQNVYLTGSVSELGNWDPAKAIGP MYNQVVYQYPNWYYDVSVPAGKTIEFKFLKKQGSTVTWEGGSNHTFTAPSSGTATINVNWQ P

Figure 1. Overview of SBD, luciferase, and luciferase-SBD expression vectors used in this study. **Panel A:** Schematic representation of pTrcHisB/SBD vector used for SBD expression in *E. coli*. The pBIN19/SBD, pBIN19/Luc, and pBIN19/Luc-SBD binary vectors were used for SBD, luciferase, and Luc-SBD fusion protein expression in potato plants. In these cases, the genes are under the control of a potato GBSS I promoter. Amyloplast entry is mediated by the potato GBSS I transit peptide. **Panel B:** The amino acid sequence of SBD and that preceding SBD in pTrcHisB is shown. Underlined amino acids (thick line) represent the SBD sequence, which is identical to that of the SBD of the *Bacillus circulans* CGTase. A black box with white characters shows the affinity tag for protein purification. A gray box indicates antiXpress antibody epitope. Italic characters in as gray box represent the enterokinase recognition site. An upwards arrow shows enterokinase cleavage site. Other amino acids are not present in the bacterial gene, and are either part of the vector sequence or introduced as a result of the cloning strategy. **Panel C:** The amino acid sequence of SBD and that preceding SBD in pBIN19 is shown. Underlined amino acids (thick line) represent the sequence, which is identical to that of the SBD of the *Bacillus circulans* CGTase. Underlined amino acids (dotted line) represent the sequence of the potato GBSS I transit peptide. A downwards arrow indicates the splice site for amyloplast entry.

The splice site for amyloplast entry of the Luc-SBD construct is the same as the one in SBD (see Figure 1C). The pBIN19/Luc vector for the expression of the luciferase gene in potato plants served as a control to demonstrate that an enzyme without SBD (but with amyloplast-targeting) was not incorporated in starch granules.

Characterization of SBD transformants

Potato cv. Kardal (KD) and cv. Karnico (KN), and the amylose-free (*amf*) potato mutant (often referred to as 1029-31; Jacobsen *et al*., 1989) were transformed with the pBIN19/SBD. The transgenic potato plants are referred to as KDSxx, KNSxx, and *amf*Sxx, respectively (S represents the SBD gene and xx refers to the clone in that particular series of transformants). Untransformed control plants are referred to as KD-UT, KN-UT, and *amf*-UT, respectively. Thirty-two kanamycin-resistant, transformed lines from each genotype were grown in the greenhouse to generate tubers. The morphology of plants and tubers, as well as the tuber-yield of all transgenic plants was comparable to that of the control plants (results not shown).

The expression of SBD in the KDS series of transformants was analyzed by Northern blot and Western dot blot analyses. With both methods positive transformants were found. However, the RNA level of the transformants did not always correlate with the amount of SBD protein accumulated. Figure 2A shows the results of Northern blot and Western dot blot analyses from three selected transformants. Transformant KDS49, containing the highest level of SBD protein had a low level of SBD mRNA. Probably, the lower amount of transcript helps to avoid post-transcriptional silencing, consequently leading to the higher protein amount (Elmayan and Vaucheret, 1996). The other two series of transformants were scored by Western dot blot analysis, because this procedure is simpler, and the results are directly related to the amount of protein accumulated in granules. The potato plants of each transgenic line were divided into 4 classes, Based on the amount of SBD that was associated with the starch granules: –, +, ++ and +++ represent no, low, intermediate and high levels of SBD accumulation, respectively (see Figure 2B). SBD accumulation in the three genotypes is summarized in Figure 2C. The figure clearly shows that transformants with high levels of SBD are more abundant in the *amf*S series than in the amylose-containing KDS and KNS series. The latter two gave similar results with respect to SBD accumulation levels. Based on the Western dot blot results, *amf*S51 (–), *amf*S45 (+), *amf*S36 (++), and *amf*S48 (+++) were further investigated by SDS gel electrophoresis combined with Western blot analysis. These results, which are shown in Figure 2D, demonstrate that the accumulation

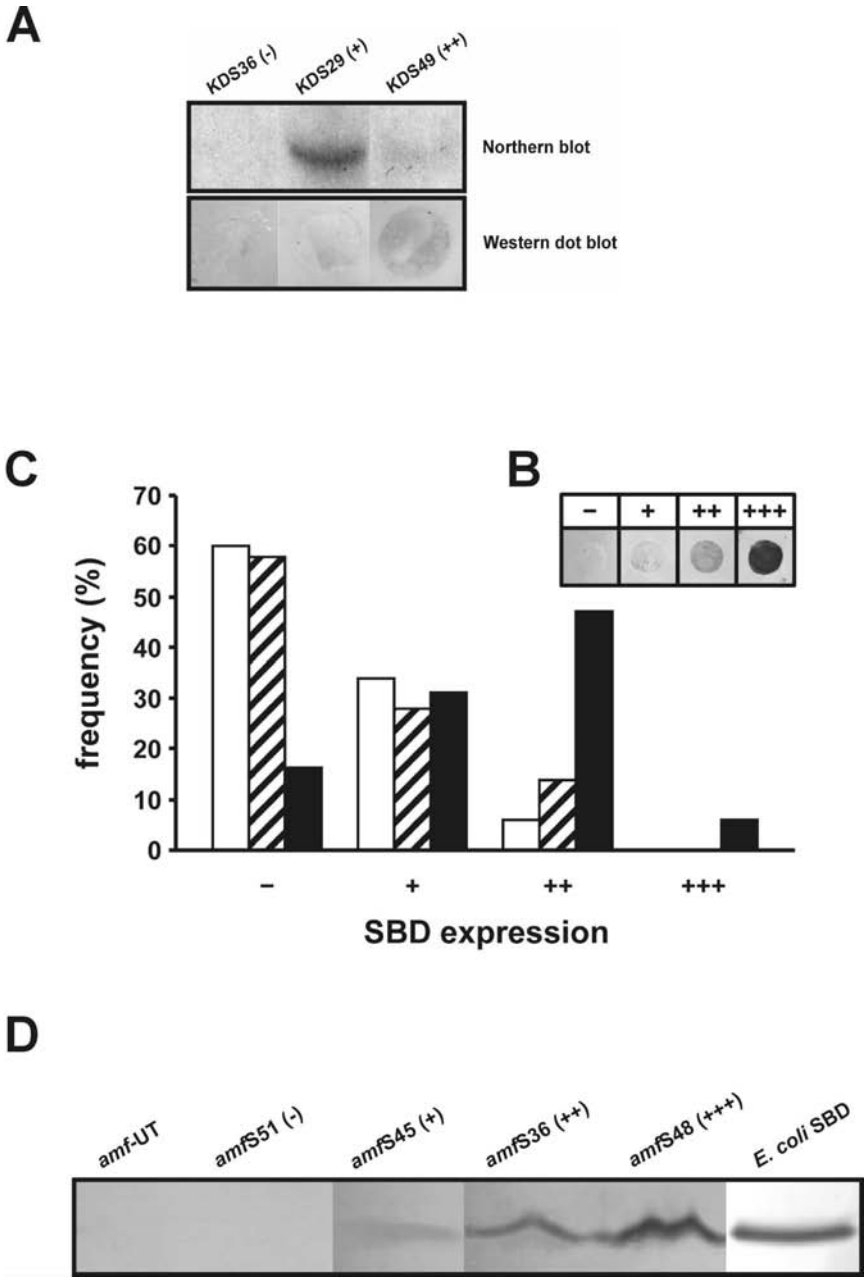


Figure 2. Different levels of SBD accumulation in potato starch granules. **Panel A** shows the results of Northern blot and Western dot blot analyses from three selected transformants. **Panel B** shows that four categories of SBD-expression were defined, based on the results with Western dot blot analysis, where –, +, ++ and +++ stand for no, low, intermediate, and high SBD expression, respectively. **Panel C** shows the distribution of the individual transformants over the four categories for the three different potato backgrounds. The white, hatched, and black bars represent the KDS, KNS and *amf*S transformant series, respectively. **Panel D** shows that an appropriately sized SBD protein is detected by Western blot analysis in three different transformants of the *amf*S series. The *E. coli*-produced SBD protein serves as a positive control.

levels of these transformants were consistent to that determined with the dot blot analysis. This confirmed that Western dot blot analysis is a rapid and reliable method for the screening of SBD accumulation levels in starch granules. The bands of transgenic *amf*S starches on the Western blot have a strange appearance, contrary to transgenic KDS and KNS starches (see Figure 3). To this end, we do not have an explanation for this phenomenon.

In order to estimate the amount of SBD protein accumulated in the starch granules, a dot blot was made with gelatinized starch from KDS49, KNS10, and *amf*S48 (showing the highest SBD accumulation in granules of that genotype). On the same blot, different amounts of purified SBD (produced in *E. coli*) were applied for calibration (data not shown). The protein concentration of the *E. coli* SBD solution was determined spectrophotometrically at 280 nm on the basis of Lambert-Beer's law (see Experimental Protocol for calculation of the molar extinction coefficient). The densities of the dots of the SBD starches were compared with those of the calibration samples. The amount of SBD in KDS49, KNS10 and *amf*S48 was estimated to be 2 mg, 2 mg and 16 mg per gram of dried starch, respectively. The SBD protein accumulation in *amf*S48 is about 8 times higher than that in the best of the KDS and KNS series.

The amount of SBD was also compared to the amount of endogenous proteins, notably GBSS I, in potato starch granules. For this purpose, the proteins present in KDS49 starch (having the highest SBD accumulation in a amylose-containing background) were separated by SDS gel electrophoresis. After Coomassie staining, it was estimated that GBSS I was at least 3 times more abundant in the granule than SBD (data not shown).

Is SBD present in soluble phase or granule-bound?

In order to determine whether SBD is also present in the soluble phase of the potato tuber, 10 times concentrated potato juices of KDS29 and *amf*S48 were subjected to Western blot analysis. No SBD protein was found in the potato tuber juices (data not shown). This showed that SBD occurred exclusively in the granule fraction, and that SBDs are efficient tools for targeting to the starch granule.

Is SBD present inside starch granules or at their surface?

We also addressed the question whether the SBDs accumulated inside the starch granule or at the granule surface. To investigate this, starch granules from transformants KDS29 (+) and *amf*S48 (+++) were treated with a protease (trypsin). The resulting digests were analyzed by Western blot

analysis with antiSBD as a probe. Samples without a trypsin treatment served as a control. The results are shown in Figure 3. The SBD bands on the blot had a similar intensity and size with and without trypsin treatment of the granules (Figure 3A lanes 3-4, and 3B lanes 3-4). To exclude the possibility that granule-bound SBD was not degraded by trypsin, the following experiment was done. KD-UT and *amf*-UT starches were incubated with the purified, native SBD protein, produced in *E. coli*. The SBDs will bind the surface of the granule and the following complexes were obtained: [KD-UT/SBD] and [*amf*-UT/SBD]. Subsequently, these samples were digested withtrypsin to investigate whether the surface-bound SBD proteins could be digested. The same complexes without trypsin treatment served as controls. Figure 3 clearly shows that the SBD band disappears upon protease-treatment of the granules (compare lanes 1 and 2 in Figure 3A and 3B). These results show that trypsin is able to digest granule surface-bound SBD. The observation that SBDs, associated with the transgenic granules, are protected from the protease demonstrates that here SBD is present inside the granules.

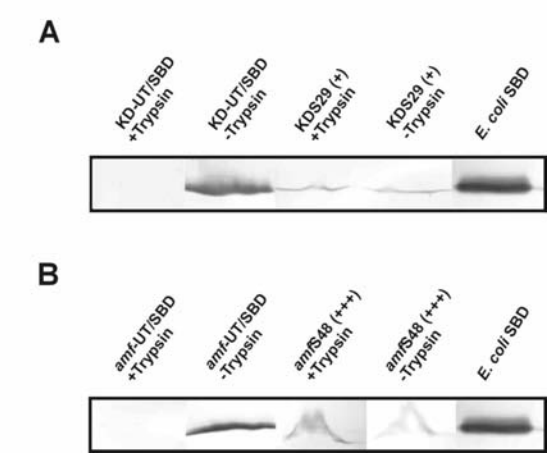


Figure 3. Western blot analysis of transgenic starches KDS29 (+) and *amf*S48 (+++) with and without trypsin treatment. The complexes [KD-UT/SBD] and [*amf*-UT/SBD] were obtained by incubating the KD-UT and *amf*-UT starches with the purified (native) SBD protein, produced in *E. coli*. These complexes served as a control, in which SBD is bound to the surface of the granules.

Granule morphology

Granules from transgenic Kardal and Karnico plants stained blue when treated with an iodine solution, similar to the granules of their untransformed controls. Contrary to the controls, a high proportion of the transgenic starches (35% and 36% for KDS29 and KNS10, respectively) showed a more pronounced staining in the hilum of these granules (arrows in Figure 4B and 4D). Analysis of

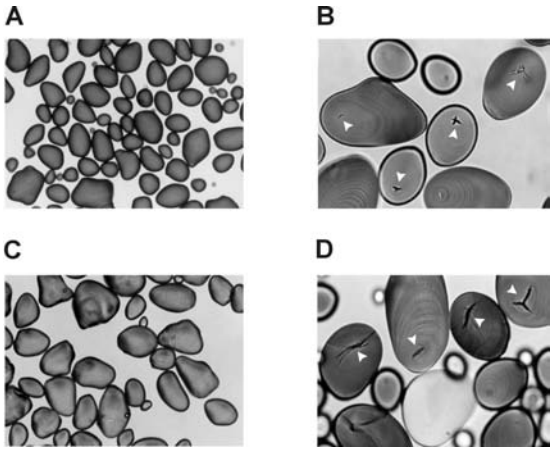


Figure 4. Micrographs showing the morphology of untransformed (KD-UT, KN-UT) and transgenic (KDS29, KNS10) starch granules. The granules were stained with a 20× diluted Lugol solution. (A) KD-UT (200×). (B) KDS29 (800×). (C) KN-UT (200×). (D) KNS10 (800×). The white arrows (in B and D) indicate a more pronounced staining in the hilum of the granules.

these granules by scanning electron microscopy (SEM) showed that their surface was smooth (data not shown), indicating that the pronounced staining does not represent cracks on the surface, but rather a slightly altered internal organization. The granule morphology of transgenic *amf*S starches was similar to that of untransformed controls after staining with an iodine solution.

One transformant in the KDS and KNS series, KDS32 and KNS22 showed a typical phenotype when stained with iodine: red granules with blue cores of varying size (data not shown). This phenotype was comparable to that of granules from antisense GBSS I potato transformants (Kuipers *et al*., 1994; Flipse *et al*., 1996). Since these transformants accumulated no or little SBD, the "blue core" phenotype does not seem to be related to the presence of SBD. These observations might be explained by co-suppression, a phenomenon often occurring in transgenic plants in which endogenous genes or parts thereof are present in the introduced construct (Flipse *et al*., 1996). In our case, co-suppression might be caused by the choice for the promoter and transit peptide sequence, both derived from the (endogenous) potato GBSS I.

Granule size distribution, gelatinization behaviour, and amylose content

The impact of SBD accumulation in granules on the physicochemical properties of starch was also investigated. Within each genetic background, one control, and one expresser of each class (–, +, ++, +++) were selected for further study. Table 1 summarizes the results on granule size distribution (d_{50}), granule-melting behaviour (T_0 and ΔH), and apparent amylose content (AM%). As can be seen from the table, no consistent differences were observed between starches from the three series of transgenic plants and their corresponding controls.

Table 1. Gelatinization characteristics (T_0, ΔH), apparent amylose content (AM%) and starch granule size (d_{50}) measuments of starches from transgenic lines and controls. Data (± SD) are the average of three independent measurements.

Clone	T_0 (°C)[a]	ΔH (kJ/g)[b]	AM (%)	d_{50} (µm)[c]
KD-UT	68.6 (± 0.2)	10.8 (± 0.7)	19.9 (± 0.4)	33
KDS36	67.9 (± 0.1)	11.5 (± 0.5)	18.9 (± 0.6)	30
KDS29	67.1 (± 0.2)	11.2 (± 0.4)	20.9 (± 0.3)	30
KDS49	69.4 (± 0.1)	11.4 (± 1.0)	19.8 (± 0.4)	31
KN-UT	71.3 (± 0.2)	11.6 (± 1.1)	18.6 (± 0.5)	23
KNS5	68.9 (± 0.1)	14.0 (± 1.8)	18.6 (± 0.4)	27
KNS20	68.0 (± 0.2)	11.6 (± 2.0)	21.7 (± 0.3)	18
KNS10	70.7 (± 0.4)	11.1 (± 1.1)	20.3 (± 0.1)	16
amf -UT	74.6 (± 0.4)	12.6 (± 0.7)	3.8 (± 0.2)	20
*amf*S51	75.0 (± 0.2)	11.8 (± 0.4)	3.7 (± 0.2)	19
*amf*S45	74.9 (± 0.1)	12.0 (± 0.2)	3.9 (± 0.1)	21
*amf*S36	75.2 (± 0.2)	11.9 (± 0.3)	3.7 (± 0.2)	19
*amf*S48	75.1 (± 0.1)	11.9 (± 0.1)	3.9 (± 0.3)	18

[a] Temperature of onset of starch gelatinization.
[b] Enthalpy released.
[c] Median value of the granule size distribution.

The two “blue core” transformants mentioned above showed a reduced amount of amylose compared to their controls. Amylose content was 4.8%, similar to the amylose content of the *amf* control (see Table 1).

Characterization of starch containing Luc and Luc-SBD fusion proteins

After transforming cv. Kardal with pBIN19/Luc and pBIN19/Luc-SBD, 45 kanamycin-resistant transformed lines were obtained for both series, which were grown in the greenhouse to generate tubers. Luciferase expression in the KDL (L refers to luciferase) and KDLS (LS refers to the Luc-SBD fusion) transformants was first determined by measuring the luciferase activity in tuber slices with a luminometer (data not shown). A number of positive transformants were found of which KDL17 and KDLS16 were the most positive ones of their respective series. Based on these results, the starches of these tubers were isolated, and the luciferase activity of the granules was determined. Figure 5 shows the total light emission and light emission images of KD-UT, KDL17 and KDLS16 tuber slices and starches after incubating the samples with the luciferase substrate. It can be seen that the KDLS16 granules have a 10 times higher luciferase activity than those of KDL17 (luciferase activity of KDL17 starch granules is comparable to that of the control), although the luciferase activity of the KDL17 slices was higher than that of the KDLS16 slices. This result clearly demonstrates that Luc-SBD fusion protein can accumulate in starch granules with retention of activity of the luciferase, and that the presence of SBD as an integral part of the protein is a prerequisite for anchoring the luciferase to the starch granule. The higher activity observed in the KDL17 slices compared to the KDLS16 ones might be explained by a higher level of expression in the former. However, it is also possible that the C-terminal presence of the SBD reduces the activity of the luciferase, or that the luciferase activity is lower because the luciferin needs to diffuse into the starch granule.

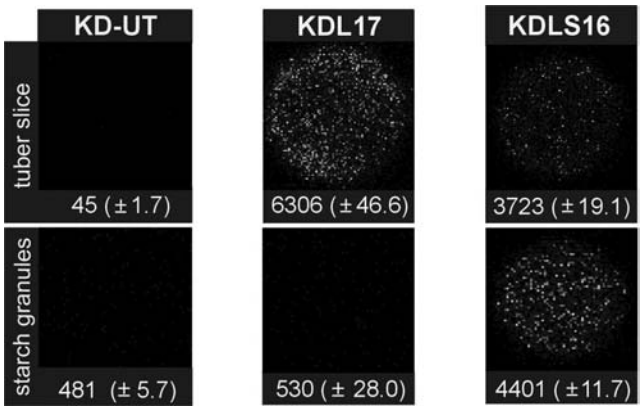


Figure 5. Luciferase activities in tuber slices (upper panel) and starch granules (lower panel) of KD-UT, KDL17, and KDLS16. The images of the slices (as well as total light emission (counts)) were recorded by a luminometer, 20 s after spraying the luciferin substrate (without ATP) onto the tuber slice (9 mm diameter, 2 mm thickness). For the granules, 10 mg of dried starch was mixed with 30 µl of Bright-Glo Luciferase Assay Substrate. The images of the starch granules (as well as total light emission (counts)) was recorded by a luminometer, 5 min after the substrate had been added. Data (± SD) are the average of three independent measurements.

KDLS16 starch was also subjected to dot blot analysis in order to estimate the amount of Luc-SBD fusion protein that accumulated in the granules. It appeared that the intensity of the dot in KDLS16 starch was comparable to that of KDS49 (data not shown). This indicates that the N-terminal presence of luciferase in the fusion protein does not affect the affinity for the starch granule of SBD to a large extent.

In order to investigate whether the SBD fusion protein accumulated inside starch granules or at granule surface, transgenic starch from KDLS16 was treated with trypsin. The resultant digest was dried, and luciferase activity was measured by the luminometer. Starch from the same transformant without a trypsin treatment served as a control. Starches with and without trypsin treatment gave similar results with respect to total light emission (data not shown). This demonstrates that the Luc-SBD fusion protein is present inside the granules.

Discussion

It was tested whether microbial SBDs can be used as an universal tool for starch modification in plant biotechnology. In their natural setting, SBDs are part of enzymes that play a role in starch degradation (Svensson *et al.*, 1989; Janeček and Ševčík, 1999). The function of the SBD is to concentrate the amylolytic enzyme on the granule surface, and possibly to open up the granular structure by a poorly understood non-hydrolytic action (Southall *et al*., 1996). The enzymes remain mainly on the outside (close to the surface) of the granule, because they are too large to penetrate. In this study, separate SBDs were targeted to the granule during the starch biosynthesis process. After isolation of the transgenic starch from potato tubers, it appeared that SBD protein was associated with the granules. The SBDs could not be removed with a protease treatment. This demonstrates that SBDs must be present inside the granule, and that they are protected from protease action by the starch crystallite. Thus, SBDs can be incorporated in the starch granule during the biosynthesis process. The physico-chemical properties of the starch are not affected by the presence of SBDs (Table 1). The melting temperature of the granules was unaltered, as was their granule size distribution and their apparent amylose content. Iodine-staining of the granules with the highest SBD content of the KDS and KNS series detected some minor differences with granules from the untransformed controls (see Figure 4). The significance of this is presently unclear.

Luc-SBD fusion genes were successfully expressed in potato tubers. The fusion proteins were present inside the starch granules, and the luciferase was still active. When the luciferase without an

attached SBD was expressed in potato tubers, only very little luciferase activity appeared to be associated with the starch granules. This demonstrates that SBDs can be used as tools to anchor effector proteins (without affinity for starch granules) inside starch granules during biosynthesis. It may be anticipated that catalytic domains other than the luciferase reporter can be fused to SBD. Dalmia *et al*. (1994) expressed a *β*-galactosidase-SBD fusion protein in *E. coli*; the SBD was used as an affinity purification tag. The purified fusion protein still had *β*-galactosidase activity. These results provide a second example that the activity of a catalytic domain is retained after fusion to SBD, but it does not necessarily mean that all effectors are compatible with SBD. It is not known whether the PT-linker is an absolute requirement to retain the activity of an effector, and whether another linker peptide would have been more effective.

SBD accumulation in starch granules appeared to be genotype-dependent. Up to 8 times more SBD could be incorporated in the *amf* background than in the amylose-containing backgrounds, Kardal and Karnico (KDS and KNS series gave comparable results). The main difference between the *amf* and KDS or KNS series is the presence of another granule-bound protein (GBSS I), which is not present in the former series, contrary to the latter two. The higher level of SBD accumulation in the *amf* granules may be explained in different ways. First, it is possible that GBSS I has a higher affinity for starch granules than SBD, and that the two proteins bind similar sites in the granule. Consequently, SBDs can be out-competed by the GBSS I protein in amylose-containing genotypes. Second, the presence of amylose may block the binding of SBD to the granule. However, the generally accepted view that amylose is synthesized downstream of amylopectin argues against this explanation (Ball *et al*., 1998); it is likely that the SBD is already bound to the granule before amylose is formed. Third, the amylopectin structure of *amf* potato starch may be different from that of wild type potato starch, although experiments thus far do not provide evidence for this (Visser *et al*., 1997). However, it cannot be excluded that there are differences in the longer amylopectin side chains, which may favour the binding of SBD to *amf* potato starch.

It may be argued that particular starch biosynthetic enzymes such as GBSS I would provide a better alternative for targeting of effectors than SBD. Although some of our data may suggest that GBSS I has a higher affinity for starch than SBD, it is important to point out that GBSS I has an approximately 5 times higher molecular weight than SBD. It is not unlikely that such a large “binding domain” can seriously compromise the activity of the effector. For this reason, we believe that SBD is, so far, the better alternative of the two. In addition to this, GBSS I has an activity (amylose synthesis) of its own.

This study demonstrates that SBDs can be used as tools to anchor effector proteins inside starch

granules, providing good perspectives for the applicability of SBD technology for starch bioengineering. Since the presence of SBD does not alter the properties of starch, it may be expected that any change in starch properties can be attributed solely to the effector. This is clearly an advantage, because it makes the impact of the effector more predictable. Preferably, the effector-SBD fusions are introduced in (potato) genotypes lacking the GBSS I protein (*amf* potato mutant), because more SBD can be accumulated in such a background. It is expected that the presented technology is widely applicable in starch bioengineering. Future research will focus on developing a number of applications in which SBDs are used to incorporate effector proteins in granules. By equipping (heterologous) biosynthetic enzymes with a SBD, one might influence the partitioning of these enzymes over the soluble and insoluble phases in the amyloplast. In this way it may be possible to concentrate previously soluble enzymes inside the granule or at the granule surface. The impact of known enzymes on starch structure/functionality may be altered in this way. Perhaps the most charming feature of SBD technology is the idea that certain post-harvest applications come within reach. Enzymes introduced in the starch granule can be activated during processing. The advantage of this is that reactions can be catalyzed which normally do not occur in amyloplasts, for instance because the appropriate reactants are lacking, or because the enzyme requires more extreme reaction conditions. This strategy may be used as an alternative for certain chemical derivatisation procedures. Also, SBD technology provides the opportunity to make starch-based protein carriers. Either enzymes or receptors can be immobilized in this way, for catalyzing particular reactions, or for affinity-purification of certain compounds, respectively.

Experimental procedures

Preparation of constructs

Four constructs were made, one for SBD expression in *E. coli* (pTrcHisB/SBD), one for SBD expression in potato plants (pBIN19/SBD), one for the expression of a luciferase-SBD fusion protein (pBIN19/Luc-SBD) in potato plants, and one for the expression of a luciferase without an attached SBD (pBIN19/Luc) in potato plants. All constructs were sequenced to verify their correctness.

The pTrcHisB/SBD construct was assembled according to the following procedure. A SBD-encoding fragment was obtained by PCR amplification with the primers 5′–TGACAACTT **GGATCC**ATTGTC–3′ and 5′–**GGTACC**TTAAGATCTCTGCCAATTCACG–3′ with CGTase

gene from *Bacillus circulans* strain 251 as a template (Lawson *et al*., 1994). Primers were designed such that the amplified fragment contained a *BamHI* restriction site at the 5′ end, and a *KpnI* site at the 3′ end. The amplied fragment was subcloned into a pGEMTeasy vector (Promega, USA). Plasmid DNA was propagated in *E. coli* DH5α and purified from the cells with the Wizard *Plus* Minipreps DNA Purification System (Promega, USA). After digestion of this plasmid, the *BamHI-KpnI* fragment was inserted into the corresponding sites of the pTrcHisB expression vector (Invitrogen, The Netherlands) to give the pTrcHisB/SBD vector (see Figure 1A). Amino acids of the original SBD, which were altered as a result of engineering this construct, are indicated in Figure 1B. The predicted molecular mass of the SBD produced in *E. coli* is 15 251 Da.

The pBIN19/SBD construct was assembled from four DNA fragments: (i) a tuber-specific GBSS I promoter (*HindIII-NcoI*) (van der Leij *et al*., 1991), (ii) a sequence encoding the GBSS I transit peptide (*NcoI-NcoI*) (van der Leij *et al*., 1991), (iii) a SBD fragment (*NcoI-SalI*), and (iv) the NOS terminator sequence (*SacI-EcoRI*) (Bevan, 1984). The first two fragments were subcloned into pMTL23. The orientation of the *NcoI-NcoI* fragment was verified by sequencing. Subsequently, the *NcoI* site connecting the promoter region and transit peptide-encoding sequence was destroyed by mutagenesis with a QuickChange site-directed mutagenesis kit (Stratagene, UK) according to the manufacturer's instructions. The *NcoI-SalI* fragment was amplified from the pTrcHisB/SBD vector by PCR with 5′–**CCATGG**CCGGAGATCAG–3′ and 5′–**GTCGAC**TTATGGCTGCCAATTCAC –3′ as primers. The combined fragments (i)-(ii) and fragment (iii) were cloned into a pBIN19 vector, which already contained the NOS terminator sequence. The plasmid is referred to as pBIN19/SBD (see Figure 1A). Amino acids of the original SBD, which were altered as a result of the cloning strategy, are indicated in figure 1C. The plant SBD has a predicted molecular mass of 19 899 Da. After cleavage of the transit peptide the predicted molecular mass is 11 567 Da.

Standard cloning procedures were used to assemble the pBIN19/Luc-SBD construct (Figure 1A) from four DNA fragments: (i) a luciferase fragment (*NcoI-BglII*), (ii) a SBD fragment (*EcoRI-BamHI*), (iii) an artificial PT-linker (*BglII-EcoRI*), and (iv) a pBIN19 plasmid, which already contained the potato GBSS I promoter, the potato GBSS I transit peptide and the NOS terminator sequence. The linker was similar to the sequence joining the catalytic and cellulose-binding domain of a *Cellulomonas fimi* exoglucanase (Cex). The DNA sequences used to connect the luciferase and the SBD corresponded to: 5′–**GATCT**CCGACGCCGACGCCCACCACGCCGACCCCGACGCC CACGACGCCGACGCCGACCCCGTCGACC**G**–3′ and 3′–**A**GGCTGCGGCTGCGGGTGGTGC GGCTGGGGCTGCGGGTGCTGCGGCTGCGGCTGGGGCAGCTGG**CTTAA**–5′. The Luc-SBD fusion protein accumulated in potato tubers has a predicted molecular mass of 74 847 Da. For

making the pBIN19/Luc construct, a *HpaI-KpnI* fragment (corresponding to part of the transit peptide-coding sequence and the full-length luciferase gene) was obtained by PCR amplification from the pBIN19/Luc-SBD vector with 5′–AGGTAT**GTTAAC**AAGCTTGATGGG–3′ and 5′–**GGTACC**TTACAATTTGGACTTTCCGC–3′ as primers. The second primer contained an appropriately positioned stop codon. This fragment was cloned in a pBIN19 vector after digesting pBIN19/Luc-SBD with *HpaI* and *KpnI*. The plasmid is referred to as pBIN19/Luc (see Figure 1A). The luciferase protein accumulated in potato tubers has a predicted molecular mass of 60 845 Da.

Production of SBD in E. coli and its purification

Recombinant *E. coli* Top 10 cells (Invitrogen, The Netherlands), containing the pTrcHisB/SBD construct, were grown in LB with 50 μg/ml ampicillin at 30 °C for 4 h. Expression of SBD protein was induced by adding of IPTG to a final concentration of 1 mM; subsequently, the cells were grown at 30 °C for 2 h. Cells were harvested by centrifugation (1300 × *g*, 10 min, 4 °C), frozen in liquid nitrogen, and stored at –80 °C. The proteins were purified under both denaturing and native conditions. Under denaturing conditions, one volume of cells was lysed with 3 volumes of a 8 M urea solution (containing 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 8.0). Lysis was started with a 30 s sonification pulse. After 3 h at room temperature (with thorough mixing at 30 min intervals), the lysate was centrifuged at 1300 × *g* for 10 min. The supernatant, containing the His-tagged SBDs, was applied onto a ProBond Resin Column (Invitrogen, The Netherlands). The SBD proteins were eluted from the column using buffers of decreasing pH as described by the manufacturer (Invitrogen, The Netherlands). Finally, the fractions containing SBD were pooled and desalted on a NAP-25 column (Pharmacia, Sweden) according to the manufacturer's instructions.

For protein purification under native conditions, 1 ml of the frozen cells was added to 1 ml of 100 mM sodium phosphate buffer pH 7.0, containing half a tablet of protease inhibitor (Complete EDTA-free, Boehringer Mannheim, Germany). The cells were lysed in a French Press. The lysate was centrifuged for 10 min at 1300 × *g* at 4 °C. Protein was purified as described above.

Preparation of a polyclonal antibody against SBD

Polyclonal antibodies against SBD produced in *E. coli* were raised by injecting a mixture of 0.5 ml purified (denaturing conditions) SBD (0.5 μg/μl) and 0.5 ml SPECOL into a rabbit. This procedure was repeated twice, after 30 and 45 days after the first immunization. The antibody titer was

analyzed by Western blot analysis. Sera were collected and stored at –20 °C. This antiserum is referred to as antiSBD.

Plant transformation and regeneration

Plasmid pBIN19/SBD was transformed into *Agrobacterium tumefaciens* according to the three-way mating protocol described by Visser *et al*. (1991). The acceptor strain *Agrobacterium tumefaciens* LBA 4404 with pAL4404 (rifampicine), the conjugation-proficient strain *E. coli* HB101 with pRK2013 (kanamycin) and donor strain *E. coli* DH5α with pBIN19/SBD (kanamycin) were used for the conjugation. Internodal stem segments from two tetraploid potato cultivars (cv. Kardal and cv. Karnico), and one diploid amylose-free (*amf*) potato mutant were used for *Agrobacterium*-mediated transformation (Visser, 1991) with pBIN19/SBD. 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, 32 transgenic, root-forming shoots were multiplied and transferred to the greenhouse for tuber development. In addition, 10 untransformed controls of each background were grown in the greenhouse. The plant transformation with pBIN19/Luc and pBIN19/Luc-SBD was conducted in the same way as described for pBIN19/SBD. In this case, 45 positive shoots from each series were multiplied and transferred to the greenhouse for tuber development.

RNA analysis

Total RNA extraction was performed according to Kuipers *et al*. (1994). Total RNA extracted from 5 g (fresh weight) of potato tuber material was ground in liquid nitrogen and suspended in a mixture of 3 ml RNA extraction buffer (2% SDS, 10 mM EDTA, 50 mM Tris, pH 9.0) and 3 ml phenol. After centrifugation (1300 × *g*, 10 min), the supernatant was extracted with 3 ml phenol/$CHCl_3$/isoamyl alcohol (25:24:1). Subsequently, RNA was precipitated with 3 ml isopropanol. The pellet was re-suspended in 1125 µl water. RNA was precipitated by the addition of 375 µl 8 M LiCl and incubation at 0 °C overnight. After centrifugation (7500 × *g*, 10 min), the pellet was dissolved in 400 µl water and reprecipitated with 40 µl 3 M sodium acetate and 1 ml ice-cold ethanol. The final pellet was dissolved in 50 µl water.

Based on a spectrophotometric RNA determinations, 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}P]-labelled *NcoI-SalI* DNA fragment of SBD as a probe; labelling was performed with a *redi*prime II kit (Amersham) according to the manufacturer's instructions.

Isolation of tuber starch

Tuber tissue was homogenized in a Sanamat Rotor (Spangenberg, The Netherlands). The homogenate was filtered through a sieve to remove particulate material. The resulting homogenate was allowed to settle for 20 min at 4 °C, and the tuber juice was decanted, concentrated 10 times in a freeze-drier (LABCONCO, The Netherlands), and stored at −20 °C for later use. The starch sediment was washed three times with water, and finally air-dried at room temperature.

Determination of SBD content of transgenic starches by dot blot analysis

A 12.5% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel (50 mm × 50 mm × 3 mm) with 9 equally spaced holes (9 mm diameter) was placed in contact with a similar-sized Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, UK). (Transgenic) starch (20 mg) was boiled for 5 min with 200 µl of a 2× SDS sample buffer (Laemmli, 1970). After cooling to room temperature, the starch gel was transferred into one of the holes. SBD proteins from transgenic starch gels were blotted onto the membrane with PhastSystem (Pharmacia, 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 (TBS is 20 mM Tris, 500 mM NaCl pH 7.5) at room temperature. Subsequently, the blot was washed in TTBS (0.05% Tween-20 in 1 liter TBS) for 5 min, and incubated for 2 h at room temperature with a 1:500 dilution of the primary antibody (antiSBD) in a 0.96% blocking solution in TTBS. After this the blot was washed twice in TTBS for 5 min, and incubated for 1 h at room temperature with a 1:2000 dilution of Goat Anti-Rabbit IgG (H+L) Alkaline Phosphatase Conjugate (Bio-Rad, USA) 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 with a 0.1 M $NaHCO_3$ solution pH 9.8 containing 1% NBT/BCIP (Roche Molecular Biochemicals, Germany), and 0.01 M $MgCl_2$.

Western blot analysis

A 20 mg portion of starch was boiled for 5 min in 200 µl of 2× SDS sample buffer. After cooling to room temperature, the starch gel was loaded onto a 12.5% SDS-PAGE gel (145 mm × 95 mm × 3 mm), and the proteins were separated by electrophoresis. SBD protein bands were transferred onto a membrane and stained as described above for the dot blot procedure.

For the potato juice fraction, 50 µl of a 10× concentrated juice was boiled for 3 min with 50 µl of 2× SDS sample buffer. The rest of the procedure was conducted in the same way as described for starch analysis.

Analysis of SBD location: inside the granule, or at its surface

In order to investigate whether SBD (or Luc-SBD) was present inside transgenic starch granules or at their granule surface. A 20 mg portion of starch was suspended in 1 ml 50 mM Tris buffer pH 8.2, containing 20 mM $CaCl_2$, and treated for 2.5 h with 20 µl of trypsin (3260 U/ml) (Sigma, The Netherlands) at room temperature with continuous mixing. Samples without trypsin added served as a control. The samples were centrifuged (7500 × *g*, 5 min), the supernatants discarded, and the pellets re-suspended in 1 ml water. This procedure was repeated three times. The pellets were boiled for 5 min with 200 µl of 2× SDS sample buffer. The result of trypsin digestion was evaluated by Western blot analysis.

Control samples with SBD bound to the granule surface were prepared as follows. A 20 mg portion of KD-UT or *amf*-UT potato starch was first incubated for 30 min (room temperature) with 4 µl of purified (under native conditions) SBD protein (219 µg/ml), produced in *E. coli*, in 1 ml 50 mM Tris buffer pH 8.2, containing 20 mM $CaCl_2$. The resulting starch-SBD complexes were treated with trypsin as described above.

Determination of SBD protein concentration

The protein concentration of a SBD solution was determined spectrophotometrically at 280 nm on the basis of Lamber-Beer's law ($A_{280} = \varepsilon CL$). The molar extinction coefficient, ε, was calculated with the following formula: $\varepsilon_{280} = (5700n_{Trp} + 1300n_{Tyr})/M$, where M is the molecular weight, n_{Trp} the number of tryptophan residues, and n_{Tyr} the number of tyrosine residues of the protein (Charles

and Paul, 1980). The ε_{280} of the *E. coli*-produced SBD is 2.092 (ml mg^{-1} cm^{-1}), that of the plant-produced one after truncation is 2.646.

Analysis of physicochemical properties of starch granules

Average granule size and granule size distribution of the starches were determined with a Coulter Multisizer II, equipped with an orifice tube of 200 µm (Beckman-Coulter, UK). Approximately 10 mg of starch was dispersed in 160 ml of Isoton II. The granule size distribution was recorded by counting about 50 000 particles. The coincidence (the frequency of two granules entering the tube at the same time, and consequently being counted as one) was set at 10%.

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

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, The Netherlands), equipped with a Neslab RTE-140 glyco-cooler. The instrument was calibrated with indium (melting point 156.6 °C) and zinc (melting point 419.47 °C). Before DSC analysis, the moisture content of starch samples was determined by drying them overnight in an oven at 80 °C, and weighing them before and after drying. Precisely 10 mg of starch was transferred to a stainless-steel pan, and the starch content of the pan was adjusted to 20% by adding an appropriate amount of water. The pan was sealed and allowed to equilibrate for at least 1 h. The samples were heated from 40 °C to 100 °C at a scanning rate of 10 °C/min. An empty sample pan was used as a reference. For each endotherm, the onset temperature of gelatinization (T_0), as well as the difference in enthalpy (ΔH), were computed automatically.

Measurement of luciferase activity in tuber slices and starch

Screening of KDL and KDLS transformants for the presence of Luc and Luc-SBD proteins, respectively, was done by measuring the luciferase activity of tuber slices with a diameter of 9 mm and a thickness of 2 mm. The total light emission from the samples was detected by a luminometer (Hamamatsu Argus-50 Image Processor and II Controller; Camera Lens: Nikon, Nikkor 50mm

1:1.2, Japan) 20 s after spraying the luciferin substrate (0.15 mg/ml, without ATP) onto the slices (20 °C). To measure the luciferase activity in isolated starch granules, 10 mg of dried starch was mixed with 30 µl of Bright-Glo Luciferase Assay Substrate (Promega, USA) containing ATP, and the total light emission from the sample was detected by the luminometer after 5 min at 20 °C.

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Chapter 3

Microbial starch-binding domains are superior to granule-bound starch synthase I for anchoring luciferase to potato starch granules

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Summary

Microbial starch-binding domains (SBD) and granule-bound starch synthase I (GBSSI) are proteins which can be accumulated in potato starch granules. The efficiency of SBD and GBSSI for targeting active luciferase reporter proteins to granules during starch biosynthesis was investigated. These sequences were fused to the N- or C-terminus of the luciferase (LUC) gene, via an artificial Pro-Thr encoding linker sequence. The genes were introduced in an amylose-containing potato cultivar (cv. Kardal) and/or the amylose-free (*amf*) potato mutant. It appeared that SBD was superior to GBSSI as a targeting sequence, mainly because the luciferase retained higher activity in the SBD-containing fusion proteins than in the GBSSI-containing ones. Furthermore, both in N- and C-terminal position, SBD was capable of granule-targeting during starch biosynthesis, although it seemed to be more efficient at the C-terminus.

Key words: fusion protein, granule-bound starch synthase I, luciferase, starch-binding domain, transgenic potato

Introduction

In a previous paper, we have described a method for incorporating foreign proteins in starch granules during the biosynthesis process (Ji *et al*., 2003). The use of microbial starch-binding domains (SBDs) is a key feature of this technology. We have shown that SBD could be accumulated in potato starch granules, either by itself or as part of a SBD luciferase fusion protein. The luciferase was not incorporated in starch without SBD, indicating that it had no affinity for starch of its own. Furthermore, the luciferase retained its activity in the fusion protein, which is promising for applications in starch bioengineering in which an effector protein is tagged with SBD. It was also found that more SBD protein could be accumulated in the starch granules of the amylose-free (*amf*) potato mutant than in those of wild-type potato plants, demonstrating that the background in which SBD is expressed is a factor of importance.

Various SBDs have been described in the literature, and they have been grouped in protein families according to their predicted secondary structure similarities and position in modular enzymes (http://afmb.cnrs-mrs.fr/CAZY/; Sumitani *et al*., 2000). Currently, three SBD families are known: CBM20, CBM21 and CBM25, where CBM stands for carbohydrate-binding module. In our experiments we have used the SBD of *Bacillus circulans* cyclodextrin glycosyltransferase (CGTase), which belongs to the CBM20 family. The amino acid sequences of CBM20 and CBM21 proteins are similar. The main difference between these two families is that CBM20s are found at the C-terminus of modular proteins, whereas CBM21s are positioned at the N-terminus (Janeček and Ševčík, 1999; Sumitani *et al*., 2000; Svensson *et al*., 1989). An example of a CBM21 is the SBD of glucoamylase from *R. oryzae* (Ashikari *et al*., 1986). CBM25s often occur as repeated sequences, but their position in the modular protein can be different. The maltopentaose-producing amylase from an unidentified bacterium contains three CBM25 repeats at its C-terminus (Candussio *et al*., 1990), whereas the amlC protein from *Streptomyces lividans* has two CBM25s at its N-terminus (Yin *et al*., 1998). In the β/α-amylase from *B. polymyxa* the two catalytic domains are separated by two internal CBM25s (Uozumi *et al*., 1989).

Granule-boundness of enzymes is also encountered with some starch biosynthetic enzymes (Kok-Jacon *et al*., 2003). Granule-bound starch synthase I (GBSSI) is probably the most well-known example of such an enzyme. Extensive sequence comparisons of starch-, glycogen- and sucrose synthases suggest that the amino acid sequence of the mature GBSSI corresponds to a single module (MacGregor, 2002). Therefore, it seems likely that the amino acids conferring granule-boundness of GBSSI are an integral part of the catalytic domain, and that the enzyme does

not contain separate starch-binding domain, which is in contrast with many starch-degrading enzymes.

Cornett *et al*. (2003) have shown that when the C-terminal SBD of glucoamylase is moved to the N-terminus of the protein, both the affinity and the activity towards raw starch of the mutant enzymes are affected. From this, they concluded that the position of SBD with respect to the catalytic domain matters, and that the two domains seem to work in a coordinated way. For *in planta* starch bioengineering, we intend to use SBDs in a non-native setting, where the concerted action of domains is probably less important. However, it remains to be established whether the position of SBD in a fusion protein is a factor of importance. In the current study, we have investigated whether the C-terminal SBD of CGTase can also be used as an N-terminal anchor. For this, SBD was fused to the N-terminus of luciferase, the construct was expressed in wild-type potato, and the resulting transformants were compared to the luciferase-SBD transformants (SBD at C-terminus of fusion protein) described previously (Ji *et al*., 2003). Additionally, we investigated whether potato GBSSI can be used as an alternative for SBD. For this, GBSSI was fused to the N- or C-terminus of luciferase, and these constructs, together with the SBD/luciferase ones, were introduced in the *amf* background. The results of the various anchors and anchor positions are discussed in terms of starch-binding efficiency and luciferase activity.

Results

Characterization of transformants with luciferase fusion proteins

Potato cv. Kardal (KD) was transformed with pBIN19/LUC, pBIN19/SBD-LUC and pBIN19/LUC-SBD. The resulting transgenic potato clones are referred to as KDLxx, KDSLxx and KDLSxx, respectively; L, SL and LS represent the LUC, SBD-LUC and LUC-SBD genes, respectively, and xx refers to the clone number in the series of transformants. The amylose-free (*amf*) potato mutant was transformed with pBIN19/LUC, pBIN19/SBD-LUC, pBIN19/LUC-SBD, pBIN19/GBSSI-LUC and pBIN19/LUC-GBSSI (Figure 1). The resulting transgenic clones are referred to as *amf*Lxx, *amf*SLxx, *amf*LSxx, *amf*GLxx and *amf*LGxx, respectively. L, SL, LS, GL and LG represent the LUC, SBD-LUC, LUC-SBD, GBSSI-LUC and LUC-GBSSI genes, respectively. Forty-five (Kardal background), or 17-27 (*amf* background) kanamycin-resistant, transformed clones of each construct were grown in the greenhouse to generate tubers. During growth the transgenic plants appeared to be phenotypically comparable to the controls (data not shown).

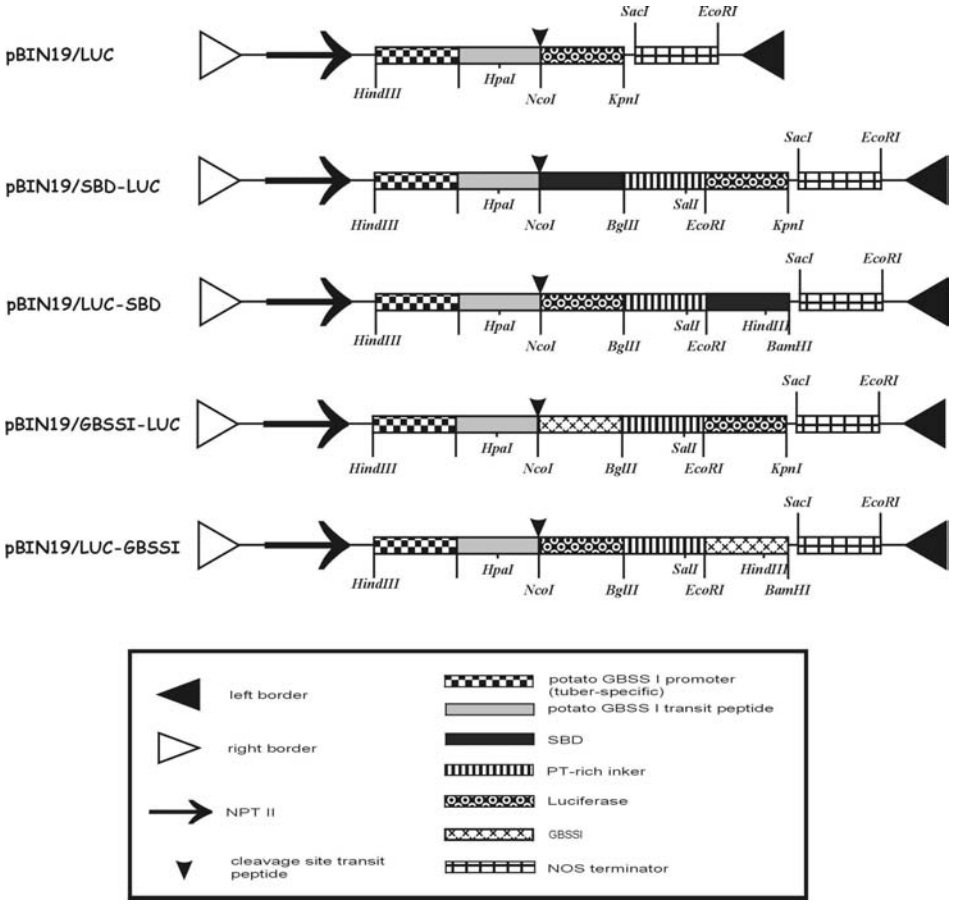

Figure 1. Schematic representation of pBIN19/LUC, pBIN19/SBD-LUC, pBIN19/LUC-SBD, pBIN19/GBSSI-LUC and pBIN19/LUC-GBSSI binary vectors used for expression of (fusion) proteins in potato plants. In all cases, the genes were under the control of the tuber-specific potato GBSSI promoter. Amyloplast entry was mediated by the potato GBSSI transit peptide.

Expression of luciferase and luciferase-containing fusion proteins in the wild-type (WT) and *amf* backgrounds was first analyzed by measuring the luciferase activity in tuber slices with a luminometer. Figure 2A shows the total light counts and the light emission images of selected (transgenic) tuber slices after incubating the samples with the luciferase substrate. Figure 2B shows luciferase activities in tuber slices of the *amf*L, *amf*SL and *amf*LS s eries. It can be seen that

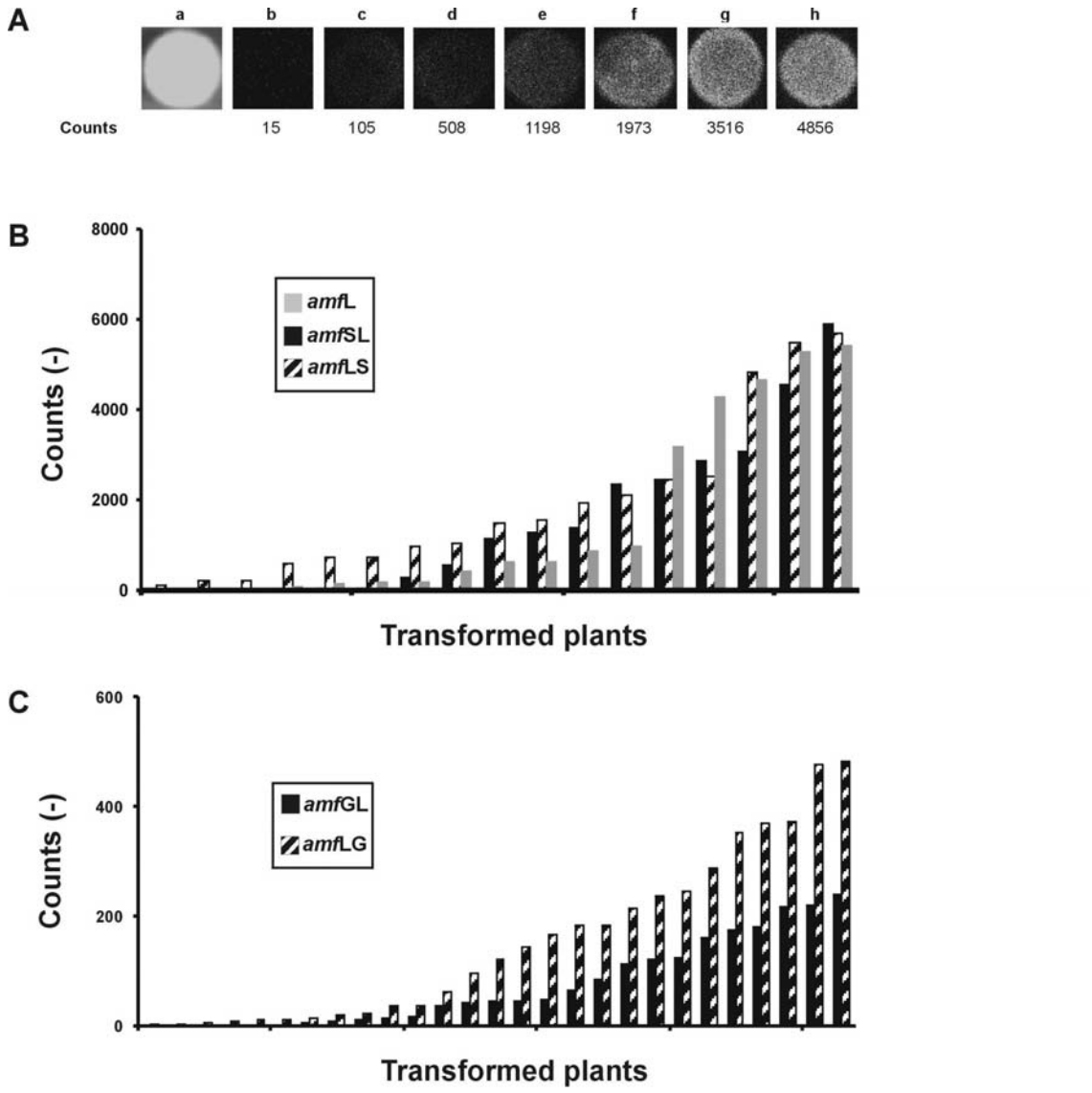

Figure 2. Luciferase activity in slices of transgenic potato tubers expressing SBD-containing and GBSSI-containing gene fusions. **Panel A** shows images of luciferase activity from selected (transgenic) tuber slices with a diameter of 9 mm and a thickness of 2 mm. Image a, tuber slice without adding the luciferin substrate; Image b, untransformed tuber slice (background); Images c-h, transgenic tuber slices of different transformants with increasing luciferase activity. **Panel B** shows the number of photon counts after 20 s in the slices of 17 transgenic lines of the *amf*L, *amf*SL and *amf*LS series. The measurements were performed immediately after spraying the luciferin substrate (without ATP) onto slices at 20 °C. **Panel C** shows the number of photon counts after 20 s in the slices of 27 transgenic lines of the *amf*GL and *amf*LG series. The measurements were performed in the same way as mentioned above.

lucifease activity in tuber slices of the most positive *amf*L transformant was similar to that in the most positive transformant from *amf*SL and *amf*LS series. Luciferase activities in tuber slices of *amf*SL and *amf*LS series appeared more or less even. The luciferase activities in tuber slices of the corresponding series in the WT background were slightly lower than in the *amf* background (data not shown).

We also investigated whether GBSSI can be used for targeting luciferase to the starch granule during starch biosynthesis. This series of experiments was only done in the *amf* background, in order to exclude competition between native GBSSI (present in WT granules, but not in *amf* ones) and the introduced GBSSI-containing fusion proteins. Luciferase activities in tuber slices of *amf*GL and *amf*LG transformants are shown in Figure 2C. It is clear that luciferase activities of tuber slices in *amf*LG and *amf*GL transformants are 10-20 times lower than those in *amf*SL and *amf*LS series.

Characterization of luciferase-containing transgenic starch granules

Based on the results of luciferase activities of tuber slices, transgenic starches were isolated from the most positive transformants of the various series, and luciferase activity of the starch granules was determined with a luminometer. The results are summarized in Table 1. Very little luciferase activity is accumulated in the *amf*L32 granules, demonstrating that luciferase does not have an affinity for amylose-free starch granules. This is consistent with our previous results for KDL17 (Ji *et al*., 2003), and shows that appending an SBD to luciferase is essential for association with starch granules. For comparison, the luciferase activities of KDL17 and KDLS16 (the transformants with the highest luciferase activity from the KDL and KDLS series; Ji *et al*., 2003) starch granules are also included in this table. The luciferase activity in *amf*SL12 was approximately 4 times higher than that in KDSL23, suggesting that a larger amount of fusion protein can be incorporated in the granules of the *amf* background than in those of the WT. The granules of the Kardal background containing the fusion protein with SBD at the N-terminus (KDSL23) seemed to display a higher luciferase activity than those with the fusion protein with SBD at the C-terminus (KDLS16); typically, this was the opposite in the *amf* background. The granules with the GBSSI-containing fusion proteins have a much lower luciferase activity than those with the SBD-containing fusion proteins. This showed that GBSSI is inferior to SBD in anchoring active luciferase in starch granules.

The levels of SBD-LUC and LUC-SBD fusion proteins accumulated in transgenic starch granules of both backgrounds were also investigated by Western dot blot analysis using antiSBD (Ji

et al., 2003) and Anti-Luciferase pAb (Promega, USA) antibodies (see Table 1). Estimation of the amount of fusion proteins accumulated in transgenic granules was performed according to the dot intensity scheme in Table 1. *Amf*S48, the highest SBD accumulator (3+ class) from the *amf*S series (Ji *et al*., 2003) was used as a control for calibrating the dot intensities. Higher levels of SBD-containing fusion proteins can be accumulated in the *amf* background than in Kardal, *i.e*. *amf*SL12 granules contained more SBD than KDSL23 granules, and *amf*LS13 granules more than KDLS16 granules. Furthermore, it appeared as if fusion proteins with SBD at the C-terminus had a higher affinity for the starch granule (compare KDLS16 with KDSL23, and *amf*LS13 with *amf*SL12). Western dot blot analysis of the transgenic SBD-containing starches with an anti-luciferase antibody was consistent with the results obtained with the antiSBD antibody (Table 1).

In order to investigate whether the accumulation of luciferase and SBD-containing fusion proteins in the Kardal background affected the accumulation of native GBSSI in the starch granules, the GBSSI contents of the selected transgenic starches from the KDL, KDLS and KDSL series were determined by Western dot blot analysis with an antiGBSSI (Vos-Scheperkeuter *et al*., 1986) antibody. Starch from untransformed Kardal and *amf* tubers served as controls. The results showed that the amount of GBSSI in the transgenic granules was comparable to that of Kardal starch (see Table 1).

To estimate the amount of GBSSI-containing fusion proteins in starch granules, *amf*GL31 and *amf*LG30 starch were subjected to Western dot blot analysis using the anti-luciferase and antiGBSSI antibodies. Starch from an *amf* mutant complemented with potato GBSSI, *amf*COMP, (often referred to as pWAM101; Flipse *et al*., 1996) served as a positive control when the antiGBSSI antibody was used. It can be seen from Table 1 that the amount of GBSSI in both *amf*GL31 and *amf*LG30 transgenic granules was much lower than in those of *amf*COMP. Western dot blot analysis with the anti-luciferase antibody demonstrated that the amount of luciferase accumulated in the *amf*LG30 and *amf*GL31 granules was comparable to that in the *amf*SL12 and *amf*LS13, respectively. However, it should be noted that the luciferase activity of granules with the GBSSI-containing fusion proteins is much lower than those with the SBD-containing ones.

Table 1. Luciferase activity and amount of SBD- and GBSSI-containing luciferase fusion proteins accumulated in the transgenic starch granules of the most luciferase-positive transformants of each series.

Clone	Counts[a]	Amount of protein[b]		
		AntiSBD	Anti-luciferase	AntiGBSSI
WT-UT	0	n.t.[c]	n.t.	+++++
amf-UT	0	n.t.	n.t.	–
KDL17	49 (± 28.0)	n.t.	–	+++++
KDLS16	3,920 (± 11.7)	++	+	+++++
KDSL23	7,117 (± 20.2)	+	±	+++++
*amf*L32	22 (± 11.2)	n.t.	–	–
*amf*LS13	50,076 (± 33.2)	+++	++	–
*amf*SL12	29,225 (± 51.1)	++	+	–
*amf*COMP[d]	n.t.	n.t.	n.t.	++++
*amf*LG30	164 (± 7.2)	n.t.	+	+
*amf*GL31	61 (± 3.0)	n.t.	++	++

[a] 10 mg of dried starch was mixed with 30 µl Bright Glo Luciferase Assay Substrate (luciferin with ATP). The activity was measured for 5 min at 20 °C after adding the substrate. Data (±SD) are the average of three independent measurements.

[b] The amount of the fusion proteins present in the transgenic granules was estimated by Western dot blot analysis with various antibodies, according to the dot intensity scheme indicated below.

[c] Not tested.

[d] This transformant represents an *amf* potato mutant, which is complemented with the full-length potato GBSSI gene, including its promoter (Flipse *et al*., 1996).

Discussion

In the present study, we compared the efficiency of two granule-targeting sequences, SBD and GBSSI, for directing effector proteins (in this case the reporter luciferase) to the growing starch granule in potato tubers. All proteins were equipped with the same amyloplast-targeting sequence, consisting of the potato GBSSI transit peptide with two extra amino acids (MASIT…SATIVC↓TM), to allow a good comparison between SBD and GBSSI in targeting luciferase to the starch granule. From previous research it was known that this transit peptide can efficiently direct proteins into the amyloplast (Ji *et al*., 2003; 2004). We also determined whether the position (N- or C-terminal) of SBD or GBSSI in the fusion protein could affect the activity of the effector and the binding affinity of the targeting domains. This knowledge is important for engineering efficient fusion proteins that can be used for genetic modification of starch. The results of this study confirmed our previous findings that luciferase does not have affinity for starch granules of its own (Ji *et al*., 2003). Our results with tuber slices in the *amf* background indicate that the presence of an appended SBD does not influence the activity of the luciferase so much. The *amf* granules had a 4 to 12-fold higher luciferase activity than those of Kardal, which corresponds roughly to the amount of fusion protein in the granules (Table 1). This is consistent with previous results, which demonstrated that SBD targeting is more efficient in the *amf* background compared to the Kardal (Ji *et al*., 2003).

From Table 1, it seems as if both luciferase activity and affinity for starch of the fusion proteins depend on their modular arrangement. Our data show that fusion proteins with SBD at the C-terminal end accumulate to higher levels in starch granules than SBD-LUC. The amount of LUC-SBD in the granules of the most positive transformants of the Kardal and *amf* series is similar to that of SBD alone (thus, without an attached luciferase) in the two backgrounds (Ji *et al*., 2003). This indicates that SBD's affinity for starch is not compromised in the LUC-SBD fusion protein. In accordance with Cornett *et al*. (2003), we show that a CBM20, which is present at the C-terminus of the native protein, can be used as an N-terminal anchor as well. It is evident that SBD in this position is less efficient in binding starch. It could be that the physical presence of the attached luciferase shields the exposed aromatic amino acids of SBD, which are known to mediate starch-binding (Kok-Jacon *et al*., 2003). With respect to luciferase activity, it is more difficult to conclude which is the preferred position of SBD in the fusion protein. In the Kardal background, SBD-LUC is the most active fusion protein, whereas in the *amf* background this is LUC-SBD. In principle, we would have expected to find a higher luciferase activity in the KDLS16 granules (or lower luciferase activity for KDSL23 granules), which would have been more consistent with the amount

of luciferase and SBD determined by Western dot blots. We do not have a good explanation for this observation. Possibly, the presence of amylose may alter the conformation of fusion proteins, the effect of which yields a less active LUC-SBD.

With GBSSI as a targeting sequence, it was also possible to accumulate luciferase protein in starch granules, approximately to a similar amount as with SBD. However, it seems that GBSSI's affinity for starch is greatly impaired by the attached luciferase, because much less GBSSI protein was detected by Western dot blot analysis in the *amf*LG30 and *amf*GL31 starch granules than in *amf* granules complemented with the native GBSSI. Furthermore, the luciferase activity of the GBSSI-containing fusion proteins was much lower than that of the SBD-containing ones, which was apparent from the chemiluminiscence measurements of both tuber slices and starch granules. These results demonstrate that GBSSI and luciferase are poorly compatible partners in a fusion protein. It seems likely that this is related to the size of GBSSI, which has a roughly 5 times higher molecular weight than SBD. It could be that the large fusion protein is folded incorrectly. Another possibility is that the two domains interact with each other, thereby shielding the important amino acid residues for granule-binding of GBSSI and compromising the accessibility of the active site of luciferase. Our results do not mean that GBSSI is an inferior granule-targeting sequence per se. It is possible that GBSSI can be truncated to a minimum starch-binding sequence, which is much smaller than the native protein, and which has an affinity for starch comparable to the native protein.

In this study we compared the usefulness of two granule-targeting sequences, SBD and GBSSI. Our results show that the small microbial starch-binding domain is the preferred sequence of the two. Furthermore, we found that the position of SBD in the modular protein matters; in terms of starch affinity the SBD is best put at the C-terminus of the fusion protein, whereas in terms of activity SBD's preferred position is ambiguous. For application in *in planta* starch bioengineering it is probably better to test both the N-terminal SBD fusion protein and the C-terminal one, the more so as it is possible that other effector proteins may respond differently to the presence of an attached SBD.

Experimental procedures

Preparation of constructs

Five different constructs were used in this study. The pBIN19/SBD-LUC and pBIN19/LUC-SBD

plasmid were used for the expression of the SBD-LUC (N-terminal SBD) and LUC-SBD (C-terminal SBD) fusion proteins in potato plants (WT and *amf* background), respectively. The pBIN19/GBSSI-LUC and pBIN19/LUC-GBSSI plasmids were used for the expression of GBSSI-LUC (N-terminal GBSSI) and LUC-GBSSI (C-terminal GBSSI) fusion proteins in the mutant *amf* potato plants, respectively. The pBIN19/LUC construct served as a control in which lucifease was directed to the amyloplast without granule-targeting (WT and *amf* background).

The pBIN19/SBD-LUC construct (Figure 1) was assembled from four DNA fragments: (i) a SBD fragment (*NcoI-BglII*), (ii) an artificial PT-linker (*BglII-EcoRI*), (iii) a luciferase fragment (*EcoRI-KpnI*), and (iv) a pBIN19 plasmid, which already contained the potato GBSSI promoter, the potato GBSSI transit peptide, and the NOS terminator sequences (see also Ji *et al*., 2003). The luciferase fragment was amplified from the pLuk07 plasmid (kindly provided by dr. Mankin, North-Carolina State University; Mankin *et al*., 1997) by PCR with the following primers: 5′–GGCC**GTCGAC**CGAATTCATGGAAGACGCCAAAAACAT–3′ and 5′–CGCTGAATACA**GGTACC**TTTTACAAT–3′, which contained *SalI* and *KpnI* sites, respectively. The construct was verified by DNA sequencing. The SBD-LUC fusion protein accumulated in potato tubers has a predicted molecular mass of 74 790 Da, excluding the transit peptide. The pBIN19/LUC-SBD and pBIN19/LUC constructs were prepared as described previously (Ji *et al*., 2003).

For making pBIN19/GBSSI-LUC plasmid, a GBSSI-encoding fragment (*NcoI-BglII*) was amplified. This fragment was used to replace the SBD fragment (also *NcoI-BglII*) in the pBIN19/SBD-LUC plasmid, giving the pBIN19/GBSSI-LUC plasmid (Figure 1). The GBSSI-LUC fusion protein accumulated in potato tubers has a predicted molecular mass of 121 795 Da, excluding transit peptide. The pBIN19/LUC-GBSSI plasmid was assembled using a similar procedure as for making the pBIN19/GBSSI-LUC. A GBSSI-encoding fragment (*EcoRI-KpnI*) was amplified and exchanged with the SBD fragment in pBIN19/LUC-SBD plasmid (Ji *et al*., 2003). The resulting plasmid is referred to as pBIN19/LUC-GBSSI (Figure 1). The LUC-GBSSI fusion protein accumulated in potato tubers has a predicted molecular mass of 121 664 Da, excluding the transit peptide. The constructs were verified by DNA sequencing.

Plant transformation and regeneration

To investigate the effect of SBD position in luciferase fusion proteins in terms of catalytic activity and starch-binding properties, the pBIN19/SBD-LUC plasmids was transformed in *Agrobacterium tumefaciens* according to the three-way mating protocol described by Visser *et al*. (1991).

Internodal stem segments from the amylose-containing potato cultivar (cv. Kardal), and the amylose-free (*amf*) potato mutant (often referred to as 1029-31; Jacobsen *et al*., 1989) were used for *Agrobacterium*-mediated transformation (Visser, 1991). For comparison, the pBIN19/LUC-SBD and pBIN19/LUC plasmids (Ji *et al*., 2003) were also transformed into the cv. Kardal (Ji *et al*., 2003) and the *amf* backgrounds. More than 50 independent shoots were harvested for each construct and each genotype. Shoots were tested for root growth on a kanamycin-containing (100 mg/l) MS30 medium (Murashige and Skoog, 1962). For each construct in the Kardal background, 45 transgenic, root-forming shoots were multiplied and five plants of each transgenic line were transferred to the greenhouse for tuber development; in the *amf* background, this was 17 for each construct. In addition, 10 untransformed controls of each background were grown in the greenhouse.

To investigate whether GBSSI could be used as an alternative for SBD, the pBIN19/LUC-GBSSI and pBIN19/GBSSI-LUC plasmids were transformed into the *amf* potato plants, following the same procedure as mentioned above. For each construct, 27 transgenic plants were multiplied and transferred to the greenhouse for tuber development. Ten untransformed *amf* plants served as controls.

Starch isolation from potato tubers

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

Measurement of luciferase activity in tuber slices, starch granules

Screening of the transformants was done by measuring the luciferase activity of tuber slices with a diameter of 9 mm and a thickness of 2 mm. One similar-sized mature potato tuber was harvested from each greenhouse-grown clone of the various series. The tubers were sliced longitudinally, and subsequently a round slice was sampled, each time from the same position of the section. The total light emission from the samples was detected by a luminometer (Hamamatsu Argus-50 Image

Processor and II Controller; Camera Lens: Nikon, Nikkor 50 mm 1:1.2, Japan) 20 s after spraying the luciferin substrate (0.15 mg/ml, without ATP) onto the slices (20 °C). To measure the luciferase activity in isolated starch granules, 10 mg of dried starch was mixed with 30 µl of Bright-Glo Luciferase Assay Substrate (Promega, USA) containing ATP, and the total light emission from the sample was recorded by the luminometer after 5 min at 20 °C (Ji *et al*., 2003).

Western dot blot analysis

The amount of luciferase and fusion proteins accumulated in transgenic starches was estimated with a Western dot blot procedure as described by Ji *et al*. (2003). Anti-Luciferase pAb (Promega, USA), antiSBD and antiGBSSI polyclonal antibodies were used to visualize their respective immunogens by chemiluminescence. For SBD-containing fusion proteins, a 1:500 dilution of antiSBD was used as the primary antibody. For GBSSI-containing fusion proteins, a 1:250 dilution of antiGBSSI was used as the primary antibody. In both cases, a 1:2000 dilution of Goat Anti-Rabbit IgG (H+L) Alkaline Phosphatase Conjugate (Biorad, USA) was used as the secondary antibody for detection. For luciferase and luciferase-containing fusion proteins, a 1:500 dilution of Anti-Luciferase PAb was used as the primary antibody. A 1:2000 dilution of Donkey Anti-Goat lgG Ap (Promega, USA) was used as the secondary antibody for detection.

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Chapter 4

Reduction of starch granule size by expression of an engineered tandem starch-binding domain in potato plants

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Summary

Granule size is an important parameter when using starch in industrial applications. An artificial tandem repeat of a family 20 starch-binding domain (SBD2) was engineered by two copies of the SBD derived from *Bacillus circulans* cyclodextrin glycosyltransferase via the Pro-Thr-rich linker peptide from Xyn10A from *Cellulomonas fimi*. SBD2 and a single SBD were introduced into the amylose-free potato mutant, *amf,* using appropriate signal sequences. The accumulation of SBD2 into transgenic starch granules was much higher than that of SBD. In a number of transformants, particularly *amf*SS3, the starch granules were much smaller than in control plants. The *amf*SS3 mean granule size was 7.8 μm compared with 15.2 μm in the control, whereas other starch properties were unaltered. This new starch combines the advantage of the high purity of potato starch with that of the small granule size of other crop species, such as cassava, taro and wheat. This starch may find application in the manufacture of biodegradable plastic films. Both genes were also expressed in *Escherichia coli* and the affinity for soluble starch of the purified recombinant proteins was determined. SBD2 had an approximately 10-fold higher affinity for starch than SBD, indicating that the two appended SBDs act in synergy when binding to their target polysaccharide ligand.

Key words: tandem starch-binding domain, granule size, starch biosynthesis, transgenic potato, starch affinity.

Introduction

Starch is the major storage carbohydrate in many plants and an important raw material for food and industrial applications. Starches can be subjected to different kinds of chemical derivatization procedures to improve their properties. In food, starch can be used as a thickening agent. In paper manufacturing, it is used for surface sizing, in which a continuous film of gelatinized starch is deposited on a sheet to enhance the surface properties for writing and printing. In textile manufacturing, yarns are coated with starch polymers to increase their tensile strength during weaving. Starches are also used by the pharmaceutical industry to create a slow-release matrix in which therapeutic compounds are dispersed. The suitability of starch for the above-mentioned applications is determined by its granule size, the physicochemical properties of the granule (such as the amylopectin to amylose ratio and the chain length distribution) and the presence of non-starch components, such as lipids and proteins. It is known that starch properties can vary enormously between different species. For instance, the diameter of starch granules can range from 1 to 40 μm in wheat, 3 to 26 μm in maize and 5 to100 μm in potato (Ellis *et al*., 1998). The mechanisms controlling granule size are not known, although there is considerable industrial interest in this. For maize, increased granule size is often desirable, because this may improve the wet-milling efficiency, and thus starch yield (Gutiérrez *et al*., 2002). Potato starch granules are often too large to produce high-quality noodles (Chen *et al*., 2003) and to manufacture biodegradable plastics, in which the starch is used as a dry filler of the plastic film (Lim *et al*., 1992).

A number of starches with new or improved functionalities have been tailored *in planta* using recombinant DNA technology. We have previously described a method that anchors foreign proteins to potato starch granules during the biosynthesis of the granule (Ji *et al*., 2003) via the non-catalytic starch-binding domain (SBD) of *Bacillus circulans* cyclodextrin glycosyltransferase (CGTase). Interestingly, larger amounts of SBD were accumulated in the granules of the amylose-free (*amf*) potato mutant than in those of the wild-type potato (Ji *et al*., 2003). It is envisaged that the use of this technology to incorporate appropriate effector proteins into starch granules will have numerous applications in starch bioengineering (Kok-Jacon *et al*., 2003).

In this paper, we investigate the possibility of depositing more protein into the starch granule during biosynthesis by using a higher-affinity anchor than SBD. It is well established that modular glycosyl hydrolases have acquired a series of non-catalytic carbohydrate-binding modules (CBMs) to increase their affinity for the target polysaccharide. Examples include *Cellulomonas fimi*

xylanase 11A, which has two family 2b xylan-binding CBMs (Bolam *et al*., 2001), and a *Piromyces equi* protein with two family 29 glucomannan-binding CBMs (Freelove *et al*., 2001). In both proteins, the tandem CBMs displayed affinity for the target ligand which was more than 10-fold higher than that of the individual CBMs, showing that there was significant co-operativity between these linked modules. We have adopted nature's strategy to increase the affinity of starch-binding CBMs for their ligands by engineering an artificial protein comprising two identical SBDs (referred to as SBD2) that are joined by a Pro-Thr-rich linker sequence. The SBD belongs to CBM family 20 and, to our knowledge, no such CBM20 repeat structures have been found in nature. The affinity for starch of this tandem repeat SBD was compared to that of a single SBD. The introduction of SBD2 into the *amf* potato mutant had a pronounced effect on starch granule size, and the potential of this approach in starch bioengineering *in planta* is discussed.

Results

Binding affinity of SBD and SBD2

In the tandem SBD construct, SBD2, two identical family 20 SBDs were joined via a Pro-Thr-rich linker peptide derived from the *C. fimi* Xyn10A (formerly known as Cex; Gilkes *et al*., 1991; Tomme *et al*., 1995) (Figure 1). The linker provides sufficient flexibility for each SBD to bind to starch independently. After expression in *Escherichia coli*, the SBD (single domain) and SBD2 (duplicated domain) proteins were purified to electrophoretic homogeneity by metal ion affinity chromatography. The capacity of the purified proteins to bind to soluble starch was investigated by isothermal titration calorimetry (ITC). The data (Figure 2) show that both proteins interact strongly with soluble starch. As the concentration of binding sites in soluble starch is unknown, the SBDs were treated as the ligand. Both titrations were fitted using a one-binding-site model; the two starch-binding sites on each SBD molecule displayed similar affinity for ligand, as reported previously for a family 20 CBM (Belshaw and Williamson, 1993), preventing accurate measurement of the affinity of the individual binding sites. Thus, the data gives an average affinity for all the binding sites in the SBD.

A comparison of the K_a values showed that the SBD2 has approximately 10-fold higher binding affinity than SBD for starch (Table 1). The greater than twofold increase in the association constant

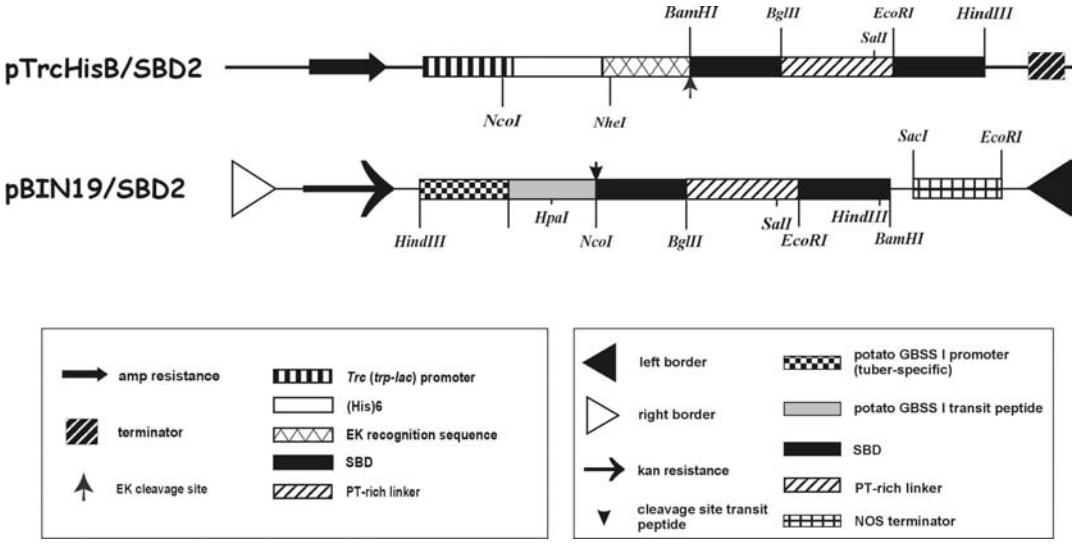

Figure 1. Schematic representation of pTrcHisB/SBD2 vector used for expression of the tandem starch-binding domain (SBD2) in *Escherichia coli*. The pBIN19/SBD2 binary vector was used for SBD2 expression in potato plants. In this case, the engineered SBD2 gene was under the control of the potato granule-bound starch synthase I (GBSS I) promoter. Amyloplast entry was mediated by the potato GBSS I transit peptide.

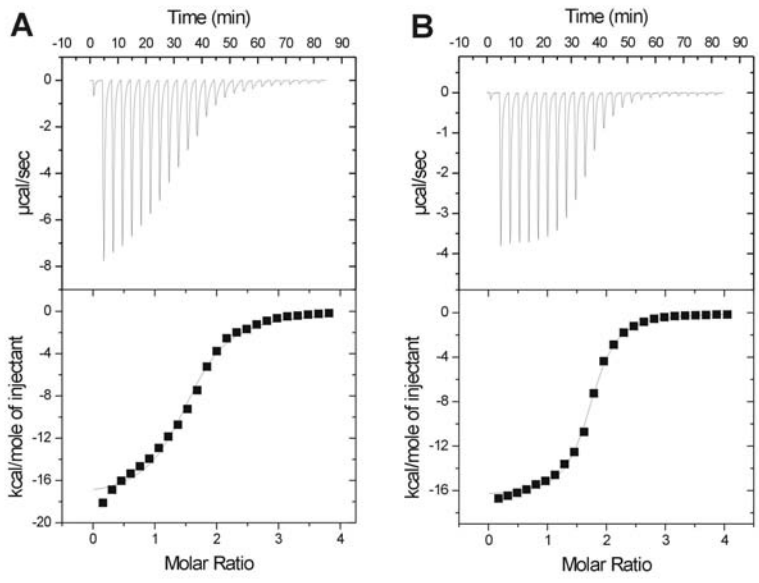

Figure 2. Isothermal titration calorimetry (ITC) of the binding of the starch-binding domain (SBD) (A) and tandem starch-binding domain (SBD2) (B) to soluble starch. The top half of each panel shows the calorimetric titration of ligand into protein, and the lower half shows the integrated heats from the upper panel, corrected for heats of dilution. The full line is the curve of the best fit that was used to derive parameters K_a and ΔH. All titrations were performed in 50 mM sodium phosphate buffer, pH 7.0 at 25 °C.

cannot be explained solely by duplication of the binding sites in SBD2. When maltohexaose instead of soluble starch was used as the ligand, SBD2 displayed only twofold higher affinity than SBD for the oligosaccharide, as would be expected for a protein with twice the number of binding sites (data not shown). This strengthens the hypothesis that the two appended SBDs act synergistically rather than additively, as maltohexaose is probably too small to span the two linked CBM20s in SBD2.

Table 1. Affinity of the starch-binding domain (SBD) and tandem starch-binding domain (SBD2) for soluble starch, as determined by isothermal titration calorimetry (ITC). Data (±SD) are the average of three independent measurements.

	K_a (M^{-1})	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	$T\Delta S$ (kcal mol^{-1})
SBD	$1.3\ (\pm 0.2) \times 10^5$	−7.0 (±0.1)	−21.6 (±1.6)	−14.6 (±1.7)
SBD2	$1.4\ (\pm 0.1) \times 10^6$	−8.4 (±0.0)	−28.2 (±1.2)	−19.8 (±1.2)

Characterization of SBD2 transformants

The *amf* potato mutant was transformed with the pBIN19/SBD2 construct, and the resultant transgenic potato plants are referred to as *amf*SSxx (where SS represents the SBD2 gene and xx refers to the clone in the series of transformants). Untransformed control plants are referred to as *amf*-UT. Fifty kanamycin-resistant, transformed lines were grown in the greenhouse to generate tubers. During growth, the transgenic lines appeared phenotypically normal with respect to plant size, shape and colour, as well as the number and size of tubers produced (results not shown).

The levels of SBD2 accumulation in transgenic granules were investigated by Western dot blot analysis. The SBD2 accumulating lines were grouped into seven classes (ranging from 0+ to 6+), based on the amount of SBD2 protein that was associated with the starch granules (see Figure 3A). The 6+ class (not shown in Figure 3A) represents the transgenic granules giving a similar signal in the Western dot blot analysis as the 5+ class, with half the amount of starch. SBD2 accumulation levels in the transgenic granules are summarized in Figure 3B. For comparison, single SBD accumulation in the *amf* background (*amf*S series) is also indicated in this figure (Ji *et al.*, 2003). It is apparent that, in the *amf*SS series, many more transformants are found in the classes (4+, 5+ and 6+) with high SBD2 accumulation, whereas, for the *amf*S series, these classes are not observed at all. These results demonstrate, also in *planta*, that SBD2 is a higher-affinity binding domain than SBD.

In order to estimate the amount of SBD2 protein accumulated in transgenic granules, *amf*S48, the highest SBD accumulator (3+ class) from the *amf*S series (Ji *et al*., 2003) was used as a control for comparison with the density of dots from the *amf*SS series in Western dot blot analysis. The amount of SBD2 in the highest accumulator (6+ class) was estimated to be approximately fivefold higher than that of *amf*S48 (approximately 80 mg of SBD2 protein per gram of dried starch). This is also consistent with results from ITC analysis.

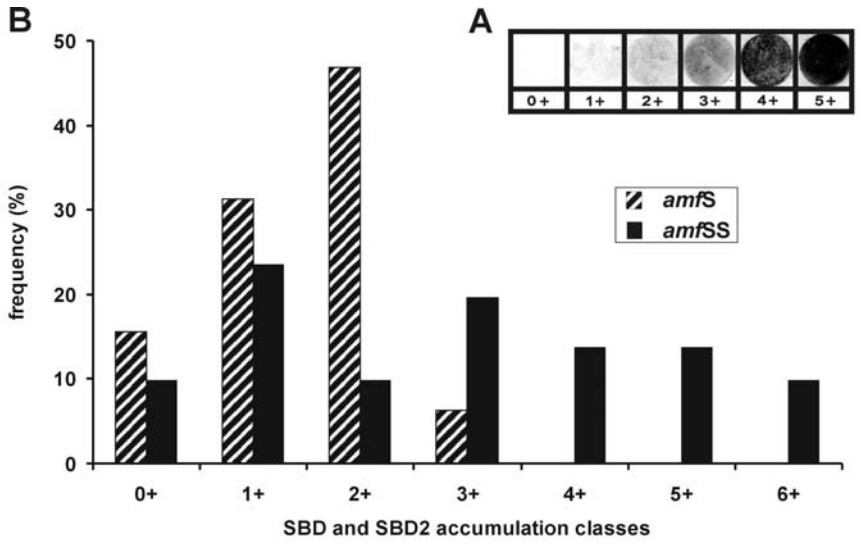

Figure 3. Accumulation levels of the starch-binding domain (SBD) and tandem starch-binding domain (SBD2) in potato starch granules isolated from amylose-free (*amf*) genotypes.
Panel A defines the classes of SBD accumulation in potato starch granules. This classification is based on the results of a Western dot blot analysis with various starch samples. The 6+ category represents the transgenic granules which gave a similar signal in Western dot blot analysis to the 5+ class with half the amount of starch. **Panel B** shows the distribution of the individual transformants over the seven classes of SBD2 accumulation in the *amf* background (*amf*SS series). For comparison, single SBD accumulation in transgenic *amf* potato starch granules is also indicated (*amf*S series).

SBD2 accumulation in the soluble fraction of potato juice was also determined by Western dot blot analysis. In none of the juices of the transgenic lines SBD protein was detected, with the exception of juices belonging to the 6+ class of transformants. The amount of SBD found in these juices corresponded to the dot with an intensity of 2+ in Figure 3A. Thus, only in the class with the highest amount of SBD2 in the granules, can SBD2 be detected in the juice. This suggests that the

amount of SBD2 in the 6+ class is saturating, *i.e.* all binding sites for SBD2 in the starch granules have been occupied.

Granule size is altered by SBD2 expression

Microscopic examination of starch granule morphology from 50 transformants revealed that the size of the starch granules of some transgenic lines was substantially smaller than that of the control. This observation was further substantiated by measuring the granule size distributions of all the transgenic starches. This analysis confirmed that some of the transgenic lines had smaller granules. A number of lines, including *amf*SS3 (see further), showed a shift to smaller granules in their granule size distribution. For a number of other lines, the granule size distribution was bimodal (Figure 4A); the larger granules, indicated by the second peak, were approximately similar in size to the *amf*-UT (non-transgenic control) starch granules. The valley point in the bimodal distributions was, in all cases, approximately 12 µm. Figure 4B gives an impression of the granule size in the various SBD2 accumulation classes. For each class, the percentage of transformants in which 50% and 65% of the granules were smaller than 12 µm is indicated (i.e. for these transformants, the grey area in Figure 4A should represents more than 50% and 65% of the total area, respectively). For

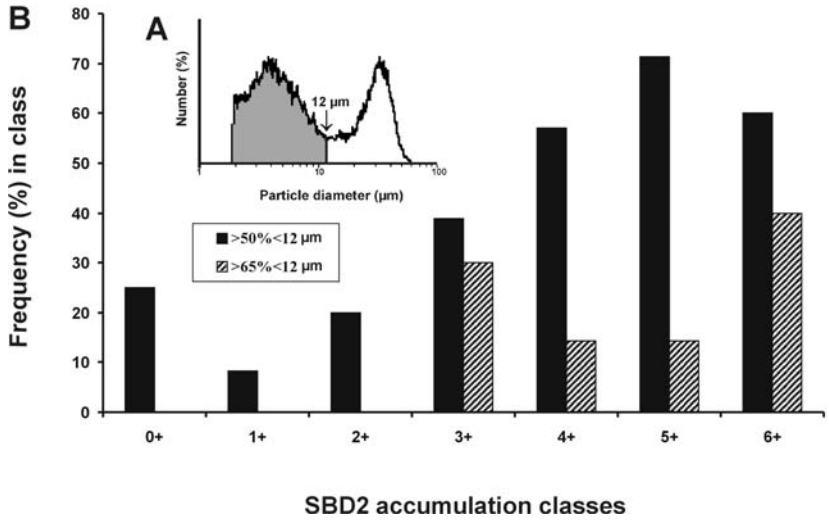

Figure 4. Relationship between the occurrence of small granules and tandem starch-binding domain (SBD2) accumulation.
Panel A shows the bimodal distribution of a representative *amf*SS starch. The 12 µm point represents the valley point in bimodal distributions, which was observed in a number of the transgenic SBD2 starches. **Panel B** shows the frequency of transformants with small granules in the various SBD2 accumulation classes.

instance, in the 3+ class, 40% of the transformants showed that over 50% of the granules were smaller than 12 µm, and 30% of the transformants showed that over 65% of the granules were smaller than 12 µm. Thus, it can be clearly seen that a high SBD2 accumulation correlates with more small granules. However, the lines with the smallest granules typically belong to the 3+ class.

Because *amf*SS3 had the most pronounced reduction in granule size of all transformants, the starch of this transformant was subjected to a more extensive investigation. Analysis of *amf*SS3 granules by scanning electron microscopy (SEM) (Figure 5A) showed two size-classes of granules for this transformant: large ones and a large number of very small ones (sometimes organized in clusters). The micrograph of *amf*-UT starch shows a normal distribution of granule sizes (having small, many intermediate and large granules). Figure 5B shows the granule size distribution of *amf*SS3 starch in comparison with starch from *amf*-UT. The mean granule size by number of *amf*-UT starch was 15.2 µm, whereas the mean of *amf*SS3 starch was 7.8 µm, which is approximately 2 times smaller than the control. It should be noted that the granule size distribution of *amf*SS3 is relatively wide compared to that of *amf*-UT, which is in accordance with the SEM results. Although it seems that the large granules of *amf*SS3 dominate the scanning electron micrograph at first sight,

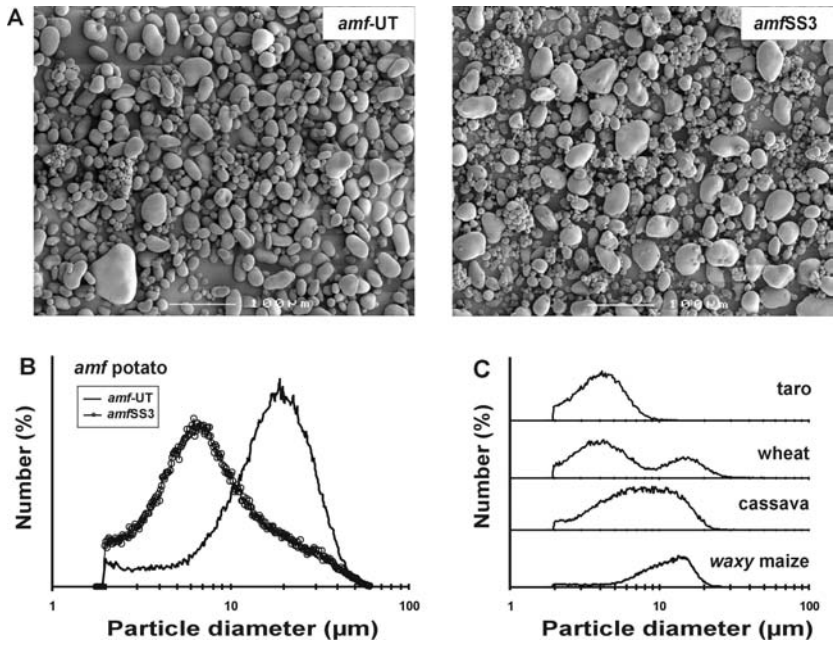

Figure 5. Scanning electron microscopy (SEM) of starch granules from wild-type (WT) and transgenic tubers, and particle size distributions of selected starches.
Panel A shows SEM analysis of starch granules from *amf*-UT and *amf*SS3 tubers at magnifications of 200 times. **Panel B** shows the particle size distributions of *amf*-UT and *amf*SS3 starches. **Panel C** shows the particle size distributions of taro, wheat, cassava and waxy maize starches for comparison.

their abundance is low compared with the small granules, and consequently they contribute relatively little to the granule size distribution.

For comparison, the granule size distributions of a number of important crop plants are shown in Figure 5C. Taro starch has a mean of 4.0 μm, which is, to our knowledge, the smallest starch granule known in a crop plant. Wheat starch has a bimodal granule size distribution with large granules of the A-type crystallite and smaller ones with a B-type crystallite (French, 1984). The mean of wheat starch is 4.0 μm for the smaller granules, and 15.0 μm for the larger ones. Maize and cassava are major starch sources for the industry. The mean of maize starch is 10.1 μm, whereas that of cassava is 7.3 μm. Our results show that the granule size of amylose-free potato starch can be decreased to that of cassava by SBD2 expression.

Starch content of line amfSS3 and characterization of its starch

The impact of SBD2 accumulation in granules on the starch content and the physicochemical properties of the starch were also investigated. The starch content of *amf*SS3 (13.7±1.6 w/w% fresh weight) was comparable with that of *amf*-UT (15.0±1.1 w/w% fresh weight), which shows that starch yield is not affected by the reduction in granule size. Granule–melting behaviour (T_0 and ΔH) of *amf*-UT and *amf*SS3 starch were studied by differential scanning calorimetry (DSC). The pasting

Table 2. Gelatinization properties (T_0, ΔH), pasting properties (*Tg*, *Tp*, *peak* and *end visicosity*) and apparent amylose content (AM, %) of starches from *amf*SS3 and *amf*-UT. Bohlin data are the mean of two measurements. Differential scanning calorimetry (DSC) and AM data (± SD) are the average of three independent measurements.

	gelatinization properties		pasting properties				
clone	T_0[a]	ΔH[b]	*Tg*[c]	*Tp*[d]	*peak visc.*	*end visc.*	AM
	(ºC)	(kJ/g)	(°C)	(°C)	(Pas)	(Pas)	(%)
***amf*-UT**	68.6 (±0.3)	8.0 (±1.4)	61.8	74.7	36.2	26.3	3.6 (±0.2)
***amf*SS3**	68.2 (±0.5)	9.4 (±1.2)	63.5	75.2	37.3	24.5	3.3 (±0.2)

[a] Temperature at onset of starch gelatinization (DSC).

[b] Enthalpy released (DSC).

[c] Starch gelatinization temperature (Bohlin).

[d] Peak temperature (Bohlin).

properties of the two starches were determined by Bohlin rheometry, which involves measuring viscosity changes whilst the starch suspension is heated and then cooled with constant stirring. The highest viscosity of a starch paste is referred to as the peak viscosity, whereas the viscosity after cooling of the starch paste is called the end viscosity. Also, the amylose content of both samples was analyzed. The results are summarized in Table 2. From the data, it can be seen that there are no consistent differences in the various parameters for the two starches.

Discussion

In the present study, we have engineered an artifical tandem repeated SBD (CBM20) and determined its affinity for starch, both *in vitro* and *in planta*. The ITC data indicate that SBD2 can bind to soluble starch with an approximately 10-fold higher affinity than the individual SBD, indicating that the two SBDs act in synergy to bind the ligand. This increased affinity of SBD2 for starch was also evident *in planta*. When SBD2 was introduced into *amf* potato plants, an approximately fivefold higher protein accumulation in the starch granule could be achieved compared with *amf* potato starch granules accumulating individual SBDs.

To our knowledge, this paper provides the first report of synergy between two appended CBM20s. It should be noted that particular microbial starch-degrading enzymes have been identified, which contain a triple repeat SBD motif (Sumitani *et al*., 2000). However, these binding domains are different from those used in this study, and have been classified as family 25 CBMs. To this end, there are no data available on the binding affinity of the individual family 25 modules, or on that of a series of CBM25s. The binding characteristics of appended CBMs have been studied more extensively in plant cell wall-degrading enzymes, such as cellulases and xylanases. For instance, Linder *et al*. (1996) showed that an artificial tandem CBM1 exhibited much higher affinity for insoluble cellulose than the individual CBM1s, indicating a co-operative effect between the two domains. Another example is the two CBM2bs from *C. fimi* xylanase 11A, covalently joined via a flexible linker, which showed an approximately 18-20 fold increase in the affinity for xylan when compared with the individual modules (Bolam *et al*., 2001). Similar observations were made for two appended CBM29s, which have affinity for cellulose, mannan and glucomannan (Freelove *et al*., 2001). However, there are also examples of appended CBMs which do not show this synergy in binding the substrate; these include the repeated CBM4s of *C. fimi* endoglucanase *CenC* and those of *Rhodothermus marinus* endoxylanase *Xyn10A* (Abou-Hachem *et al.*, 2000; Tomme *et al*., 1996).

Linker sequences in cellulases and xylanases can vary considerably in length (16 to 59 amino

acids) and in amino acid composition (Gilkes *et al*., 1991). In this study, the two SBDs were separated by a Pro-Thr-rich linker of 22 residues in length derived from *C. fimi* Xyn10A. Such linker sequences are quite common in modular proteins (Gilkes *et al*., 1991), and those containing proline may display some rigidity (Janeček *et al*., 2003). In contrast, the linker in *Aspergillus niger* glucoamylase 1, which links the SBD to the catalytic module, is relatively long (68 amino acids), glycine-rich, and thus flexible (Janeček *et al*., 2003). It is possible that the nature of the linker may influence the binding characteristics of the SBDs, and it is therefore possible that a linker sequence other than the one used in this study could enhance the co-operative binding displayed by the modules in SBD2.

The expression of SBD2 in *amf* mutant potato plants resulted in a number of clones containing starch with smaller granules. This phenomenon was not observed when single SBD was expressed in the *amf* background (Ji *et al*., 2003). The *amf*SS3 transformant had the smallest granules of all transgenic lines. Starch yield of this transformant was comparable with that of the untransformed control, suggesting that this line accumulates more small granules and that starch biosynthesis is not inhibited. To a certain extent, the occurrence of small granules in SBD2 transformants is correlated with the amount of SBD2 accumulated in the granule (Figure 4). Typically, the smallest granules are obtained with a transformant belonging to the 3+ class, having intermediate levels of SBD2 accumulation. This result may be explained by Figure 6, which illustrates schematically the interaction of SBD2 with starch at different SBD2 accumulation levels [low (*e.g.* the 1+ class), intermediate (*e.g.* the 3+ class) and high (*e.g.* the 6+ class)]. Our ITC measurements show that SBD2 interacts strongly with starch. We hypothesize that SBD2 binds instantaneously to the growing starch granule when entering the amyloplast, thereby “coating” the granule surface. At low SBD2 levels, the surface is not fully covered, and the enzymes involved in starch biosynthesis are not hindered greatly in attaching material from the stroma to the growing granule. At intermediate SBD2 levels, the granule surface may be fully covered with SBD2. We postulate that, in this situation, both domains of each protein are attached to the same granule. As a consequence, the SBD2s may hinder synthases from elongating amylopectin side-chains at the granule surface, and/or branching enzymes from attaching side chains to the growing granule. In this way, additional nucleation sites for granule formation may be enforced by SBD2. The starch-synthesizing potential then needs to be distributed over more nucleation sites, which could explain our observation of more small granules in this class.

At high-level SBD2 accumulation, the surface of the granule is also fully covered but, in this situation, the binding mode of SBD2 is different. We speculate that high concentrations of SBD2

increase the chance that only one of the two domains of SBD2 is attached to the granule surface, whereas the other sticks out in solution (see Figure 6). Our results indicate that the amount of SBD2 in these transformants seems to be saturating, since SBD2 was also detected in the potato juice. Interestingly, the high accumulation class has approximately twofold more SBD2 than the intermediate class, in agreement with the proposed binding modes. We further speculate that the exposed SBD of the SBD2 protein is available for capturing soluble α-glucans in the amyloplast. If these α-glucans are sufficiently long, they may bind more than one SBD2, possibly attached to different growing granules. Potentially, this could "cross-link" different nucleation sites, which may explain why no reduction in granule size is observed in the high SBD2 accumulation classes; *i.e.* the three indicated nucleation sites may become part of one larger granule.

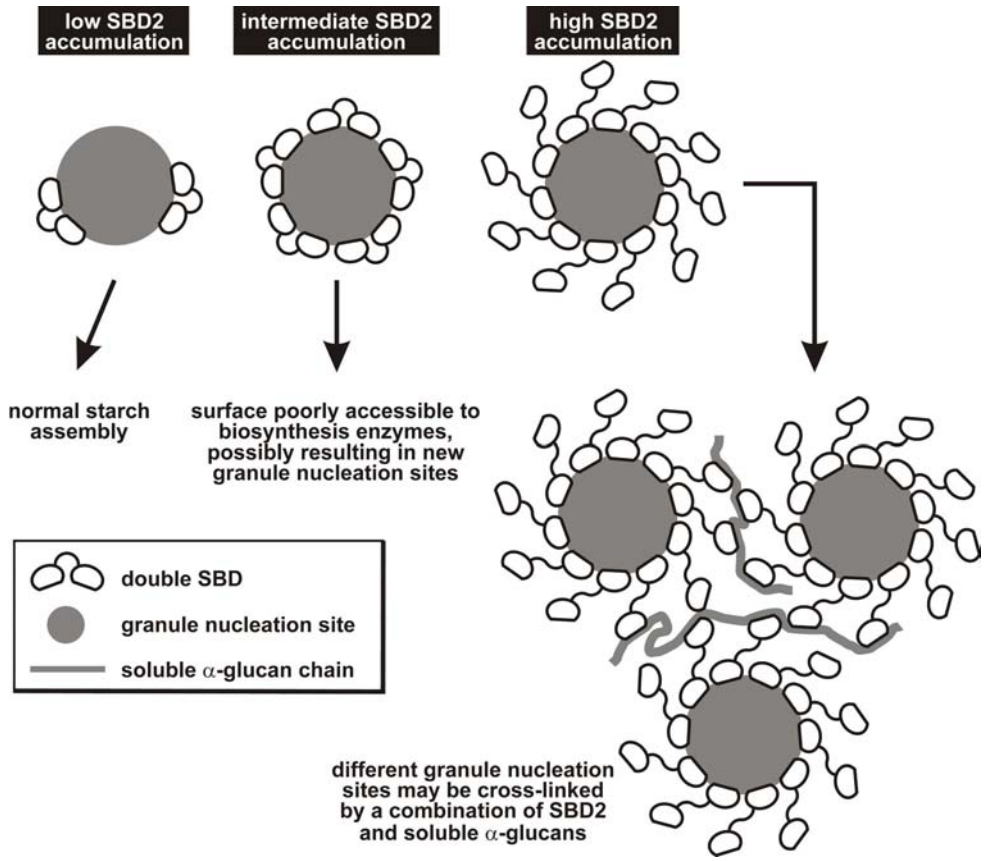

Figure 6. Schematic representation of tentatively different binding modes of the tandem starch-binding domain (SBD2) at various accumulation levels. For simplicity, only the SBD2 proteins at the granule surface are shown. During the biosynthesis process, SBD2 protein is continuously incorporated into the granules as subsequent α-glucan layers are deposited. For details, see text.

In this paper, we have shown that it is possible to produce smaller potato starch granules *in planta*, without accompanying changes in other starch properties and without a yield penalty. Potato starch is often preferred over starches from other sources because of its low level of contaminants, such as proteins and lipids. In certain applications, however, they can not be used due to their large granule size. An example is the production of starch noodles. Chen *et al*. (2003) have shown that native potato starch is not suitable for starch noodle preparation due to the abundance of large granules. However, it was demonstrated that a fraction of small-sized potato starch granules (<20 µm) could be used for making consistently long noodle strands with good quality. Native potato starch only contains a small proportion of such granules (approximately 5%). Another example is the manufacturing of films, in which starch can be used as a biodegradable additive (Gage, 1990). It has been shown that films made with small granules are thinner and have higher elongation rate and tensile strength (Lim *et al*., 1992). In principle, the smaller, disk-shaped wheat starch granules could be used for this type of application. Wheat starch, however, has the disadvantage of containing considerable amounts of non-starch contaminants, including proteins (Baldwin, 2001), which, at elevated temperatures, can participate in so-called Maillard reactions that can cause discoloration of the film (Griffin, 1989). Alternative sources of small-granule starch are amaranth or partially degraded corn starch (acid treatment, followed by milling; Jane *et al*., 1992). However, these sources are relatively expensive, or they require extra processing. Our SBD2 starch combines the purity of potato starch with the small granule found in wheat and corn, which may make this transgenic starch particularly suitable for making starch noodles and films. Another advantage of small-granule potato starch might be in chemical starch modification, such as cross-linking. The more favourable surface-to-volume ratio of the small granules could improve the diffusion rate of the modifying agent into the granule, thereby decreasing the reaction time and possibly reducing the amount of chemicals required.

Experimental procedures

Preparation of constructs

Two constructs were made, one for SBD2 expression in *E. coli* [pTrcHisB/SBD2], and one for SBD2 expression in potato plants [pBIN19/SBD2]. The pTrcHisB/SBD2 construct was assembled from the pTrcHisB/SBD plasmid (Ji *et al*., 2003). A sequence similar to SBD in pTrcHisB/SBD and

an artificial Pro-Thr-rich linker (Ji *et al*., 2003) were inserted into pTrcHisB/SBD. The second SBD-encoding sequence was obtained by polymerase chain reaction (PCR) amplification with the primers 5′–CAACTTCGAG**GAATTC**TACG–3′ and 5′–**AAGCTT**ATGGCTGCCAATTCAC–3′, which contain *EcoRI* and *HindIII* sites at their 5′ ends, respectively. The CGTase gene (CGT13A) from *Bacillus circulans* strain 251 was used as a template (Lawson *et al*., 1994). The amplified fragment was subcloned into a pGEMTeasy vector (Promega, USA). Plasmid DNA was propagated in *E. coli* DH5α and purified from the cells with the Wizard *Plus* Midipreps DNA purification system (Promega, USA). After digestion of this plasmid, the *EcoRI-HindIII* SBD fragment was inserted into the corresponding sites of the pTrcHisB/SBD expression vector to give the pTrcHisB/SBDI-SBDII vector. The Pro-Thr-rich linker used to connect the two SBDs contained a *BglII* site at 5′ end and an *EcoRI* site at 3′ end. The linker was inserted into the corresponding sites of the pTrcHisB/SBDI-SBDII vector to give the pTrcHisB/SBD2 expression vector (see Figure 1).

Using standard cloning procedures, the pBIN19/SBD2 vector was assembled from four DNA fragments: (i) the potato tuber-specific granule-bound starch synthase (GBSSI) promoter (*HindIII-NcoI*) (van der Leij *et al*., 1991); (ii) a sequence encoding the potato GBSSI transit peptide for amyloplast entry (*NcoI-NcoI*) (van der Leij *et al*., 1991); (iii) a SBD2 fragment (*NcoI-BamHI*); and (iv) the NOS terminator sequence (*SacI-EcoRI*) (Bevan, 1984). The first two fragments were subcloned in pMTL23 (Ji *et al*., 2003). The *NcoI-BamHI* SBD2 fragment was amplified by PCR with the primers 5′–**CCATGG**CCGGAGATCAG–3′ and 5′–CTTCTC**GGATCC**GCCAAAAC–3′ using pTrcHisB/SBD2 as a template. The combined fragments (i)-(ii) and fragment (iii) were cloned in a pBIN19 vector, which already contained the NOS terminator sequence. The plasmid is referred to as pBIN19/SBD2 (see Figure 1). The splice site for cleavage of the transit peptide was the same as that used previously for SBD targeting (Ji *et al*., 2003). The SBD2 protein, which will be accumulated in potato tubers, has a predicted molecular mass of 25 371 Da, excluding transit peptide. Both constructs were sequenced to ensure no mutations had occurred during the cloning procedure.

Production of SBD2 protein in E. coli and its purification

Recombinant *E. coli* Tuner cells (Invitrogen, The Netherlands), containing the pTrcHisB/SBD or pTrcHisB/SBD2 construct, were grown in Luria-Bertani (LB) with 100 μg/ml ampicillin at 37 °C until $0.8 < OD_{600} < 1.0$ (OD, optical density). SBD expression was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 2 mM, and by growing the cells for 12 h at 29

°C. Cells were harvested by centrifugation (1300 × *g*, 10 min, 4 °C), re-suspended in Talon buffer (20 mM Tris, 300 mM NaCl, pH 8.0), and frozen at −80 °C. The cells were lysed by sonification for 2 min, and after centrifugation (7500 × *g*, 45 min, 4 °C), the cell-free extract was applied onto an immobilized metal affinity column (6 ml bed volume; TALON Clontech, USA). The SBD proteins were eluted from the column using buffer with increasing imidazole concentration (10, 15, 20, 30 and 50 mM imidazole in Talon buffer). Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to check the eluted fractions for presence of the appropriate polypeptide. Finally, the fractions containing the SBD proteins were pooled and desalted by dialysis against 50 mM sodium phosphate buffer (pH 7.0). The concentration of the purified proteins was determined by UV absorbance at 280 nm, using a calculated molar extinction coefficient of 31 900 $M^{-1}cm^{-1}$ for SBD and 63 800 $M^{-1}cm^{-1}$ for SBD2.

Isothermal titration calorimetry (ITC)

ITC measurements were taken at 25 °C using a MicroCal Omega titration calorimeter. During a titration, the protein sample (0.04-0.1 mM), stirred at 310 r.p.m. in a 1.4331 mL reaction cell, was injected with 25 successive 10 µl aliquots of either 0.2-0.4% (w/v) soluble starch (Sigma Chemical Co.) or 10 mM maltohexaose (Sigma) at 200 s intervals. The proteins were dialysed extensively against 50 mM sodium phosphate buffer, pH 7.0, prior to the titration, and the ligands were dissolved in the same buffer to minimize heats of dilution. The binding data were corrected for the heat of dilution of both proteins and ligand. Integrated heat effects were analyzed by non-linear regression using a single set of sites binding model (MicroCal ORIGIN v5.0), yielding independent values for K_a and ΔH. The equation, $-RT \ln K_a = \Delta G = \Delta H - T\Delta S$, was used to derive the other thermodynamic parameters.

Plant transformation and regeneration

The pBIN19/SBD2 plasmid was transformed into *Agrobacterium tumefaciens* according to the three-way mating protocol described by Visser (1991). Internodal stem segments from the diploid amylose-free (*amf*) potato mutant (often referred to as 1029-31; Jacobsen *et al*., 1989) were used for *Agrobacterium*-mediated transfomation containing the plasmid (Visser, 1991). More than 50 independent shoots were harvested. Shoots were tested for root growth on a kanamycin-containing (100 mg/l) MS30 medium (Murashige and Skoog, 1962). Fifty transgenic, root-forming shoots were

multiplied and five plants of each clone were transferred to the greenhouse for tuber development. In addition, 10 untransformed controls were grown in the greenhouse.

Starch isolation from potato tubers

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

Determination of SBD2 content of transgenic starches by dot blot analysis

A 12.5% sodium dodecylsulfate-polyacrylamide gel (50 mm × 50 mm × 3 mm), with 9 equally spaced holes (∅ = 9 mm), was placed in contact with a similar-sized Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, UK). Twenty mg of (transgenic) starch was boiled for 5 min with 200 µl of a 2× SDS sample buffer (Laemmli, 1970). After cooling to room temperature, the starch gel was transferred into one of the holes. SBD2 proteins from transgenic starch gels were blotted onto the membrane with PhastSystem (Pharmacia, Sweden; 20 V, 25 mA, 15 °C, 45 min) (Ji *et al*., 2003). The protein was identified with antiSBD antibody according to the method described by Ji *et al*. (2003).

SBD2 proteins in the soluble fraction were determined as follows. 500 µl of tuber juice was freeze-dried. The dried material was dissolved in 200 µl of 2× SDS sample buffer. In order to make the sample suitable for the Western dot blot procedure, the mixture was boiled for 5 min in the presence of 20 mg starch from the control samples. The starch gel obtained was applied to one of the holes in the sodium dodecylsulfate-polyacrylamide gel. The rest of the procedure was conducted in the same way as described for granule-bound SBD2.

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; 20 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.

Analysis of potato tuber starch

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

Average granule size and granule size distribution of the control and transgenic starches were determined in triplicate with a Coulter Multisizer II, equipped with an orifice tube of 100 µm (Beckman-Coulter, UK). Approximately 10 mg of starch was dispersed in 160 ml of Isoton II. The number percentages of the differently sized granules in the sample were recorded by counting approximately 50 500 (±500) particles. The coincidence (the frequency of two granules entering the tube at the same time, and consequently being counted as one) was set at 10%.

The apparent amylose content of starches 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 DSC using a Perkin-Elmer Pyris 1 (Perkin-Elmer, The Netherlands), equipped with a Neslab RTE-140 glyco-cooler (Ji *et al*., 2003). Dynamic rheological properties of 5% (w/v) starch suspensions at small deformations were determined by applying a small oscillating shear deformation (5 s^{-1}) using a Bohlin CVO rheometer (Mettler Toledo, The Netherlands). The suspensions were preheated to ~40 °C with gentle stirring and loaded into the sample cell (preheated at 40 °C). After this, the cell was subjected to the following temperature programme: heating to 90 °C, 15 min at 90 °C, cooling to 20 °C, and 15 min at 20 °C. Heating and cooling were performed at a rate of 2 °C min^{-1}. Data were collected automatically every 10 s.

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Chapter 5

Glucan polymerization and granule packing uncoupled during starch biosynthesis in potato plants

To be submitted

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Summary

This study investigates whether it is possible to produce an amylose-free potato starch by displacing the amylose enzyme, granule-bound starch synthase (GBSSI), from the starch granule by an engineered, high-affinity, tandem-repeat of a family 20 starch-binding domain (SBD2) [Ji *et al.*, (2004) *Plant Biotechnol. J.* 2, 251-260]. SBD2 was introduced in the amylose-containing potato cultivar (cv. Kardal), and the starch of the resulting transformants was compared with that of SBD2-expressing amylose-free potato lines. We have obtained evidence that SBD2 and GBSSI compete for binding sites at the granule surface, and that GBSSI has the highest affinity for starch of the two. A reduction in amylose content was not achieved. Most striking were the observations that SBD2 expression could affect physical processes underlying poorly understood starch characteristics such as granule morphology, size, crystallinity, and the formation of growth rings, without altering the primary structure of the constituent starch molecules.

Key words: fusion protein, granule-bound starch synthase I, starch-binding domain, transgenic potato, granule size and morphology, granule assembly

Introduction

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.*, 1999). The fact that GBSSI has a lower affinity (higher Km) for ADP-glucose in comparison with (at least some) other starch synthases may explain this observation (Edwards *et al.*, 1999). 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). It was also shown that more SBD could be incorporated in the granules of amylose-free (*amf*) potato mutant. One explanation for this observation is 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 Pro-Thr rich linker peptide (SBD2). It was shown that SBD2 had an approximately 10-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). In this study we investigated whether it is possible to obtain an amylose-free starch by introducing SBD2 in a wild-type potato background. Our hypothesis was that two appended SBDs might be strong enough to displace GBSSI from the starch

granule. Besides, the characteristics of SBD2-containing starch of both the WT and *amf* background are compared.

Results

Characterization of SBD2 transformants

The pBIN19/SBD2 was transformed into potato cv. Kardal (KD), and the amylose-free (*amf*) potato mutant plants. The resulting transgenic potato plants are referred to as KDSSxx and *amf*SSxx, respectively (SS represents the SBD2 gene and xx refers to the clone in that particular series of transformants). Untransformed control plants are referred to as KD-UT and *amf*-UT, respectively. Fifty kanamycin-resistant, transformed lines from each genotype were grown in the greenhouse to generate tubers. The morphology of plants and tubers, as well as the tuber-yield of all transgenic plants was comparable to that of the 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 5 classes (ranging from 0+ to 4+; see Figure 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 both KDS and *amf*S series (Ji *et al*., 2003) and SBD2 accumulation in the *amf*SS series (Ji *et al*., 2004) are also indicated in Fig. 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).

The SBD2 protein concentration was also determined (Western dot blot analysis) in the potato juice of one representative of each class from the KDSS series. The results are summarized in Figure 2. For comparison, the SBD2 concentration in the soluble fraction of representative *amf*SS potato tubers of each class (Ji *et al*., 2004) are also indicated in the figure, as well as the amount of SBD in the respective starch granules. The amount of SBD2 found in class 1+ and 2+ KDSS tuber juice corresponded to the dot with an intensity of 1+ (see Figure 1A), and that in class 3+ and 4+ KDSS

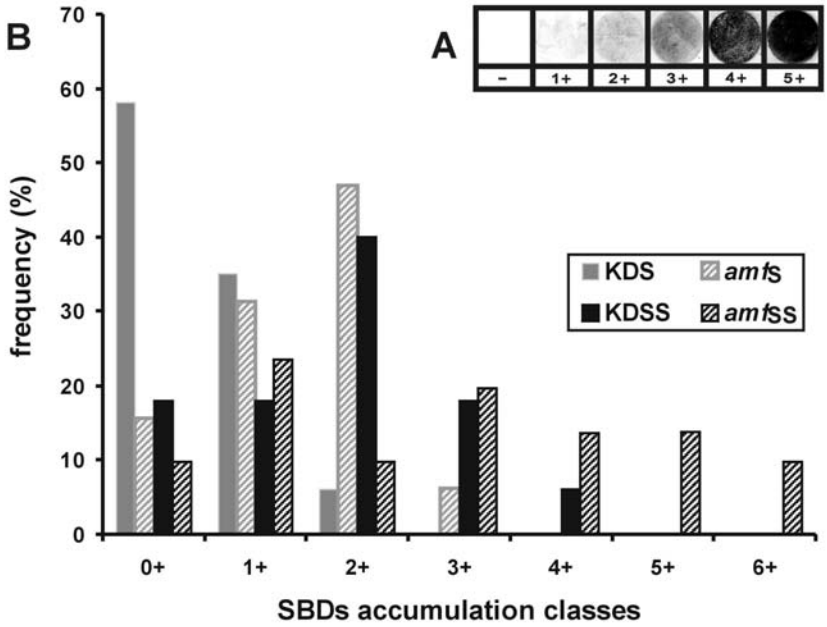

Figure 1. Accumulation levels of SBD and SBD2 in potato starch granules isolated from wildtype (WT) and amylose-free (*amf*) genotypes.
Panel A defines the classes of SBD2 accumulation in potato starch granules. This classification is based on the results of a Western dot blot analysis with various starch 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. **Panel B** shows the distribution of the individual transformants over the seven classes of SBD2 accumulation in WT background (KDSS series). SBD2 accumulation in transgenic *amf* background (*amf*SS series) and SBD in both backgrounds (KDS and *amf*S series) are indicated for comparison.

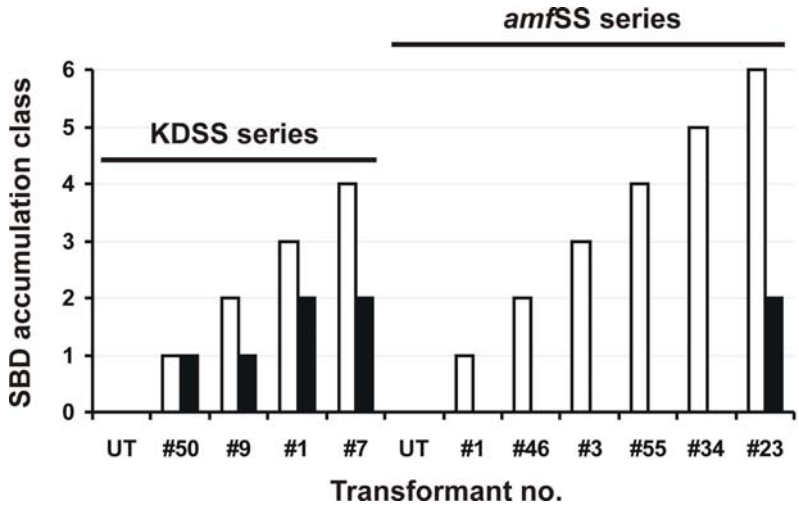

Figure 2. Accumulation levels of SBD2 protein in starch granules (open bars) and juices (closed bars) of selected (transgenic) potato tubers.

tuber juice to 2+. In the *amf*SS series, SBD2 can only be detected in the tuber juice derived of the 6+ class transformants. Our data suggest that amylose-containing granules were saturated with SBD2 at lower concentrations than the *amf* ones.

Based on the Western dot blot results, one transgenic line of each SBD2 accumulation-class of the KDSS and *amf*SS series, as well as their respective controls, were selected for Northern blot analysis (Figure 3). The SBD2 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 4+ class (KDSS series, Figure 3A) or the 6+ class (*amf*SS series, Figure 3B) had much higher amounts. SBD2 transcripts were absent in the untransformed controls.

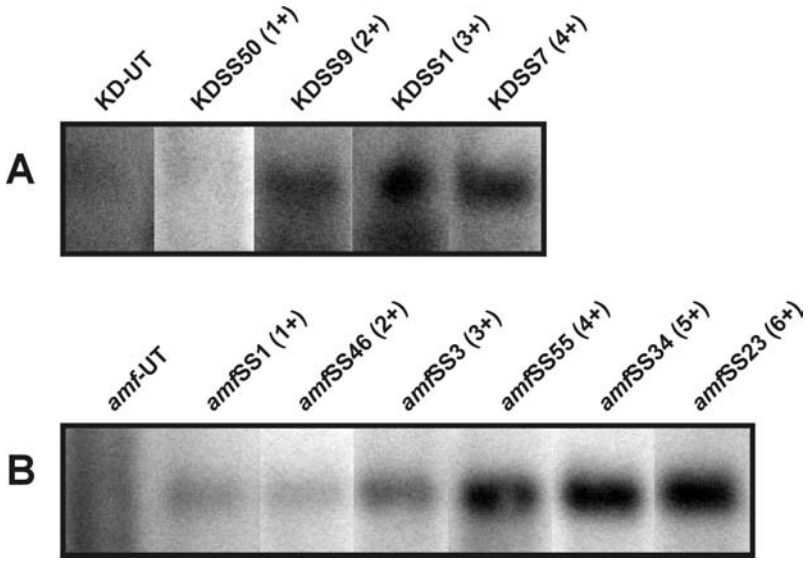

Figure 3. Northern blot analysis of SBD2 expression in selected transgenic lines of KDSS (A) and *amf*SS series (B), and their respective controls. Each line contains 40 µg total RNA.

Amylose content

The starch granules of each transformed line of the KDSS series were stained with a Lugol's 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 KDSS starch samples of each SBD2 accumulation class. In all samples an amylose content (approximately 20%) similar to the untransformed control was found. These data demonstrate that introduction of SBD2 in an amylose-containing background does not reduce the amylose content.

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 KDSS starch granules by Western dot blot analysis. In each class of the KDSS series, a similar amount of GBSSI protein as in KD-UT was found (data not shown). These results suggest that the high-affinity SBD2 is unable to displace GBSSI from the starch granule during the biosynthesis process.

Granule morphology

Starch granule morphology of each transformed clone from both the KDSS and *amf*SS series was investigated by light microscopy (LM). The selected micrographs of various transgenic granules and their respective controls are shown in Figure 4. It can be seen that the morphology of KD-UT and KDSS50 (1+) starch granules is more or less similar, except for the presence of apparent cracks in the granules. Further analysis of these granules by scanning electron microscopy (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 (Ji *et al.*, 2003). Higher SBD2 accumulation levels in KDSS series showed altered granule morphology in both light and scanning electron micrographs. Granules were sometimes organized as a bunch of grapes (further referred to as “grape-like structures”). It seemed that this phenomenon was most pronounced in KDSS1 (3+) and KDSS7 (4+), although it could also be observed KDSS9 (2+) (see Figure 4A and Figure 5A, B).

Analysis of *amf*SS granules by LM and SEM revealed that the granule morphology of the various transformants was altered in comparison with the control (Figure 4B). From this figure, it can be seen that granules are sometimes organized in large clusters of very small granules (further referred to as “large clusters”), particularly in *amf*SS3 starch, which shows a relatively low level (3+) of SBD2 accumulation. Two other transformants in the 3+ class also showed this phenomenon, but the large clusters were not found in any of the transformants of the other classes of the *amf*SS series. Also, in none of the transformants of the KDSS series these large clusters were observed.

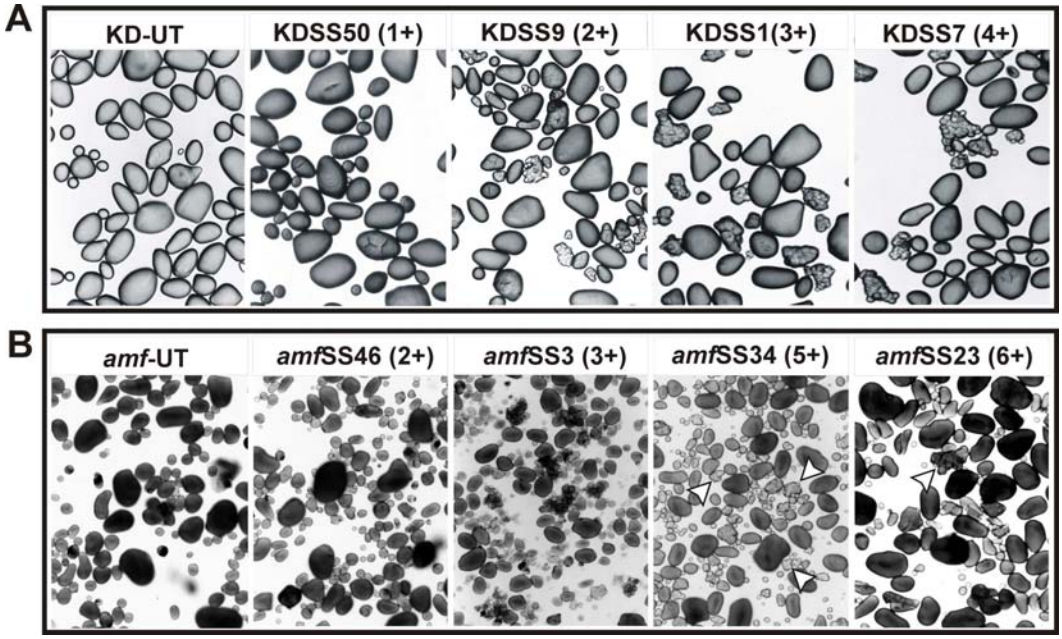

Figure 4. Light micrographs showing the morphology of control and transgenic starch granules. The granules were stained with a 20× diluted Lugol solution. **Panel A** shows the micrographs of representative granule preparations of selected plants of the KD-UT and the KDSS series. **Panel B** shows the micrographs of representative granule preparations of selected plants of the *amf*-UT and the *amf*SS series. Magnification of the starch granules is 200 times.

Furthermore, a high magnification scanning electron micrograph of *amf*SS3 granules showed that the large clusters are more loosely associated than the grape-like structures in the amylose-containing background (Figure 5C, D). In *amf*SS34 and *amf*SS23 with higher levels of SBD2 than *amf*SS3, the large clusters were not observed, but smaller grape-like structures were encountered (Figure 4B, indicated with arrowheads). Higher magnification scanning electron micrographs of the high SBD2 accumulators showed that the surface of the granules can be rough and contain deep grooves, particularly in the *amf*SS23 (6+ class) (Figure 5E, F). We have never observed such phenotypes before in any of the transgenic starches we have generated over the years. These micrographs suggest that SBD2 can interfere in the process of assembling the starch granule, at least when it is present in large amounts.

In the starch granules from a number of transformants of the KDSS series, we observed the contour lines of grape-like structures inside larger starch granules (Figure 6). 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 grape-like structures can both occur in the starch of

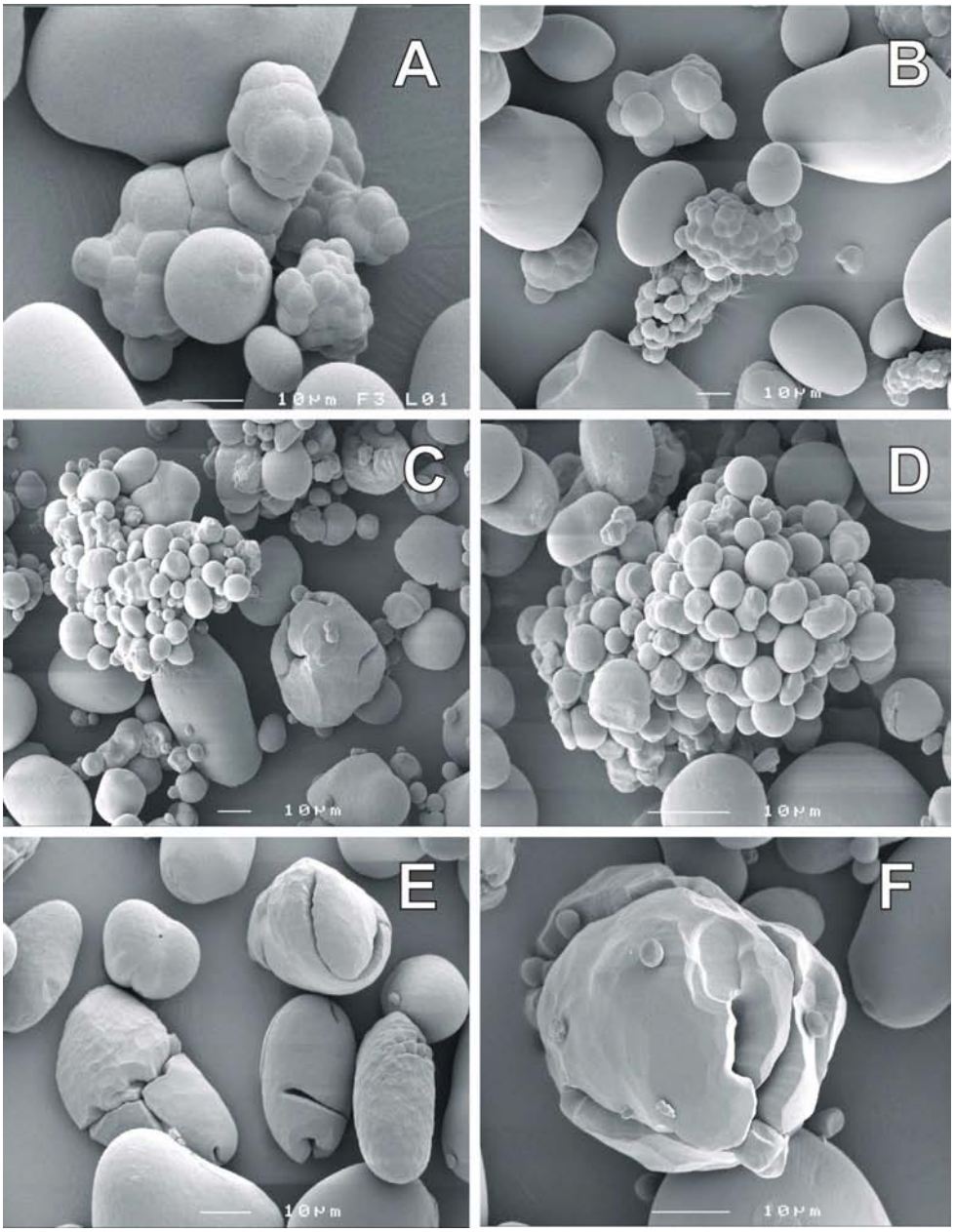

Figure 5. SEM analysis of transgenic KDSS and *amf*SS starch granules. (A) KDSS1: 3+, 1400 ×; (B) KDSS7: 4+, 800 ×; (C) *amf*SS3: 3+, 800 ×; (D) *amf*SS3: 2000 ×; (E) *amf*SS23: 6+, 1220 ×; (F) *amf*SS23: 1900 ×.

KDSS transformants. This phenomenon was not observed in the starch granules from any of the *amf*SS transformants. These data suggest that the irregular grape-like structures can grow out to round or oval granules.

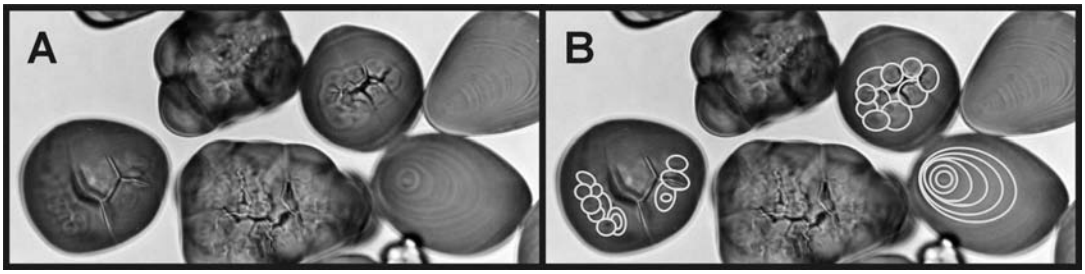

Figure 6. Light micrograph of starch granules (KDSS2 (2+ class); 800 × magnification) stained with a 10× diluted Lugol solution. **Panel A** shows the original image of the starch granules. **Panel B** shows the same image, highlighting in white the contour lines of grape-like structures, which seem to be incorporated in larger starch granules, as well as the concentric growth-rings of apparently normally assembled granules.

Since the morphology of the granules appeared very different, a number of the starches were investigated by microscopy under polarized light (Figure 7). The untransformed controls, KD-UT and *amf*-UT, clearly showed the characteristic "Maltese cross" in the starch granules. Both the starches from the KDSS and the *amf*SS transformants 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.

Chain-length distribution of SBD2 starches

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-performance 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 mutually, and between transgenic starches and the respective untransformed controls (data not shown).

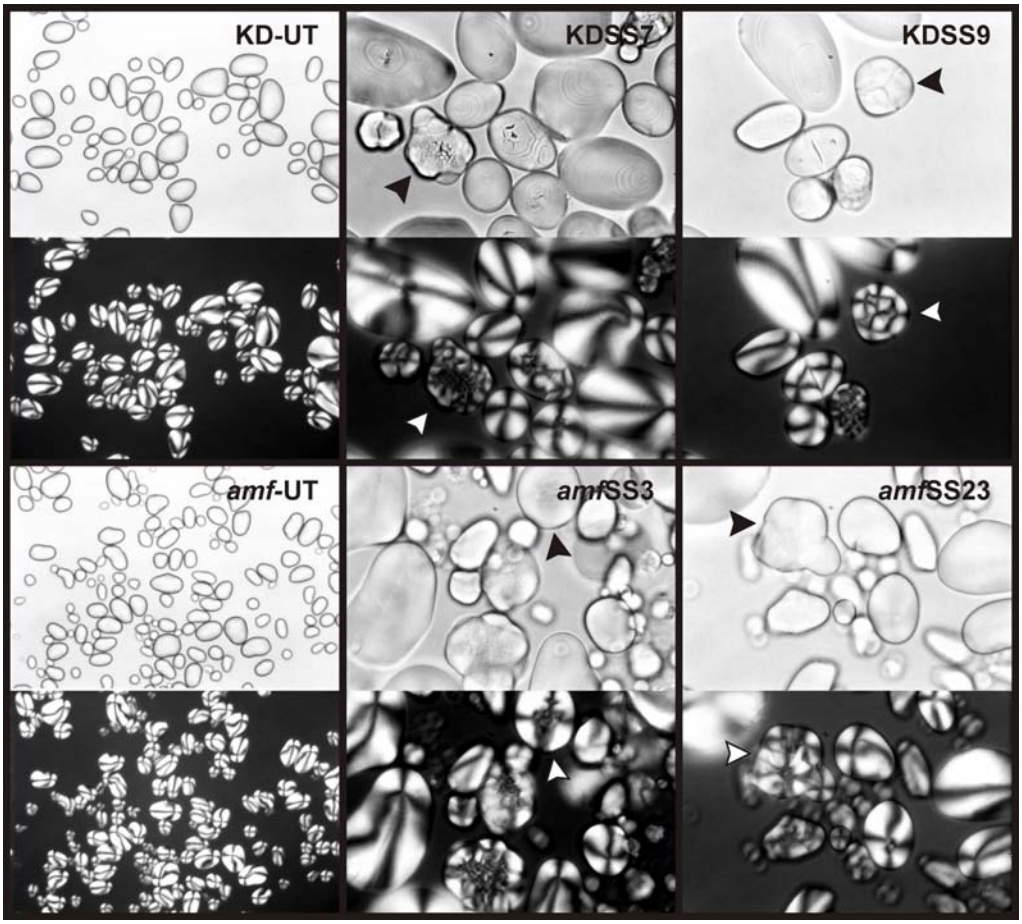

Figure 7. Light micrographs of starch granules from various transformants viewed under normal and polarized light. For each starch, a pair of micrographs is shown in which the upper panel shows the normal light micrograph, and the lower panel the polarized light micrograph of the same granules. Control starches: 200 × magnification. Transgenic starches: 800 × magnification.

Increased crystallinity of transgenic starches

Since the granule packing appeared to be affected by the presence of SBD2, three *amf* starches were selected for X-ray diffraction (XRD) 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 43%, that of *amf*SS3 (3+) 44%, and that of *amf*SS23 (6+) 47%. This demonstrates that an increased SBD2 accumulation and an increased crystallinity are correlated.

Granule size distribution

The granule size distributions of all transgenic starch lines of the two different backgrounds were investigated. All granule size distributions recorded within one class of transformants were averaged, the result of which is summarized in Figure 8A. The averaged granule size distributions of the starch of the various classes of the KDSS series were more or less similar to the profile of the control, indicating that the granule size of the KDSS starches is not affected by SBD2 accumulation in the granules. 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.

Although the triplicate measurements with the Coulter Multisizer measurements 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. An example of this is provided in Figure 8B, which shows three different granule size distributions of starches belonging to the 3+ class: *amf*SS3 has very small starch granules (7.8 µm in mean granule size), whereas *amf*SS57 has relatively large ones (13.3 µm in mean granule size). *Amf*SS25 had a bimodal distribution with peaks at 7.0 and 27.2 µm.

Fractionation of starch granules

In order to investigate the relationship between granule size and the level of SBD2 accumulation in the granule, the *amf*SS22 starch (6+ class) with a bimodal granule size distribution was fractionated into granules larger and smaller than 20 µm. 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 (Figure 9A). 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 (Figure 9B).

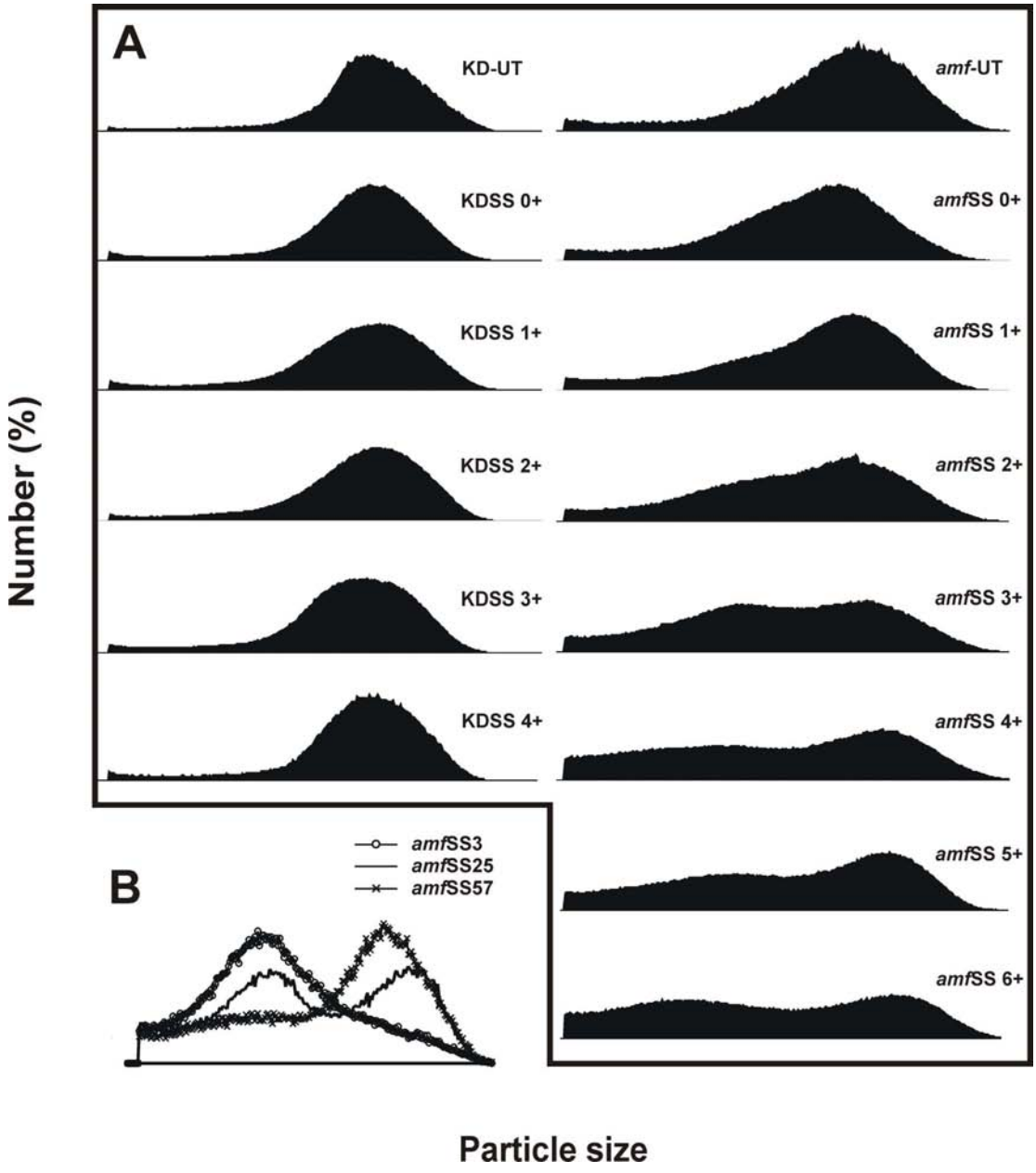

Figure 8. Relationship between particle size distributions of the various starches and their SBD2 accumulation levels. **Panel A** shows the averaged particle size distributions of the transgenic starches of the various SBD2 classes of the KDSS and *amf*SS series. Each starch sample was analyzed in triplicate, and the granule size distribution profiles of all starches belonging to the same class were averaged. **Panel B** shows particle size distributions of *amf*SS3, *amf*SS25, and *amf*SS57 transgenic starches from the 3+ class. The profiles are the average of three independent measurements.

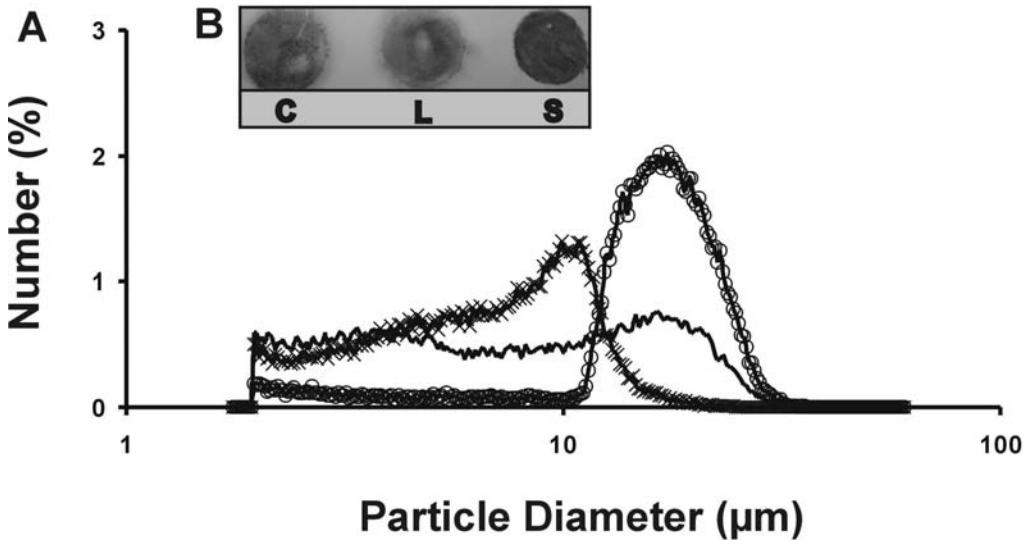

Figure 9. Relationship between particle size distribution and SBD2 protein accumulation in *amf*SS22 starch and its two constituent fractions. **Panel A** shows the particle size distribution of the unfractionated *amf*SS22 starch (solid line) and its two fractions after sieving: larger (solid line with circles) and smaller than 20 µm (solid line with crosses). **Panel B** shows the amount of SBD2 in the *amf*SS22 (C), the large granule fraction (L), and the small granule fraction (S).

Treatment of large clusters and grape-like structures with α-amylase

In order to investigate whether the association of small granules in both large clusters and grape-like structures is α-glucan-related, *amf*SS3 and KDSS7 starch, as well as that of their respective untransformed lines were treated with α-amylase. Samples without α-amylase treatment served as controls. The starch granule morphology and the granule size distribution of the *amf*SS3 starch with and without α-amylase treatment are shown in Figure 10. The large cluster structures, which can be seen in the left panel of Figure 10A, were not observed anymore in the α-amylase-treated sample (Figure 10A, right panel). This demonstrated that the subunits of the large cluster structures can be separated by α-amylase, which indicates that they are connected by α-glucans. This observation was further substantiated by measuring the granule size distributions of the treated and untreated *amf*SS3 starch (Figure 10B); the granule size distribution shifted to smaller particles in the α-amylase-treated sample, whereas this was not observed for the untreated control. Furthermore, no change in granule size distribution was observed when *amf*-UT starch was treated with the α-amylase (data not shown), indicating that the reduction in particle size of the α-amylase-treated *amf*SS3 starch is

caused by separation of the subunits of the large clusters, rather than by granule degradation in general (Figure 10B). On the contrary, the grape-like structures present in the KDSS7 starch were unaffected by α-amylase treatment (data not shown). This probably indicates that the apparent subunits of the grape-like structures are connected by α-glucans of a more crystalline nature as in the large clusters, which makes them harder to degrade by α-amylase.

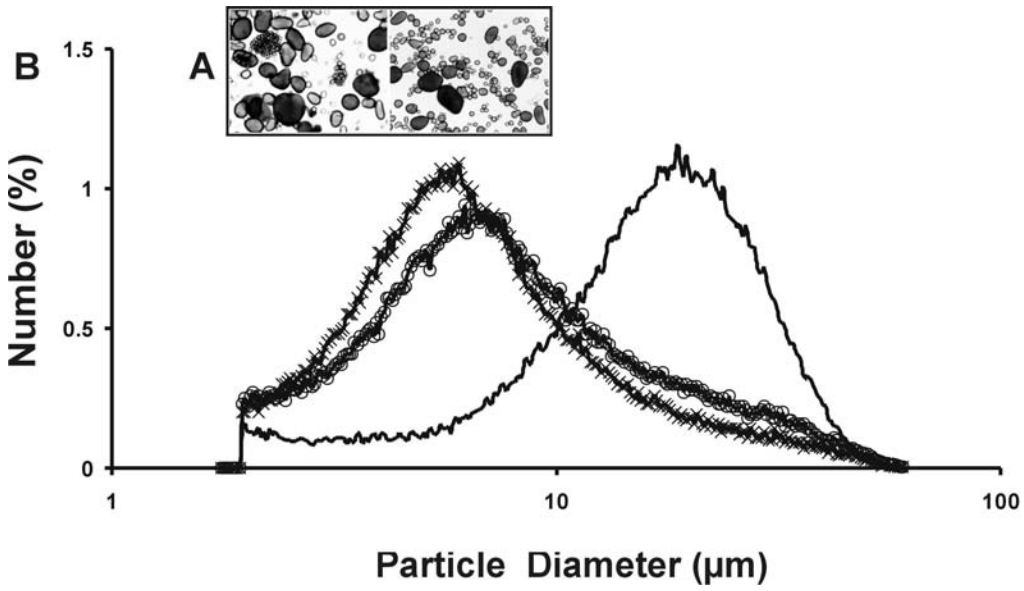

Figure 10. Granule size distribution of *amf*SS3 starch with and without α-amylase treatment. **Panel A** shows the micrographs of *amf*SS3 granules before (left) and after (right) α-amylase treatment. **Panel B** shows the particle size distribution of *amf*SS3 granules before (solid line with circles) and after (solid line with crosses) treatment with α-amylase, as well as the untransformed *amf* control (solid line). Granule size distributions are the average of the three independent measurements.

Starch content and physicochemical properties of the starches

The impact of SBD2 accumulation in granules on their physicochemical properties and on the starch content of the tubers was also investigated for the KDSS series. For this, one transgenic line from each SBD2 accumulation-class and their respective control were selected. The starch content of the selected transformants and their controls were measured. Granule-melting behaviour (T_0 and ΔH) of the selected transgenic lines and their controls were investigated by differential scanning calorimetry (DSC). The results show that there are 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).

Discussion

In this study, an engineered high-affinity tandem repeat SBD was introduced into an amylose-containing potato genotype to investigate whether it could displace GBSSI from the starch granule during biosynthesis. Our data show that it is not possible to produce an amylose-free potato starch in this way.

In both the Kardal and *amf* background, higher amounts of SBD2 than of SBD can be accumulated. Interestingly, a point of saturation was reached in the two backgrounds, because in both cases SBD2 was found in the potato juice, indicating that part of the protein was unbound. In the *amf* background, the point of saturation was reached with the 6+ class, whereas in the Kardal background this point was reached much earlier. This is consistent with our previous results, showing that more SBD can be accumulated in *amf* granules than in those of Kardal (Ji *et al.*, 2003). Typically, the granules of all classes of the KDSS transformants appeared to be saturated with SBD2; the amount of SBD2 in the juice increased proportionally with that in the granules for the transformants of the various classes (Figure 2). This result might be explained as follows. In the low SBD2 expressers, GBSSI is probably present in surplus to SBD2, and the chance of it binding to and accumulating in the granule is high; GBSSI becomes the predominant granule-bound protein. In the higher SBD2 expressers, the binding equilibrium shifts in favour of SBD2, and consequently more of this protein will be bound. However, Western dot blot analysis of KDSS starches did not reveal a reduction in the amount of GBSSI. It is possible that a reduction in GBSSI content is simply too small to be determined accurately. With respect to this, it is important to realize that SBD2 detection is probably more sensitive than that of GBSSI, since (i) it has two copies of the antigen, and (ii) it is likely that the relatively large size of GBSSI dictates a non-stoichiometric displacement by SBD2 (*i.e.* one GBSSI molecule might occupy two SBD2 binding sites). Therefore, we believe that our results are most consistent with a model in which GBSSI has a higher affinity for starch than SBD2, and in which the two proteins bind similar sites of the growing starch granule. It seems as if SBD2 (although better than SBD) is not competitive enough to keep GBSSI from the granule surface. This, together with the current view that GBSSI is present in the granule in surplus (reviewed in Denyer *et al.*, 2001), is insufficient to reduce the amylose content of potato starch granules.

Our experiments also provide new insights in starch biosynthesis in more general terms. Before this issue is elaborated, it is important to discuss the different binding modes of SBD2 (see also Ji *et al.*,

2004). Based on the discussion above, we think that SBD and GBSSI bind similar sites, and we hypothesize that they bind close to the non-reducing termini of amylopectin side chains as is indicated in Figure 11. At low SBD2 concentrations, we expect both domains to interact with the same granule surface (mode 1). At higher SBD2 concentrations, the surface area may become limiting to accommodate both domains on the same surface. As a result of this, the SBD2 proteins may have one SBD attached to the cluster, whereas the other one is available for interaction with soluble glucans (mode 2). In the Kardal background, the situation is more complex because of the presence of GBSSI. We assume that GBSSI always binds a cluster of amylopectin side chains in the same way. Malto-oligosaccharides can dock at the GBSSI protein, and are subsequently elongated. SBD can bind sites, which are not occupied by GBSSI (no displacement), whereas SBD2 and GBSSI can coexist in the same amylopectin cluster (competition for binding sites). It is unlikely that SBD2 only binds the Kardal starch according to mode 2, because both SBDs are required for effective competition with GBSSI. Therefore, SBD2 probably interacts with the Kardal starch in both modes.

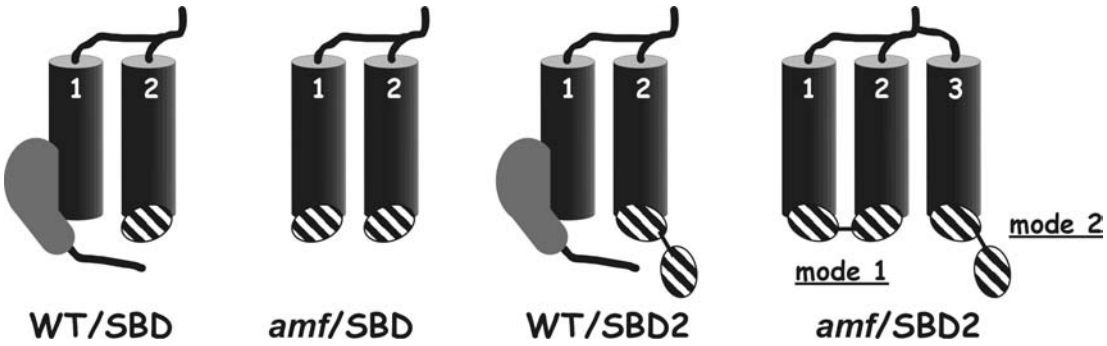

Figure 11. Schematic representation of possible binding sites and modes of SBD (hatched), SBD2, and GBSSI (grey). Parallel double helix structures are indicated as black cylinders; single-chain α-glucans are shown as thick black lines.

SBD2 seems to interfere with various aspects of the starch biosynthesis process, *i.e.* granule packing and granule morphology (Kardal and *amf*), the formation of growth rings (only apparent in Kardal), and granule size (*amf*). Most striking is the observation that SBD2 expression can affect the granule distribution in the *amf* background from class 2+ onwards. In a previous paper (Ji *et al.*, 2004), we have suggested that the binding mode of SBD2 plays a pivotal role in this phenomenon. At low

SBD2 concentration, mode 1 predominates, making the granule surface less accessible for enzymes involved in starch biosynthesis. New granule nucleation sites may be formed, a process in which isoamylases have recently been shown to play a major role (Bustos *et al.*, 2004). At high SBD2 concentration, mode 2 predominates, and it is suggested that the exposed SBD of SBD2 can capture soluble glucans from the stroma. In principle, different granule nucleation sites can be cross-linked in this way. The observation that the "large clusters" observed in the *amf*SS transformants can be separated by α-amylase treatment provides evidence for such a mechanism, because it shows that the cross-link between these loosely associated, small granules are an α-glucan (Figure 10). Typically, we have not observed a decrease in granule size with any of the KDSS starches. This can not be related to the amount of SBD2 accumulation, because 3+ and 4+ KDSS starches did not show the effect, whereas 2+ *amf*SS starches did. One explanation for this is that a larger proportion of SBD2 is present in mode 2 in the Kardal background, which is consistent with the higher surface area occupation due to the presence of GBSSI (additional to SBD2). Another possibility (not excluding the first one) is that GBSSI elongates malto-oligosaccharides at the granule surface. Because of the processive character of GBSSI, these malto-oligosaccharides are more or less granule-bound, which may provide extra cross-linking potential. It might be argued that this is not in accordance with the widely accepted view that amylose production occurs inside the crystalline matrix of the starch granule (Denyer *et al.*, 2001; Tatge *et al.*, 1999). However, it can not be excluded that amylose biosynthesis already starts directly at the granule surface.

In both the Kardal and the *amf* background, it was observed that the packing of the starch granules was altered. This is most clearly seen in Figure 7; the multiple "Maltese crosses" within one granule suggest that these granules were assembled from many smaller ones, which seems to be in accordance with α-glucans cross-linking different granule nucleation sites (see above). Because the starch yield of all transformed and control lines was similar, and because no differences in chain-length distributions of transgenic and controls starches were found, it was concluded that it is possible to uncouple glucan polymerization and granule packing during the starch biosynthesis process by SBD2 expression. The crystallinity of the transgenic granules appeared to increase with SBD2 content, but the altered packing did not influence their melting temperature. The morphological changes were more predominant in the small granules, which is in line with their higher SBD2 content (Figure 9). We hypothesize that the "grape-like structures" (Kardal) and "large clusters" (*amf*) are actually very similar structures, differing only in the presence of amylose. We think that amylose effectively fills the gaps between loosely associated granules, making these structures more or less resistant to α-amylase treatment. The larger granules seem to have over-grown the clustered appearance, and they develop (in most cases) into

normal-looking ones. Evidence for this is presented in Figure 6, which shows that the grape-like structures are part of the larger Kardal granules. We expect that this is also true for the *amf*SS starches, although we have not been able to visualize this directly by light microscopy. Thus, it seems as if SBD2 is present in non-limiting amounts at the onset of starch biosynthesis. Depending on the amount of SBD2 expressed, SBD2 becomes limiting at a certain time point in starch biosynthesis, the clustering stops, and the granules develop normally. In principle, this is very similar to what happens in partial antisense GBSSI potato lines. GBSSI, which is under the control of the same promoter as SBD2 in this study, accumulates in starch granules until its resources in the stroma are depleted. The subsequent layers deposited on the growing starch granule do not contain this enzyme anymore, and consequently only the cores of these granules stain blue with iodine (Denyer *et al.*, 2001; Kuipers *et al.*, 1994; Tatge *et al.*, 1999). Figure 6 also shows that the growth ring pattern can be altered upon SBD2 expression. Many factors have been shown to contribute to the control of ring formation, but the exact mechanism is not understood (Pilling and Smith, 2003). A reduced activity of a major isoform of starch synthase (SSIII in potato) may disrupt the regularity of ring patterns (Fulton *et al.*, 2002; Tatge *et al.*, 1999). Here we show that physical events, *i.e.* modifying the interaction of glucan chains by the presence of SBD2, can also alter ring formation. Only at very high SBD2 expression in the *amf* background (6+ class), it was observed that the assembly of oval-shaped granules was impaired (Figure 5E, F). An 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 (Bustos *et al.*, 2004; Fulton *et al.*, 2002; Schwall *et al.*, 2000; Tatge *et al.*, 1999).

In the present paper, we have shown that it is possible to influence a number of starch characteristics, the origin of which is not well understood, by SBD2 expression. For this reason, we expect that SBD2 is an important tool for studying the starch biosynthesis process, because it offers the opportunity to uncouple the glucan polymerization process from granule assembly *in planta*. In other words, SBD2 can affect physical processes underlying granule morphology, size, crystallinity, and growth ring formation, without altering the primary structure of the constituent starch molecules.

Experimental procedures

Construct 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.

Plant transformation and regeneration

The pBIN19/SBD2 plasmid was introduced into the amylose-containing potato cultivar (cv. Kardal), and the amylose-free (*amf*) potato mutant (often referred to as 1029-31; Jacobsen *et al*., 1989) via *Agrobacterium*-mediated transfomation 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 line were transferred to the greenhouse for tuber development. In addition, 10 untransformed controls of each genotype were grown in the greenhouse.

Determination of SBD2 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 an 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, England). The membrane was hybridized with a [^{32}P]-labeled NcoI-BamHI DNA fragment of SBD2 as a probe; labelling was performed using a *redi*prime II kit (Amersham, England) 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 Peeler (Spangenberg, The Netherlands). The peeled tubers were homogenized in a Sanamat Rotor (Spangenberg), and filtered through a sieve to remove particulate material. The resulting homogenate was allowed to settle for 20 min at 4 °C, and the tuber juice was collected for later use, and stored at –20 °C. The starch sediment was washed three times with water, and finally air-dried at room temperature (Ji *et al*., 2004).

Determination of SBD2 and GBSSI content of transgenic starches by dot blot analysis

A 12.5% sodium dodecyl sulfate-polyacrylamide gel (50 mm × 50 mm × 3 mm) with nine equally spaced holes (∅ = 9 mm) was placed in contact with a similar-sized Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, UK). For determining the SBD2 content, 20 mg of (transgenic) starch was boiled for 5 min with 200 µl of 2× SDS sample buffer (Laemmli, 1970). After cooling to room temperature, the starch gel was transferred into one of the holes. SBD2 proteins from transgenic starch gels were blotted to the membrane with PhastSystem (Pharmacia, Sweden; 20 V, 25 mA, 15 °C, 45 min) (Ji *et al*., 2003). The protein was identified with antiSBD antibody according to the method described by Ji *et al*. (2003). For determining the GBSSI content of the granules, a similar procedure as outlined above was used, except that the 20 mg starch sample consisted of 2.5 mg of the transgenic starch and 17.5 mg of *amf*-UT starch, and that the (primary) antiGBSSI polyclonal antibody (Vos-Scheperkeuter *et al*., 1986) was used in a 1:250 dilution.

Determination of SBD2 content of potato juice

SBD2 proteins in the soluble fraction of potato tubers were determined as follows. 500 µl of tuber juice was freeze-dried. The dried material was dissolved in 200 µl of 2× SDS sample buffer. 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 µm (for KDSS series) or 100 µm (for *amf*SS series) (Beckman-Coulter, 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 (±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, Germany). Starch granules were stained with a 20× diluted Lugol's solution (1% I_2/KI).

For determining the birefringence of the granules the polarized light device was used. For scanning electron microscopy (SEM, JEOL 6300F, 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 DSC using a Perkin-Elmer Pyris 1 (Perkin-Elmer, 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 (Artenay, France) spectrometer working at 40 kV and 30 mA, operating in the Debye-Scherrer transmission mode. The X-ray radiation CuKα_1 (λ = 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° (2θ). 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 by Wakelin *et al.* (1959). The percentage of crystallinity was taken as the slope of the line $(I_{sample}-I_{amor})_{2\theta} = f\ (I_{crys}-I_{amor})_{2\theta}$ where I_{sample}, I_{amor} and I_{crys} are the diffracted intensity of the sample, the amorphous and the crystalline standards, respectively.

Determination of chain-length distribution

Five mg of (transgenic) starch was suspended in 250 µl of dimethylsulfoxid (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 µl of 50 mM NaAc buffer (pH 4.0), containing sufficient isoamylase (Sigma) 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 (7500 × *g*, 2 min). For HPAEC, the supernatant was diluted 5 times with a 25% DMSO solution; for high-performance size-exclusion chromatography, the samples were used as such (HPSEC).

HPSEC was performed on a P680 HPLC pump system (Dionex, Sunnyuale, Cal. USA) equipped with three TSKgel SWXL columns in series (a G3000 and two G2000; 300 mm × 7.5 mm; Montgomeryville, USA) in combination with a TSKgel SWXL guard column (40 mm × 6 mm) at 35 °C. Aliquots of 100 µl were injected using a Dionex ASI-100 Automated Sample Injector, and subsequently eluted with 10 mM NaAc buffer (pH 5.0) at a flow rate of 0.35 ml/min (3 h run). The effluent was monitored using a RID-6A refractometer (Shimadzu, The Netherlands). The system was calibrated using dextran standards (10, 40, 70, 500 kDa; Pharmacia). Dionex Chromeleon software version 6.50 SP4 Build 1000 was used for controlling the HPLC system and data processing.

HPAEC was used to obtain a better separation of the smaller amylopectin side chains (in the range of 2 to 45 glucose residues). HPAEC was performed on a GP40 gradient pump system (Dionex) equipped with a CarboPac PA 100 column (4 mm × 250 mm; Dionex) at 35 °C. The flow rate was 1.0 ml/min and 20 µl sample was injected with a Dionex AS3500 automated sampler. Two eluents were used, eluent A (100 mM 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→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 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. 20 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 (Ji *et al*., 2004).

α-amylase treatment of starch granule preparations

Twenty milligrams of *amf*SS3 or KDSS7, as well as their respective control starches, was suspensed in 1 ml of 50 mM NaAc buffer (pH 6.9), and treated with 5 U of porcine pancreas α-amylase (Sigma, the Netherlands) for 4 h at 25 °C. Samples without α-amylase addition served as controls. After incubation, the samples were centrifuged (7500 × *g*, 10 min) Subsequently, 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 line *amf*SS22 was fractionated by sieving (20 µm DIN-ISO 3310/1, Retsch, 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.

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Chapter 6

General Discussion

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In their natural setting, starch-binding domains (SBDs) are part of starch-degrading enzymes (Svensson *et al*., 1989; Janeček and Ševčík, 1999). It is generally believed that the primary role of SBDs in starch degradation is to potentiate catalytic activity by bringing the attached hydrolase into prolonged and intimate contact with the target substrate; the enzymes remain mainly on the outside of the granules during the course of starch degradation (Figure 1A). We anticipated that it could also be advantageous to equip biosynthetic enzymes with a SBD and introduce this gene with the appropriate signal sequences in potato plants. It is expected that the fusion protein will be incorporated into granules. To test this, the experiments described in this thesis were performed.

For this, a family 20 starch-binding domain (SBD) derived from *Bacillus circulans* cyclodextrin glycosyltransferase (CGTase) was used as an anchor for engineering artificial granule-bound proteins. After introduction of the separate SBD gene (thus without a protein attached to it) into an amylose-containing and the amylose-free mutant potato plants, it was observed that the SBDs accumulated inside the starch granules, and not at the surface of granules. Furthermore, the physicochemical properties of the transgenic starch were not affected by accumulation of SBDs in these granules (Chapter 2). The SBD fused to the luciferase gene via a Pro-Thr-rich linker, and a similar construct without the linker and SBD were introduced into potato plants. Our data showed that the SBD/luciferase fusion protein can be incorporated into granules during starch biosynthesis with retention of luciferase activity. The luciferase alone did not accumulate in the granules, indicating that luciferase did not have affinity for starch granules of its own, and that an anchor is essential for making the association with granules (Chapters 2 and 3).

Potential applications of SBD technology

For SBD technology, we distinguish three main fields of application: (i) starch modification *in planta*, (ii) post-harvest starch modification, and (iii) production of starch-based carriers for various proteins.

- **Starch modification *in planta*.** In this case, the effector is an enzyme involved in the synthesis or degradation of starch polymers. The effector may be native to potato, but may also be derived from another species (including microorganisms). It is expected that the partitioning of the catalytic activity between stroma and granule can be influenced by the presence of an attached SBD. In this way, it is possible to concentrate enzymes previously soluble inside the granule or at the granule surface. A granule with a different structure from wild type may be formed from the initial stages of starch biosynthesis (Figure 1B). The impact on starch structure and functionality of the granule-bound enzyme may be higher (or different) than that of the “soluble” protein.

- **Post-harvest modification of starch.** The objective of this application is also to modify the starch polymers. The main difference with the previous application is that the effector is not active in the plant during the biosynthesis process, but that it can be made active after harvest (Figure 1C). The advantage of this may be that reactions can be catalysed which normally do not occur in amyloplasts, for instance because either appropriate reactants are lacking, or the enzyme requires more extreme reaction conditions. After exposure to an external trigger, which can be a substrate or a change in pH value, the modification of the starch structure is initiated. This strategy may be used as an alternative for certain chemical derivatization procedures, or to produce sufficiently high amounts of starch before altering it. Also, certain starch-degrading enzymes may be incorporated in the starch granule to generate a more porous starch structure.

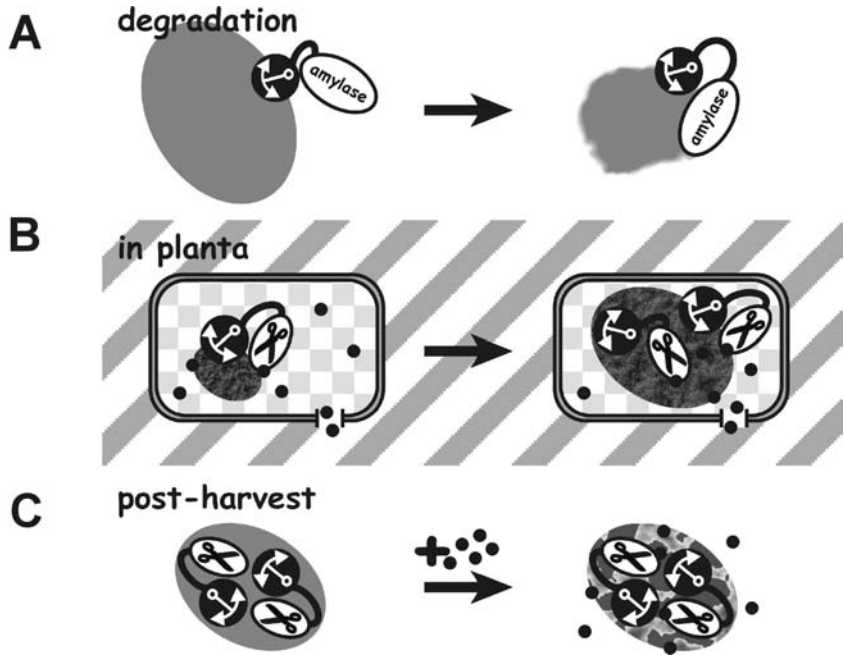

Figure 1. Cartoon summarizing the possibilities for application of SBD technology. **Panel A:** Starch-degrading enzymes are usually equipped with a SBD for the degradation of raw starch granules. The presence of the SBD determines whether the water-insoluble substrate can be degraded. **Panel B:** SBDs can be fused to a protein of choice, and the fusion protein is incorporated in starch granules (in the amyloplast) during the biosynthesis process. The effector protein is thus concentrated at the granule surface and in the granule. When the effector protein has access to its substrate, a modified starch will accumulate in the plant. **Panel C:** In some cases the effector may not have access to its substrate, for instance because translocators for substrate import are lacking in the amyloplast membrane. In such cases, the substrate can be offered to the effector protein post-harvest. Supply of substrate will then initiate starch modification. Anchors represent the SBD, scissors an enzyme activity.

- **Starch-based protein carriers.** In principle, this is also a post-harvest application. The main difference with the previous application is that the effector does not (need to) have activity towards starch polymers. For instance, enzymes or receptors may be immobilised in starch granules in order to catalyse certain conversions or to affinity-purify certain compounds, respectively. It is important that substrates and products can diffuse freely in and out the granule. Molecules as large as ADP-Glc and maltotriose can enter the granule to provide GBSSI with substrate (van de Wal *et al*., 1998; Denyer *et al*., 1999; van de Wal, 2000). Another possibility is to accumulate vaccines attached to SBD in starch granules. Plants are more and more considered as a suitable production system for such molecules (Giddings *et al*., 2000; Ma, 2000). Besides the fact that it is relatively easy to recover starch from plant material, other advantages of vaccines in starch granules are the possibility for oral supply, and the potential for a slow-release application.

Putting SBD technology into practice

SBD is superior to GBSSI as a targeting sequence

In this study, we have shown that the SBD derived from CGTase is superior to a native potato GBSSI as a granule-targeting sequence for luciferase to starch granules. The luciferase activities of SBD-containing fusion proteins were much higher in comparison with GBSSI-containing ones (Chapter 3). We speculate that this is related to the size of GBSSI, because GBSSI has an approximately 5 times higher molecular weight than SBD. It could be that the large fusion protein is folded incorrectly or two domains interact with each other, thereby shielding the important amino acid residues for granule-binding of GBSSI and the active site of luciferase. MacGregor *et al*. (2002) suggest that the amino acid sequence of the mature GBSSI corresponds to a single module, indicating the enzyme does not contain a separate starch-binding domain, like the CGTase. Affinity of GBSSI for starch granules is probably due to a certain region of the protein. However, it is probably difficult to truncate a minimum starch-binding sequence from the existing GBSSI structure. For this reason, we believe that SBD is, sofar, a better alternative of the two granule-targeting sequences.

SBD2 is a higher-affinity anchor than SBD

In this study, we also investigated whether more protein can be accumulated in granules by using an artificial tandem repeated starch-binding domain (SBD2) (Chapters 4 and 5). The results show that SBD2 is a higher-affinity domain for starch compared with the single SBD. We also observed that the two domains of SBD2 were acting in synergy to bind their target substrates. This is the first report, which demonstrates synergy between two appended SBD from CBM family 20, although similar results between CBMs have been reported for other CBM families, such as CBM family 1 (Linder *et al*., 1996), CBM2 (Bolam *et al*., 2001), and CBM29 (Freelove *et al*., 2001). Therefore, we expect that SBD2 can also be used in SBD technology for accumulation more proteins in the granules. However, it should be noted that expression of SBD2 in the granules could interfere with certain aspects of starch biosynthesis, which led to changes in granule packing and granule morphology (Kardal and *amf*), the formation of growth rings (only apparent in Kardal), and granule size (only observed in *amf*).

Effects of SBD's position in fusion proteins on activity and binding properties

Effects of the SBD position in the fusion proteins on the activity of luciferase and the binding affinity of SBD were investigated in both the WT and *amf* backgrounds. The results show that the C-terminal SBD can also be used as an N-terminal anchor for incorporating luciferase in granules with retention of its activty. However, the affinity for starch of these fusion proteins depends on the arrangment of their domains. The LUC-SBD protein (SBD at the C-terminus of fusion protein) accumulated more in starch granules than the SBD-LUC (SBD at the N-terminus of fusion protein). The amount of LUC-SBD protein accumulated in the granules of the most positive transformants of the Kardal and *amf* series is similar to that of the separate SBD in the two backgrounds (Chapter 3). This indicates that when the SBD was fused to the C-terminus of luciferase, its affinity for starch granules is not affected by the appended luciferase. However, for luciferase activity, it is difficult to conclude which is the preferred position of SBD in fusion protein. So based on the observation in this study, we propose that for application of the SBD technology in starch modification *in planta*, it is better to test both the C- and N-terminal SBD-containing fusion proteins.

Effect of genotype on levels of protein accumulation in granules

The single SBD and SBD2, as well as SBD/luciferase fusion genes were expressed in potato tubers. The different proteins were present inside the starch granules. However, the levels of their accumulation in granules appeared to be genotype-dependent. These results showed that much higher levels can be accumulated in the *amf* background than in the WT backgrounds, demonstrating that the background in which genes were expressed is a factor of importance (Chapters 3 and 4). The main reason for lower levels of protein accumulation in the amylose-containing granules is most probably the presence of another abundant granule-bound protein (GBSSI), in the WT genotype. It is speculated that GBSSI and SBD proteins might bind similar sites in the granule. Also, GBSSI seemed to have a higher affinity for starch granules than SBD and SBD2, such that SBD proteins cannot compete successfully with the GBSSI for binding sites in amylose-containing granules (Chapter 2). The possibility of having different sites for the two proteins can, however, not be excluded. If the two binding sites are close to each other, the presence of GBSSI might affect the binding of SBDs to granules.

Final comments

This thesis research has explored the possibilities of using microbial SBDs as an anchor to target any protein into potato starch granules during their biosynthesis. This may lead to potato starches with new or improved functionalities, either generated *in planta* or post-harvest.

Most of the starch utilized world-wide is derived from a relatively small number of crops, the most important ones being maize, potato, wheat and cassave. Potato starch is often preferred over starches from other sources because of its low protein and lipid content, and the high degree of amylose and amylopectin polymerization. However, potato starch as raw material is used far less for industrial purposes (7.6%) than maize starch (78.5%) (http://www.starch.dk/isi/stat/raw-material.html). Now we have obtained proof of concept for applying SBD technology in potato plants, it will be interesting/challenging to extrapolate this technology to other important starch-producing crops such as maize.

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SUMMARY

Starch is the major storage carbohydrate in many plants. It is deposited as crystalline granules which consist of two polysaccharides, an essentially linear amylose (20-30%) and a highly branched amylopectin (70-80%). Starch granules can vary enormously in size, shape, composition and properties between different species. To tailor starch to suit a multitude of applications in textile, paper, biodegradable plastics, pharmaceutical and food industries, chemical or physical modification after isolation can be used, but also genetic modification. Modification of the starch biosynthesis pathway holds an enormous potential for tailoring granules or polymers with new functionalities. Over the years, a number of modified starches have been generated *in planta* using recombinant DNA technology. In most cases certain biosynthetic enzymes were inhibited. For instance, an amylose-free (*amf*) starch was obtained by the introduction of an antisense granule-bound starch synthase I (GBSSI) construct. However, there are also examples in which heterologous enzymes were added to the biosynthetic machinery. For instance, a more heavily branched amylopectin was generated by the introduction of *E. coli* glycogen branching enzyme in an *amf* potato background.

The aim of this thesis research was to explore the possibility of engineering artificial granule-bound proteins by using starch-binding domains (SBDs) of starch-degrading enzymes. The principle of this technology is that by fusing a SBD to an effector protein and introducing this construct with the appropriate signal sequences in plants, SBD-containing fusion proteins might be incorporated into granules during starch biosynthesis. In this way, starches with new or improved functionalities may be generated *in planta*, but also post-harvest. A number of important aspects were studied in this thesis research: (i) can SBDs be accumulated in starch granules?; (ii) can active enzymes be targeted to starch granules by fusion to SBD?; (iii) can potato GBSSI be used as an alternative for SBD?; (iv) is it possible to engineer a higher affinity SBD with which more protein can be accumulated in granules, and (v) does the anchor's position (C- or N-terminal) in fusion proteins influence the activity of the effector and binding affinity of the anchor? Normal potato plants, as well as an amylose-free genotype, were chosen for these studies.

A family 20 SBD derived from *Bacillus circulans* cyclodextrin glycosyltransferase (CGTase) was used as the anchor for engineering artificial granule-bound proteins. To determine whether microbial SBD can be incorporated into starch granules, a separate SBD (thus without a protein

attached to it) was expressed in the tubers of two amylose-containing potato cultivars (Kardal and Karnico) and the amylose-free (*amf*) potato mutant under the control of the potato GBSSI promoter. The potato GBSSI transit peptide mediated amyloplast entry of the SBD. Our data showed that SBD protein accumulated inside starch granules and not at the granule surface. However, the accumulation levels of SBD in granules appeared to be genotype-dependent. The amount of SBD protein accumulated in the *amf* background was estimated to be approximately 8 times higher than that in the two amylose-containing backgrounds. The latter two gave similar results on SBD accumulation levels. The physicochemical properties of the transgenic starches were not affected by accumulation of SBDs in granules, except for apparent cracks (after staining with an iodine solution) in the granules of the highest SBD-accumulators in both backgrounds. Our results demonstrated that SBDs could be incorporated inside the granules during starch biosynthesis (Chapter 2).

The SBD in its natural setting is present at the C-terminus of CGTase. To investigate whether active enzymes can be targeted to starch granules by fusion to SBD, and whether the C-terminal SBD can be used as an N-terminal targeting sequence for enzymes to granules, the SBD was fused to the C- or N-terminus of the luciferase (LUC) gene via a PT-linker. A similar construct without the linker and SBD was used as a control. All proteins were equipped with the same amyloplast-targeting sequence as that for SBD, and introduced in the Kardal and *amf* backgrounds (Chapters 2 and 3). Our results showed that both LUC-SBD (SBD at the C-terminus) and SBD-LUC (SBD at the N-terminus) fusion proteins could be accumulated inside granules with retention of the activity of the luciferase. The LUC-SBD fusion proteins accumulated to higher levels in granules than the SBD-LUC proteins. The amount of LUC-SBD in the granules of the most positive clones of the Kardal and *amf* series is similar to that of the separate SBDs in the two backgrounds. This indicates that SBD's affinity for starch is not affected by the attached luciferase. When luciferase alone was expressed in the two backgrounds, only very little activity was found in the granules, and the amount of luciferase protein accumulated in granules was undetectable by Western dot blot analysis, indicating that luciferase did not have affinity for starch granules of its own. Based on these results, it can be concluded that SBDs can be used as tools to anchor effector proteins (without affinity for starch granules) to granules. The C-terminal SBD can also be used as an N-terminal anchor for attaching luciferase to granules. For starch affinity, the SBD is best put at the C-terminus of the fusion protein, whereas for luciferase activity, it is difficult to conclude which is the preferred position of SBD in the fusion protein.

The efficiency of two granule-targeting sequences, SBD and GBSSI, for attaching luciferase to *amf* granules was compared in Chapter 3. The GBSSI was fused to C- and N-terminus of luciferase. The LUC-GBSSI and GBSSI-LUC fusion proteins were equipped with the same amyloplast-targeting sequence as the SBD/luciferase fusion proteins. Luciferase activities in granules were measured for both series. The luciferase activity in the most positive transformant of each series was much lower than that of the SBD-containing fusion proteins. In addition, the amount of GBSSI protein accumulated in these granules was much less than that in *amf* granules complemented with the native potato GBSSI, indicating that a large GBSSI protein in fusion proteins is not compatible with luciferase. It can be concluded that the small microbial SBD is superior to native potato GBSSI as a granule-targeting sequence for luciferase to starch granules.

To investigate whether an artificial tandem-repeat of a family 20 starch-binding domain (SBD2) is a higher-affinity anchor than SBD, the affinity of SBD and SBD2 for starch was tested both *in vitro* and *in planta*. For *in vitro* experiments, SBD and SBD2 were expressed in *E. coli* and the ability of the purified proteins to bind to soluble starch was investigated by isothermal titration calorimetry (ITC). The K_a (association constant) obtained from ITC analysis showed that SBD2 had an approximately 10-fold higher binding affinity for starch than SBD, indicating that the two domains of SBD2 act in synergy when binding to their target substrates. The larger than 2-fold increase in affinity for starch cannot be explained by just a duplication of the binding sites in the SBD2 (Chapter 4). The increased affinity of SBD2 for starch was also observed *in planta*. Higher levels of SBD2 could be accumulated in Kardal and *amf* granules in comparison with SBD in the two backgrounds (Chapters 4 and 5). These results demonstrate that SBD2 is a higher-affinity anchor than SBD.

It was observed that expression of SBD2 in potato tubers seems to interfere with certain aspects of the starch biosynthesis process, which resulted in changes in granule packing, morphology, size, crystallinity, and the formation of growth rings. In both the KDSS and *amf*SS series, sometimes, small granules organized in large clusters were observed. These granules showed an altered birefringence pattern compared with the normal granules, *i.e.* multiple “Maltese crosses” within one granule were observed. In addition, expression of SBD2 in the *amf* background led to a number of transformants, particularly *amf*SS3, containing starch with smaller granules (7.8 μm in mean granules size) in comparison with the *amf* control (15.2 μm in mean granule size). Also, the granule size distribution of the *amf*SS series became bimodal with increasing SBD accumulation. This was

not observed with the KDSS series. Further, the data from X-ray diffraction analysis showed that SBD2 expression in the *amf* background did not alter the crystal type (B-type) , but the crystallinity of the granules was slightly changed. The crystallinity of the *amf*-UT, *amf*SS3 (3+), and *amf*SS23 (6+) were 43%, 44%, and 47%, respectively, which demonstrates that an increased SBD2 accumulation and an increased crystallinity are correlated. However, the accumulation of SBD2 in the KDSS and *amf*SS starch granules did not affect the starch content or primary structure of the constituent starch molecules.

The results of the research described in this thesis demonstrate that the SBD technology has a great potential for tailoring starches with new or improved functionalities *in planta* as well as post harvest.

SAMENVATTING

Zetmeel is het belangrijkste opslag koolhydraat in planten. Het wordt afgezet in de vorm van kristallijne korrels bestaande uit twee polysacchariden; een lineair amylose (20- 30%) en sterk vertakte amylopectine (70-80%). Zetmeelkorrels kunnen enorm variëren in omvang, vorm, samenstelling en eigenschappen tussen verschillende plantensoorten. Om zetmeel geschikt te maken voor praktische toepassingen zoals in textiel en papier, in biologisch afbreekbare plastics en in de farmaceutische- en levensmiddelenindustrie, kunnen chemische en fysische modificaties worden uitgevoerd na isolatie. Door het zetmeel via genetische modificatie te veranderen kunnen sommige modificaties overbodig worden. Modificatie van de biosynthese route van zetmeel geeft een enorm aantal mogelijkheden om zetmeelkorrels of -polymeren voor nieuwe doeleinden (geschikt) te maken. In de laatste 15 jaren, is een verscheidenheid aan gemodificeerde zetmelen gecreëerd *in planta* met behulp van recombinant-DNA technologie. In de meeste gevallen werden specifieke biosynthese enzymen uitgeschakeld of ingebracht. Zo werd bijvoorbeeld een amylose-vrij (*amf*) zetmeel verkregen met behulp van een antisense korrel-gebonden zetmeel synthase (GBSS) construct. Er zijn echter ook voorbeelden te noemen waarbij heterologe enzymen werden toegevoegd aan de biosynthese machinerie. Een sterker vertakt amylopectine werd bijvoorbeeld gecreëerd door introductie van *E. coli* glycogeen vertakkingsenzym in een *amf* aardappel achtergrond.

Het doel van deze studie was te onderzoeken of er mogelijkheden zijn om kunstmatig korrel-gebonden eiwitten te vervaardigen door gebruik te maken van een zetmeel-bindend domein (SBD) van een zetmeelafbrekend enzym. Het principe van deze technologie berust op het samenvoegen van een SBD en een effector eiwit. Dit fusie-eiwit, voorzien van de juiste signaalsequenties, wordt vervolgens in een plant geintroduceerd, waarbij de SBD-bevattende fusie-eiwitten gedurende zetmeelbiosynthese in de korrels opgenomen worden. Op deze manier kan zetmeel met nieuwe of verbeterde eigenschappen *in planta* worden gegenereerd, maar ook na de oogst. Een aantal belangrijke aspecten zijn in dit onderzoek bestudeerd: (I) kunnen SBDs worden geaccumuleerd in zetmeelkorrels?; (II) kunnen actieve enzymen naar de zetmeelkorrel gedirigeerd worden door fusie met SBD?; (III) kan aardappel GBSS worden gebruikt als een alternatief voor SBD?; (IV) is het mogelijk om een SBD te creëren waarmee meer eiwit in korrels kan worden geaccumuleerd, en (V) heeft de positie van SBD (C- of N-terminaal) in fusie-eiwitten een effect op de affiniteit van het anker? Normale aardappelplanten, alsmede een amylose-vrij genotype, zijn voor deze studie gebruikt.

Een family 20 SBD, afkomstig van *Bacillus circulans* cyclodextrine glycosyltransferase (CGTase), is gebruikt als anker voor het maken van kunstmatige korrel-gebonden eiwitten. Om vast te stellen of microbiële SBDs in zetmeelkorrels ingebracht kunnen worden, is een aparte SBD tot expressie gebracht in de knollen van twee aardappelcultivars (Kardal en Karnico) en de amylose-vrije (*amf*) aardappelmutant onder controle van de aardappel GBSS promoter. Het aardappel GBSS transit-peptide zorgde voor dat import van SBD in de amyloplast. Onze resultaten laten zien dat SBD in de zetmeelkorrel ophoopt. De accumulatieniveaus van SBD in korrels bleek genotype afhankelijk te zijn. De hoeveelheid SBD in de *amf* korrel bleek circa acht keer hoger dan de hoeveelheid in de twee amylose bevattende achtergronden. De twee laatst genoemden gaven overeenkomstige resultaten. De fysisch-chemische eigenschappen van het transgene zetmeel werden niet beïnvloed door de aanwezigheid van SBDs in de korrels, afgezien van de schijnbare scheuren (na kleuring met een joodoplossing) in de korrels van de hoogste SBD accumulators in beide achtergronden. Ons onderzoek wees uit dat SBDs geïncorporeerd kunnen worden in korrels gedurende de zetmeelbiosynthese (hoofdstuk 2).

Het SBD is aanwezig aan de C-terminus van het CGTase. Om te onderzoeken of actieve enzymen gefuseerd met SBD aan zetmeel gebonden kunnen worden, en of de C-terminale SBD gebruikt kan worden als een N-terminaal anker, werd de SBD gefuseerd aan de C- of N-terminus van het luciferase (LUC) gen via een Pro-Thr-linker. Alle eiwitten werden voorzien van dezelfde "amyloplast-targeting" sequentie als beschreven voor SBD, en werden geïntroduceerd in de Kardal en *amf* achtergrond (hoofdstuk 2 en 3). De resultaten laten zien dat zowel LUC-SBD (SBD aan de C-terminus) en SBD-LUC (SBD aan de N-terminus) fusie-eiwitten binnenin de korrels geaccumuleerd kunnen worden met behoud van de luciferase activiteit. De LUC-SBD fusie-eiwitten accumuleerden in hogere mate in korrels dan de SBD-LUC eiwitten. De hoeveelheid LUC-SBD in de korrels van de meest positieve klonen van de Kardal en *amf* serie is vergelijkbaar met dat van SBD in de twee achtergonden. Dit laat zien dat de affiniteit van SBDs voor zetmeel niet aangetast wordt door de aanwezigheid van luciferase in hetzelfde eiwit. Wanneer alleen luciferase (dus zonder SBD) in de twee achtergronden tot expressie werd gebracht, werd maar weinig activiteit gevonden in de korrels. De hoeveelheid luciferase activiteit geaccumuleerd in de korrels was heel laag, wat aangeeft dat luciferase op zich geen affiniteit had voor zetmeelkorrels. Op basis van deze resultaten kan geconcludeerd worden dat SBDs gebruikt kunnen worden als een gereedschap om effectoreiwitten (zonder affiniteit voor zetmeelkorrels) aan de korrels te binden. De C-terminale SBD kan ook gebruikt worden als een N-terminaal anker voor luciferase aan korrels. Voor zetmeel affiniteit is SBD bij voorkeur gebonden aan de C-terminus van het fusie-eiwit, waarbij het voor wat

betreft luciferase activiteit moeilijk is vast te stellen welke de favoriete positie is van SBD in het fusie-eiwit.

De efficiëntie van twee "granule-targeting" sequenties, SBD en GBSS, om luciferase aan *amf* korrels te binden is ook vergeleken (in hoofdstuk 3). De GBSS werd gefuseerd aan de C- en N-terminus van luciferase. De LUC-GBSS en GBSS-LUC fusie-eiwitten werden uitgerust met dezelfde amylopast-targeting sequentie als de SBD/luciferase fusie-eiwitten. De luciferase activiteit werd gemeten in beide series. De luciferase activiteit in de meest positieve transformant van elke serie was veel lager dan die van de SBD-bevattende fusie-eiwitten. Daar komt bij dat de hoeveelheid GBSS eiwit geaccumuleerd in deze korrels veel lager was, dan die van *amf* korrels gecomplementeerd met het oorspronkelijke aardappel GBSS. Dit geeft aan dat een groot GBSS eiwit in fusie-eiwitten niet erg compatibel is met luciferase. Men mag concluderen dat het kleine microbiële SBD superieur is aan het oorspronkelijk aardappel GBSS als granule-targeting sequentie voor luciferase aan zetmeel korrels.

Om te onderzoeken of een kunstmatig dubbel zetmeel-bindend domain (SBD2) een hogere affiniteit heeft dan SBD, werd de affiniteit voor zetmeel van SBD en SBD2 getest *in vitro* en *in planta*. Voor de *in vitro* experimenten werden SBD en SBD2 in *E. coli* tot expressie gebracht, en de capaciteit van de gezuiverde eiwitten om aan oplosbaar zetmeel te binden is onderzocht door middel van een calorimetrische titratie (ITC). De K(a) (associatie constante) gevonden met behulp van de ITC analyse liet zien dat SBD2 een circa tienmaal hogere bindingsaffiniteit voor zetmeel had dan SBD, wat aangeeft dat de twee domeinen van SBD2 synergistisch werken wanneer zij aan hun substraat binden (hoofdstuk 4). De toename in affiniteit van SBD2 voor zetmeel is ook onderzocht *in planta*. Hogere hoeveelheden SBD2 kunnen worden geaccumuleerd in Kardal en *amf* zetmeel, in vergelijking met SBD in de twee achtergronden (hoofdstukken 4 en 5). Deze resultaten tonen aan dat SBD2 een sterker anker is dan SBD.

Observaties zijn gedaan waarbij SBD2-ophoping in aardappelknollen lijken te storen op bepaalde aspecten in de zetmeelsynthese, wat leidde tot veranderingen in korrelpakking, morfologie, grootte, kristalliniteit, en het groeiringenpatroon. In zowel de KDSS als de *amf*SS series werden soms kleine korrels gezien, georganiseerd in grote clusters. Deze korrels vertoonden een veranderd brekingspatroon onder gepolariseerd licht in vergelijking met gewone korrels; meervoudige “Maltezer kruizen” werden waargenomen in één korrel. Daar komt bij dat de expressie van SBD2 in de *amf* achtergrond van invloed was op de korrelgrootte, specifiek *amf*SS3 die klein-korrelig zetmeel bevatte (7.8 μm in gemiddelde korrelgrootte). Ook werd in sommige planten van de *amf*SS serie een bimodale korrelgrootteverdeling gevonden, hetgeen correleerde met

toenemende SBD accumulatie. Dit werd niet gevonden in de wildtype (KDSS) serie. Verder toonden de uitkomsten van de röntgendiffractie-analyse aan dat SBD2 expressie in de *amf* achtergrond niet het kristal type veranderde, alhoewel de kristalliniteit van de korrels wel iets veranderde. De kristalliniteit van de *amf*-UT, *amf*SS3 (3+), en *amf*SS23 (6+) waren respectievelijk 43%, 44% en 47%, wat aangeeft dat toenemende SBD2 accumulatie en toenemende kristalliniteit gecorreleerd zijn. Desondanks beïnvloedde de accumulatie van SBD2 in de KDSS en *amf*SS zetmeelkorrels niet de zetmeelhoeveelheid in de knol, en ook niet de fijnstructuur van de zetmeel polymeren.

De resultaten van het onderzoek demonstreren dat de SBD technologie een grote potentie heeft bij het genetisch modificeren van zetmeel in planten.

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Qin Ji

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CURRICULUM VITAE

Qin Ji was born on Oct. 3, 1957 in Jiangsu, P.R. China. She graduated from Nanjing Agricultural University (NAU) and received a Bsc degree in Horticulture in 1982. After graduation, she worked as a teaching assistant (from 1982) in the Horticulture Department, and then as a lecturer (from 1987) in the Department of Food Science and Technology at NAU. In 1992, she enrolled as an MSc student of Wageningen University. She obtained her MSc degree in Biotechnology in 1994. In the same year, she became an associate professor in the Department of Food Science and Technology at NAU. From 2000, she started her PhD research on the project of "Microbial starch-binding domains as a tool for modifying starch biosynthesis" in the Laboratory of Plant Breeding at Wageningen University.