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

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Abstract It has been shown previously that mutan can be co-synthesized with starch when a truncated mutansucrase (GtfICAT) is directed to potato tuber amyloplasts. The mutan seemed to adhere to the isolated starch granules, but it was not incorporated in the starch granules. In this study, *GtfICAT* was fused to the N- or C-terminus of a starch-binding domain (SBD). These constructs were introduced into two genetically different potato backgrounds (cv. Kardal and *amf*), in order to bring GtfICAT in more intimate contact with growing starch granules, and to facilitate the incorporation of mutan polymers in starch. Fusion proteins of the appropriate size were evidenced in starch granules, particularly in the *amf* background. The starches from the various GtfICAT/SBD transformants seemed to contain less mutan than those from transformants with GtfICAT alone, suggesting that the appended SBD might inhibit the activity of GtfICAT in the engineered fusion proteins. Scanning electron microscopy showed that expression of SBD-GtfICAT resulted in alterations of granule morphology in both genetic backgrounds. Surprisingly, the *amf* starches containing SBD-GtfICAT had a spongeous appearance, i.e., the granule surface contained many small holes and grooves, suggesting that this fusion protein can interfere with the lateral interactions

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of amylopectin sidechains. No differences in physico-chemical properties of the transgenic starches were observed. Our results show that expression of granule-bound and ''soluble'' GtfICAT can affect starch biosynthesis differently.

Keywords Mutan · Glucansucrase · Starch-binding domain · Granule-boundness · Transgenic potato · Microscopy · Granule morphology

Introduction

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

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

Materials and methods

Preparation of constructs containing *GtfICAT* and SBD

pPF and pPF*GtfICAT* (Kok-Jacon et al. 2005a) were used as starting material for cloning the SBD-linker and linker-SBD fragments, resulting in pPF*SBDGtfICAT* and pPF*GtfICATSBD*, respectively. The SBD-linker and linker-SBD fragments were obtained from the pTrcHisB/SBD2 plasmid (Ji et al. 2004) that was used as a template for PCR amplification. The SBD fragment originated from the *CGTase* gene of *Bacillus circulans* strain 251 (Lawson et al. 1994), and the linker fragment is similar to the Pro-Thr-rich linker of the *Cellulomonas fimi* exoglucanase (*Cex*) (Gilkes et al. 1991). For the construction of pPF*SBDGtfICAT*, the SBD-linker fragment was obtained by PCR amplification with a forward primer, containing a *Sca*I restriction site (5′-<u>AGTACT</u>ATGGCCGGAGATCAGGTC-3′), and a reverse primer, containing a *Nru*I restriction site (5′-<u>TCGCGA</u>CGACGGGGTC-3′), and cloned into the *Sma*I restriction site of pPF*GtfICAT*. For the construction of pPF*GtfICATSBD*, the linker-SBD fragment was obtained by PCR amplification with a forward primer, containing an *EcoR*V restriction site (5′-<u>GATATC</u>TCCGACGCCGACGC-3′), and a reverse primer, containing a *Sma*I restriction

site (5′-CCCGGGATCCACCAAAAC-3′), and cloned into the *EcoR*V restriction site of pPF, resulting in pPFSBD. In order to remove the stop codon in the original *GtfICAT* sequence, the *GtfICAT* fragment was amplified with a forward primer, containing a *Sma*I restriction site (5′-CCCGGGACTGAAAACTGTTAG-3′), and a reverse primer, containing a *Nru*I restriction site (5′-TCGCGAACATTGAGGTACTTG-3′), and cloned into the *Sma*I restriction site of pPFSBD, resulting in pPF*GtfICATSBD*. pPF*SBDGtfICAT* and pPF*GtfICATSBD* were completely sequenced in one direction by Baseclear (The Netherlands) to verify the correctness of the construct. pPF*SBDGtfICAT* and pPF*GtfICATSBD* were digested with *Xho*I and ligated into a pBIN20 binary vector (Hennegan and Danna 1998), resulting in pPFSBDIC and pPFICSBD (Fig. 1). The predicted molecular mass of the fusion proteins excluding the signal peptide is 125,870 Da and 125,390 Da for SBD-GtfICAT and GtfICAT-SBD, respectively.

Transformation and regeneration of potato plants

pPFSBDIC and pPFICSBD were transformed into *Agrobacterium tumefaciens* strain LBA 4404 using electroporation (Takken et al. 2000). Internodal stem segments from the tetraploid (cv. Kardal (KD)) and diploid mutant (*amf*) potato genotypes were used for *Agrobacterium*-mediated transformation, which was performed as described by Kok-Jacon et al. (2005b).

Starch isolation

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

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

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

Determination of morphological and physicochemical properties of starch granules

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

Fig. 1 Schematic representation of pPFSBDIC (**A**) and pPFICSBD (**B**) binary vectors used for potato plant transformation
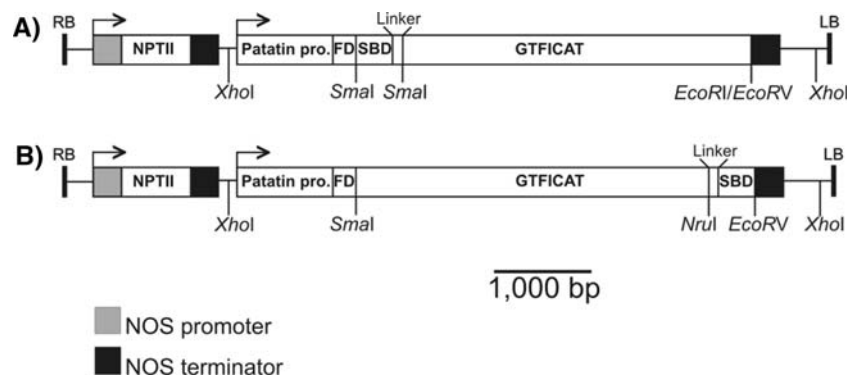
butions (HPSEC, HPAEC) were determined as described by Kok-Jacon et al. (2005b).

Quantification of SBD transgenic starches and tuber juices

Western dot blot analysis was performed according to the method described by Ji et al. (2003). A 12.5% sodium dodecylsulphate-polyacrylamide gel (50 mm × 50 mm × 3 mm), with nine equally spaced holes (9 mm diameter), was placed in contact with a similarly sized Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, UK). Twenty milligrams of (transgenic) starch was boiled for 5 min with 200 μl of 2× SDS sample buffer, containing 5% (v/v) β-mercaptoethanol. After cooling to room temperature, the starch gel was transferred into one of the holes. SBD proteins from transgenic starch gels were blotted onto the membrane with PhastSystem (Pharmacia, Sweden; 20 V, 25 mA, 15°C, 45 min). The blot was incubated overnight in a 1% blocking solution (10 ml 10× western blocking reagent; Roche, Germany) in 90 ml TBS (20 mM Tris, 500 mM NaCl pH 7.5) at room temperature. Subsequently, the blot was washed in TTBS (0.05% Tween-20 in TBS) for 5 min, and incubated for 2 h at room temperature with a 1:500 dilution of the primary antibody (antiSBD) in a 0.96% blocking solution in TTBS. After this, the blot was washed twice in TTBS for 5 min, and incubated for 2 h at room temperature with a 1:2000 dilution of goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate (BioRad, 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 for 15 min in dark with a 0.1 M NaHCO$_3$ solution of pH 9.8, containing 1% NBT/BCIP (Roche Molecular Biochemicals, Germany), and 0.01 M MgCl$_2$.

The amount of SBD-containing protein in potato tuber juice was determined as described by Nazarian et al. (2006a).

Electrophoretic separation of granule-bound proteins

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

Post-harvest determination of mutansucrase activity

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

HPAEC was performed as described previously except that the column temperature was 28°C and a different gradient was used. Three eluents were used, eluent A (100 mM NaOH), eluent B (1 M NaAc in 100 mM NaOH) and eluent C (H$_2$O) with the following gradient: $0 \rightarrow 12$ min, $25 \rightarrow 85\%$ eluent A and $75 \rightarrow 15\%$ eluent C (linear gradient); $12 \rightarrow 25$ min, $0 \rightarrow 10\%$ eluent B and $15 \rightarrow 5\%$ eluent C (linear gradient); $25 \rightarrow 25.1$ min, $85 \rightarrow 0\%$ eluent A, $10 \rightarrow 100\%$ eluent B, and $5 \rightarrow 0\%$ eluent C (linear gradient); $25.1 \rightarrow 30$ min, 100% eluent B (rinsing phase); $30 \rightarrow 45$ min, $0 \rightarrow 25\%$ eluent A, $100 \rightarrow 0\%$ eluent B, and $0 \rightarrow 75\%$ eluent C (equilibration phase). The eluents were monitored by an ED40 electrochemical detector in the pulsed amperometric mode (Dionex).

Results

The *SBD-GtfICAT* and *GtfICAT-SBD* fragments were cloned in frame to the plastidic protein targeting sequence (FD) (Gerrits et al. 2001), and driven by the highly tuber-expressed patatin promoter (Wenzler et al. 1989), resulting in the pPFSBDIC and pPFICSBD constructs (Fig. 1). These constructs were used for *Agrobacterium*-

mediated transformation of amylose-containing (cv. Kardal (KD)) and amylose-free (amf) mutant potato plants; thirty independent transgenic potato clones, per background and construct, were obtained, which were named KDSICxx, KDICSxx, amfSICxx and amfICSxx. SIC and ICS represent the SBD-GtfICAT and GtfICAT-SBD genes, respectively, and xx the clone number. The untransformed genotypes are referred to as KD-UT and amf-UT. During the growth period in the greenhouse, the transgenic plants were morphologically similar to the controls (data not shown).

Classification of transformants by RT-PCR analysis

GtfICAT expression was determined by RT-PCR analysis. Based on the band intensity of the PCR products, the transformants were divided in different classes (Fig. 2). The ubiquitin-ribosomal gene expression (Ubi3), which is known as constitutive (Garbarino and Belknap 1994), was used as an internal control. The (N), (L), and (H) classes represent plants with no, low, and high levels of mRNA, respectively. From the KDSIC series, the KDSIC30 (N), KDSIC15 (L), KDSIC19 (L), KDSIC1 (H), KDSIC2 (H), and KDSIC10 (H) transformants were selected for further analysis; from the amfSIC series, amf-SIC10 (N), amfSIC4 (L), amfSIC5 (H), amf-SIC14 (H), and amfSIC17 (H) (see Fig. 2A, B) were selected. From the KDICS series, the KDICS10 (N), KDICS25 (N), KDICS5 (L), KDICS28 (L), KDICS4 (H), and KDICS27 (H) transformants were subjected to further characterization; from the amfICS series, amfICS29 (N), amfICS27 (L), and amfICS6 (H) (see Fig. 2A, B) were selected. Given the intensity of the Ubi3 PCR product, KDICS5 might also be assigned to the (H) class. As expected, no GtfICAT mRNA was detected in the KD-UT and the amf-UT control plants.

Accumulation of SBD-containing proteins in the transformants

In the selected transformants (based on RT-PCR analysis), the amount of SBD-containing protein in the granule was analyzed by Western dot blot

analysis. Quantification was performed by using starch from the (+), (++) and (+++) classes of the amfS series of transformants (Ji et al. 2003) for calibration. Subsequently, the dot intensities obtained for the starches of the selected transformants were compared to these positive controls. Starch from amf transformants showed higher SBD accumulation than those from Kardal. The amfS IC5, amf SIC14, amf SIC17, and amf ICS6 starches showed a dot intensity comparable to that of the (++) class, whereas the dot intensities of the KDSIC10 and amfSIC4 starch were comparable to that of the (+) class. The starch of the other selected transformants showed a dot intensity comparable to that of either KD-UT or amf-UT, in which no SBD-containing protein was present. The results of the Western dot blot analysis correlated well to the results obtained by RT-PCR analysis.

Also the tuber juices of the selected transformants were analyzed for the presence of the fusion protein. None of the juices gave a positive response in the Western dot blot assay, indicating that the amount of SBD-containing protein was too low to be detected.

SBD-containing fusion proteins of expected size in starch granules

In order to determine whether the fusion proteins in the starch granules had the correct size, the granule proteins of the amf starches with the highest SBD content (amfSIC14 and amfICS6, representing both series) were separated by SDS polyacrylamide gel electrophoresis, followed by Western blotting using the SBD antibody (Fig. 3). For both starches, a band of almost 140 kDa was found, which corresponded well with the predicted molecular weight of 125 kDa for the fusion proteins (excluding the transit peptide). This showed that the fusion protein was correctly imported into the amyloplast, and incorporated into the starch granules.

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

As described previously (Kok-Jacon et al. 2005a), mutan can be visualized using an erythrosine red
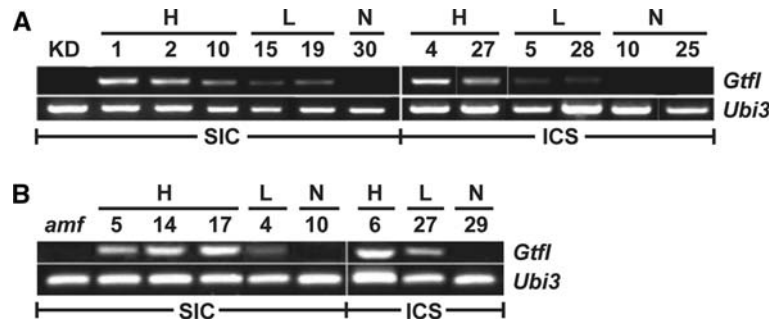
Fig. 2 RT-PCR analysis of the selected transformants in Kardal (**A**) and *amf* (**B**) and their corresponding untransformed control plants. In each series, the upper panels show the PCR products using the primers designed on the *GtfI* sequence. The lower panels show the PCR products using the primers designed on the *Ubi3* sequence, and served as an internal control
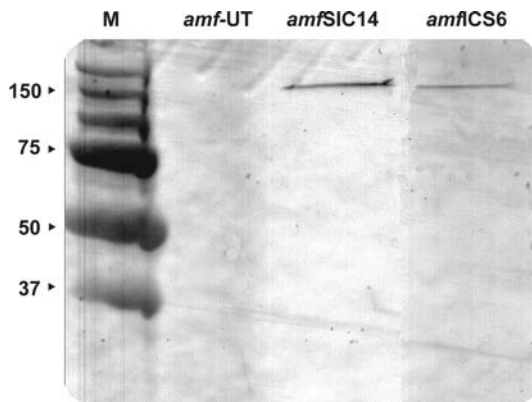

Fig. 3 Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of granule-proteins of the starches from *amf*ICS6 and *amf*SIC14 transformants, followed by antibody detection. Lane M shows the migration of the marker proteins. Molecular mass of the proteins is indicated in kDa

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

Fig. 4 LM analysis of starch granules (800×) from KD-UT (**A**) purified mutan (**B**) and KDSIC10 (+) (**C**) stained with erythrosine. The granules of KDSIC10 (**D**) and KDIC15 (**E**) are shown after treatment with 25 mU of exo-mutanase
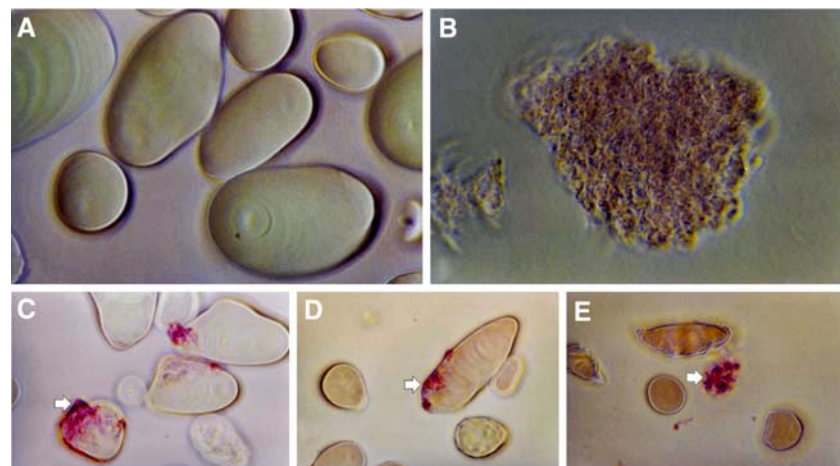
(Fig. 4E). From Fig. 4D, it can be seen that most of the mutan remained attached to the granule, which suggests that a proportion of the mutan might be present inside the starch granules, and therefore inaccessible to the exo-mutanase.

Accumulation of SIC and ICS fusion proteins interferes with granule packing

SEM analysis was performed on the selected transgenic starches. The presence of altered starch granule surfaces was visualized in the (H) class of the KDSIC series (Fig. 5B, F–H) in contrast to the (N) class, which was comparable to

that of KD-UT (Fig. 5A). It can be seen that surfaces of the starch granules were irregular (Fig. 5F–H). In addition, small protrusions on the granular surface were present. Such granule phenotypes were not observed in the (H) class of the KDIC transformants (Fig. 5D, I). For the (H) class of the KDICS series (Fig. 5C), starch granules were not altered, and similar to those of KD-UT. In the *amf* background, granules with altered morphology were found in both the SIC and ICS series (Fig. 6). Also, the position of SBD in the fusion protein seems to matter with respect to morphological changes. For instance, the surface of *amf*SIC granules (Fig. 6B, E) appeared
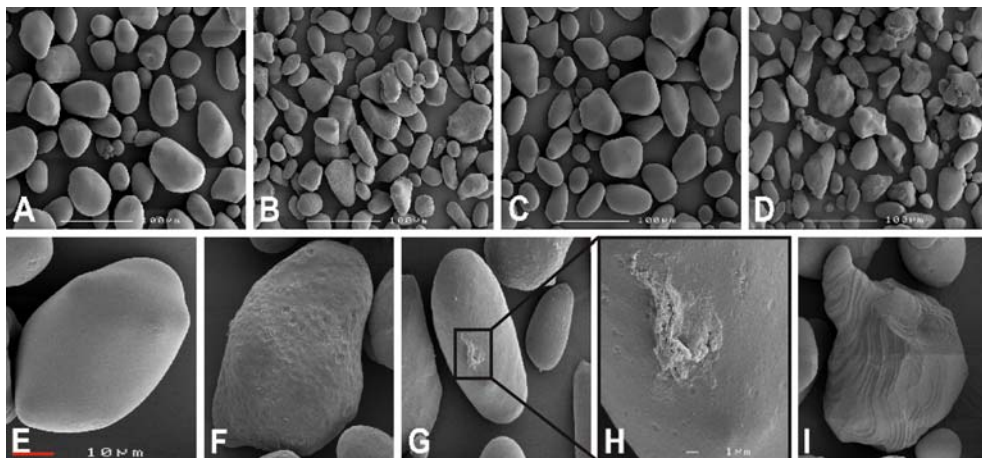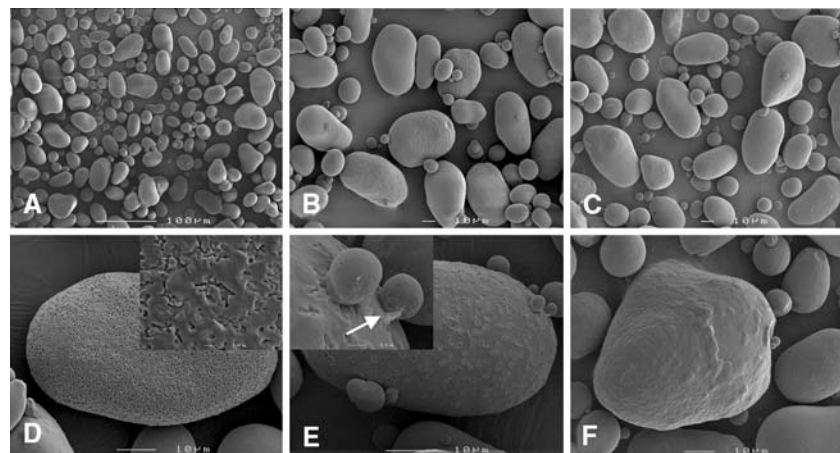

Fig. 5 SEM analysis of starch granules from KD-UT (**A**) KDSIC10 (**B**) KDICS27 (**C**) KDIC15 (**D**) and close-ups of the granule surface of KD-UT (**E**) KDSIC10 (**F**) KDSIC10 (**G**) KDSIC10 (**H**) and KDIC15 (**I**)

Fig. 6 SEM analysis of starch granules from *amf*SIC4 (+) (**A**, **D**) *amf*SIC14 (++) (**B**, **E**) and *amf*ICS6 (++) (**C**, **F**). Arrow (**E**) might indicate mutan, which is co-deposited with starch

spongeous or porous, just as if a crust of material was deposited on top of the surface. The surface of *amf*ICS granules (with (++) level of SBD accumulation) showed a bumpy and rough appearance (Fig. 6F). The different phenotypes observed for the two potato backgrounds might find their origin in the presence, or in the fact that in the *amf* background more of the fusion protein is accumulated in the starch granules.

From the SEM pictures, the number of altered granules was scored by counting a population of 100 starch granules in triplicate for one transformant of the (N) and two transformants of the (H) class of each series. This quantification is shown in Fig. 7 for the selected transformants. The highest numbers of altered starch granules were found in the (H) class of the *amf*SIC and KDSIC series, 25.5% ± 3.5 and 24.7% ± 3.8 for *amf*SIC17 and KDSIC10, respectively. In the (H) class of the *amf*ICS and KDICS series, the number of altered starch granules was similar, ranging from 12.0% ± 2.8 in *amf*ICS6 to 10.7% ± 2.3 in KDICS27. For the (N) class of transformants, the frequency of altered starch granules was less than 7%. In general, it seemed as if SBD-GtfICAT affected starch granule morphology more than GtfICAT-SBD. However, in the Kardal background, the granule morphology of the best SBD-GtfICAT transformants was less affected than that of the best KDIC transformants, in which 31.3 ± 2.3% of the starch gran-
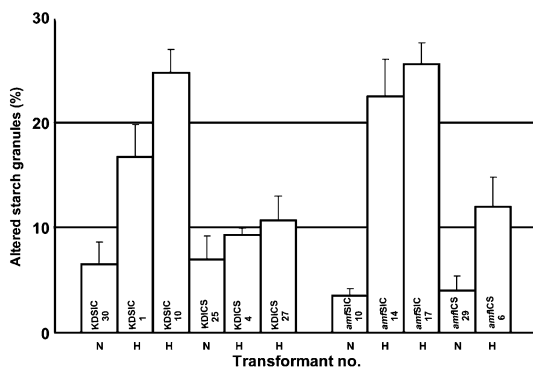

Fig. 7 Percentage of starch granules with altered morphology of various transformants. A population of 100 granules was counted in triplicates. Bars indicate the mean of three independent counts, error bars represent the standard deviation. Classification of transformants based on RNA (N, H) is indicated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

ules exhibited altered morphologies (Kok-Jacon et al. 2005a).

GTFICAT/SBD does neither influence starch properties nor content

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

GTFICAT with appended SBD does not seem to alter starch fine structure

The chain length distribution was determined in order to detect deviations in starch structure, which may indicate the presence of α-(1 → 3)-linked glucosyl residues. After complete debranching of starch with isoamylase, no consistent changes were detected with HPSEC and HPAEC in comparison to the control starches (data not shown). In addition, isoamylase debranched starches were further incubated with α-amylase, and analyzed with HPAEC in order to detect the presence of possible α-(1 → 3) linkages. No consistent changes in amylopectin fine structure were detected in comparison to control starches (data not shown). These results suggest that mutan is not covalently attached to the starch polymers, but rather is present as a separate carbohydrate.

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

In an effort to produce more mutan, starch granules of the best transformants were incubated with an excess of sucrose at 37°C, which is the optimal temperature for GtfICAT, and at 45°C.

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

Transformant	RNA[a]	SBD[b]	d_{50} (μm)	To (°C)	Amylose Content (%)	Starch content (mg/g FW)
KD-UT	N	–	28.8 (±0.5)	66.7 (±0.1)	20.1 (±0.5)	242.9 (±93.5)
KDSIC10	H	+	25.1 (±0.8)	69.5 (±0.1)	18.0 (±0.2)	179.7 (±38.3)
KDICS27	H	–	28.6 (±0.2)	67.4 (±0.1)	20.0 (±0.2)	191.6 (±29.4)
KDIC15	H	–	20.2 (±0.4)	67.2 (±0.1)	19.7 (±0.2)	81.3 (±37.3)
amf-UT	N	–	13.1 (±0.2)	70.1 (±0.1)	3.3 (±0.1)	140.8 (±24.2)
*amf*SIC4	L	+	13.9 (±0.1)	69.4 (±0.1)	3.0 (±0.1)	161.0 (±17.5)
*amf*SIC5	H	++	14.9 (±0.1)	71.4 (±0.2)	3.0 (±0.0)	152.0 (±17.5)
*amf*SIC14	H	++	15.1 (±0.4)	72.3 (±0.1)	3.5 (±0.0)	133.8 (±21.3)
*amf*SIC17	H	++	11.7 (±0.2)	70.9 (±0.1)	3.6 (±0.0)	109.6 (±8.6)
*amf*ICS27	L	–	12.3 (±0.1)	70.6 (±0.1)	3.6 (±0.1)	96.5 (±21.9)
*amf*ICS6	H	++	11.8 (±0.1)	70.5 (±0.1)	3.2 (±0.1)	125.2 (±9.1)

[a] Based on RT-PCR analysis

[b] Based on Western dot blot analysis

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

Discussion

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

Granule targeting of GtfICAT without covalent attachment of mutan

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

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

Interestingly, the starch from the KDSIC10 transformant had mutan attached, which seemed more difficult to degrade or detach from the granule than that of transformants in which the mutan was synthesized by GtfICAT without an appended SBD. Our experiments in which starch polymers were treated with isoamylase and α-amylase only gave the common maltooligosaccharide degradation products, without indications for the presence of more peculiar oligosaccharides having α-1,3 linkages. These results lend some support to the idea that, with an appended SBD, it is possible to co-deposit novel polymers with starch in granules, although the more intimate contact between the growing starch granule and mutansucrase do not seem to lead to covalent connections between the polymers.

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

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

In the Kardal background, only the SIC transformants showed altered granule morphology, although the spongeous appearance was not observed. Possibly the small holes in the granule surface are filled with amylose (it is considered very unlikely that the different ploidy level and

alleles of starch biosynthetic enzymes of Kardal and the *amf* potato mutant are responsible for the observed altered granular morphology). The alterations are less severe and at lower frequency than in the *amf* background, which is consistent with the lower accumulation of SBD-containing fusion proteins in Kardal. It has been shown that GtfICAT alone also gives an altered granular phenotype (Fig. 5I, and Kok-Jacon et al. 2005a), which is different from that of GtfICAT with appended SBD. It is believed that, in case of GtfICAT alone, the morphology of the granule is determined by the presence of mutan, whereas, in case of GtfICAT with appended SBD, the phenotype is determined by the presence of the fusion protein interfering with lateral interactions of amylopectin side chains.

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

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