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

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Abstract Microbial starch-binding domains (SBD) and granule-bound starch synthase I (GBSSI) are proteins which are accumulated in potato starch granules. The efficiency of SBD and GBSSI for targeting active luciferase reporter proteins to granules during starch biosynthesis was compared. GBSSI or SBD 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 into an 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.

Keywords: luciferase, GBSSI, SBD, *amf* potato.

In a previous paper, we have described a method for incorporating foreign proteins in starch granules during the biosynthesis process[1]. The use of microbial starch-binding domains (SBD)-encoding region of cyclodextrin glycosyltransferase (CGTase) from *Bacillus circulans* is a key of this technology. We have shown that SBD could be accumulated in potato starch granules, either on itself or as part of a SBD luciferase fusion protein. The luciferase could not be incorporated into 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.

Granule-boundness of enzymes is also encountered with some starch biosynthetic enzymes[2]. Granule-bound starch synthase I (GBSSI) is probably the most well-known example of such an enzyme. From previous studies we found that native GBSSI and recombinant SBD proteins may bind similar sites in the granule and that GBSSI has a higher affinity for starch granules than SBD[1].

In this study, we explored that (1) whether potato GBSSI can be used to engineer artificial granule-bound luciferase proteins as an alternative for SBD, which might be incorporated into granules during starch biosynthsis, (2) whether luciferase retains its activity in the GBSSI-containing fusion protein, and (3) whether more fusion proteins can be accumulated inside starch granules. For these, GBSSI was fused to the luciferase, and the constructs were introduced into the amylose-free (*amf*) potato mutant[3]. The results of the various anchors and anchor positions are discussed in terms of starch-binding efficiency and luciferase activity.

1 Materials and methods

1.1 Materials

The plasmids, gene fragments, *amf* potato mutant plants[3] and *amf*COMP starch[4] used in this study were from Laboratory of Plant Breeding, Wageningen University.

1.2 Methods

1.2.1 Preparation of constructs

In order to investigate the effect of GBSSI position (C- or N- terminal) in luciferase fusion proteins in terms of catalytic activity and starch-binding properties, two constructs were made. The pBIN19/GBSSI-LUC and pBIN19/LUC-GBSSI plasmids were used for the expression of the GBSSI-LUC (N-terminal GBSSI) and LUC-GBSSI (C-terminal GBSSI)

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fusion proteins in the mutant *amf* potato plants, respectively. The pBIN19/LUC[11] (Fig. 1) plasmid served as a control in which luciferase was directed to the amyloplast without granule-targeting.

For making pBIN19/GBSSI-LUC plasmid, a GBSSI-encoding fragment was amplified from a pUC19/GBSSI plasmid by the polymerase chain reaction (PCR) with the following primers: GL1 (5′-TAAG**CCATGG**GAAAGGGAATGAACTTGATC-3′) and GL2 (5′-CGT**AGATCT**GGGAGTGGCTACATTTTCCTTG-3′), which contained *Nco*I and *Bgl*II sites, respectively. The GBSSI *Nco*I-*Bgl*II fragment was used to replace the SBD fragment (also *Nco*I-*Bgl*II) in the pBIN19/SBD-LUC[5], giving the pBIN19/GBSSI-LUC plasmid (Fig. 1). The pBIN19/LUC-GBSSI plasmid was assembled using a similar procedure as for making the pBIN19/GBSSI-LUC. A GBSSI-encoding fragment was amplified from the pUC19/GBSSI plasmid by PCR with the following primers: LG1 (5′-AACC**CTCGAG**CGAATTCGGAAAGGGAATGAACTTGAT-3′) and LG2 (5′-CGAATTCCCATAT**GGTACC**C-3′), which contained *Xho*I and *Kpn*I sites, respectively. The GBSSI *Xho*I-*Kpn*I fragment was replaced by the SBD fragment in pBIN19/LUC-SBD plasmid[11]. The resulting plasmid is referred to as pBIN19/LUC-GBSSI (Fig. 1). Two constructs were verified by DNA sequencing.

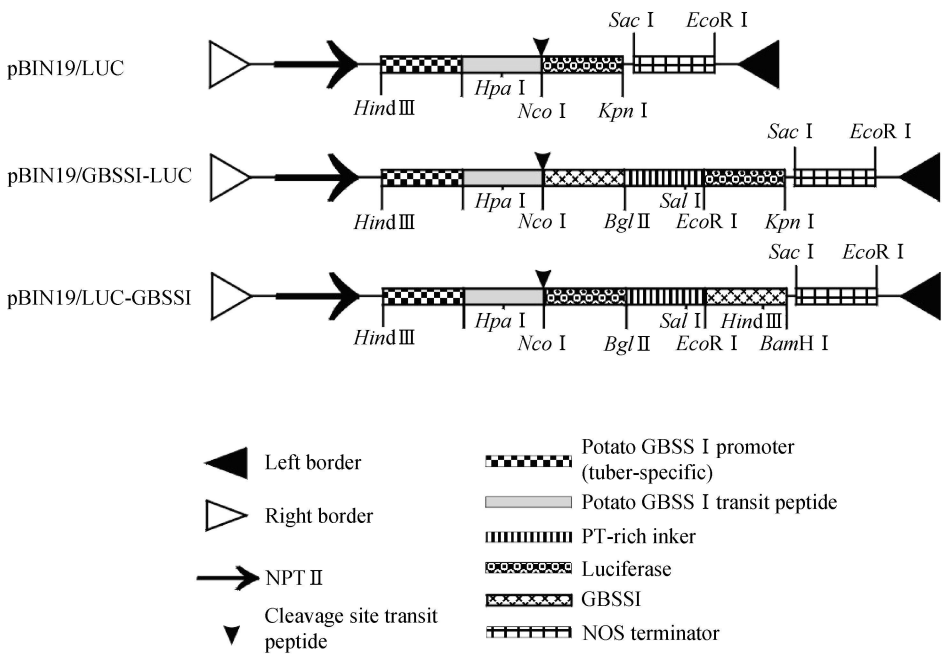

Fig. 1. Schematic representation of pBIN19/LUC, pBIN19/GBSSI-LUC and pBIN19/LUC-GBSSI binary vectors used for expression of (fusion) proteins in potato plants. In all cases, the genes were under the control of the tuber-specific potato GBSSI promoter. Amyloplast entry was mediated by the potato GBSSI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

1.2.2 Plant transformation and regeneration

The pBIN19/GBSSI-LUC, pBIN19/LUC-GBSSI and pBIN19/LUC plasmids were transformed into *Agrobacterium tumefaciens* according to the three-way mating protocol described by Visser et al.[6]. Internodal stem segments from the *amf* potato mutant were used for *Agrobacterium*-mediated transformation[6]. More than 50 independent shoots were harvested for each construct. Shoots were tested for root growth on a kanamycin-containing (100 mg/L) MS30 medium[7]. For each construct, 27 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 *amf* plants were grown in the greenhouse and served as controls.

1.2.3 Measurement of luciferase activity in tuber slices, starch granules

The transformants were screened 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

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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℃)[1]. Each measurement was repeated three times and the untransformed tuber slices were served as controls. 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℃[1]. The untransformed starch was used as controls.

1.2.4 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.[1]. Anti-Luciferase pAb (Promega, USA) and antiGBSSI[8] polyclonal antibodies were used to visualize their respective antigens by chemiluminescence. For GBSSI-containing fusion proteins, a 1∶250 dilution of antiGBSSI was used as the primary antibody. 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.

2 Results

2.1 Characterization of transformants with luciferase fusion proteins

The *amf* potato mutant was separately transformed with pBIN19/LUC, pBIN19/GBSSI-LUC and pBIN19/LUC-GBSSI (Fig. 1). The resulting transgenic clones are referred to as *amf*Lxx, *amf*GLxx and *amf*LGxx, respectively. L, GL and LG represent the LUC, GBSSI-LUC and LUC-GBSSI genes, respectively. Twenty-seven 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).

Expression of luciferase and luciferase-containing fusion proteins in the *amf* background was first analyzed by measuring the luciferase activity in tuber slices with a luminometer. Luciferase activities in tuber slices of the *amf*L, *amf*GL and *amf*LG series are summarized in Fig. 2. For comparison, luciferase activities in tuber slices of the *amf*SL and *amf*LS series[5] are also indicated in Fig. 2. It can be seen that (1) luciferase 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, indicating that luciferase activity is not impaired by the attached SBD; (2) it is clear that luciferase activities of tuber slices in *amf*LG and *amf*GL transformants were 10—20 times lower than those in *amf*SL and *amf*LS series, suggesting that luciferase activity is impaired by the attached GBSSI; (3) it appeared as if fusion proteins with GBSSI at the C-terminus had a slightly higher luciferase activity in the starch granule (compare *amf*LG with *amf*GL series).

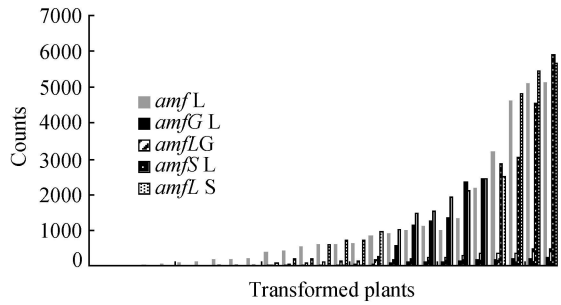

Fig. 2. Luciferase activity in slices of transgenic potato tubers expressing SBD-containing and GBSSI-containing gene fusions. The figure shows the number of photon counts after 20 s in the slices of transgenic lines of different series. The measurements were performed immediately after spraying the luciferin substrate onto slices at 20℃.

2.2 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. For comparison, the luciferase activities of *amf*LS13 and *amf*SL12 (the transformants with the highest luciferase activity from the *amf*LS and *amf*SL series) starch granules[5] are also included in this table. From the table it can be seen that

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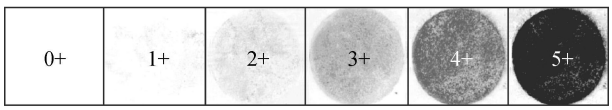
very little luciferase activity is accumulated in the *amf*L7 granules, demonstrating that luciferase does not have an affinity for amylose-free starch granules. This is consistent with our previous results for KDL17[1], and shows that appending an "anchor" to luciferase is essential for association with starch granules. It is clear that the granules with the GBSSI-containing fusion proteins have a much lower luciferase activity than those with the SBD-containing fusion proteins. This shows that GBSSI is inferior to SBD in anchoring active luciferase in starch granules.

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)]	
		anti-Luciferase	anti-GBSSI
amf-UT	0	n. t.	n. t.
*amf*LS13	50076(±33.3)	2+	n. t.
*amf*SL12	29225(±51.1)	1+	n. t.
*amf*L7	28(±12.6)	0+	n. t.
*amf*COMP[d)]	n. t.	n. t.	4+
*amf*LG30	164(±7.2)	1+	1+
*amf*GL31	61(±3.0)	2+	2+

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℃ 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) n. t. means not tested.

d) This transformant represents an *amf* potato mutant, which is complemented with the full-length potato GBSSI gene, including its promoter[4].

The levels of LUC and LUC/GBSS fusion proteins accumulated in transgenic starch granules were also investigated by Western dot blot analysis using anti-Luciferase pAb and antiGBSSI antibodies, respectively (see Table 1). Starch from an *amf* mutant complemented with potato GBSSI, *amf*COMP, served as a positive control when the antiGBSSI antibody was used. Estimation of the amount of fusion proteins accumulated in transgenic granules was performed according to the dot intensity scheme[5]. It can be seen that the amount of GBSSI in both *amf*GL31 and *amf*LG30 transgenic granules was much lower than that in *amf*COMP, indicating that starch-binding efficiency of GBSSI in the fusion proteins is impaired by the attached luciferase. 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.

3 Discussion

In the present study, we compared the efficiency of two granule-targeting sequences, SBD and GBSSI, for directing luciferase to the growing starch granule in potato tubers. We also determined whether the position (N-or C-terminal) of GBSSI in the fusion protein could affect the activity of the luciferase and the binding affinity of the GBSSI. 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)[1], 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[1,5].

In order to exclude competition between native GBSSI (present in wildtype granules, but not in *amf* ones) and the introduced GBSSI-containing fusion proteins, the *amf* mutant potato plants were used for experiments.

With GBSSI as a targeting sequence, both in N- and C-terminal position, GBSSI was capable of granule-targeting during starch biosynthesis. However, affinity for starch of GBSSI in fusion proteins 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,

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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. Extensive sequence comparisons of starch-, glycogen- and sucrose synthases suggest that the amino acid sequence of the mature GBSSI corresponds to a single module[9]. 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 a separate starch-binding domain, which is in contrast with many starch-degrading enzymes. For this reason, the full-length GBSSI gene was used for making fusion genes in this study. However, 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.

References

1 Ji Q., Vincken J. P., Suurs L. C. J. M. et al. Microbial starch-binding domains as a tool for targeting proteins to granules during starch biosynthesis. Plant Mol. Biol., 2003, 51(5): 789—801.

2 Kok-Jacon G. A., Ji Q., Vincken J. P. et al. Towards a more versatile α-glucan biosynthesis in plants. J. Plant Physiol., 2003, 160: 765—777.

3 Jacobsen E., Hovenkamp-Hermelink J. H. M., Krijgsheld H. T. et al. Phenotypic and genotypic characterization of an amylose-free starch mutant of potato. Euphytica, 1989, 44(1): 43—48.

4 Flipse E., Straatman-Engelen I., Kuipers A. G. J. et al. GBSS T-DNA inserts giving partial complementation of the amylose-free potato mutant can also cause co-suppression of the endogenous GBSS gene in a wild-type background. Plant Mol. Biol., 1996, 31: 731—739.

5 Ji Q., Vincken J. P., Raemakers K. et al. Effect of SBD's position in fusion protein on activity and binding properties. J. Nanjing Normal Univ. (Natural Science), 2005, 28(1): 88—92.

6 Visser R. G. F. Regeneration and transformation of potato by *Agrobacterium tumefaciens*. In: Plant Tissue Culture Manual. Dordreche: Kluwer, 1991, B5: 1—9.

7 Murashige T. and Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant, 1962, 15(3): 473—497.

8 Vos-Scheperkeuter G. H., de Boer W., Visser R. G. F. et al. Identification of granule-bound starch synthase in potato tubers. Plant Physiol., 1986, 82: 411—416.

9 MacGregor E. A. Possible structure and active site residues of starch, glycogen, and sucrosesynthases. J. Prot. Chem., 2002, 21: 297—306.

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