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ORIGINAL PAPER

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# Efficiency of transcriptional gene silencing of GBSSI in potato depends on the promoter region that is used in an inverted repeat

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**Abstract** Transcriptional gene silencing (TGS) of the endogenous GBSSI promoter in potato was induced by inverted repeat constructs containing different regions of the GBSSI promoter. Clear differences in silencing efficiency were observed. The 35SGBP-IR construct, containing sequences from −766 to −168 bp relative to the transcription initiation site (TIS), induced weak silencing effects in 57–60% of the transformants. Weak silencing effects were also induced by the ASP-IR construct harbouring allele-specific sequences covering the region from −531 to −330 bp relative to the TIS, but only in a low percentage (4–5.5%) of the transformants. These percentages are too low to distinguish effects between the two potato cultivars. Therefore, this approach cannot be used to induce allele-specific TGS. Strong silencing effects were obtained in 49% of the transformants harbouring the full promoter inverted repeat construct. This construct contained sequences from −766 to +194 bp relative to the TIS. In the strongly silenced transformants no GBSSI mRNA could be detected by Northern blot analysis. This was accompanied by the accumulation of GBSSI promoter-specific small interfering RNAs. Methylation studies revealed that, in the weakly silenced 35SGBP-IR transformants, the *Hpa*II site at −213 bp relative to the TIS was methylated. Apparently, methylation of this sequence does not result in strong silencing effects. In the full promoter transformants, both CG methylation and CNN methylation were detected. We show that, to obtain strong TGS, it is important to include sequences in the vicinity of the TIS.

**Keywords** Promoter inverted repeat · RNA-directed DNA methylation · Transcriptional gene silencing · Potato · Granule-bound starch synthase

**Abbreviations** RdDM: RNA-directed DNA methylation · TIS: Transcription initiation site · dsRNA: Double-stranded RNA · siRNA: Small interfering RNA · PTGS: Post-transcriptional gene silencing · TGS: Transcriptional gene silencing · GBSSI: Granule-bound starch synthase I

## Introduction

Post-transcriptional gene silencing (PTGS) involves sequence-specific RNA degradation and can be induced by producing double-stranded RNA (dsRNA) of coding sequences. When dsRNA of promoter sequences is produced, transcriptional gene silencing (TGS) can be induced. In this case, alterations at the DNA or chromatin level prevent transcription of the targeted genes.

Methylation is an alteration at the DNA level which occurs both in PTGS and in TGS. The methylation of coding sequences does not seem to affect transcription, while the methylation of promoter sequences usually results in promoter inactivation (Sijen et al. 2001). Methylation is induced by RNA signals and is therefore described as RNA-directed DNA methylation (RdDM). RdDM leads to the de novo methylation of almost all cytosine residues within the region of sequence identity between the triggering RNA and homologous DNA. RdDM has been shown to require dsRNA that is cleaved to small interfering RNAs (siRNAs) 21–26 nt in

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length (Matzke et al. 2004). It is not known whether the siRNAs or dsRNA guide methylation of the homologous DNA sequences, although there are indications that the longer class of siRNAs, 24–26 nt in length, is involved in this process (Hamilton et al. 2002). The first example of RdDM was described by Wassenegger et al. (1994) who demonstrated that cDNA copies of a viroid that had been integrated in the plant genome became methylated as a consequence of the presence of a replicating viroid RNA.

Mette et al. (2000) demonstrated that dsRNA of the NOS promoter led to silencing of the NOS promoter-driven *nptII* gene in tobacco. They showed that the NOS promoter dsRNA could be degraded to small RNAs in a manner similar to dsRNAs that induce PTGS. The same approach was tested in *Arabidopsis* where a NOS promoter inverted repeat also resulted in a high frequency of silencing (Mette et al. 2000).

Sijen et al. (2001) demonstrated that TGS and PTGS are initiated by a similar dsRNA pathway. They showed that the 35S promoter could be silenced in *Petunia* by inverted repeat constructs harbouring sequences of the 35S promoter. Silencing efficiency depended on the targeted sequences. A construct harbouring a minimal promoter was less efficient than constructs harbouring the enhanced or full promoter. For the latter two constructs, 35S promoter dsRNA, 35S small RNAs and methylation of the 35S promoter were detected.

The same authors also induced transcriptional silencing of the promoter of the endogenous flower pigmentation gene *dihydroflavonol 4-reductase A* (*dfrA*) by introducing a 35S promoter-driven *dfrA* promoter inverted repeat into wild-type *Petunia*. These targeted *dfrA* promoter sequences did not include a transcription initiation site (TIS) or a TATA box. The absence of *dfrA* mRNAs, observed in the transformants showing reduced pigmentation, showed that the *dfrA* gene was transcriptionally silenced. This phenomenon was accompanied by the production of *dfrA* promoter dsRNA, production of small RNAs and methylation of the *dfrA* promoter (Sijen et al. 2001).

So far, TGS of transgene promoters has been described in *Petunia*, tobacco and *Arabidopsis*. If a promoter is partially methylated, this does not necessarily prevent the promoter from being active, as was demonstrated for the 35S promoter by Sijen et al. (2001).

In potato, the granule-bound starch synthase I (GBSSI) gene has been efficiently silenced by PTGS. Even though transformants obtained through the use of antisense or inverted repeat constructs show strong silencing of GBSSI, there is still GBSSI mRNA present (Kuipers et al. 1994; Heilersig et al. 2006). In order to obtain a transformant with no GBSSI mRNA, transcription has to be prevented. Therefore, TGS could be a useful tool. To induce allele-specific silencing, TGS could also be a good approach since the four classes of GBSSI alleles are highly homologous in the coding region but vary in promoter sequences (van de Wal et al. 2001). A promoter sequence specific for the A2, A3 and A4 GBSSI allele, but absent in the A1 allele, was selected to design an ''allele-specific'' promoter inverted repeat construct. Two other promoter inverted repeat constructs targeting different regions of the GBSSI promoter were made. Results obtained with these constructs indicated that TGS of GBSSI can be induced by promoter inverted repeats. The silencing efficiency varied for the different sequences. The most efficient silencing efficiency was induced with the inverted repeat construct containing the full promoter sequence.

## Materials and methods

### Plant material and growth conditions

Potato cultivars Karnico and Ponto (Averis Seeds, Valthermond, The Netherlands) were grown in vitro on MS medium (Murashige and Skoog 1962), with 30 g/l sucrose and 8 g/l agar at 24°C, and a photoperiod of 16 h light at an irradiance of 40 μmol $m^{-2}$ $s^{-1}$.

### DNA constructs

To make the GBSSI promoter (GBP) inverted repeat construct, the primers behpromf1 (5′-CTCCGTTT TGTTCATTACTT-3′) and behpromr1 (5′-ATTCACG GCTGGACTTCAAC-3′) were used to amplify a 599-bp product (see Fig. 1a; primers P1 and P4) from pWAM10, a pUC18-based plasmid containing the class A1 potato GBSSI promoter (van der Leij et al. 1991). This PCR product was cloned in pGEM-Teasy (Promega) followed by subcloning in pMTL25 (Chambers et al. 1988). The product was excised from pMTL25 by digestion with *Bam*HI and subsequently cloned behind the GBSSI promoter in the binary vector pPGB-1s (Kuipers et al. 1995). To avoid self ligation of the *Bam*HI-digested pPGB-1s, the vector was treated with shrimp alkaline phosphatase (Roche). After ligation in the binary vector, clones were checked for the correct orientation by restriction analysis and PCR. Binary vector GBP-IR contains the 599-bp promoter fragment in an inverted repeat orientation. The spacer is 266 bp long and contains the TIS and core promoter boxes of the GBSSI promoter as well as polylinker sequences from the cloning vectors (Fig. 1b).

By inserting a 2×35S promoter in the GBP-IR construct through the *Cla*I and *Hin*dIII restriction sites, a new binary vector, 35SGBP-IR, was created (Fig. 1b). The 2×35S promoter (700 bp) was isolated from pJIT65 (John Innes Institute, Norwich, UK) and subcloned in pMTL23 (Chambers et al. 1988). From this vector it was excised by *Cla*I and *Hin*dIII.

To design the allele-specific promoter inverted repeat construct (ASP-IR), a 200-bp fragment was amplified from the DNA isolated from a dihaploid potato line (90-027-6) harbouring two A2 GBSSI alleles, using the PCR conditions as described by van de Wal et al. (2001) for

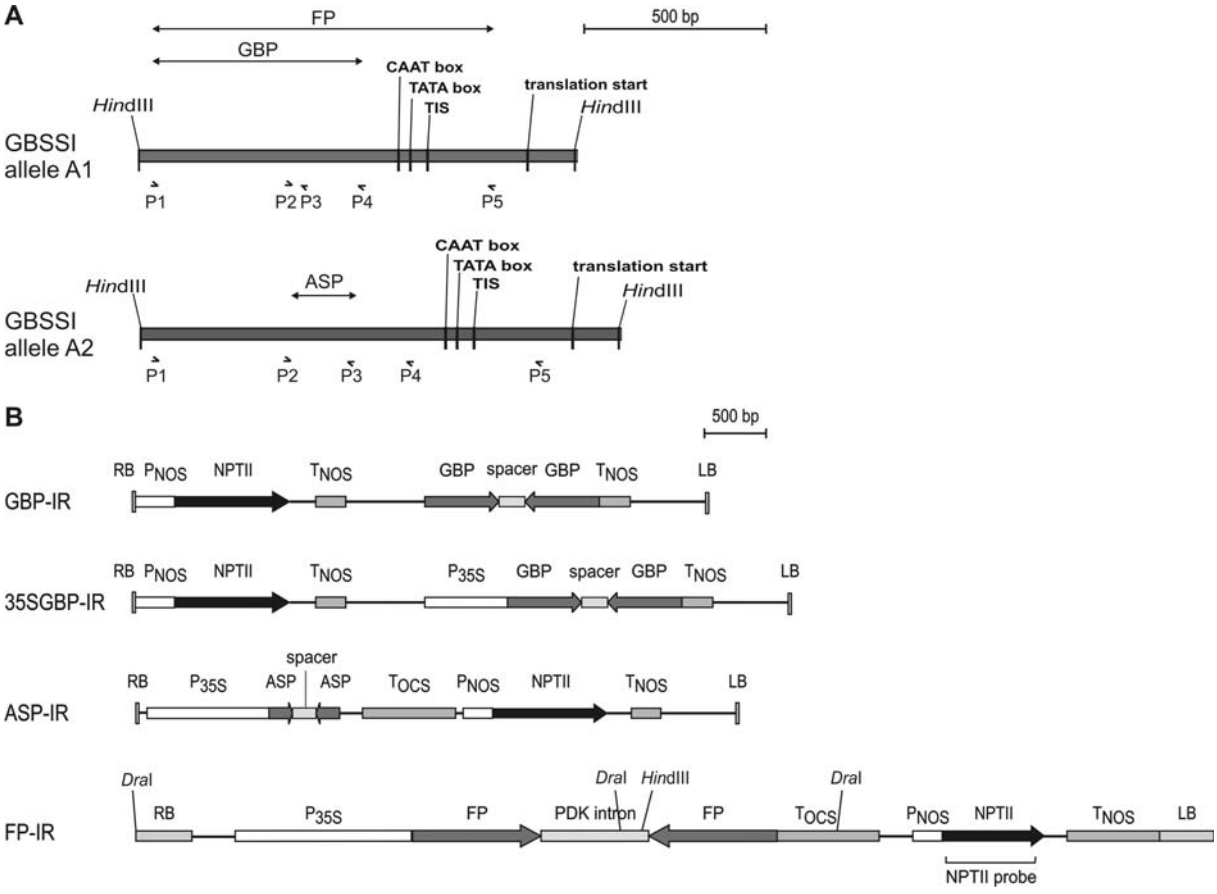


**Fig. 1** **a** Schematic overview of the promoters of endogenous GBSSI alleles A1 and A2 of potato. The regions used to construct promoter inverted repeats are GBP and FP from allele A1, and ASP from allele A2. Primers used to amplify promoter regions are indicated. *P1* behpromf1, *P2* cdf1mod, *P3* cdf2mod, *P4* behpromr1, *P5* mwpr4, *TIS* Transcription initiation site. **b** DNA constructs designed to produce GBSSI promoter dsRNA. GBP-IR, GBSSI promoter inverted repeat without a transcribing promoter. 35SGBP-IR, GBSSI promoter inverted repeat driven by the 35S promoter. ASP-IR, Allele-specific GBSSI promoter inverted repeat harbouring 200 bp of the GBSSI A2 allele. FP-IR, Full promoter inverted repeat. The NPTII probe used for Southern hybridisation is indicated. *RB* right border, *LB* left border, $P_{NOS}$, promoter of the nopaline synthase gene, *NPTII* kanamycin resistance gene, $T_{NOS}$ terminator of the nopaline synthase gene, $P_{35S}$ 35S promoter, $T_{OCS}$ terminator of the octopine synthase gene, *PDK intron* intron 3 of the pyruvate orthophosphate dikinase gene from *Flaveria trinervia*

the primer combination cdf1/cdf2. Modified cdf primers were used for the amplification (primers P2 and P3 in Fig. 1a). CDF1mod (5′-ATCCATGGCTGCAGTGC ATCTCAATCTT-3′) contained an *Nco*I and a *Pst*I site at the 5′ end, whereas CDF2mod (5′-ATCCGCGGA CTAGTACTGGTCCCTCC-3′) contained a *Spe*I and a *Sac*II site at the 5′ end. The resulting PCR product ASP was cloned in pGEM-Teasy (Promega). As a spacer a 158-bp fragment, containing 121 bp of the 3′ end of the luciferase gene and 37 bp of a linker sequence, was amplified from pBIN19/Luc-SBD (Ji et al. 2003) using the primers spacerfor (5′-CCGGAATTCAAAGTTGC GCGGAGGAGTTGTGTT-3′) and spacerrev (5′-CG GGGTACCGTCGGGGTCGGCGTCGTG-3′). The amplified fragment contained an *Eco*RI site at the 5′ end and a *Kpn*I site at the 3′ end. This fragment was cloned in pGEM-T and named pSPAC. Subsequent cloning of the ASP promoter fragment in pSPAC through the *Spe*I and *Pst*I restriction sites resulted in a vector harbouring the antisense ASP fragment downstream of the spacer. The sense ASP fragment was then subcloned upstream of the spacer using the *Nco*I and *Eco*RI restriction sites. The resulting inverted repeat was excised by *Nco*I and *Sal*I and cloned into pMTL25 (Chambers et al. 1988). Subsequently, the inverted repeat was excised by *Xho*I and *Hin*dIII and transferred to the pHANNIBAL vector (Wesley et al. 2001). By doing so, the inverted repeat was placed behind the 35S promoter and replaced the original intron in pHANNIBAL. The construct was then subcloned as a *Not*I fragment into pART27 (Gleave 1992). At all stages, the correct orientation was checked by restriction analysis. The ASP-IR construct is shown in Fig. 1b.

To make the full promoter inverted repeat construct, Gateway technology was used. A 960-bp PCR product was amplified using a modified behpromf1 primer and the mwpr4 primer (van de Wal et al. 2001) (P1 and P5 in Fig. 1a). The behpromf1 primer was modified through the addition of a CACC site at the 5′ end, allowing the cloning of the PCR product in the pENTR/D-TOPO

vector supplied by Invitrogen. Subcloning of the full promoter sequence was verified by sequence analysis. The full promoter sequence was then recombined in the silencing vector pHELLSGATE8 (Helliwell et al. 2002) by LR reaction. All Gateway reactions were performed as described by Invitrogen. Correct orientation of the final full promoter inverted repeat construct (FP-IR) (Fig. 1b) was checked by restriction analysis.

All constructs, except the Gateway-based construct, were transformed into *E. coli* DH5α (Invitrogen, Breda, The Netherlands) using the selectable markers kanamycin (50 mg/l) for the (35S)-GBP-IR constructs and spectinomycin (100 mg/l) for the ASP-IR construct. pHELLSGATE8 was propagated in *E. coli* strain DB3.1, which is resistant to the toxic ccdB product (Bernard and Couturier 1992). The FP-IR construct obtained after the LR reaction was transformed into *E. coli* DH5α (using spectinomycin (100 mg/l) as a selectable marker. All constructs were transformed to *Agrobacterium tumefaciens* strain AGL0 (Lazo et al. 1991) by electroporation (Takken et al. 2000).

## Transformation and regeneration

Internodal cuttings from in vitro-grown plants of the potato cultivars Karnico and Ponto were used for transformation by *A. tumefaciens*. These explants were precultured for 1 day on solid R3B medium (MS medium with 30 g/l sucrose, 1 mg/l BAP, 2 mg/l NAA), supplemented with 2 ml PACM (MS with 30 g/l sucrose, 2.0 g/l casein hydrolysate, 1 mg/l 2,4-D, 0.5 mg/l kinetin, pH 6.5). Explants were incubated for 5 min in a 16 h-grown culture of *A. tumefaciens*, after which they were blotted on paper and co-cultivated on R3B medium for 2 days. Then, explants were transferred to MS medium with 20 g/l sucrose, 1 mg/l zeatin, 200 mg/l cefotaxime, 200 mg/l vancomycin and 100 mg/l kanamycin. Explants were transferred to fresh medium every 2–3 weeks. When shoots appeared, they were harvested and transferred to MS medium containing 30 g/l sucrose, 200 mg/l cefotaxime and 100 mg/l kanamycin. Shoots rooting on this medium were considered transgenic.

## In vitro tuberisation

Microtubers were induced on in vitro-grown stem segments containing axillary buds. These were placed on petri dishes with MS medium containing 80 g/l sucrose and 5 μM BAP (Hendriks et al. 1991). Incubation of these petri dishes in the dark at 18°C resulted in the formation of microtubers after 2–3 weeks.

## Starch staining

Microtubers were cut and stained with a 1:2 LUGOL:$H_2O$ solution (LUGOL is a 5% (w/v)iodine and 10% (w/v) potassium iodide solution). Staining of the starch granules was examined microscopically. Starch granules containing amylose and amylopectin will stain blue whilst starch granules that only contain amylopectin will stain red with a faint blue core at the hilum. If the amount of amylose is reduced, the starch granule stains red with a blue core that can vary in size. The size of this blue core is closely related to the amylose content (Kuipers et al. 1994). Transformants of which granules showed completely blue staining were classified as not silenced. Transformants showing granules with a large blue core and a small red outer layer were classified as weak silencers. Transformants with granules having a small blue core and a large red outer layer were classified as strongly silenced. Transformants with granules with varying sizes of cores were classified as medium silenced. Per transformant, three microtubers were stained and examined microscopically.

If starch granules with different levels of silencing were found within one microtuber, the amylose percentage was determined spectrophotometrically in 2 mg isolated starch according to the method described by Hovenkamp-Hermelink et al. (1988). Besides the starch of Karnico, starch of the amylose-free mutant (*amf*) (Hovenkamp-Hermelink et al. 1987) was used as a control.

## Number of T-DNA integrations

Genomic DNA of in vitro-grown FP-IR transformants was isolated from 0.5 to 2.0 g of leaves, as described by Chen et al. (1992). DNA (3 μg) was digested with *Dra*I and *Hin*dIII, electrophoresed on a 0.8% agarose gel for 16 h at 30 V, and subsequently vacuum blotted (Pharmacia) onto a Hybond N+ membrane (Amersham) in 10× SSC. A 722-bp fragment amplified with the *npt*II primers npt3 (5′-TCGGCTATGACTGGGCACAAC AGA-3′) and npt 4 (5′-AAGAAGGCGATAGAAG GCGATGCG-3′) was used as a probe (NPTII) to check for the integration of T-DNA sequences near the LB of the FP-IR construct (Fig. 1b).

## Northern analysis

Total RNA was isolated from microtubers using Trizol agent (Sigma). RNA concentrations were measured spectrophotometrically. Northern blotting and hybridisation were carried out as described by Sambrook et al. (1989), using 20 μg of total RNA. The membranes were hybridised with a 2.4-kb fragment containing the complete GBSSI cDNA (Visser et al. 1989). To check for equal loading, membranes were probed with a 2.3-kb *Eco*RI fragment of a potato 28S ribosomal RNA gene (Landsmann and Uhrig 1985).

## Reverse transcription-polymerase chain reaction

Total RNA was isolated from microtubers using Trizol agent (Sigma). 3 μg RNA was treated with 7 U

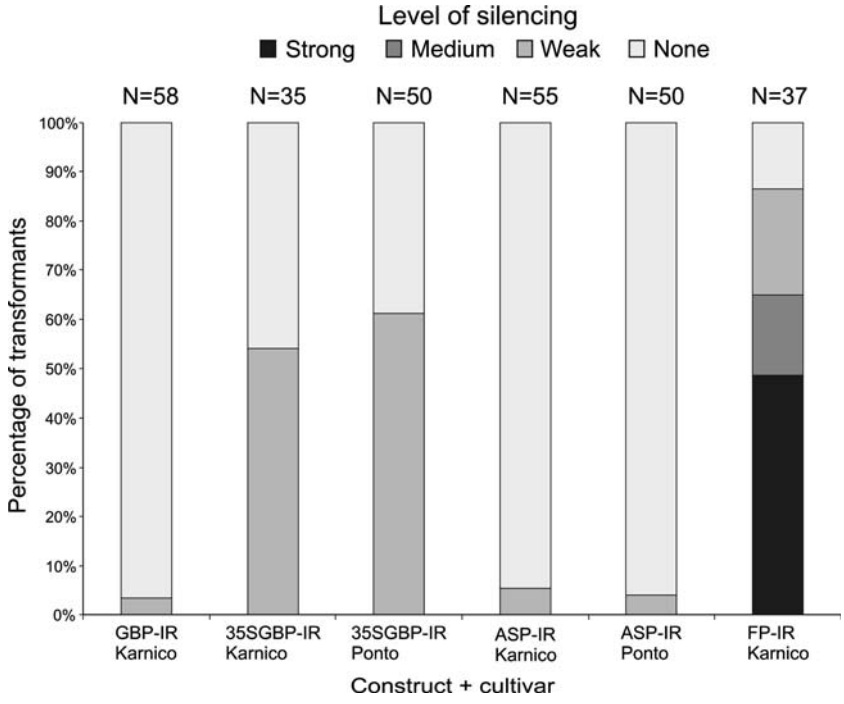


**Fig. 2** Silencing of GBSSI in Karnico and Ponto transformants by promoter inverted repeat constructs. *N* total number of transformants per construct/genotype combination

RNAse-free DNAse (Amersham) for 10 min at 37°C after which DNAse was inactivated through incubation for 15 min at 65°C in 0.0025 M EDTA. cDNA was synthesised on 750 ng RNA using Superscript II Reverse Transcriptase (Invitrogen) and 100 ng oligo-dT primer in a total volume of 20 µl. 2 µl was used for PCR amplification in a volume of 50 µl. Ubiquitin was amplified using the primers Ubifor (5′-GTCAGGCC CAATTACGAAGA-3′) and Ubirev (5′-AAGTTCCA GCACCGCACTC-3′) ($T_m$ 55°C, 40 cycles). To detect premature mRNA, intron-specific primers GBSS4 (5′-CAGGAATAGGCAAAATAAAGATGA-3′) and GBSS11 (5′-GTTCCCTTACATTTCCTGATTC-3′) were used ($T_m$ 55°C). For the detection of mature RNA, exon-specific primers MF1 (5′-GCAAGCTTATCTGGACA ATGAACTTA-3′) and MR1 (5′-CTGGATCCTTCT GCTCCTCAAGTCTG-3′) were used ($T_m$ 55°C). For both primer combinations, 35 and 45 amplification cycles were performed. To visualise the PCR products, 10 µl was electrophoresed on a 1% agarose gel. To verify the effectiveness of the DNAse treatment, RNA (100–150 ng) as well as DNAse-treated RNA (100–150 ng) were used as templates in the PCR reaction with intron-specific primers.

## Small RNA analysis

Total RNA was isolated from microtubers using Trizol agent (Sigma). Enrichment for small weight RNA was performed according to Hamilton et al. (2002) with modifications. High molecular weight RNA was precipitated by adding polyethylene glycol (MW 8,000) and sodium chloride to final concentrations of 5% and 500 mM, respectively. After incubation on ice for 30 min, high molecular weight RNA was precipitated by centrifugation. Low molecular weight RNA was then precipitated from the remaining supernatant by precipitation with sodium acetate and ethanol. RNA concentrations were measured spectrophotometrically. From every sample, 20 µg of low molecular weight RNA was electrophoresed on a 15% polyacrylamide gel for 1.5 h at 100 V using a vertical gel system (Biorad). RNA was then transferred onto Hybond N membrane (Amersham) by overnight electro blotting at 25 V (Biorad). As a probe, the GBP fragment (Fig. 1a) was used, which was labelled with the Megaprime DNA labelling system (Amersham). Hybridisations were performed in glass bottles in a Hybaid hybridisation oven, at 50°C for 16 h. The blots were rinsed twice with 2× SSC, 1% SDS, followed by a rinse with 1× SSC, 1% SDS.

## Methylation of the GBSSI promoter

Genomic DNA was isolated from 0.5 to 2.0 g of leaves of in vitro-grown transformants, as described by Chen et al. (1992). DNA isolated from the 35SGBP-IR transformants (9 µg) was digested with *Hin*dIII and *Eco*RI, after which one-third was digested with the methylation-sensitive enzyme *Hpa*II and one-third was digested with another methylation-sensitive enzyme *Msp*I. The remaining one-third was used as a control. As a reference, the DNA of the construct 35SGBP-IR and of wild-type Karnico and Ponto were subjected to the same treatments.

DNA isolated from FP-IR transformants (5 µg) was digested with *Hin*dIII and *Xba*I. Digested samples were

divided in three portions; one-third was subsequently digested with *Hpa*II, the second portion with *Hae*III and the remaining was used as a control. As a reference, DNA of the construct FP-IR and of the wild type Karnico was subjected to the same treatments.

After precipitation, samples were electrophoresed on a 0.8% agarose gel for 16 h at 30 V and subsequently vacuum blotted (Pharmacia) onto Hybond N+ membrane (Amersham) in 0.4 N NaOH. For the 35S GBP-IR transformants, the GBP fragment (Fig. 1a) (a 599-bp PCR fragment obtained with primers behpromf1 and behpromr1) was used as a probe. To detect fragments harbouring sequences corresponding to the FP-IR construct, the FP sequence (Fig. 1a) (a 960-bp PCR fragment obtained with the primers behpromf1 and mwpr4) was used as a probe. Labelling and hybridisation experiments were performed as described for small RNA analysis using a hybridisation temperature of 65°C.

## Results

### Silencing effects of partial and full promoter inverted repeat constructs

To test whether TGS of the endogenous GBSSI gene could be induced by inverted repeats containing GBSSI promoter sequences, a general GBSSI promoter inverted repeat construct (GBP-IR) was made. This GBP-IR construct contained a 599-bp sequence derived from the A1 GBSSI allele from −766 to −168 bp relative to the TIS (Fig. 1a). The GBP-IR was placed under the control of a 35S promoter resulting in a second construct named 35SGBP-IR (Fig. 1b).

Endogenous GBSSI alleles are highly homologous in their coding sequences (over 98% identity) but show variability in their promoter sequences (van de Wal et al. 2001). The degree of homology between the promoters of the A2, A3 and A4 classes is over 90%, while the A1 promoter only shows 80% homology to the other classes of GBSSI alleles. It was hypothesised that selective silencing of GBSSI alleles could be obtained by targeting allele-specific promoter sequences. To test this hypothesis, a promoter sequence present in the A2, A3 and A4 alleles (Fig. 1a, ASP), but not in the A1 allele, was selected and used to create an ''allele-specific'' 35S-driven promoter inverted repeat construct (ASP-IR) (Fig. 1b). This fragment contained the sequences from −531 to −330 bp relative to the TIS in the GBSSI A2 allele.

Construct GBP-IR was transferred to the potato cultivar Karnico whilst 35SGBP-IR and ASP-IR were transferred to the potato cultivars Karnico and Ponto. These cultivars differ in the GBSSI allele composition and are therefore useful to test the specificity of the ASP-IR construct. The GBSSI allele composition of Karnico is A1A1A1A4 whilst Ponto contains alleles A1A2A3A4 (van de Wal et al. 2001). The number of transformants varied from 35 to 58 per construct. From every transformant, microtubers were induced after which the level of silencing was determined by staining starch granules with iodine solution. Depending on the size of the blue core in starch granules, transformants were classified into four silencing classes: strong, medium, weak or none. Figure 2 shows the effect of the three described constructs on the percentage of transformants showing different levels of silencing. Only transformants showing weak silencing were observed for the three tested partial promoter inverted repeat constructs. However, clear differences in the percentages of transformants showing weak silencing effects were observed. The highest percentage of transformants showing an effect (57–60%) was obtained with the 35SGBP-IR construct. No significant difference was observed between the Karnico and Ponto transformants for this construct. The GBP-IR construct, which has the same sequences but does not have a transcribing promoter, resulted in 2% of the transformants showing an effect. The allele-specific construct (ASP-IR) resulted in low percentages (4–5.5%) of transformants showing a weak silencing effect. The latter percentage is too low to distinguish effects between the different potato cultivars Karnico and Ponto.

The 35SGBP-IR construct targeted the region from −766 to −168 bp relative to the TIS. It did not contain the CAAT, TATA box and the TIS. In the full promoter inverted repeat construct (FP-IR), containing the region from −766 to +194 bp relative to the TIS, these boxes were present. This region was cloned into pHELLSGATE8, resulting in a 35S promoter-driven full promoter inverted repeat construct (FP-IR) (Fig. 1b). The effect of this construct is shown in Fig. 2.

Strong silencing was observed in 49% of the transformants. Within a microtuber of a single transformant, starch granules with different silencing levels were sometimes found, which complicated the phenotyping. Therefore, transformants were phenotyped by spectrophotometrically determining the amylose percentages in the microtuber-derived starch. Using this analysis, low amylose contents are overestimated. Even in the amylose-free control (*amf* mutant) an amylose content of 2.3% was measured. Depending on the amylose percentage, transformants were classified in the four previously described silencing classes. All transformants that had amylose contents lower than 3% were classified as strongly silenced. Table 1 shows the phenotypic observations as well as the amylose contents of 12 transformants that are discussed in more detail in this article. When starch granules with different levels of silencing were found within a microtuber of a single transformant, the percentages of starch granules showing the different levels of silencing are indicated. T-DNA integration numbers were determined in eight of these transformants by digesting DNA with *Dra*I (and *Hin*dIII), followed by hybridisation with the NPTII probe (see Fig. 1b). Three transformants with 1 T-DNA integration were detected. Two of these transformants showed strong silencing.

**Table 1** Level of silencing in relation to amylose content and number of T-DNA integrations in FP-IR transformants

| Transformant | Level of silencing[a] | Percentage of amylose in starch | No. of T-DNA integrations |
|---|---|---|---|
| Fp1 | Strong | 1.8 | 9 |
| Fp2 | Weak | 14.5 | NT |
| Fp3 | Strong | 2.0 | 2 |
| Fp4 | 55% strong<br>3% medium<br>31% weak<br>11% none | 3.2 | NT |
| Fp6 | 93% strong<br>5% medium<br>2% weak | 2.3 | NT |
| Fp8 | 99% strong<br>1% weak | 2.1 | 1 |
| Fp13 | Strong | 2.2 | 4 |
| Fp16 | Strong | 1.9 | NT |
| Fp24 | 95% strong<br>1% medium<br>4% weak | 2.7 | 1 |
| Fp25 | Weak | 13.6 | 1 |
| Fp33 | Strong | NT | 3 |
| Fp39 | Strong | 2.2 | 3 |
| Amylose-free control (*amf* mutant) | Strong | 2.3 | – |
| Wild type (Karnico) | None | 16.4 | – |

[a]When percentages are indicated, they represent the percentages of starch granules showing different levels of silencing within a microtuber of a single transformant
*NT* not tested

Transformants with interesting phenotypes were transferred to the greenhouse. Transformants of ASP-IR that showed weak silencing effects in microtubers did not show a silencing effect in greenhouse tubers. The silencing levels in the greenhouse-grown tubers of 35SGBP-IR and FP-IR transformants, however, were similar to the silencing levels observed in the microtubers.

## mRNA levels in FP-IR transformants

The presence of GBSSI mRNA was investigated by a Northern blot analysis. Total RNA was hybridised with a GBSSI cDNA probe. Results are shown in Fig. 3.

No transcript could be detected in transformants showing strong silencing whereas a transcript was present in the weakly silenced transformant Fp2 as well as in the wild-type Karnico. To apply a more sensitive method, RT-PCR was performed on RNA from wild-type Karnico, three FP-IR transformants and one strongly silenced PTGS transformant (IRMA-S-55; see Heilersig et al. 2006). The accumulation of mature and premature GBSSI was determined by using GBSSI

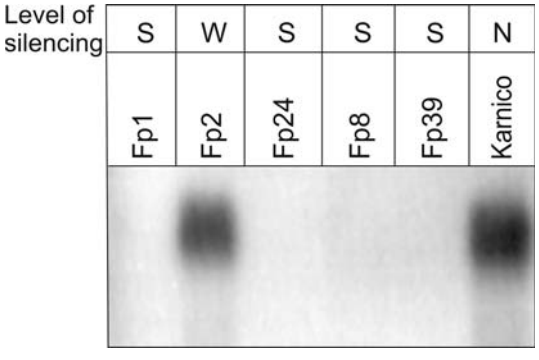


**Fig. 3** Northern blot analysis demonstrating the absence of GBSSI mRNA in strongly silenced FP transformants after hybridisation with a GBSSI cDNA probe. *N* non-silenced, *S* strongly silenced, *W* weakly silenced

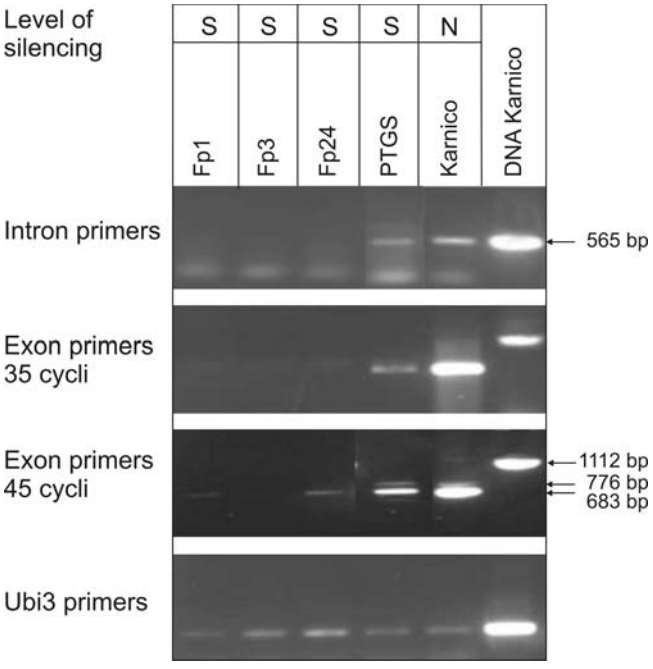


**Fig. 4** RT-PCR analysis on Fp1, Fp3, Fp24, a strongly silenced PTGS transformant and wild type Karnico RNA. As a reference, wild-type Karnico DNA was included. Amplification with intron primers GBSS4 and GBSS11 (565 bp) was used to detect premature RNA. Exon primers MF1 and MR1 (683 bp) were used to demonstrate the presence of mature RNA. The 776-bp fragment is most likely derived from mature RNA from which intron 9 had not been spliced. Amplification of the genomic GBSSI DNA with the exon primers MF1 and MR1 resulted in a fragment of 1,112 bp. The presence of cDNA was demonstrated by amplification with Ubi3 primers. *N* non-silenced, *S* strongly silenced

intron and exon primers (see Fig. 4). Neither mature nor premature GBSSI was found in the FP-IR transformants when 35 amplification cycles were used. However, when 45 cycles were used, products were found for the exon primer combination for transformants Fp1 and Fp24. This indicates that there is a minimal amount of transcript present. A correlation was found between the silencing effect and the intensity of the PCR fragment. Transformant Fp3 only showed strongly silenced starch granules, whereas 5% of the starch granules of transformant Fp24 showed medium or weak silencing. In transformant Fp1, all granules were strongly silenced but the size of the blue-staining core was slightly larger than in Fp3. In transformant Fp3, no fragment is visible in the 45-cycles PCR whilst in transformant Fp24, a clear fragment is visible. These two samples were also used in a 40-cycle PCR reaction; no fragment could be amplified in transformant Fp3 whereas a fragment was found in transformant Fp24 (data not shown).

In these FP-IR transformants, the quality of cDNA was verified by amplification with ubiquitin primers. Furthermore, PCRs on RNA and DNAse-treated RNA of all samples showed that the DNAse treatment worked efficiently (data not shown). In the PTGS transformant both mature and premature GBSSI RNA were found (Fig. 4). For Karnico and the PTGS transformant, amplification with the exon primers resulted in two fragments. The intense fragment with a size of 683 bp represents mature mRNA without intron nine whereas the 776-bp fragment most likely represents mature mRNA from which intron nine has not been spliced.

Total RNA isolated from the FP-IR transformants was analysed for the presence of GBSSI promoter-derived siRNAs. For this purpose, total RNA was enriched for siRNAs, which were electrophoresed and hybridised with a promoter-specific probe. Figure 5 shows that GBSSI promoter-derived siRNAs accumulate in the strongly silenced transformants Fp3, Fp8, Fp16 and Fp24, but not in wild type Karnico. GBSSI promoter-derived siRNAs were also detected in medium silenced transformant Fp22 (data not shown).

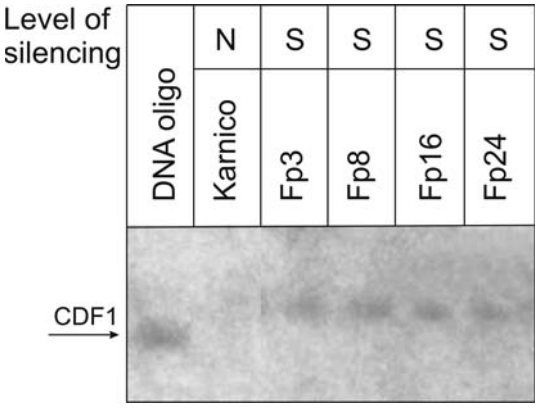


**Fig. 5** Production of GBSSI promoter-derived siRNAs in silenced FP transformants. siRNAs were detected by hybridisation with the GBP probe. As DNA oligo, the 20-nt CDF1 primer was used. Since DNA oligos migrate approximately 10% faster than RNA oligos of equal length (Sambrook et al. 1989, p. 7.76), the position of primer CDF1 corresponds to that of an RNA oligo of approximately 18 nt. *N* non-silenced, *S* strongly silenced

## Methylation status of transgene and endogenous GBSSI promoter in 35SGBP-IR transformants

To test whether the introduced 35SGBP-IR construct induced methylation of the transgene and the endogenous GBSSI sequences, DNA of the transformants was subjected to restriction analysis with methylation-sensitive enzymes. To distinguish between endogenous and transgene sequences, the DNA was first digested with *Eco*RI and *Hin*dIII. Fragments of 883 and 619 bp were obtained for the 35SGBP-IR construct while the endogenous GBSSI sequence gave rise to fragments of 1,202 and 1,346 bp (see Fig. 6a, b). The two endogenous fragments are derived from different alleles. The promoter of the GBSSI A1 allele gives rise to the 1,202-bp fragment whilst the promoter of the other alleles gives rise to a 1,346-bp fragment. In Fig. 6d, restriction fragments obtained before and after restriction with methylation-sensitive enzymes *Hpa*II or *Msp*I are shown for two weakly silenced transformants as well as for the wild type Ponto and the 35SGBP-IR construct. Since Ponto has the allele composition A1A2A3A4, it is not surprising to see that the intensity of the A1-derived fragment is lower than that of the A2-, A3-, and A4-derived fragments. For Karnico, containing three A1 alleles, the intensity of the 1,202-bp fragment was higher than that of the 1,346-bp fragment supporting the 3:1 ratio in intensity (Fig. 6e). *Hpa*II and *Msp*I both cleave at the recognition sequence CCGG. However, *Hpa*II will not cut if the inner C is methylated and cuts very weakly if the outer C is methylated. In contrast, *Msp*I cuts if the inner C is methylated but will not cut if the outer C is methylated (Jeddeloh and Richards 1996). Sensitivity to *Hpa*II indicates lack of methylation at CG or CNG sites, whereas sensitivity to *Msp*I indicates lack of methylation at only CNG sites.

If the *Hpa*II site of the GBP sequence was methylated, the same fragments would be obtained before and after restriction with *Hpa*II. For the transgene-derived antisense 619-bp fragment, this was indeed found, but the size of the transgene-derived sense 883-bp fragment was slightly reduced. Further analysis revealed that there was an additional *Hpa*II site in the spacer sequence which could explain the slight reduction in size to a 868-bp fragment (see Fig. 6b, *Hp*• and M•, and Fig. 6d, T868•). Apparently, this *Hpa*II site in the spacer is not methylated. This indicates that the methylation is restricted to the targeted sequences. In Fig. 6d, transformant 35SGBP-72 shows the accumulation of an additional fragment in the *Hpa*II or *Msp*I-digested fragments of approximately 582 bp indicating partial methylation of the *Hpa*II site. In total, 22 35SGBP-IR transformants were analysed of which 14 showed com-

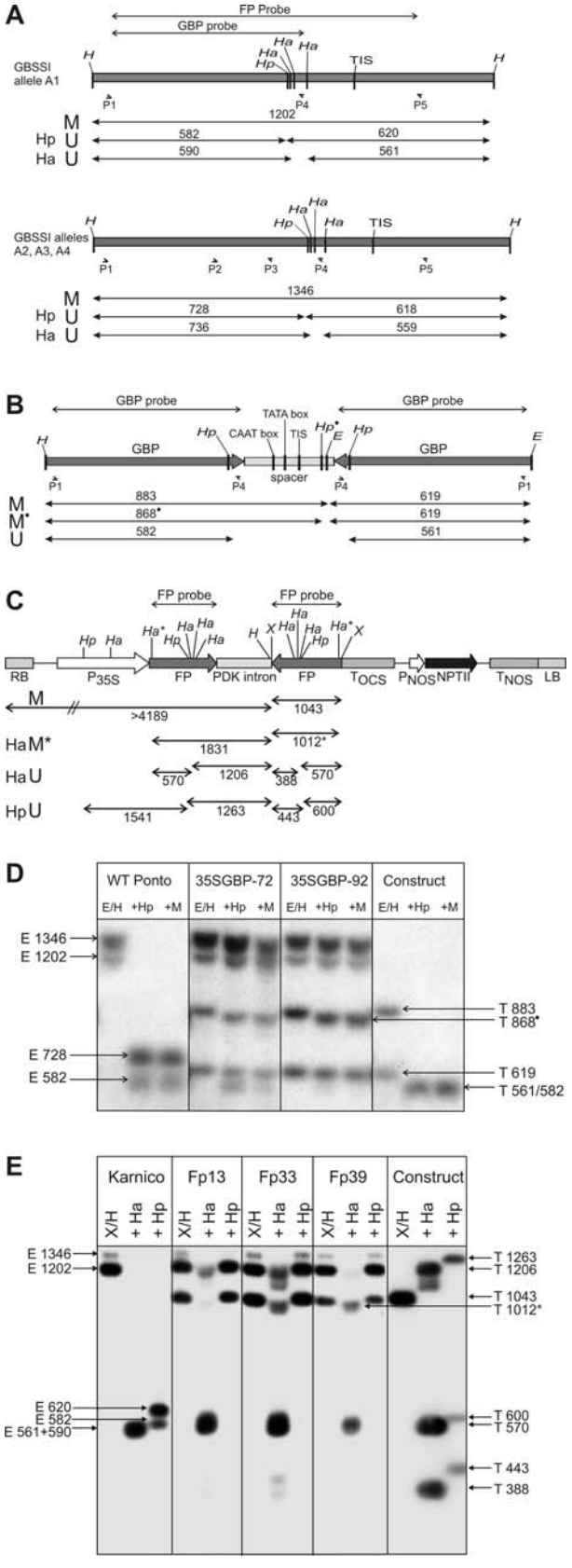


**Fig. 6** DNA methylation analysis in 35SGBP-IR and FP-IR transformants. **a** Expected hybridizing endogenous (E) fragments after digestion with *Hin*dIII and *Hpa*II or *Hin*dIII and *Hae*III. Fragments derived from the A1 allele as well as for the A2, A3 or A4 allele are indicated. *M* methylated, *U* unmethylated. Probes are indicated. P1, P2, P3, P4, P5, primers as indicated in Fig. 1. **b** Expected transgene (T)-derived fragments after digestion of 35SGBP-IR transformants with *Hin*dIII, *Eco*RI and *Hpa*II. *M* all *Hpa*II sites methylated, *M*•, no methylation of the *Hpa*II site in spacer, *U* all *Hpa*II sites unmethylated. **c** Expected transgene (T)-derived fragments for FP-IR transformants after digestion with *Xba*I/*Hin*dIII plus *Hae*III or *Hpa*II. Only relevant *Hae*III and *Hpa*II sites are indicated. *M* all *Hpa*II or *Hae*III sites methylated; *Ha M** all *Hae*III sites methylated but Ha* can be digested, *Ha U* all *Hae*III sites unmethylated, *Hp U* all *Hpa*II sites unmethylated. **d** Southern blot analysis showing the methylation of *Hpa*II and *Msp*I sites in 35S GBP-IR transformants. The GBP sequence was used as a probe. *First lane of each panel* shows a *Hin*dIII/*Eco*RI double digest (E/H). The *second lane* represents the triple digests with *Hin*dIII/*Eco*RI plus *Hpa*II (+Hp), and in the *third lane* the results of the triple digests with *Hin*dIII/*Eco*RI plus *Msp*I (+M) are shown. Sample 35SGBP-72 in the second panel is an example of partial methylation where accumulation of an additional fragment of about 582 bp is visible. In the third panel, an example of complete methylation (35SGBP-92) is shown. Wild-type Ponto and construct 35SGBP-IR are shown in the first and last panels, respectively. **e** Southern blot analysis showing complete or partial methylation of *Hpa*II and *Hae*III in FP-IR transformants. The FP sequence was used as a probe. *First lane of each panel* shows an *Xba*I/*Hin*dIII double digest (X/H). The *second lane* represents the triple digests with *Xba*I/*Hin*dIII plus *Hae*III (+Ha), and in the *third lane* the results of the triple digest with *Xba*I/*Hin*dIII plus *Hpa*II (+Hp) are shown. H, *Hin*dIII; E, *Eco*RI; Ha, *Hae*III; Ha*, *Hae*III sites followed by a G resulting in methylation insensitivity; Hp, *Hpa*II; X, *Xba*I. E fragments, endogenous GBSSI-derived fragments; T fragments, transgene-derived fragments. Sizes are indicated in bp

plete methylation and 8 showed partial methylation of the *Hpa*II site in the GBP region. No difference between the patterns of *Msp*I and *Hpa*II was observed indicating that the outer C in the CCGG sequence is always methylated, otherwise *Msp*I would be able to cut. No correlation between the silencing level and the level of methylation was observed (data not shown). Methylation of the *Hpa*II site at −213 bp relative to the TIS in the GBSSI promoter only influences the expression of the GBSSI gene to a certain extent since all transformants showed only weak silencing.

## Methylation status of transgene and endogenous GBSSI promoter in FP-IR transformants

DNA of FP-IR transformants was digested with *Hin*dIII and *Xba*I followed by hybridisation with the FP probe (Fig. 1a). This gave rise to transgene-derived fragments of 1,043 and >4,189 bp (Fig. 6c) while fragments of 1,202 and 1,346 bp (Fig. 6a) were obtained for the endogenous GBSSI sequences. The DNA was subsequently digested with methylation-sensitive restriction enzymes *Hpa*II and *Hae*III. Since *Hpa*II and *Msp*I gave the same methylation patterns in the analysis of the 35SGBP-IR transformants, it was decided to use only *Hpa*II to analyse CG methylation in the FP-IR transformants. For the detection of CNN methylation, the *Hae*III enzyme was used. The recognition sequence for

*Hae*III is GGCC. When the outer C of this sequence is methylated digestion still occurs. However, if the inner C is methylated digestion is prevented.

In Fig. 6a, c, the expected sizes of the *Hpa*II and *Hae*III-digested fragments are shown for the endogenous and the transgene-derived sequences, respectively. In Fig. 6e, the results of the Southern blot are shown. Endogenous (E) and transgene-derived (T) fragments are indicated with their sizes in bp. Similar to the analysis of the 35GBP-IR transformants, fragments of 1,202 and 1,346 bp were obtained for the endogenous GBSSI sequence. In wild-type Karnico, both fragments are converted to smaller fragments after digestion with *Hpa*II and *Hae*III. The lack of conversion to smaller fragments after digestion with *Hpa*II in the FP-IR transformants indicates that the *Hpa*II site is methylated in the endogenous as well as in the transgene-derived sequences. The endogenous fragments seem to disappear after digestion with *Hae*III but transgene-derived fragments appear at almost the same height as the original 1,202-bp A1 fragment. However, the intensity is much lower than the intensity of the endogenous 1,202-bp fragment. This indicates that the *Hae*III restriction sites in the A1 endogenous allele are not or only partially methylated. It is clear that the endogenous A4 allele (E 1346) is never methylated at all three internal *Hae*III sites simultaneously, since the 1,346-bp fragment has disappeared after *Hae*III digestion. The intense signal observed at the height of 560–590 bp most likely represents non-methylated endogenous as well as transgene-derived fragments. The presence of non-methylated transgene-derived products indicates that the methylation of transgene fragments at *Hae*III sites is partial.

The 1,043-bp transgene fragment (T 1043) seems to shift to the position of a slightly smaller fragment. Analysis of the *Hae*III restriction sites revealed that the inner Cs of the three *Hae*III sites in the vicinity of the *Hpa*II site are all in a CNN context. However, the *Hae*III site close to the *Xba*I site in the antisense FP sequence (Ha* in Fig. 6c) is followed by a G, implicating that the cleavage at this *Hae*III site cannot be blocked by methylation (New England Biolabs, Catalogue 2004/2005). Observation of the slightly smaller transgene fragment of 1,012 bp (see Fig. 6c, Ha M*, and Fig. 6e, T1012*) thus implies that methylation of all three *Hae*III sites close to the *Hpa*II site in the FP region occurs. The same phenomenon was found for the *Hae*III site that is followed by a G in the sense FP sequence (also indicated by an asterisk in Fig. 6c). Digestion of the FP-IR transformants with *Hae*III resulted in a 1,831-bp fragment (Fig. 6c, Ha M*), which indicates that the three *Hae*III sites close to the *Hpa*II site within the sense FP sequence could not be digested, but the *Hae*III site at the extreme 5′ end (Ha*) could (data not shown).

Six FP-IR transformants were analysed with *Hae*III and *Hpa*II. Five strongly silenced transformants showed complete methylation of *Hpa*II, whereas one weakly silenced transformant showed partial methylation of *Hpa*II. The *Hae*III sites were partially methylated in all transformants. From these data, it seems that CG methylation is complete whilst methylation at the non-CG sites is only partial.

To address whether methylation could spread outside the inverted repeat region in the FP-IR transgenes a new approach with another CNN methylation-sensitive enzyme was used. DNA was first digested with *Dra*I and *Hin*dIII, resulting in an antisense-specific and a sense-specific transgene-derived fragment. The second digest was then performed with *Dde*I which also has restriction sites outside the inverted repeat region. Four transformants varying in T-DNA integration number were subjected to these treatments together with wild type and FP-IR construct DNA. Again, partial methylation was observed in all transformants. However, in Fp1 spreading of methylation beyond the region capable of producing dsRNA was detected. No spreading of methylation was found in the other three transformants (data not shown).

## Discussion

### GBSSI partial promoter inverted repeats

The use of the ASP inverted repeat in order to selectively silence some GBSSI alleles did not lead to efficient silencing. The percentages of transformants showing weak silencing effects (4–5.5%) were too low to distinguish effects between the two potato cultivars that varied in allele composition. Therefore, this construct cannot be used to selectively silence GBSSI alleles. It would have been preferable to test the ASP inverted repeat construct in a potato cultivar without an A1 allele. However, among 52 potato cultivars and genotypes analysed, not one lacked the A1 allele. The role of the allele-specific sequence in the A2, A3 and A4 alleles is not known and homology to any known sequences other than the GBSSI promoters was not found by BLAST searches. If this sequence is not important in the functioning of the GBSSI promoter, its methylation will not affect the expression of GBSSI.

On the other hand, silencing of the A2, A3 and/or A4 allele does not necessarily have to influence the amylose content since the expression of one GBSSI allele can still be sufficient to obtain GBSSI activity comparable to wild type. Flipse et al. (1996) tested a gene-dosage population that varied in the number of functional GBSSI alleles. Even a simplex genotype with one functional GBSSI allele and three mutated GBSSI alleles (Aaaa) showed amylose percentages of 16% or higher. Compared to wild type where the amylose percentage is ±20% (Shannon and Garwood 1984), this percentage is high. In a few plants containing one functional GBSSI allele, starch granules were completely blue with a small red outer layer. This phenotype was also found in a few of our ASP transformants which might be caused by the expression of the A1 allele.

Allele-specific silencing might be analysed by allele-specific RT-PCR. However, in the case of the GBSSI alleles the coding regions and even the 3′ UTRs are highly homologous (97–99% identical). This means that it is difficult to design an allele-specific primer. Alternatively, real-time PCR might be performed to analyse the extent of reduction of GBSSI transcripts in the weakly silenced transformants compared to the wild-type genotypes. It would also be interesting to compare the level of reduction between the Ponto transformants and the Karnico transformants. It would be expected that reduction in GBSSI transcription is the highest in the Ponto transformants, which contain three non-A1 alleles, whereas the Karnico transformants only contain one non-A1 allele.

A more efficient silencing was obtained with the 35SGBP-IR construct targeting the region from −766 to −168 bp relative to the TIS. In this case, a weak silencing effect was observed in 57–60% of the transformants. The same sequence was included in GBP-IR where no transcribing promoter immediately upstream of the IR was present. Of the 58 GBP-IR transformants, only one showed a weak silencing effect. Apparently, production of dsRNA through read through transcription of neighbouring promoters, such as the NOS promoter that drives the expression of the *nptII* gene, only occurs at low frequency.

## Inclusion of sequences surrounding the TIS enhances silencing efficiency

The most efficient silencing was observed in the FP-IR transformants. In these transformants, the sequences −766 bp until +194 bp relative to the TIS were targeted. Since efficient silencing was obtained with the latter construct, we believe that inclusion of sequences in the vicinity of the TIS are important. Van der Steege et al. (1992) describe promoter-controlled GUS expression using different regions of the GBSSI promoter. They demonstrated that the sequence –346 bp until +54 bp relative to the TIS was still functional. This indicates that this sequence or part of it is important for functionality of the GBSSI promoter. If this sequence is not silenced, it is likely that the promoter will maintain its activity. This sequence was not included in the ASP-IR which might explain the low silencing efficiency obtained with this construct.

Mette et al. (2000) induced TGS of the NOS promoter by production of NOS promoter dsRNA through an inverted repeat. The region used in this inverted repeat comprised the region −264 to +34, relative to the TIS. They also created NOS promoter inverted repeats in planta by site-specific recombination. For this purpose, they used a direct repeat (DR) comprising two fragments in sense orientation; one from position −264 to −1 followed by a second one from position −264 to −67. One of these was flanked by loxP sequences. Following conversion of the NOS promoter DR into an IR by crossing in the *Cre* gene, transcriptional silencing of the NOS promoter was induced. This indicates that dsRNA targeting the region from position −264 to −67 is sufficient to induce silencing of the NOS promoter. From these results, it becomes clear that silencing of the NOS promoter can be obtained, regardless of whether the TIS is included in the inverted repeat. Sijen et al. (2001) tested several regions of the 35S promoter for their ability to silence a 35S promoter driving a *chsA* inverted repeat in *Petunia*. Promoter inverted repeats containing the full promoter (35Sfull) and enhancer sequences (35Senh) were able to induce silencing of the 35S promoter whilst promoter inverted repeats targeting the minimal 35S promoter (−90 to +30, relative to the TIS) only led to partial silencing of the 35S promoter in three out of five transformants. The 35S-full sequence (−614 to +36, relative to the TIS) does contain a TIS whilst the 35S-enh sequence (−614 to −65, relative to the TIS) does not. Both sequences were able to cause silencing of the 35S promoter. As was found for the NOS promoter, it seems that silencing of the 35S promoter can be obtained with or without inclusion of the TIS in the dsRNA. The low silencing efficiency obtained with the minimal 35S promoter construct might be caused by a failure of dsRNA production since no dsRNA of the 35S promoter could be detected in the transformants.

Sijen et al. (2001) described silencing of the endogenous *dfrA* promoter in *Petunia*. For this purpose, they used the promoter sequences from position −1,823 to −35, relative to the TIS. They were able to induce TGS using this sequence. At a later stage, TGS of the same promoter was induced by other promoter inverted repeat constructs (J. Kooter, personal communication). Promoter sequences that were located more than 600 bp upstream of the TIS were not able to inactivate the promoter, despite methylation of these sequences. Instead, constructs targeting a 500-bp region upstream of the TIS were efficient silencing inducers (J. Kooter, personal communication). Thus, it seems important to include regions in the vicinity of TIS in order to obtain efficient TGS.

In some of the FP transformants, different silencing levels were found within a microtuber of a single transformant. We believe that the starch granules showing the same level of silencing are of clonal origin since they seem to be derived from the same area in the microtuber. This variegated pattern is probably caused by the clonal and cell-autonomous character of TGS (Vaucheret et al. 1998; Qin et al. 2003). A similar observation was made by Sijen et al. (2001) who found that *Petunia* transformants in which the *dfr*A promoter was silenced had flowers containing sectors of white and light purple cells.

## Methylation studies

Examination of the methylation status of the GBSSI promoter in 35SGBP-IR transformants revealed that the

*Hpa*II site within the targeted 599-bp region was completely or partially methylated. An example of partial methylation is shown in Fig. 6d where an additional fragment approximately 582 bp in size accumulates in the transformant 35SGBP-72 (panel 2). However, the intensity of the endogenous (1,202 and 1,346 bp) and transgene-derived (883 and 619 bp) fragments was similar in *Hpa*II-treated and non-treated DNA in all the transformants indicating that the majority of the *Hpa*II sites are completely methylated. Apparently, the methylation of this site only inhibits the activity of the promoter to a small extent since transformants showed only weak silencing effects. That methylation of promoter sequences does not necessarily lead to silencing has been demonstrated before by Sijen et al. (2001). They showed that partial methylation of a 35S promoter did not prevent the promoter from being active.

Methylation of *Hpa*II indicates CG methylation. CG methylation at the *Hpa*II site was also found in the silenced FP-IR transformants. A clear difference between CG and CNN methylation was found in the FP-IR transformants. Whilst CG methylation at the *Hpa*II site was complete, CNN methylation at the *Hae*III sites was only partial. CNN methylation was also tested at the *Dde*I sites, which showed partial methylation as well. The methylation studies were performed with DNA isolated from in vitro-grown plants. Possibly, DNA from mature greenhouse-grown plants would be more extensively methylated at asymmetric Cs (in a CNN context).

The methylation studies with *Dde*I suggested that methylation could spread outside the inverted repeat region. Methylation is thought to be primarily restricted to the region of RNA–DNA sequence identity. There is no or hardly any spreading of methylation into the adjacent DNA sequences (Wassenegger 2000; Aufsatz et al. 2002; Vogt et al. 2004). In transformant Fp1, we detected spreading of CNN methylation within the T-DNA sequence in the 5′ direction over a long distance of 1,000 bp. This transformant harboured nine T-DNA integrations. No spreading of methylation was found in three other FP transformants harbouring one, two or three T-DNA integrations. The spreading of CNN methylation in the Fp1 transformant could be a consequence of the high T-DNA integration number. A complex locus, for example, might be responsible for the induction of methylation of Cs in the T-DNA outside the promoter inverted repeat sequence.

## Concluding remarks

We have shown that an endogenous promoter in potato can be transcriptionally silenced in transformants harbouring promoter inverted repeat constructs. The full GBSSI promoter inverted repeat construct containing sequences from −766 to +194 bp relative to the TIS induced the most efficient silencing with 49% of the transformants showing strong silencing. Although the 35SGBP-IR construct induced CG methylation of the *Hpa*II site at position −213 bp relative to the TIS, only weak silencing effects were observed. We observed that it is important to include sequences in the vicinity of the TIS. No GBSSI transcript could be detected in the strongly silenced FP-IR transformants by Northern blot analysis indicating that silencing is very effective.

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