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Post-transcriptional gene silencing of GBSSI in potato: effects of size and sequence of the inverted repeats

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Abstract

In the past, silencing of granule-bound starch synthase (GBSSI) in potato was achieved by antisense technology, where it was observed that inclusion of the 3′ end of the GBSSI coding region increased silencing efficiency. Since higher silencing efficiencies were desired, GBSSI inverted repeat constructs were designed and tested in potato. First, large inverted repeats comprising the 5′ and the 3′ half of the GBSSI cDNA were tested. The 5′ IR construct gave a significantly higher silencing efficiency than the 3′ IR construct. Since it was not known whether the observed difference was due to the sequence or the orientation of the inverted repeat, the GBSSI cDNA was divided into three regions, after which each region was tested in small inverted repeats in two orientations. To this end large numbers of independent transformants were produced for each construct. The results suggested that there was no effect of inverted repeat orientation on silencing efficiency. The percentage of transformants showing strong inhibition varied from 48% for a 3′-derived construct to 87% for a 5′ as well as a middle region-derived construct. Similar to the large inverted repeats, the 3′ sequences induced the least efficient silencing implying that the observed differences in silencing efficiency are caused by sequence differences. The small inverted repeat constructs with a repeat size of 500–600 bp and a spacer of about 150 bp were more efficient silencing inducers than the large inverted repeat constructs where the size of the repeat was 1.1 or 1.3 kb whilst the size of spacer was 1.3 or 1.1 kb. The results presented here show that size and sequence of the inverted repeat influenced silencing efficiency.

Abbreviations: dsRNA, double-stranded RNA; GBSSI, granule-bound starch synthase I; IR, inverted repeat; PTGS, post-transcriptional gene silencing; RISC, RNA-induced silencing complex; siRNA, small interfering RNA; UTR, untranslated region; VIGS, virus-induced gene silencing

Introduction

In plants, post-transcriptional gene silencing (PTGS) is a mechanism which causes degradation of specific mRNA sequences. The phenomenon was initially described in studies on transgenic plants in which the introduction of a transgene led to loss of expression from the corresponding endogenous gene (Napoli *et al.*, 1990; van der Krol *et al.*, 1990a). Similar findings were described in *Neurospora crassa* where the phenomenon was called quelling (Cogoni *et al.*, 1996) and in animals in which it was called RNA interference (Fire *et al.*, 1998).

A key molecule in PTGS is double-stranded RNA (dsRNA), which is processed into small interfering RNAs (siRNAs) by the action of an RNAse III-like enzyme. These siRNAs, 21–25 nt in length, are incorporated into a multiprotein complex named the RNA-induced silencing complex (RISC). Activation of RISC results in the unwinding of the double-stranded siRNAs, which are used as guides to identify complementary RNAs. The target RNA is then cleaved opposite the center of the guide siRNA. Finally, the cleaved RNA is degraded by exoribonucleases (Cerutti, 2003).

In plants, dsRNA can be produced in different ways; first of all, single-stranded RNA viruses have double-stranded replication intermediates which are potent activators of PTGS (Angell and Baulcombe, 1997; Waterhouse *et al*., 2001). The second class of PTGS activators is represented by transgenic constructs containing inverted repeats. Such an inverted repeat encodes dsRNA, which triggers the onset of PTGS (Smith *et al*., 2000). The third source of dsRNA is a single-copy transgene, from which the mRNA is converted to dsRNA either by the action of an RNA-dependent RNA polymerase or by read-through transcription of promoters in the transgene or flanking DNA (Sijen and Kooter, 2000).

To study gene silencing in potato, several constructs for down-regulation of granule-bound starch synthase I (GBSSI) have been tested. GBSSI catalyses the synthesis of amylose in amyloplasts. Starch comprises two different glucose polymers: amylose and amylopectin. In wild type potato plants starch usually consists of approximately 20% amylose and 80% amylopectin (Shannon and Garwood, 1984). Amylose is composed of linear chains of α(1–4)-linked glucose residues with very few α(1–6) branches. Amylopectin, on the other hand, is a highly branched glucan with a specific clustered distribution of α(1–4) and α(1–6) linkages (Hizukuri, 1986; Manners, 1989).

In potato, inhibition of GBSSI has been achieved by transformation with antisense and sense GBSSI constructs. Silencing of GBSSI was more efficient with antisense constructs than with sense constructs (Wolters and Visser, 2000). For antisense inhibition, a positive correlation between the T-DNA integration number and the effect on silencing was found (Kuipers *et al*., 1995). Kuipers *et al*. (1995) compared eleven antisense constructs of different GBSSI sequences for their silencing ability in potato. In this study, they found that a sequence comprising 0.6 kb of the 3′ end of the GBSSI cDNA was important for GBSSI inhibition since constructs lacking this sequence resulted in substantially lower silencing efficiencies. The most efficient silencing was obtained with construct pKGBA50, which harboured the complete GBSSI cDNA in antisense orientation behind the GBSSI promoter. Depending on the cultivar used for transformation, this construct resulted in percentages of transformants showing strong silencing that varied from less than 1% to 23% (Heeres *et al*., 2002). In potato cultivar Karnico, this percentage was only 14%. Therefore, attempts to increase the silencing efficiency in potato cultivar Karnico were made by designing various GBSSI cDNA inverted repeat constructs.

Wesley *et al*. (2001) demonstrated that different regions of cDNA can induce silencing using (intron-spliced) hairpin RNA constructs. They described one example in which two regions of a cDNA were tested; the complete and the 3′ two-thirds of the cDNA of the *Arabidopsis* flowering repression gene FLC1. Both constructs resulted in 100% silencing indicating that the 3′ two-thirds of cDNA were sufficient to induce silencing of the FLC1 gene.

These observations show that different regions of cDNA can induce silencing when used in inverted repeats. However, reports in which cDNA sequences within one gene are compared, are limited. In this study, eight different inverted repeat constructs harbouring different regions of the potato GBSSI cDNA in sense–antisense or antisense–sense orientation relative to the promoter were made and tested in potato. By doing so, the effect of size and sequence on silencing of GBSSI could be determined. The results suggested that there was no effect of inverted repeat orientation on silencing efficiency, but the size and sequence of the inverted repeat did show an effect. We found that the 3′ sequences were less efficient silencing inducers than the sequences from the 5′ and middle region of the GBSSI cDNA. Small size inverted repeats turned out to be more efficient silencing inducers than the large size inverted repeats.

Materials and methods

Plant material and growth conditions

Potato cultivar Karnico (Averis Seeds, Valthermond, the Netherlands) was 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

For the design of the constructs IR 1.1S–A and IR 1.3A–S, the antisense GBSSI construct pKGBA50 was used (Kuipers *et al*., 1995). The 1.1 kb 5′ half of the GBSSI cDNA was excised from pWx1.1 (Visser *et al*., 1991) and cloned as an *Eco*RI fragment into vector pMTL25 (Chambers *et al*., 1988), resulting in plasmid pMTL1.1 (de Vetten *et al*., 2003). A 1.2-kb *Sal*I fragment was excised from this vector and cloned in the binary vector pKGBA50 resulting in vector IR 1.1S–A (Figure 1). In a similar way, the 1.3-kb 3′ region of the GBSSI cDNA was cloned from pWx1.3 (Visser *et al*., 1991) into the vector pMTL25, after which the fragment was excised with *Bam*HI. Cloning of this fragment into the binary vector pKGBA50 resulted in vector IR 1.3A–S (Figure 1).

To design the six small inverted repeat constructs, primers were designed on three different regions of the GBSSI cDNA (Visser *et al*., 1989; sequence deduced from the genomic sequence published by van der Leij *et al*., 1991). Primers used to amplify the different regions of the GBSSI cDNA and the sizes of the PCR products are as follows: 5F1 (5′ CAGAATTCCATTCGTTGTTT GTCATC 3′) and 5R1(5′ CAGGATCCATAGA TTTTTGAACCAGT 3′) (674 bp), 5F2 (5′ ACGAATTCTAGGACTCAGGAACCATA 3′) and 5R1 (548 bp), 5F1 and 5R2 (5′ CAGGATCCG CATCTTTGTATTGGTCA 3′) (519 bp), MF1 (5′ GCAAGCTTATCTGGACAATGAACTTA 3′) and MR1 (5′ CTGGATCCTTCTGCTCCTC AAGTCTG 3′) (761 bp), MF2 (5′ CGAAGCT TTCTCATTCCTTGCTACTT 3′) and MR1 (618 bp), MF1 and MR2 (5′ CTGGATCCTT CACAATCCCAGTTATG 3′) (488 bp), 3F1 (5′ CTGAATTCTTTGAGCAGGAGATTGAA 3′) and 3R1 (5′ CAGGATCCCCTTACCTACAAAA TCAT 3′) (680 bp), 3F2 (5′ CAGAATTCCCTT GTGGTCTCATTCAG 3′) and 3R1 (548 bp) and finally 3F1 and 3R2 (5′ TAGGATCCAGGGAGT GGCTACATTTT 3′) (504 bp), see Figure 1A.

For each region of the GBSSI cDNA, three PCR fragments were amplified. An *Eco*RI or a *Hin*dIII site was included at the 5′ part of each forward primer, whereas a *Bam*HI site was created at the 5′ part of each reverse primer. PCR products were cloned in pGEM-T or pGEM-Teasy (Promega) vectors followed by ligation of two PCR products in pBluescript SK+ (Stratagene), pMTL25 or pMTL24 (Chambers *et al*., 1988). Ligation took place either through the *Bam*HI site or through the *Eco*RI or *Hin*dIII site. Depending on the restriction site through which ligation was performed, antisense–sense or sense–antisense inverted repeats were obtained for the three cDNA fragments. The sense–antisense inverted repeats contained the sequence between the R2 and R1 primers as a spacer, while in the antisense–sense inverted repeats the sequence between the F1 and F2 primers acted as a spacer (Figure 1A). Inverted repeats were subcloned behind the potato GBSSI promoter in the binary vector pPGB-1s (Kuipers *et al*., 1995) through the *Xba*I or the *Bam*HI site (Figure 1B).

All constructs were transformed into *E. coli* DH5α (Invitrogen, Breda, the Netherlands). The large IR constructs (IR 1.1S–A and IR 1.3A–S) and the three small IR constructs with an antisense–sense orientation were transformed into *A. tumefaciens* strain AGL0 (Lazo *et al*., 1991) by triparental mating. The other three constructs were transformed to *A. tumefaciens* strain AGL0 by electroporation (Takken *et al*., 2000).

Transformation and regeneration

Internodal cuttings from *in vitro* grown plants of potato cultivar Karnico were used for transformation by *Agrobacterium tumefaciens*. These explants were precultured for one day on solid R3B (MS with 30 g/l sucrose, 1 mg/l BAP, 2 mg/l NAA) medium, 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 two 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

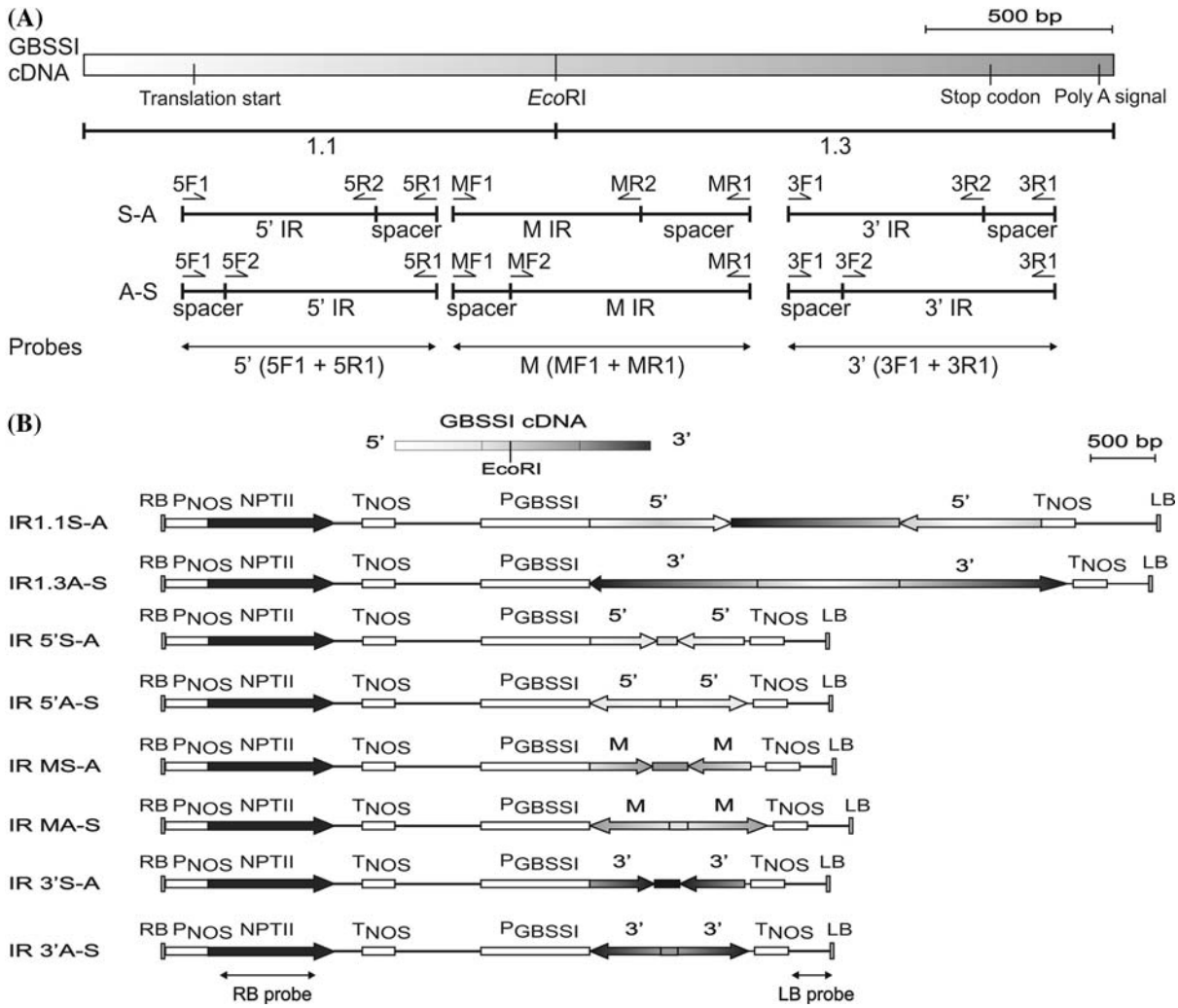

Figure 1. (A) Origin of potato GBSSI cDNA sequences used for the construction of the inverted repeats. Primers used to amplify the region-specific fragments are indicated. S–A, sense–antisense; A–S, antisense–sense. (B) Inverted repeat constructs of potato GBSSI cDNA. Inverted repeat constructs were cloned behind the potato GBSSI promoter in binary vector pPGB-1s. 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}GBSSI, promoter of GBSSI. RB and LB probes used for Southern analysis are indicated.

100 mg/l kanamycin. Explants were transferred to fresh medium every two to three 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*

Transgenic shoots were propagated *in vitro* on 50 ml MS medium with 30 g/l sucrose. After 3–4 weeks, 20 ml of liquid tuber-inducing medium was added. This medium (Duchefa, Haarlem, the Netherlands) contained 325 g/l sucrose and 1.75 g/l chlorocholine chloride (CCC). Transformants were then incubated at 18 °C in the dark. After 4–6 weeks, microtubers had developed on most shoots. This method was used to induce microtubers of the transformants of IR 1.1S–A, IR 1.3A–S and the small IR constructs in antisense–sense orientation. For the other transformants and transformants derived from repeated transformations, microtubers were induced by means of an

alternative microtuber induction method. Using this method, *in vitro* grown stem segments containing axillary buds were placed on MS medium containing 80 g/l sucrose and 5 μM BAP (Hendriks *et al.*, 1991). Incubation 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 the blue core is closely related to the amylose content (see Figure 4 in Kuipers *et al.*, 1994): the larger the blue core, the higher the amylose content. In starch granules with a small blue core the amylose content was close to 0%, indicating a reduction of 100%.

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. The transformants with granules with varying sizes of cores were classified as medium silenced. Examples of these classes are shown in Figure 2. Per transformant, three microtubers were stained and examined microscopically. Starch staining was also performed on granules of greenhouse grown tubers.

Southern analysis

Genomic DNA of the greenhouse-grown transformants was isolated from 0.5 to 2.0 g of leaves, as described by Tanksley *et al.* (1992). DNA (5 μg) was digested with *Hin*dIII, electrophoresed on a 0.8% agarose gel for 16 h at 30 V and subsequently vacuum blotted (Pharmacia) onto Hybond (N+) membranes (Amersham) in 0.4 M NaOH. A 722-bp fragment amplified with the *npt*II primers NPT3 (5′ TCGGCTATGACTGGGCACAACAGA 3′) and NPT4 (5′ AAGAAGGCGATAGAAGGCGATG CG 3′) was used as probe to check for integration of T-DNA sequences near the RB. For the LB probe, a 529-bp product amplified with primers NOStF (5′ATGAGATGGGTTTTTATGAT 3′) and NOStR (5′TTGAGTGTTGTTCCAGTTTG 3′) was digested with *Eco*RI. The 405-bp fragment, covering the T-DNA sequence near the LB, was excised and used as a probe. Probes were radioactively labelled with the Megaprime DNA labelling system (Amersham). Hybridisations were performed in glass bottles in a Hybaid hybridisation oven, at 65 °C for 16 h. The blots were rinsed twice with 2× SSC, 1% SDS, followed by a rinse with 1× SSC, 1% SDS.

Northern analysis

Total RNA was isolated from microtubers using Trizol agent (Sigma). RNA concentrations were measured using Ribogreen dye (Jones *et al.*, 1998). 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).

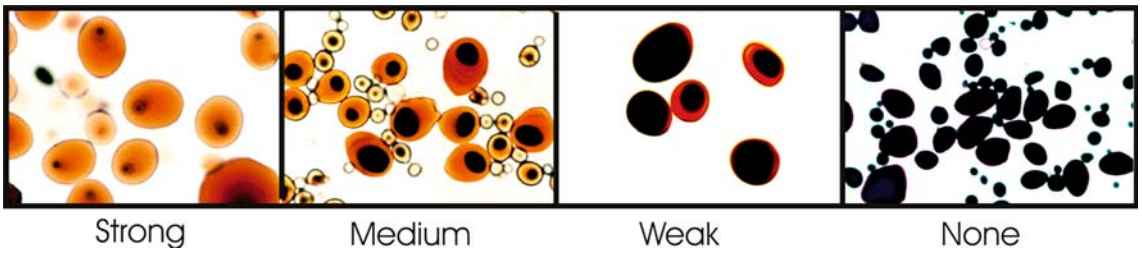

Figure 2. Visualisation of silencing effect of GBSSI in potato tuber starch granules of transformants showing strong, medium, or weak silencing, and of the wild type potato, stained with iodine solution.

Small RNA analysis

Total RNA was isolated from microtubers using Trizol agent (Sigma). From every sample, 20 μg of total RNA was electrophoresed, at first on a 6% polyacrylamide gel, and later on 15% polyacrylamide gels, for 1.5 h at 100 V using a vertical gel system (Biorad). To check for equal loading the same volume of RNA was electrophoresed on an agarose gel and visualised by ethidium bromide staining. RNA on the polyacrylamide gels was transferred onto Hybond N by overnight electro blotting at 25 V (Biorad). As probes, PCR fragments of the GBSSI cDNA were used (the 5′, M and 3′ probes in Figure 1A). Labelling and hybridisation experiments were performed as described for Southern analysis, using a hybridisation temperature of 50 °C. As a size indicator a DNA primer of 26 nt present in the analysed region (5′, M, or 3′) was included on the RNA gel. DNA oligos migrate approximately 10% faster than RNA markers of equal length (Sambrook *et al*., 1989, p. 7.76; Bonifacio *et al*., 1997; Hamilton *et al*., 2002; Miki *et al*., 2005). Therefore, the position of the 26-nt DNA oligo is expected to correspond to a 23-nt RNA oligo.

Statistical analysis

The effect of transformation experiment on silencing efficiency was tested with the Kruskal–Wallis One-Way Analysis of Variance using an α of 0.05. To test whether the silencing effects between constructs were significantly different, a binomial test was used whereby P^s is the number of strongly silenced transformants/total number of transformants, P^t is the number of silenced transformants/total number of transformants. The null hypothesis of no difference between proportion of (strongly) silenced transformants was rejected at an experiment-wise type I error of 0.05.

Results

Inverted repeats differ in silencing efficiency

Potato variety Karnico was initially transformed with two inverted repeat constructs; IR 1.1S–A harbouring an inverted repeat of the 5′ half of the GBSSI cDNA in sense–antisense orientation and IR 1.3A–S containing an inverted repeat of the 3′ half of the GBSSI cDNA in antisense–sense orientation. Construct IR 1.1S–A contained the 3′ half of the GBSSI cDNA as a spacer, whereas in construct IR 1.3A–S the 5′ half of the cDNA functioned as a spacer (Figure 1). Both inverted repeats were transcribed from the GBSSI promoter. In antisense GBSSI constructs, the use of this promoter resulted in a higher percentage of transformants showing strong inhibition than the use of the 35S promoter (Kuipers *et al*., 1995).

After transferring these constructs to potato, microtubers were induced and starch granules were stained with iodine to determine the percentage of transformants in which the GBSSI gene was silenced. Depending on the size of the blue core in starch granules, transformants were classified into four silencing groups: strong, medium, weak or none (Figure 2).

When compared with the efficiency of antisense construct pKGBA50 (Kuipers *et al*., 1995; Heeres *et al*., 2002), both inverted repeat constructs resulted in an almost 2-fold increase in frequency of silenced transformants (Figure 3, Table 1). However, if we consider the percentage of transformants that show strong silencing, a significant difference was observed between the two inverted repeat constructs. The IR 1.1S–A construct showed strong silencing in 62% of the transformants whereas this percentage was only 20% in IR 1.3A–S transformants.

Effect of orientation, size and sequence on silencing efficiency

Since it was not clear whether the differences between IR 1.1S–A and IR 1.3A–S were caused by the orientation of the inverted sequences or by their different sequences, new constructs varying in orientation and sequence were designed. Six inverted repeat constructs with a repeat size of 500–600 bp and a spacer of about 150 bp were made. These constructs contained inverted repeats of the 5′ part, the middle part and the 3′ part of the cDNA in two different orientations relative to the promoter: sense–antisense or antisense–sense orientation. The inverted repeats of the two constructs per region were not completely matched, since different parts of the largest PCR fragment per region were used as a spacer in the two different orientations (Figure 1A). The six small

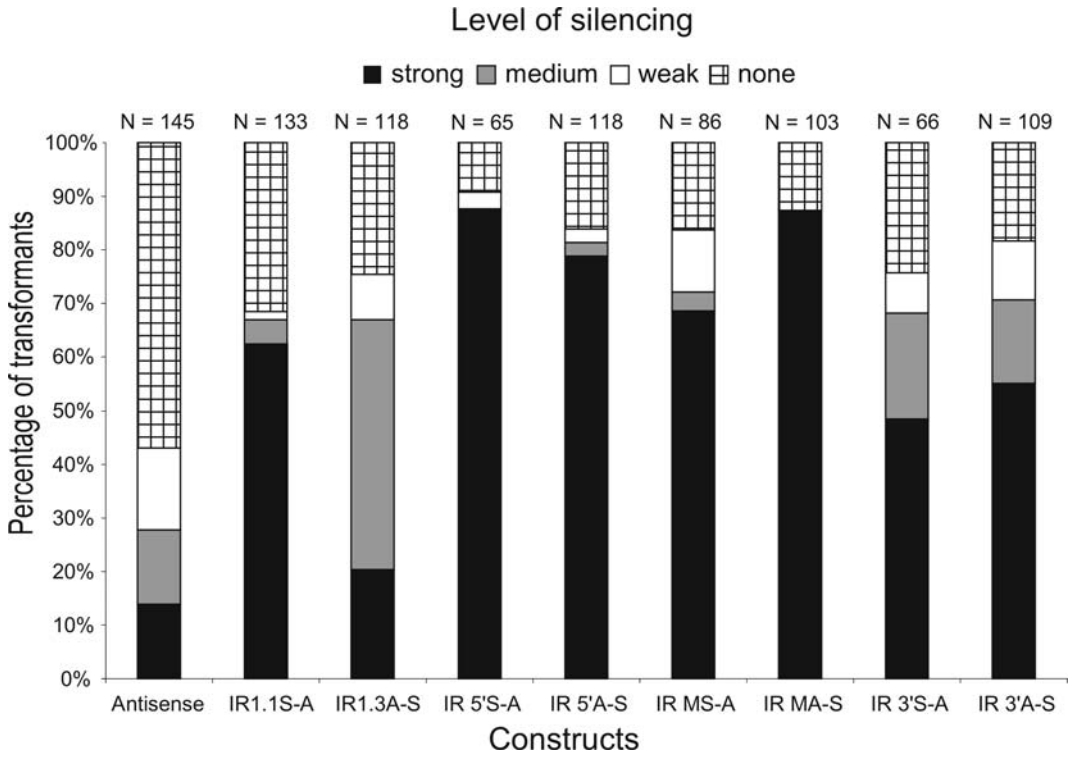

Figure 3. Silencing of GBSSI in Karnico transformants obtained with the antisense construct pKGBA50 (Heeres *et al*., 2002), the large cDNA inverted repeat constructs IR 1.1S–A and IR 1.3A–S, and the small cDNA inverted repeat constructs IR 5′S–A, IR 5′A–S, IR MS-A, IR MA-S, IR 3′S–A and IR 3′A–S. N, number of transformants.

Table 1. Total number of transformants, and number (and percentage) of transformants showing strong, medium, weak or no silencing per construct.

Construct	No. of transformants	Level of silencing			
		No. strong (%)	No. medium (%)	No. weak (%)	No. none (%)
Antisense (pKGBA50)[a]	145	20 (14)	20 (14)	22 (15)	83 (57)
IR1.1S–A	133	83 (62)	6 (5)	2 (2)	42 (31)
IR1.3A–S	118	24 (20)	55 (47)	10 (8)	29 (25)
IR 5′S–A	65	57 (88)	0 (0)	2 (3)	6 (9)
IR 5′A–S	118	93 (79)	3 (2.5)	3 (2.5)	19 (16)
IR MS-A	86	59 (69)	3 (3)	10 (12)	14 (16)
IR MA-S	103	90 (87)	0 (0)	0 (0)	13 (13)
IR 3′S–A	66	32 (48)	13 (20)	5 (8)	16 (24)
IR 3′A–S	109	60 (55)	17 (16)	12 (11)	20 (18)

[a] The antisense construct pKGBA50 is described in Kuipers *et al*. (1995). The data from this construct is from Heeres *et al*. (2002).

inverted repeat constructs were tested in two independent transformation experiments performed in different years. Since the obtained results did not significantly differ between transformation experiments, as tested by the Kruskal–Wallis test, results of the two transformation experiments were cumulated per construct. The number of independent transformants per construct varied between 65 and 118. The effects of these six constructs on silencing of GBSSI in microtubers are shown in Figure 3. If we consider the percentage of transformants showing silencing (strong + medium + weak, see Figure 3, Table 1), all six inverted repeat constructs gave

rise to high percentages varying from 76% (IR 3′S–A) to 91% (IR 5′S–A). In contrast, the antisense construct only resulted in a silencing percentage of 43%.

However, the transformants showing strong silencing are the most interesting. Therefore, we analysed the influence of construct on the proportion of strongly silenced transformants. Here, clear differences were observed. Statistical analysis of the transformants showed that the proportion of strongly silenced transformants was significantly lower in 3′ IR transformants than in the 5′ IR and IR MA-S transformants. High silencing efficiencies were found for the IR 5′S–A and the IR MA-S construct. In both cases, 87% of transformants showed strong inhibition of GBSSI. The remaining 12% of transformants were not silenced in the case of IR MA-S, whereas for IR 5′S–A another 3% of transformants showed weak silencing, which brings the total percentage of transformants showing an effect to 91%.

No significant differences between the two orientations were observed in the six small inverted repeat constructs. When proportions of strongly silenced transformants were compared for all eight inverted repeat constructs and the antisense construct, the IR 1.3A–S construct yielded a significantly lower silencing efficiency than all other constructs with the exception of the antisense construct. The proportion of strongly silenced transformants obtained with IR 1.1S–A was significantly lower than the proportion obtained with IR 5′S–A and IR MA-S but significantly higher than the proportion obtained for IR 1.3A–S and antisense constructs.

To test whether the silencing level in microtubers was representing the silencing level in greenhouse-grown tubers, a selection of transformants was transferred to the greenhouse and silencing was assessed in starch granules of greenhouse-grown tubers. The level of silencing of GBSSI in greenhouse tubers was comparable to the level of silencing in microtubers implying that the observations in microtubers were reliable (data not shown).

For both the small and large inverted repeat constructs, the 3′ sequences of the cDNA were the least efficient silencing inducers. The results suggested that there was no effect of inverted repeat orientation on silencing efficiency. The small inverted repeat constructs were more efficient silencing inducers than the large inverted repeat constructs.

Number of T-DNA integrations and silencing

To be able to select transformants containing 1 or 2 T-DNA integrations, the number of T-DNA integrations was analysed by Southern blot hybridisation in a selection of transformants obtained with the eight inverted repeat constructs. *Hin*dIII-digested DNA of transformants was transferred to a membrane that was hybridised with an RB probe (Figure 1B) containing the *npt*II sequence. The distribution of T-DNA integration number is shown in Figure 4A. All eight constructs gave similar distributions of the number of T-DNA integrations.

In Table 2, the relation between T-DNA integration number and the number of transformants showing a silencing effect is shown for all IR constructs. Silenced transformants containing one T-DNA integration were found for all IR constructs. However, in this table, no distinction was made between transformants showing weak, medium or strong silencing.

To illustrate the relation between the number of T-DNA integrations and the level of silencing, the observations of transformants of all inverted repeat constructs were pooled and charted in Figure 4B. In every group of T-DNA integration number, all silencing classes are represented. The highest percentage of transformants showing strong silencing was found within the group harbouring more than two T-DNA integrations whereas the transformants showing no silencing mostly contained one or two T-DNA integrations. For every construct, except for IR 1.3A–S, it was possible to select strongly silenced transformants with a single T-DNA integration. The two IR 1.3A–S transformants with a single T-DNA integration showed weak silencing. Strongly silenced IR 1.3A–S transformants were found in the class harbouring two T-DNA integrations.

A selection of transformants containing 1 or 2 T-DNA insertions was further analysed for integration of T-DNA at the LB by hybridisation with the LB probe (Figure 1B). Out of nine non-silenced transformants, seven (78%) did not hybridise with the LB probe. Four out of 35 silenced transformants (11%) did not hybridise to the LB probe.

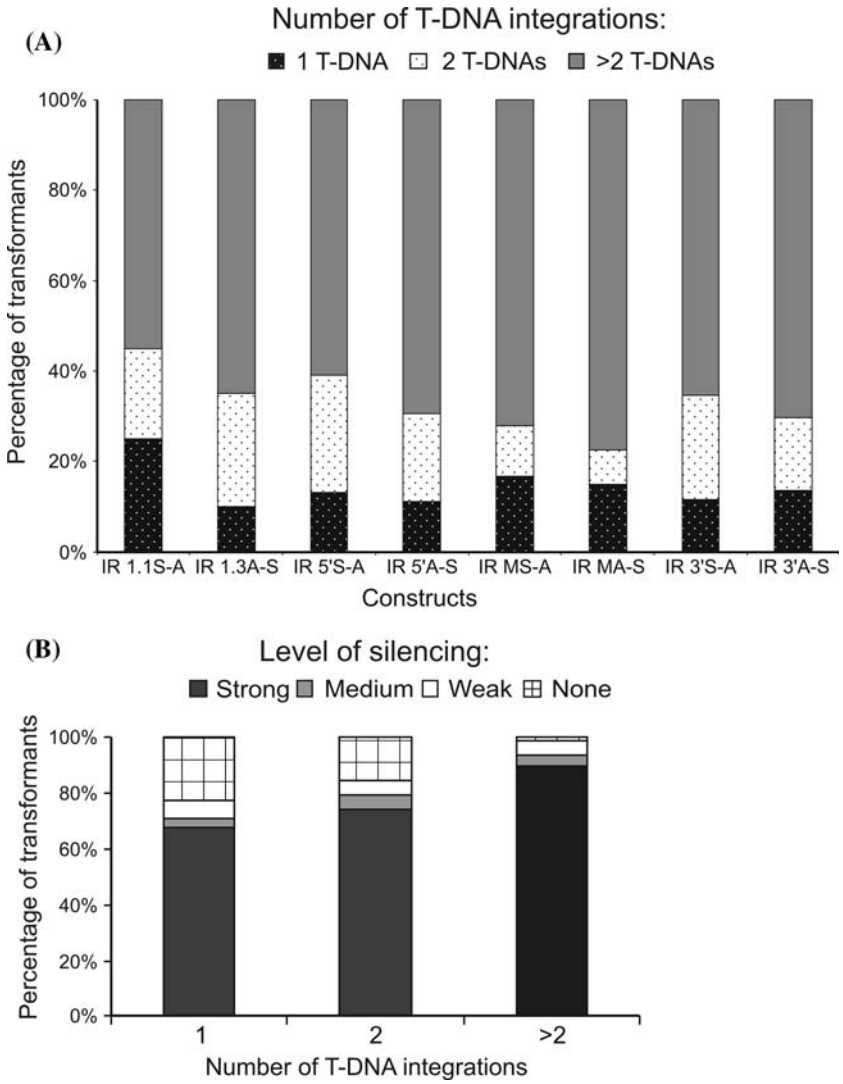

Figure 4. (A) Distribution of T-DNA integration numbers among transformants of different inverted repeat constructs. (B) Relation between number of T-DNA integrations and level of silencing in all IR transformants.

Silencing effect measured at RNA level

From the transformants of the six small inverted repeat constructs, 25 transformants with 1–2 T-DNA integrations were selected for Northern analysis. GBSSI mRNA levels were determined for these transformants by hybridising the mRNA with the complete GBSSI cDNA probe. Equal RNA loading was verified by hybridisation with a 28S ribosomal probe (data not shown).

Compared to wild type, all silenced transformants showed a reduction in mRNA level whereas non-silenced transformants produced equal amounts of transcript. In Figure 5A, the transcript level in seven transformants, harbouring inverted repeats of the middle GBSSI region, is shown. Even though GBSSI is still expressed in the silenced transformants, a reduction in relation to the expression in wild type is visible. From Figure 5A, it becomes clear that there is no distinct relation between the amount of transcript and the level of silencing. Transformant IR MS-A-43, for example, is weakly silenced but the abundance of transcript is comparable to the strongly silenced transformants.

To address whether the reduction in GBSSI mRNA level was accompanied by the production

Table 2. Number of T-DNA integrations in relation to silencing effect of transformants harbouring different inverted repeat constructs.

Construct	No. of transformants analysed	1 T-DNA		2 T-DNAs		> 2 T-DNAs	
		#sil	#non-sil	#sil	#non-sil	#sil	#non-sil
IR1.1 S–A	20[a]	5	nt	4	nt	11	nt
IR1.3 A–S	20[a]	2	nt	5	nt	13	nt
IR5′ S–A	23	3	0	5	1	14	0
IR5′ A–S	36	2	2	7	0	24	1
IRM S–A	18	3	0	1	1	12	1
IRM A–S	40	3	3	3	0	31	0
IR3′ S–A	26	2	1	4	2	17	0
IR3′ A–S	37	4	1	5	1	26	0
Total	220	24	7	34	5	148	2

sil, number of silenced transformants.
non-sil, number of non-silenced transformants.
nt, not tested.
[a] For these constructs, only silenced transformants were tested for the number of T-DNA integrations.

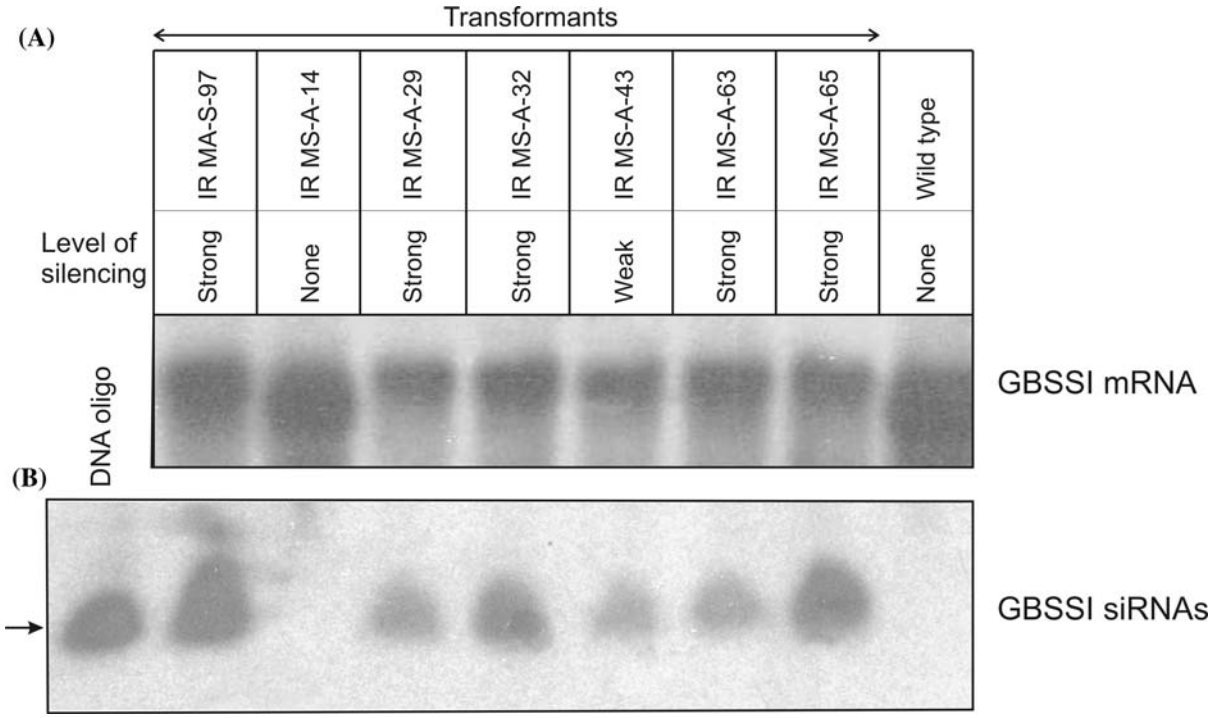

Figure 5. (A) Northern blot analysis showing the reduction of GBSSI mRNA steady state level in transformants harbouring inverted repeat constructs of the middle region of the GBSSI cDNA. Total RNA was hybridised with the complete GBSSI cDNA probe. (B) Production of small RNAs in transformants harbouring constructs of the middle region of the GBSSI cDNA. As a size indicator the 26-nt DNA primer MR2 was included. This DNA oligo is expected to migrate to a position corresponding to that of a 23-nt RNA oligo (see Materials and methods). The membrane was hybridised with the M probe (Figure 1A): a PCR fragment amplified from the middle region of the GBSSI cDNA with primers MF1 and MR1.

of siRNAs, we tested for the accumulation of siRNAs in transformants of the six small inverted repeats that contained 1 or 2 T-DNA integrations. Presence of siRNAs was detected in all 32 selected silenced transformants whereas no siRNAs were detected in the nine non-silenced transformants or wild type plants. Accumulation of siRNAs in seven transformants is shown in Figure 5B. Only

transformants showing silencing contained siRNAs. However, no clear correlation between the level of siRNAs and the level of silencing was found.

The small RNAs in Figure 5B were electrophoresed on a 6% polyacrylamide gel. Because of this, small RNAs were not very well separated. Therefore, we cannot draw a conclusion on the exact size of the GBSSI siRNAs in Figure 5B.

Discussion

Using inverted repeat constructs of the GBSSI cDNA, we were able to induce highly efficient silencing. The level of silencing may depend on several factors such as the type of construct, number of integrated T-DNAs, expression level and genetic background. All inverted repeat constructs were based on the same vector and a high number of transformants was analysed in one potato cultivar. Therefore, the variation due to position effects and genetic background is supposed to be similar for all constructs.

Strong silencing can be obtained with a single T-DNA integration of GBSSI inverted repeat

Of the inverted repeat transformants, a selection was analysed by Southern blot hybridisation with a RB-specific probe to determine the number of T-DNA insertions. Most of the non-silenced transformants contained one T-DNA integration (see Table 2, Figure 4B). This is logical since the chance that one T-DNA integration is integrated at an unfavourable position or is truncated is higher than the chance that this happens for two or more T-DNAs. However, transformants showing strong silencing were also found within the class of one T-DNA integration. This indicates that, in contrast to antisense transformants were multiple T-DNA integrations were required to obtain strong silencing in primary transformants, one T-DNA integration of an inverted repeat can cause strong gene silencing in primary transformants. In all T-DNA integration classes, transformants showing strong silencing occurred, indicating that there is no relation between the number of T-DNA integrations and the level of silencing. As shown in Figure 4A, the distribution of T-DNA integration number was similar for all constructs. Because of this, the possibility that the differences in silencing efficiency are due to differences in T-DNA integration numbers can be ruled out.

Out of nine non-silenced transformants, seven (78%) did not hybridise to a LB-specific sequence, indicating a deletion at the LB. In these seven transformants, the deletion probably extends to the region upstream of the LB probe (see Figure 1B), thereby affecting the production of dsRNA and resulting in a loss of silencing. From the 35 silenced transformants, four (11%) did not hybridise to the LB-specific sequence. In this case, small deletions at the LB that do not affect the production of dsRNA probably occurred.

mRNA levels

The strong silencing effects in inverted repeat transformants corresponded to a reduction in mRNA level, but a complete reduction was never observed. To verify that the observed signal was derived from GBSSI mRNA, RNA of three silenced transformants was analysed by RT-PCR, which confirmed the presence of both mature as well as premature GBSSI RNA (data not shown).

No correlation between the GBSSI mRNA level and the level of silencing was found. Kuipers *et al*. (1994) also could not detect such a relationship. Fluctuations in mRNA level were observed in tubers of several non-transformed potato genotypes. This may be the main cause of the lack of relationship between the GBSSI mRNA level and silencing level in transgenic plants since the amylose content, GBSSI activity and amount of GBSSI protein did correlate in several transgenic genotypes (Visser *et al*., 1991).

Orientation of inverted repeat probably does not affect silencing efficiency

Although it was originally believed that the orientation of the inverted repeat was of importance (Elbashir *et al*., 2001), our results suggest that this is not the case for silencing of GBSSI in potato. The sense–antisense and antisense–sense inverted repeats per region of the cDNA were not strictly matched. However, since the two constructs per region did not result in significantly different silencing efficiencies, this seems to indicate that the orientation of the inverted repeat

relative to the promoter does not affect the silencing efficiency. Therefore, we think that the difference in silencing efficiency between IR 1.1S–A and IR 1.3A–S are not caused by the inverted repeat orientation but rather can be ascribed to the sequence differences.

3′ GBSSI sequences are less efficient silencing inducers than 5′ sequences

All eight GBSSI cDNA IR constructs showed high silencing frequencies ranging between 68% and 91%. However, large differences were observed for the percentages of transformants showing strong silencing. These varied from 20% to 87%. The highest percentages of transformants showing strong silencing (87%) were obtained using the IR 5′S–A and the IR MA-S constructs. For the small as well as for the large inverted repeat constructs, the 3′ IR constructs resulted in the lowest percentages of transformants showing strong silencing.

To address whether the observed difference in silencing efficiency was due to the occurrence of putative polyadenylation signals, all inverted repeat constructs were screened for the presence of these signals. Polyadenylation signals are characterized by near upstream elements (NUE) and far upstream elements (FUE). Together with the cleavage site, these elements make up the plant polyadenylation signal (Rothnie, 1996). Putative polyadenylation signals were found both in constructs with a low as well as in constructs with a high silencing efficiency indicating that the observed differences could not be ascribed to the occurrence of putative polyadenylation signals. Kuipers *et al.* (1994) demonstrated that the presence of polyadenylation signals in 35S promoter-driven antisense GBSSI transformants resulted in a antisense transcript with a length smaller than expected based on the GBSSI cDNA. These smaller antisense transcripts were detected in leaves of silenced as well as in non-silenced 35S-driven antisense GBSSI transformants indicating that a premature stop of transcription did not result in the absence of antisense inhibition.

Another possible explanation for the observed lower silencing efficiency of the 3′ IR constructs might be the presence of sequences in the 3′ half of GBSSI cDNA that inhibit silencing. Reducing these inhibitory sequences by reducing the length might lead to a more efficient silencing. It is clear that no inhibitory sequences are present in the first 477 bp of the 3′ half since these sequences are present in the IR MA-S construct that gives excellent silencing. Therefore, if present, the inhibitory sequences must be present in the last 893 bp of the GBSSI cDNA.

During the different steps in the dsRNA processing pathway, several factors might influence silencing level. The position and efficiency of Dicer cleavage, which is influenced by attributes of the 3′ end structure of the dsRNA hairpin molecule, determines which siRNAs are formed. Slight 1–2 nt shifts in preferred cleavage position have been shown to dramatically alter the thermodynamic stability of siRNA termini (Vermeulen *et al.*, 2005). Schwarz *et al.* (2003) and Khvorova *et al.* (2003) have shown that the structure and thermodynamic stability of siRNAs are important for their functionality. They propose that the siRNA strand of which the 5′ end shows the weakest binding, is incorporated in RISC and guided to its complementary mRNA for cleavage. If this is the antisense strand, sense mRNA will be targeted and efficient silencing will occur provided that the target RNA is accessible. If the target RNA forms a secondary structure with a relatively low local free energy, base-pairing with the guide strand of the siRNA is prevented (Overhoff *et al.*, 2005; Schubert *et al.*, 2005). Whether the observed differences in silencing efficiency between the GBSSI inverted repeat constructs can be attributed to any of these factors or a combination of factors is not known since we did not investigate the properties of the GBSSI siRNAs.

The observation that 3′ IR constructs were the least efficient silencing inducers contrasts with the results obtained with the GBSSI antisense construct (Kuipers *et al.*, 1995) where 3′ sequences of GBSSI were more efficient than 5′ sequences. A similar phenomenon was observed for the chalcone synthase (CHS) gene in Petunia. van der Krol *et al.* (1990b) found effective inhibition of CHS when the antisense genes contained the full-length cDNA or a 3′ cDNA fragment. Recently, J. Kooter (pers. comm.) observed that when CHS was targeted by means of an inverted repeat construct, the 5′ end was a more effective silencing inducer than the 3′ end. Although in virus induced gene silencing systems, the most efficient silencing was induced by the 3′ (Braunstein *et al.*, 2002; English

et al., 1996; Sijen *et al*., 1996), the central (Marano and Baulcombe, 1998) and the 3′ region or the complete coding region (Sonoda *et al*., 1999), we feel that our results should be compared to results obtained from other stably transformed transgene systems.

Untranslated regions do not influence silencing efficiency

The cDNA used in the inverted repeat constructs was derived from the GBSSI A1 allele which is described by van der Leij *et al*. (1991). The allele composition of the tetraploid potato cultivar Karnico, which was used in our transformation experiments, is A1A1A1A4 (van de Wal *et al*., 2001). The endogenous GBSSI alleles are highly homologous (over 98%) in their coding sequences. Therefore, all alleles can be silenced using the coding region of the A1 allele as a silencing inducer. However, the alleles show variability in their untranslated regions (van de Wal *et al*., 2001). The homology between the A1 allele and a non-A1 allele (Hofvander *et al*., 1992) in the 3′ UTR sequence is 75%. Therefore, it is expected that the 3′UTR transgene-derived siRNAs will not be completely complementary to the endogenous A4 GBSSI allele. This could imply that the A4 allele is silenced less efficiently.

In two of the tested constructs, 3′ UTR sequences are present. IR 1.3A–S contains 304 bp of 3′ UTR whilst IR 3′A–S contains 176 bp of 3′ UTR. Although no 3′ UTR sequences are present in IR 3′S–A, it gives rise to a similar silencing efficiency as IR 3′A–S. Therefore, we can conclude that the inclusion of 176 bp of 3′ UTR sequences does not influence the silencing efficiency. In IR 1.3A–S, 304 bp of the 1300 bp dsRNA is represented by 3′ UTR sequences. From these 304 bp, we know that 176 bp do not affect silencing efficiency. It seems unlikely that the remaining 128 bp do affect silencing efficiency, especially not if we consider the large size of dsRNA corresponding to the homologous coding region. Therefore, we think that the lack of complementarity between the 3′ UTR sequences of the A1 and the A4 allele does not play a role in silencing efficiency.

In GBSSI IR constructs IR 1.1S–A and IR 5′S–A, 5′ UTR sequences are present. In GBSSI IR 5′S–A, 53 bp of the 5′ UTR are represented in the inverted repeat construct. The IR 1.1S–A construct comprises 258 bp of the 5′ UTR. Since efficient silencing was observed using constructs with or without 5′ UTR sequences, the presence of 5′ UTR sequences in GBSSI IR constructs does not seem to be essential for the induction of efficient silencing. Efficient silencing of 5′ UTR sequences has been observed by Wesley *et al*. (2001) who induced silencing of the 5′ UTR of Δ12-desaturase in cotton by targeting a 5′ UTR sequence of 98 bp. On the other hand, Jacobs *et al*. (1999) described that the 5′ UTR regions of the *gn1* mRNA are inefficient silencing inducers when expressed in chimeric viral RNAs.

Effect of size of GBSSI sequence in inverted repeat constructs

We found that, for GBSSI, the small inverted repeat constructs were more efficient silencing inducers than the large inverted repeat constructs. Differences in silencing efficiency were observed upon introduction of several inverted repeat constructs targeting the albino-1 gene in *Neurospora crassa* (Goldoni *et al*., 2004). Similar silencing frequencies were obtained when the length of the repeat was either 600 or 900 nucleotides but a reduction of the repeat size to 200 nucleotides produced a substantial decrease in the silencing efficiency. Akashi *et al*. (2001) tested length dependence of the RNAi effect in tobacco BY-2 cells by co-transformation of a luciferase gene construct and a luciferase dsRNA expression plasmid. No significant difference in silencing efficiency between a 300-bp and a 500-bp dsRNA expression plasmid was observed. Apparently, the presence of 300-bp dsRNA is sufficient to obtain efficient silencing and the presence of a longer dsRNA does not add to a more efficient silencing. Helliwell and Waterhouse (2003) have used gene fragments ranging from 50 bp to 1 kb to successfully silence genes. The shorter the fragments were the less frequently effective silencing was achieved. Sijen *et al*. (2001) used 1492 bp of the CHS gene in an inverted repeat with a spacer of 130 bp. The silencing obtained with this construct was comparable to the silencing obtained with the construct containing the 500 bp of the 5′ CHS region indicating that the 5′ 500-bp region was sufficient to induce silencing (J. Kooter, pers. comm.). Although Jacobs *et al*. (1999) described that

susceptibility to silencing in a viral system increased as the cDNA sequences increased in size, this did not apply to the cDNA sequences originating from the 5′ region. The silencing efficiency obtained with the complete *gn1* coding region (1294 bp) was lower than the silencing efficiency obtained with two smaller 5′ fragments. Together, these results suggest that the optimal size for an inverted repeat is 300–500 bp.

Size of spacer does not influence silencing efficiency of GBSSI

The small and large inverted repeat constructs differ in size of dsRNA as well as in size of the spacer. The size of the spacer in IR 1.1S–A and IR 1.3A–S is 1.3 kb and 1.1 kb, respectively, whereas the spacer in the small inverted repeat constructs is approximately 150 bp. If the size of the spacer would influence silencing efficiency, we should find significant differences between large and small inverted repeat constructs. Indeed, the IR 1.3A–S construct results in a silencing efficiency that is significantly lower than that found for all small inverted repeat constructs. However, of the IR 1.1S–A transformants, 62% show strong silencing which is not significantly different from the percentages obtained with two out of four of the small IR constructs that contain sequences overlapping with IR 1.1S–A. Therefore, it seems that the size of the spacer does not influence silencing efficiency of GBSSI in potato. In contrast, Xiong *et al.* (2005) reported that the spacer size did influence silencing efficiency of ACC oxidase in tomato transformants. They tested a 1002-bp and a 7-bp spacer in an ACC oxidase inverted repeat construct with a repeat size of 501 bp. The construct with the large spacer clearly gave lower percentages of transformants with complete silencing than the construct harbouring the small spacer. They did not test a spacer size in between these two extreme sizes. Since a 7-bp spacer usually does not result in stable inverted repeats this does not seem to be a generally applicable method to achieve a higher silencing efficiency.

Concluding remarks

We have observed that the 3′ half of GBSSI cDNA is a less efficient silencing inducer than the 5′ half. Comparing these observations with studies performed in systems where transgenes induced silencing of homologous sequences expressed in viruses, hardly any similarities were found. However, when we compared our finding with those found for the CHS gene in *Petunia*, we did observe similarities. Since both PTGS of GBSSI in potato and of CHS in *Petunia* are examples of transgene-induced silencing of endogenous genes, the mechanism is probably similar. Our results suggest that the orientation of an inverted repeat construct does not affect the silencing efficiency. Therefore, inverted repeat constructs can be designed in the orientation that fits best with available vectors. Finally, we demonstrated that silencing efficiency does not necessarily increase with the size of the targeted sequence. In our case, small inverted repeats of 500–600 bp separated by a spacer of approximately 150 bp were very efficient silencing inducers.

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