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Presence of an intron in inverted repeat constructs does not necessarily have an effect on efficiency of post-transcriptional gene silencing

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Abstract

The effect of introns on silencing efficiency was tested in inverted repeat constructs of Granule-Bound Starch Synthase (*GBSSI*) cDNA by comparing the silencing efficiencies induced by inverted repeat constructs with and without introns. No effect could be attributed to the presence of introns indicating that the introns neither enhance nor inhibit post-transcriptional gene silencing. The effect of a spliceable intron in the spacer was studied by comparing constructs harbouring a spliceable or a non-spliceable intron in the spacer. As opposed to the general belief that splicing of an intron increases silencing efficiency, the use of a spliceable intron in the spacer did not result in enhancement of silencing in our experimental system.

Abbreviations: dsRNA – double-stranded RNA; GBSSI – Granule-Bound Starch Synthase I; IME – intron-mediated enhancement; PTGS – Post-Transcriptional Gene Silencing; siRNA – small interfering RNA

Introduction

To study gene silencing in potato, different constructs for down-regulation of Granule-Bound Starch Synthase (GBSSI) have been tested. GBSSI catalyses the synthesis of amylose in amyloplasts. In potato, inhibition of GBSSI has been achieved by transformation with antisense *GBSSI* constructs (Visser et al. 1991; Hofvander et al. 1992; Kuipers et al. 1994; Heeres et al. 2002).

In antisense *GBSSI* silencing, genomic DNA constructs were less efficient silencing inducers than cDNA constructs. Both types of construct were tested for the full length *GBSSI* as well as for a partial *GBSSI* sequence (Kuipers et al. 1994). The authors attributed the lower efficiency of genomic DNA antisense constructs to the lower stability of the duplex formed with the endogenous mRNA. Because of the presence of introns, genomic antisense RNA will form a duplex with the mRNA with alternating stretches of higher and lower stability which will be less stable than the duplex involving the cDNA-based antisense RNA. Developments in the last decade have taught us

that it may not be the duplex between the antisense RNA and the endogenous mRNA that causes post-transcriptional gene silencing (PTGS). Fire et al. (1998) demonstrated that dsRNA is a potent trigger for RNA interference in *C. elegans*. This dsRNA is processed into small interfering RNAs (siRNAs) by the action of an RNAse III-like enzyme, named Dicer in *Drosophila* (Cerutti 2003). These small interfering RNAs were first shown to be produced in plants undergoing PTGS and were identified as a hallmark of RNA silencing pathways (Hamilton and Baulcombe 1999). The finding that genes involved in RNA interference were identified in *Drosophila*, plants, worms and fungi reflects the fact that RNA silencing phenomena in these organisms share a common underlying molecular mechanism. From these results it was clear that dsRNA triggers degradation of homologous RNA sequences in a variety of organisms. This dsRNA can be formed through the presence of multiple transgene copies arranged as inverted repeats or through read-through transcription from neighbouring promoters. Another possibility is that the transgene RNA is recognized as aberrant and is made double-stranded by the action of a RNA-dependent RNA polymerase. At present, dsRNA is often, intentionally, produced through transcription of inverted repeat constructs harbouring cDNA sequences. In general, no introns are present in cDNA sequences. The potato *GBSSI* genomic DNA contains 13 introns. If dsRNA would be made from the genomic DNA, it would contain many bulges because of antisense introns that can not be spliced. Whether these bulges affect the stability of the dsRNA is not known. On the other hand, it is known that some introns can enhance gene expression. In plants, introns can act post-transcriptionally to increase mRNA accumulation by stabilizing the transcript. Most examples of intron-mediated enhancement have been described in maize (Callis et al. 1987; Mascarenhas et al. 1990; Luehrsen and Walbot 1991; Maas et al. 1991; Rethmeier et al. 1997), but also in *Petunia* (Dean et al. 1989) and *Arabidopsis* (Rose 2002; Wang et al. 2002). Smith et al. (2000) and Wesley et al. (2001) demonstrated that the presence of an intron in the spacer of inverted repeat constructs enhanced the silencing effect. These observations might be based on the same phenomenon, i.e. a more stable production of mRNA or dsRNA through splicing of intron sequences.

To address whether the presence of introns in dsRNA either enhances or inhibits gene silencing, we compared the efficiency of a 5′ antisense-sense *GBSSI* inverted repeat with and without the second intron of *GBSSI*. The same approach was used for an antisense–sense *GBSSI* inverted repeat harbouring sequences from the middle region of the cDNA where the effect of the presence of intron 9 was tested. Besides, the effect of the splicing of an intron in the spacer was studied by comparing constructs harbouring a spliceable or a non-spliceable intron in the spacer.

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^{-1} sucrose and 8 g l^{-1} agar, at 24 °C, and a photoperiod of 16 h light at an irradiance of 40 μmol m^{-2} s^{-1}.

cDNA synthesis

Total RNA was isolated from microtubers of potato cultivar Karnico using Trizol agent (Sigma). About 2 μg RNA was treated with 5 U RNAse-free DNAse (Amersham) for 10 min at 37 °C, after which the DNAse was inactivated through incubation for 15 min at 65 °C in 0.0025 M EDTA. cDNA was synthesized on 500 ng RNA using Superscript II Reverse Transcriptase according to the manufacturer's protocol (Invitrogen). 2 μl was used for PCR amplification in a volume of 50 μl using cDNA region-specific primers (see below under 'DNA constructs'). PCR fragments were cloned in pGEM-Teasy (Promega) and then sequenced.

DNA constructs

GBSSI cDNA inverted repeat constructs were made as described by Heilersig et al. (2006). The *GBSSI* cDNA (Visser et al. 1989; sequence

deduced from the genomic sequence published by van der Leij et al. 1991) was divided in three regions; the 5′, the middle and the 3′ region. This cDNA contained introns 2 (84 bp; in the 5′ region) and 9 (93 bp; in the middle region). From the 5′ region of the *GBSSI* cDNA, two PCR fragments were amplified using the primer combinations 5F4 (5′ GCGAATTCATTCCCCTTTTTGTAGAC 3′) + 5R1 (5′ CAGGATCCATAGATTTTTGAACCAGT 3′) (670 bp) and 5F2 (5′ ACGAATTCTAGGACTCAGGAACCATA 3′) + 5R1 (563 bp). From the middle region of the *GBSSI* cDNA, three PCR fragments were amplified using the primer combinations MF1 (5′ GCAAGCTTATCTGGACAATGAACTTA 3′) + MR1 (5′ CTGGATCCTTCTGCTCCTCAAGTCTG 3′) (776 bp), MF2 (5′ CGAAGCTTTCTCATTCCTTGCTACTT 3′) + MR1 (634 bp), and MF1 + MR2 (5′ CTGGATCCTTCACAATCCCAGTTATG 3′) (503 bp). An *Eco*RI or *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 (underlined nucleotides in the primer sequences). PCR products were cloned in pGEM-T or pGEM-Teasy (Promega) vectors, followed by ligation of two different PCR products per region in pBluescript SK+ (Stratagene), pMTL25 or pMTL24 (Chambers et al. 1988). Ligation of the two PCR products to each other took place either through the *Bam*HI site of the reverse primer or through the *Eco*RI or *Hin*dIII site of the forward primer. Depending on the restriction site through which ligation was performed, antisense-sense (IR 5′A-S and IR MA-S) or sense-antisense (IR MS-A) inverted repeats were obtained. The IR 5′A-S construct contained the *GBSSI* sequence between primers 5F4 and 5F2 (113 bp) as a spacer, and had a dsRNA-forming region of 553 bp. In the IR MA-S construct the spacer consisted of the sequence between the MF1 and MF2 primers (148 bp), and the dsRNA-forming region was 624 bp. In the IR MS-A construct the sequence between the MR2 and MR1 primers (279 bp) acted as a spacer and the dsRNA-forming region was 493 bp. Inverted repeats were subcloned behind the *GBSSI* promoter in the binary vector pPGB-1s (Kuipers et al. 1995) through the *Xba*I or the *Bam*HI site.

To design IR 5′A-S and IR MA-S without introns, the same strategy was used on cDNA template that did not contain introns. In the intronless IR 5′A-S construct the dsRNA-forming region was 469 bp, while the intronless IR MA-S construct contained a 531-bp dsRNA-forming region.

Construct IR MS-Ainrev harbouring the intron in the spacer in reverse orientation was made through screening of pPGB-1s (Kuipers et al. 1995) clones in which the complete inverted repeat was cloned through the *Xba*I site. By restriction analysis, clones that harboured the spacer in antisense orientation could be distinguished from those harbouring the spacer in sense orientation.

All constructs were transformed into *E. coli* DH5α (Invitrogen, Breda, The Netherlands). IR 5′A-S and IR MA-S were transformed into *A. tumefaciens* strain AGL0 (Lazo et al. 1991) by triparental mating. All other 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 1 day on solid R3B (MS with 30 g l^{-1} sucrose, 1 mg l^{-1} BAP, 2 mg l^{-1} NAA) medium, supplemented with 2 ml PACM (MS with 30 g l^{-1} sucrose, 2 g l^{-1} casein hydrolysate, 1 mg l^{-1} 2,4-D, 0.5 mg l^{-1} 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^{-1} sucrose, 1 mg l^{-1} zeatin, 200 mg l^{-1} cefotaxime, 200 mg l^{-1} vancomycin and 100 mg l^{-1} 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^{-1} sucrose, 200 mg l^{-1} cefotaxime and 100 mg l^{-1} 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 stem

segments were placed on petridishes with MS medium containing 80 g l^{-1} sucrose and 5 μM BAP (Hendriks et al. 1991). Incubation of these petridishes 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 (Merck) 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. The transformants with granules with varying sizes of cores were classified as medium silenced. Per transformant, three microtubers were stained and examined microscopically.

Small RNA analysis

Total RNA was isolated from microtubers using Trizol agent (Sigma). About 20 μg of total RNA was electrophoresed on a 15% polyacrylamide gel 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. As a size indicator DNA oligos were included on the gels. For the analysis of IR MA-S and IR MS-A transformants a mixture of DNA oligos was used: MR2 (26 nt), MR3 (5′ CAAGTAGCAAGGAATGAGAGC 3′, 21 nt) and Infor (5′ GTAACATAAGATTTTTCCAACTCC 3′, 24 nt). 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 a 21-nt DNA oligo is expected to correspond to that of a 19-nt RNA oligo. RNA on the polyacrylamide gels was transferred onto Hybond N membrane (Amersham) by overnight electro blotting at 25 V (Biorad). Two identical blots were made. One was hybridised with a 169-bp spacer-specific probe (probe A) amplified with primers MF1 and MR3 while the other was hybridised with a 100-bp intron 9-specific probe (probe B) amplified with primers Infor and Inrev (5′ CCATGACCTGTATAGAGATTTTG 3′). Probes were radioactively 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.

Analysis of splicing efficiency of intron 9

PCR was performed using cDNA and DNA of potato cultivar Karnico as template, and primers MF1 and MR1 (see 'DNA constructs') with 35 amplification cycles, and an annealing temperature of 55 °C. PCR products were visualised on a 1.3% agarose gel. The DNA on this gel was blotted on Hybond N+ membrane (Amersham) by vacuum blotting (Pharmacia) in 0.4 N NaOH. The blot was hybridised with probe B (isolated from gel) as described for the small RNA analysis, but at a hybridisation temperature of 65 °C. The blot was rinsed once in 1×SSC, 0.1% SDS, twice in 0.5×SSC, 0.1% SDS, and once in 0.2×SSC, 0.1% SDS at 65 °C.

Statistical analysis

To test whether the silencing effects of the constructs were significantly different, a binomial test was used, whereby P^t is the number of silenced transformants divided by the total number of transformants, while P^s is the number of strongly silenced transformants divided by the total number of transformants. The null hypothesis of no difference between proportions of (strongly) silenced transformants was rejected at an experiment-wise type I error of 0.05.

Results and discussion

Effect of an intron in an inverted repeat sequence on silencing efficiency

Inverted repeat constructs were made with PCR fragments containing the 5′ and middle regions of the *GBSSI* cDNA (Figure 1). These PCR fragments were sequenced and the 5′ and middle region fragments proved to contain introns 2 and 9, respectively. Apparently, mRNA in which these introns were not spliced was used as a template for the synthesis of cDNA. To address whether the presence of these introns in the dsRNA would influence silencing efficiency, new constructs were made without introns. To do so, new cDNA was synthesized and sequenced to check for the absence of intron sequences.

Assuming correct splicing of introns in sense orientation, dsRNA structures were predicted for the IR MA-S and IR 5′A-S constructs with or without introns (Figure 1B). If the presence of loops in dsRNA influences the activity of Dicer-like enzymes, it is likely that, as a consequence, the silencing efficiency is influenced.

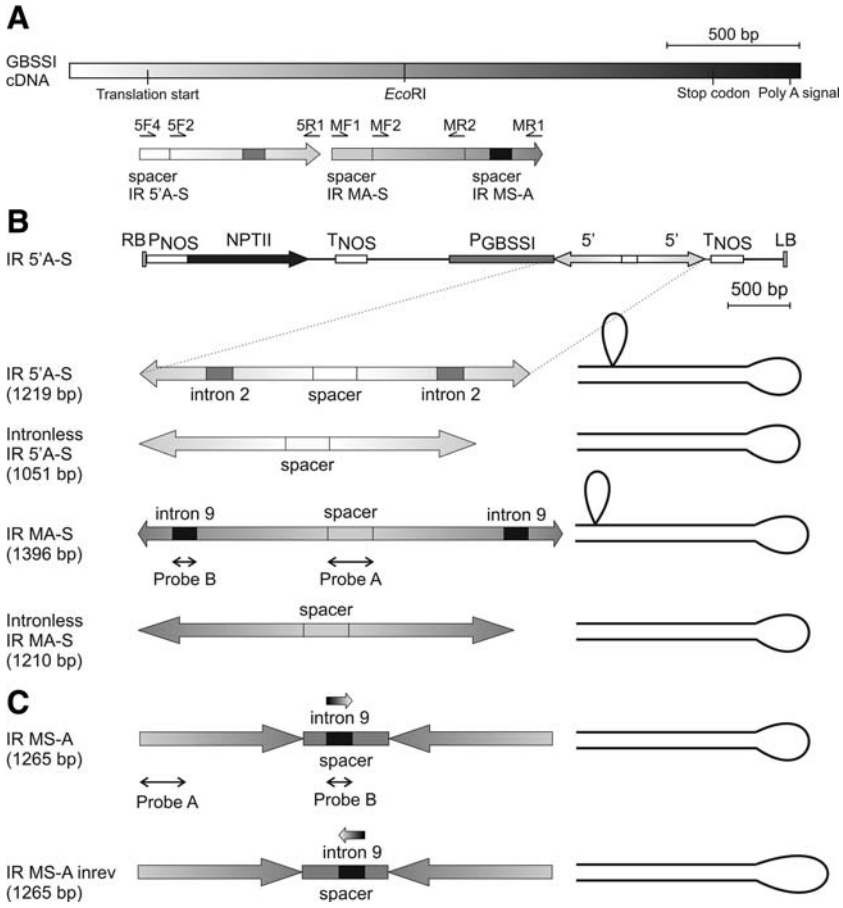

Figure 1. Composition of potato GBSSI 5′ and M region inverted repeat constructs, and predicted dsRNA structures when correct splicing of the introns in sense orientation has been achieved. (A) Potato GBSSI cDNA, primers and PCR products used in the construction of inverted repeats. (B) For construct IR 5′A-S the complete T-DNA is shown. The other constructs similarly contain the indicated inverted repeat sequence between the GBSSI promoter and NOS terminator. Inverted repeat constructs of the 5′ and M region of the GBSSI cDNA with or without an intron in the inverted repeat sequence. (C) Inverted repeat constructs of the M region with an intron in the spacer sequence in a spliceable (sense) or non-spliceable (antisense) orientation. Sizes of the inverted repeat plus spacer sequences are given below the construct names. Probes used for the detection of siRNAs are indicated. RB, right border; P_{NOS}, nopaline synthase promoter; NPTII, kanamycin resistance gene; T_{NOS}, nopaline synthase terminator; P_{GBSSI}, potato GBSSI promoter; 5′, 5′ part of the GBSSI cDNA; LB, left border.

All four constructs were tested at the same time in one transformation experiment. Per construct, 31–45 independent transformants were obtained. Microtubers were induced and analysed for the level of silencing by iodine staining followed by microscopic observation. Figure 2 shows the percentage of transformants showing different levels of silencing for the different constructs. For the inverted repeat constructs harbouring the middle region, no difference in silencing efficiency was observed. For the constructs harbouring the 5′ region, a small difference was observed but this difference was not significant. From this, we can conclude that the presence of an intron does not influence the silencing efficiency. Therefore, it is assumed that the supposed bulges in the dsRNA formed by the unspliced introns in antisense orientation do not affect the activity of the Dicer-like enzyme. It is known that Dicer preferentially cleaves dsRNAs at their termini in *C. elegans* and human but if no ends are available, Dicer will still cleave internally with lower kinetics (Zhang et al. 2002). Assuming that the Dicer-like enzymes in potato behave in a similar manner, it seems logical that it is not inhibited by loops. To address whether the dsRNA structures might be less stable because of the formation of a loop, dsRNA structures were predicted *in silico* using the Vienna RNA secondary structure server (Hofacker 2003). The differences between the predicted binding energies of dsRNA structures formed from the inverted repeat constructs with or without introns were minimal. For IR 5′A-S the predicted binding energy was −980.8 kcal mol^{-1} whilst an almost similar binding energy of −980.7 kcal mol^{-1} was predicted for the intronless IR 5′A-S. Binding energies of −1105.80 and −1100.9 kcal mol^{-1} were predicted for IR MA-S and the intronless IR MA-S. All these binding energies are very high indicating that, regardless of the presence or absence of the intron, these dsRNA structures will be very stable.

Since no significant differences between the inverted repeat constructs with and without intron were observed we can conclude that the presence of the *GBSSI* introns 2 and 9 do not inhibit silencing, and neither do they enhance silencing like introns can do in natural situations (Rose 2002).

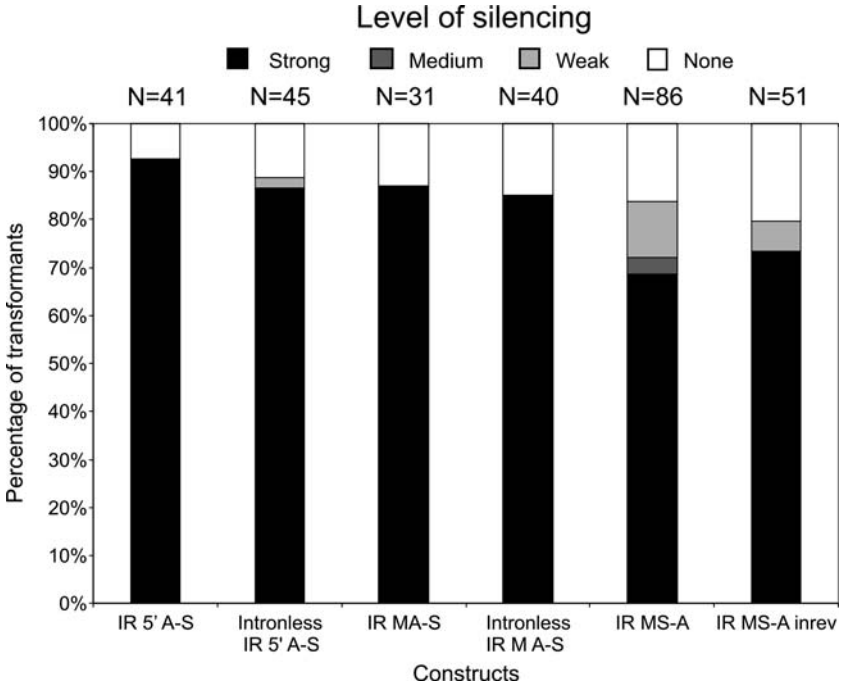

Figure 2. Silencing efficiency of the constructs shown in Figure 1 in potato transformants. Percentages of transformants displaying strong, medium, weak or no silencing of the endogenous GBSSI are indicated. N, number of independent transformants analysed.

Effect of an intron in the spacer sequence on silencing efficiency

In order to determine the importance of a spliceable intron in the spacer two constructs were made (Figure 1C). The IR MS-A construct contained the 93-bp intron 9 derived from the middle part of the *GBSSI* cDNA in a spliceable orientation in the spacer, flanked by 70 bp at the 5′ end and by 116 bp at the 3′ end. Therefore, if the intron 9 sequence is spliced out the loop of the hairpin RNA is 186 bp long. Construct IR MS-Ainrev contained the intron in the reverse, non-splicing, orientation. With this construct the loop of the hairpin RNA is 279 bp long. The effect on silencing efficiency of both constructs is shown in Figure 2. When construct IR MS-Ainrev with the non-spliceable intron was transformed into potato, 71% of the transformants showed strong silencing. Since construct IR MS-A with the spliceable intron in the spacer gave a similar silencing efficiency (69% of transformants showing strong silencing) no effect could be subscribed to the presence of a spliceable intron in the spacer.

Small RNA of IR MA-S and IR MS-A transformants was hybridised with probe A (Figure 3A), representing the spacer region in IR MA-S (Figure 1B). Figure 3B shows that no signal was present in transformants of IR MA-S, while transformants of IR MS-A did show hybridisation of siRNAs with probe A. From previous work, it was known that all these transformants, except the non-silenced transformant IR MA-S-26, showed accumulation of *GBSSI* siRNAs after hybridisation with the MF1 + MR1 PCR product of the *GBSSI* cDNA as a probe (Heilersig et al. 2006). Therefore, the lack of hybridisation with the spacer probe in transformants of IR MA-S indicates that no siRNAs of spacer sequences are formed.

If intron 9 in the spliceable (sense) orientation in construct IR MA-S is spliced after transcription no dsRNA of this sequence is formed. Therefore, we would not expect to find siRNAs from this sequence. If, on the other hand, the sense intron 9 is not spliced we should be able to demonstrate the presence of intron 9-derived siRNAs. Small RNAs from both IR MA-S and IR MS-A transformants were hybridised with probe B, representing the intron 9 region (Figure 3A). Although probe B hybridised with the Infor primer no signal was observed in transformants of both constructs (Figure 3C), indicating that the intron is indeed spliced in IR MA-S transformants. Since the surroundings of intron 9 in IR MA-S are exactly the same in the spacer of IR MS-A, we assume that this intron is spliced from the spacer as well.

To assess whether intron 9 is spliced efficiently from potato GBSSI mRNA in wild type Karnico PCR was performed with primers MF1 and MR1, using newly synthesised cDNA and DNA as templates. As is shown in Figure 4A a 1112-bp fragment is obtained when genomic DNA is used as a template. This PCR product contains 5 introns, among them intron 9. In contrast, a 683-bp fragment was obtained with GBSSI cDNA as a template. The size of this PCR product suggests that no intron is present. To prove that intron 9 is absent from this PCR product a Southern blot was made from the gel in Figure 4A. This blot was hybridised with probe B (Figure 4B). A strong signal was observed for the 1112-bp PCR product of the genomic DNA, whereas there was no hybridisation of the 683-bp PCR product of the cDNA. This suggests that intron 9 of GBSSI is efficiently spliced from the mRNA.

We did not observe an enhanced silencing effect when a spliceable intron was used in the spacer whilst Smith et al. (2000) and Wesley et al. (2001) did observe a clear enhancement of silencing when an intron was used as a spacer. Smith et al. (2000) made an inverted repeat in which PVY sequences were flanking an 800-nt spacer fragment containing *uidA* sequences. Replacing this spacer with an intron sequence resulted in an increase of the percentage of PTGS from 58 to 96%. When they replaced the spacer with the intron sequence in reverse, non-splicing orientation, the percentage of PTGS was 65% indicating that the splicing of an intron enhanced the silencing efficiency. A similar approach was tested by Goldoni et al. (2004) who tested two orientations of the *albino-1* gene (*al-1*) intron in the spacer of an inverted repeat construct targeting the *al-1* gene in *Neurospora crassa*. They also observed that intron splicing greatly enhanced dsRNA-induced silencing efficiency since the efficiency obtained with the construct harbouring the intron in reverse sequence was lower (32%) than that obtained with the construct with the spliceable *al-1* intron (77%). Wesley et al. (2001) tested a PVY inverted repeat construct in which the spacer region consisted of an intron and a

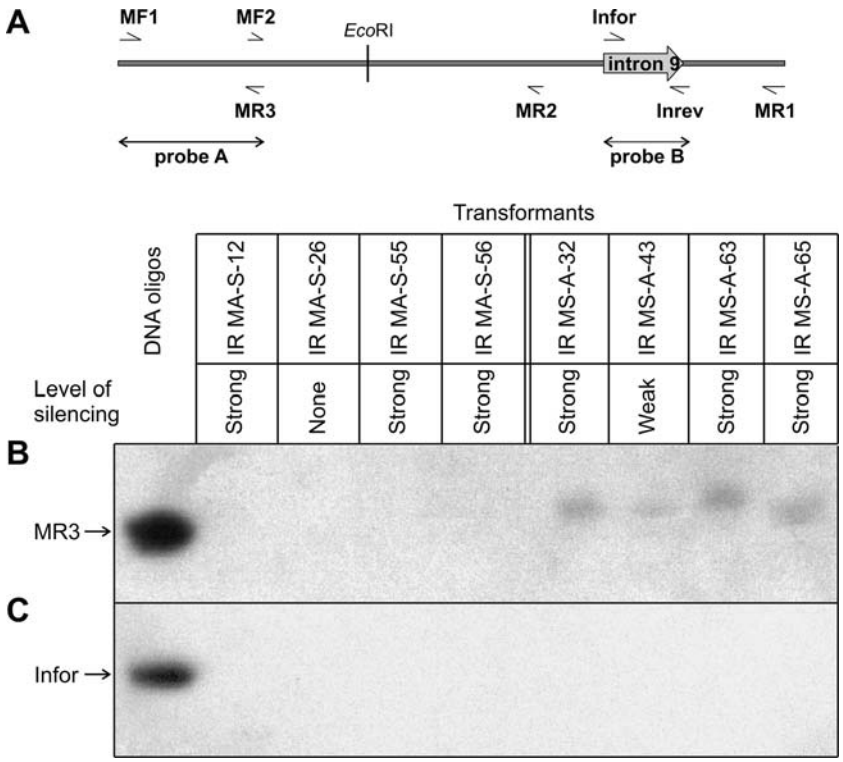

Figure 3. Small RNA analysis. (A) Primer binding sites in the 776-bp MF1 + MR1 PCR product of the GBSSI cDNA.Probe A and probe B are indicated. (B) Detection of siRNAs in IR MA-S and IR MS-A transformants by hybridisation with probe A, representing the spacer region in IR MA-S and part of the inverted repeat in IR MS-A. The DNA oligo hybridising with probe A is primer MR3 (21 nt). This DNA oligo migrates to a position similar to that of an RNA oligo of approximately 19 nt (see Materials and methods). (C) No siRNAs can be detected after hybridisation of a replicate blot with probe B, comprising the intron 9 sequence. This sequence is part of the inverted repeat sequence in IR MA-S, and part of the spacer in IR MS-A. The DNA oligo hybridising with probe B is primer Infor (24 nt).

non-spliceable sequence. Since this inverted repeat still resulted in 89% PTGS, it was suggested that the intron-enhanced silencing efficiency is not due to better alignment of the RNA arms or by presence of a tighter single-stranded RNA loop, but rather is caused by the splicing of the intron. The construct design of IR MS-A is comparable to that described by Goldoni et al. (2004) and by Wesley et al. (2001) since the spacer region contains a spliceable or a non-spliceable (reverse) intron surrounded by non-spliceable sequences. However, in our experimental system, the two different intron orientations gave similar silencing efficiencies implying that the splicing of the intron does not enhance silencing efficiency. McGinnis et al. (2005) have recently reported similar results. When they compared inverted repeat constructs with different spacer sequences, including the Petunia *CHS A* intron 1, the rice *waxy-a* intron 1, or a 335-bp non-intronic *GUS* reporter gene fragment, no differences in silencing efficiencies were observed.

Intron-enhanced expression or silencing

From the results presented here, we can conclude that inclusion of either GBSSI intron 2 or 9 in GBSSI inverted repeat constructs neither inhibits nor enhances silencing efficiency. If intron-enhanced silencing reported by others is based on a similar mechanism as the intron-enhanced expression, the features of the introns might play an important role. Rose (2002) described the requirements for intron-mediated enhancement (IME) of gene expression in *Arabidopsis*. He demonstrated that five *Arabidopsis* introns varied

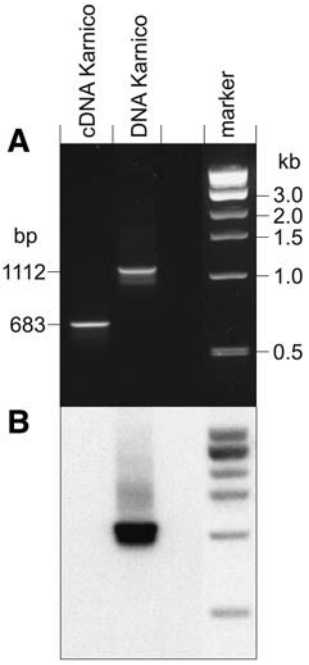

Figure 4. Splicing efficiency of intron 9. (A) Agarose gel showing the RT-PCR and PCR products with primers MF1 and MR1 using cDNA and DNA of Karnico, respectively, as template. The 1112-bp product obtained with DNA as template contains introns 5, 6, 7, 8 and 9. The 683-bp RT-PCR product obtained with cDNA as template contains none of these introns. (B) Hybridisation of a Southern blot of the gel in (A) with probe B, comprising intron 9, indicating efficient splicing of intron 9 from the GBSSI mRNA.

in their ability to increase mRNA levels even though they were all spliced with great efficiency. From the five introns tested in a PAT1:GUS system two had little or no effect on *GUS* mRNA accumulation. These introns were derived from genes whose expression is intron-independent. On the other hand, introns previously shown to stimulate expression induced *GUS* mRNA accumulation more than 10-fold. Since no obvious differences in length, nucleotide composition or splicing efficiency were found for introns that stimulated expression and those that did not, these structural components are probably not the features that determine the degree to which an intron will stimulate expression. Possibly, a combination of these features and other unknown factors are involved. Differences in IME have also been found for introns from a single gene in maize (Callis et al. 1987; Mascarenhas et al. 1990). Apparently, some introns enhance RNA accumulation whereas others do not. If the intron-enhanced silencing is based on a similar mechanism, this might explain why some introns enhance silencing whereas others do not. A comparison between silencing vectors differing in intron spacer sequences was made by Nakayashiki et al. (2005). They examined the effect of three different spacer sequences in an inverted repeat construct targeting the *eGFP* gene in *Magnaporthe oryzae*. The silencing vector with a cutinase intron spacer (147 bp) showed a higher silencing efficiency than those with a spacer of a *GUS* gene fragment (542 bp) or an intron of the chitin binding protein gene (850 bp). These differences in silencing efficiency indicate that not all introns contribute to enhancement of silencing in the same extent. The *GBSSI* introns 2 and 9 tested in our experiments seem to be introns that have no effect on silencing efficiency.

Summarizing, we can conclude that the inclusion of *GBSSI* intron 2 or 9 in *GBSSI* inverted repeat constructs does not affect gene silencing efficiency. Intron 9 was also tested in the spacer of inverted repeat constructs where both the spliceable and the non-spliceable intron gave rise to similar silencing efficiencies. We therefore postulate that it is not a general rule that inclusion of a spliceable intron in the spacer enhances silencing efficiency.

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