Factors influencing gene silencing of granule-bound starch synthase in potato

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Contents

Chapter 1

General introduction

Post-transcriptional gene silencing
In plants, post-transcriptional gene silencing (PTGS) is a control mechanism which causes degradation of specific mRNA sequences. The natural role of this mechanism is to control pathogenic RNA sequences such as viruses and viroids.

The phenomenon was initially described in studies on transgenic plants in which the introduction of extra copies of dihydroflavonol-4-reductase (*dfr*) or chalcone synthase (*chs*) led to loss of expression of the corresponding endogenous genes (van der Krol et al., 1990a). This observation was termed cosuppression since RNA of both the transgenes and the homologous endogenous genes was degraded, resulting in loss of expression. Similar findings were described in *Neurospora crassa* where the phenomenon was called quelling (Cogoni et al., 1996), and in animals in which it was referred to as RNA interference or RNAi (Fire et al., 1998). Silencing is not only triggered by transgenes but can also be initiated by virus vectors carrying portions of the host genes. This process is known as virus-induced gene silencing (Lindbo et al., 1993).

Early models for gene silencing
In first instance, it was thought that single-stranded RNAs resulting from aberrant processing of transgenes caused silencing. Such RNAs, but with antisense polarity, were hypothesized to pair with the target mRNA, leading to its subsequent destruction. However, such antisense species could not be detected by conventional RNA analyses (Tijsterman et al., 2002). Hamilton et al. (1999) were the first to detect the presence of short RNA species of around 25 nt in plants exhibiting PTGS. These short RNA species consisted of both the sense and antisense sequences of the targeted mRNAs and were called short interfering RNAs (siRNAs). The accumulation of both polarities of siRNAs suggested that these siRNAs were derived from dsRNA. The involvement of dsRNA explained why the presence of multiple T-DNA transgenes in inverted repeat orientation yielded a higher silencing efficiency than the presence of single T-DNA transgenes or multiple T-DNA transgenes in a direct repeat orientation. A model was then proposed in which dsRNA is produced prior to formation of siRNAs.

Results to support this new model were provided by Fire et al. (1998) who demonstrated that dsRNA is the potent inducer of RNA interference in *C.elegans*. In the same lab (Parrish et al., 2000), it was shown that the sequence of the dsRNA does not necessarily need to be completely identical to the target; dsRNAs with 88% of sequence identity to the target mRNA still triggered RNAi. Besides, it became clear that only a few molecules of dsRNA per cell were sufficient to completely interfere with gene expression.

The first biochemical proof for the model was provided by experiments in a cell-free system derived from *Drosophila* embryos (Hammond et al., 2000). Their data led to a simple two-step model to explain RNAi: 1) long dsRNA molecules are cleaved into 21-23 nt siRNAs by a dsRNA-specific nuclease named Dicer; 2) one of the strands of the siRNAs is incorporated into a multiprotein complex, known as the RNA Induced Silencing Complex (RISC). The complex is then guided to the target mRNA through conventional base-pairing interactions of the antisense strand of the siRNA. Subsequently, the target mRNA is cleaved by an endonuclease, and then degraded (Hammond et al., 2000).

Much progress has been made recently in the identification of molecular components of RNA silencing in different eukaryotes. The RNAi machinery is also used for the processing of micro-RNAs (miRNAs). These are endogenous single-stranded small RNAs that repress

the expression of target genes such as genes that are involved in developmental processes. They originate from imperfect stem-loop precursors produced from non-coding RNA. Like siRNAs, miRNAs can direct cleavage of their mRNA targets when the two are extensively complementary, but repress mRNA translation when they are not (Tomari and Zamore, 2005). Depending on the organism, the same Dicer or different Dicer paralogs are responsible for the cleavage of miRNA precursors and dsRNA (Tomari and Zamore, 2005). Elbashir et al. (2001) suggested that the initial direction of dsRNA processing by Dicer determined which one of two siRNA strands would be incorporated in RISC. According to their model, only the strand with its 3' terminus at the processed end would enter RISC and guide target RNA cleavage. If the antisense strand is incorporated in RISC, the complementary endogenous sense strand of mRNA will be degraded.

New insights in the RNA silencing pathways have shown that the thermodynamic properties of siRNAs also play an important role in strand entry into RISC. This was demonstrated by two groups in 2003. Schwarz et al. (2003) used a biochemical approach in *Drosophila* embryo lysates to study what are the prerequisites for uptake of one of the two siRNA strands in the RISC complex. They showed that the siRNA strand of which the 5' end shows the weakest binding, is incorporated in RISC. If this is the antisense strand, sense mRNA will be targeted and silencing will occur. For miRNAs, similar features were found; the strand with the less-tightly base-paired 5'end is usually incorporated in RISC.

Khvorova and coworkers (2003) compared the thermodynamic properties for hundreds of siRNAs and found that a low internal stability at the 5'antisense terminal position and a low internal energy across the duplex was a common feature for functional siRNAs and naturally occurring precursor/ mature miRNAs. These features for small RNAs were found in *C.elegans*, *D.melanogaster*, mouse, human and *N. benthamiana*, indicating that prerequisites for thermodynamic properties of siRNAs are a common feature in the RNA silencing machinery (Khvorova et al., 2003).

The finding that dsRNA-derived siRNAs in plants obey to the thermodynamic asymmetry rules suggests that the thermodynamic properties are more important in determining which strand is incorporated in the RISC than the direction of dsRNA processsing (Sontheimer, 2005).

In *Drosophila*, RISC assembly has been studied *in vitro* and a model for the RISC assembly pathway has been proposed (Tomari and Zamore, 2005). The Dicer enzyme involved in siRNA production, Dcr-2, is also involved in the loading of one of the two siRNA strands into RISC. Dcr-2 forms a heterodimer with R2D2, a protein with dsRNA-binding domains. This heterodimer appears to sense siRNA thermodynamic assymetry and determines which strand will be incorporated into RISC. The Dicer-interacting protein R2D2 in *D.melanogaster*, is structurally similar to *Arabidopsis* HYL1/DRB family members. Therefore, the HYL1/DRB family proteins could be considered plant orthologs of R2D2 and are likely to regulate Dicer-like protein functioning through heterodimerization (Hiraguri et al., 2005). Hiraguri et al. (2005) demonstrated that specific interactions between Dicer-like proteins and HYL1/DRB family dsRNA-binding proteins occur in *Arabidopsis thaliana*.

Inverted repeat requirements

Several inverted repeat gene constructs have been introduced in plants and animals giving rise to silencing of the targeted genes. An important feature of these inverted repeat constructs is the inclusion of a spacer, which provides stability in *E.coli* and *Agrobacterium* (Waterhouse et al., 1998). A spacer region of about 150 bp is sufficient for stable cloning of inverted repeat sequences in *E.coli* and *Agrobacterium* (J. Kooter, pers. comm).

Smith et al. (2000) tested some designs for inverted repeat constructs. The presence of a spliceable intron in the spacer was tested for two inverted repeat constructs: the first one was targeted against a viral sequence (PVY), whereas the purpose of the second construct was to silence the endogenous Δ12-desaturase gene of *Arabidopsis*. In both cases, the replacement of the spacer with an intron resulted in increased silencing efficiencies. For the endogenous gene, they even obtained 100 % silencing using the intron construct.

Involvement of an RNA-dependent RNA polymerase

A fascinating aspect of RNAi is its extreme efficiency in *C.elegans* and plants. A few trigger dsRNA molecules can inactivate a continuously transcribed target mRNA for long periods of time. To explain the potency and self sustaining nature of RNAi, a model was proposed by Sijen et al. (2001a). This model assumes that siRNAs are incorporated into RISC after which the complex is guided to the target mRNA through conventional base-pairing interactions of the antisense strand of the siRNA. Subsequently, the target mRNA can be degraded, or made double-stranded through the action of a cellular RNA-directed RNA polymerase (RdRP). The RdRP uses the antisense strands of primary siRNAs as primers on target mRNA to synthesize new dsRNA. The RdRP-synthesized dsRNA will then be recognized by Dicer and degraded to secondary siRNAs. This is illustrated in Figure 1.1 where the dsRNA-induced RNA degradation mechanism is shown as well.

Data to support this model were provided by the analysis of small interfering RNAs produced during RNAi in *C.elegans*. Secondary siRNAs, originating from RdRP-synthesized dsRNA, were detected. These secondary siRNAs were derived from regions upstream of the targeted mRNAs and were able to induce secondary RNA interference, a phenomenon called transitive RNAi (Sijen et al., 2001a). Their analyses showed a loss of transitivity and secondary siRNA signals at distances greater than several hundred basepairs from the original trigger.

More data supporting this model were presented by Lipardi et al. (2001) who used a *Drosophila* embryo extract to study RNAi. These authors showed that dsRNA-derived siRNAs serve as primers to transform target mRNA into dsRNA through the action of RdRP. The synthesised dsRNA is then cleaved by Dicer generating new siRNAs. These siRNAs have a 2-nt 3'overhang in both the sense and antisense strand. The free 3'hydroxyl group is essential for priming of the subsequent RdRP reaction. Only the antisense strand of the siRNAs is thought to act as primer on mRNA and as a consequence dsRNA synthesis takes place in the 3' to 5' direction of the mRNA (Lipardi et al., 2001). Therefore, changes in the antisense sequence of the initial trigger RNA affects silencing efficiency to a larger extent than changes in the sense sequence. This explains earlier results of Parrish et al. (2000) who showed that chemical requirements were more stringent for the antisense than for the sense strand of the initial trigger RNA.
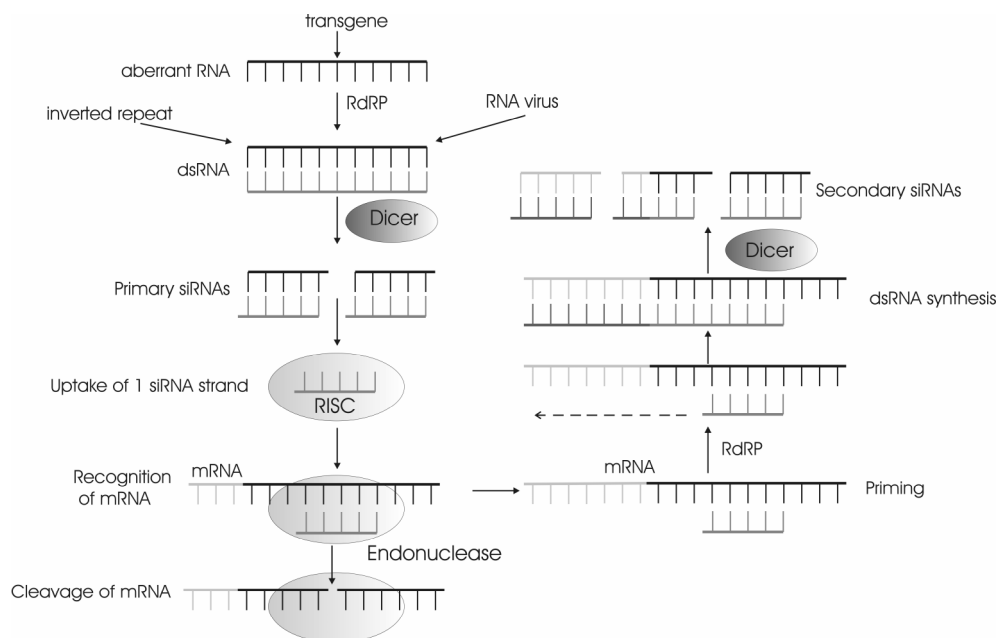
Figure 1.1. Schematic representation of the RNAi model. Primary small interfering (si) RNAs can cause degradation of complementary mRNA or be used as a primer in the RNA-dependent RNA polymerase (RdRP)-mediated synthesis of new double-stranded (ds) RNA. Cleavage of the new dsRNA leads to the formation of secondary siRNAs derived from the primary region (black/grey) or from the region upstream of the primary target region (grey/light grey). RISC=RNA-induced silencing complex. A colour illustration of this model is depicted at the rear side of this thesis.

In plants and animals, RNA silencing can propagate throughout the organism, most likely via movement of nucleic acids. This phenomenon is called systemic silencing and was shown to be related to the spreading of siRNAs in *Arabidopsis* GFP transgenes (Himber et al., 2003). In transformants where long cell-to-cell movement of silencing was observed, siRNAs located 3' from the 5' GFP initiator region were found. These siRNAs were not detected in the line carrying an RdRP null mutation, in which long cell-to-cell movement was absent.

Two constructs to silence the endogenous *Arabidopsis* RbcS and sulphur mRNAs could not induce long cell-to-cell movement of silencing indicating that transitivity of an endogenous gene could not be induced (Himber et al., 2003).

By using VIGS in *N.benthamina*, Vaistij et al. (2002) demonstrated that the target sites and the production of siRNA can spread within the transcribed region of the GFP transgene from the initiator region in both 3' and 5' directions. No spreading of target site and production of siRNAs was observed for the endogenous PDS and Rubisco genes.

Brummel et al. (2003) showed that it is possible to induce transitive silencing of an endogenous gene. These authors designed a construct in which an inverted repeat of the NOS terminator of *Agrobacterium* was placed behind the polygalacturonase (PG) transgene. In this way, they induced transitive silencing of the endogenous PG gene in

tomato. Not only were they able to silence the tomato PG gene, but also two plant transcription factors from *Arabidopsis* could be silenced using this approach.

Spreading of the siRNA production in the 3' to 5' direction is caused by the RdRP-directed elongation of the antisense siRNA primer on a sense RNA template. Spreading of the siRNA production in the 5' to 3' direction can not be ascribed to siRNA-primed RdRP-activity on a sense RNA template. Instead, this spreading might be caused by RdRP-activity on an antisense template that is produced through read-through transcription of promoters flanking the transgene. Alternatively, the spreading in the 5' to 3' direction might be caused by unprimed RdRP-activity on the sense RNA template.

Schiebel et al. (1998) were the first to isolate an RNA-directed RNA polymerase-specific cDNA clone from tomato. The tomato RdRP can perform primed as well as unprimed polymerase activity. By DNA gel blot hybridization and/or PCR amplification experiments, they showed that homologs exist in potato, tobacco, wheat, *Arabidopsis* and petunia. Other RdRP homologs were found in *Neurospora* (Cogoni and Macino, 1999), *C.elegans* (Sijen et al., 2001a; Smardon et al., 2000), *Arabidopsis* (Dalmay et al., 2000; Mourrain et al., 2000) and *Dictyostelium discoideum* (Martens et al., 2002). Surprisingly, so far no RdRP homolog has been found in *Drosophila*.

Transcriptional gene silencing

Post-transcriptional gene silencing occurs after transcription; transcripts are produced but are rapidly degraded in the cytoplasm. Silencing can also operate at the transcriptional level (TGS). In this case, alterations at the DNA or chromatin level prevent transcription of the targeted genes. TGS was initially associated with the regulation of transposons through DNA methylation in the nucleus. However, TGS can also be induced by complex transgene loci through *de novo* methylation of promoter sequences.

An example of transcriptional silencing of NOS promoter-driven transgenes was described by Matzke et al. (1994). They report how NOS promoter-driven transgenes in tobacco are efficiently inactivated by the H2 locus. This is a complex transgene locus, which comprises six scrambled copies of the NOS promoter including an inverted repeat (Jakowitsch et al., 1999). The inverted repeat (IR) and the composition of the locus are believed to trigger *de novo* methylation.

Another locus inducing *de novo* methylation is the 271 locus in tobacco (Vaucheret et al., 1996). This locus contains multiple T-DNAs of an antisense nitrite reductase (NiR) transgene driven by a CaMV 35S promoter. When this locus was combined with a single-copy 35S promoter-driven transgene, it resulted in methylation of the 35S promoter followed by its inactivation (Vaucheret et al., 1996). Both the H2 and 271 loci have been shown to generate promoter-derived small RNAs (Matzke et al., 2003).

Mette et al. (1999) introduced a chimeric gene consisting of a NOS promoter positioned downstream of the 35S promoter and flanked by a NOS terminator. Plants that normally express the *nptII* gene under control of the NOS promoter were transformed with this chimeric gene construct. One of the transformants showed silencing of the *nptII* gene and methylation in the NOS promoter region. This transformant contained an inverted repeat of two incomplete copies of the 35S promoter driven-NOS promoter. The NOS promoter sequences are located at the centre and need to be transcribed in order to inactivate unlinked NOS promoters (Mette et al., 1999).

Mette et al. (2000) demonstrated that dsRNA of the NOS promoter induced silencing of NOS promoter-driven genes in tobacco and *Arabidopsis*. In both species, a transcribed NOS promoter IR efficiently silenced target NOS promoters. Besides, it was shown that the NOS promoter dsRNA can be degraded to small RNAs in a manner similar to dsRNAs that induce PTGS.

TGS and PTGS are related

Both TGS and PTGS can be initiated by a dsRNA degradation pathway. However, TGS occurs when dsRNA is derived from promoter sequences, whereas PTGS occurs when dsRNA includes coding sequences. By using recombinant viruses, it is possible to target TGS or PTGS of a transgene, depending on whether the virus contains promoter sequences or part of a coding sequence. Since viruses replicate in the cytoplasm, an RNA signal must enter the nucleus to initiate DNA methylation and TGS (Vaucheret and Fagard, 2001).

Sijen et al (2001b) demonstrated that in *Petunia* transgenes in which the 35S promoter was silenced, dsRNA containing 35S promoter sequences was cleaved into small RNAs. This was accompanied by methylation of the 35S promoter. The small RNAs or dsRNAs are probably the signal for methylation of homologous DNA (Sijen et al., 2001b).

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-driven *dfrA* promoter inverted repeat into wild-type *Petunia*. As shown by the absence of *dfrA* mRNAs in transformants with reduced pigmentation, 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., 2001b).

Long and short siRNAs

More insight in the role of siRNAs in TGS and PTGS was provided by the findings of Hamilton et al. (2002). Among the siRNAs produced from a GFP transgene in *N.benthamiana*, two classes can be distinguished; the short (21-22 nt) and the long (24-26 nt) size class. Hamilton et al. (2002) showed that these classes of siRNAs have different roles. The long siRNAs are not required for sequence-specific mRNA degradation but correlate with systemic silencing and methylation of homologous DNA. The short siRNAs are involved in local silencing and degradation of the target mRNA. Small RNAs from endogenous retroelements in *Arabidopsis thaliana* and *Nicotiana* species are only found in the long size class. These long siRNAs are not the guide for RISC since they are not involved in the degradation of mRNA harbouring homologous retroelement sequences. There are indications that these different classes of siRNAs are produced by different Dicer activities. The *Arabidopsis* genome encodes at least four Dicer proteins involved in the production of different siRNAs. DCL1 is required for miRNA biogenesis and appears to function in the nucleus. DCL2 probably plays a role in the accumulation of viral siRNAs whilst DCL3 is required for RNA-dependent DNA methylation (RdDM). Besides, DCL3 is involved in the generation of endogenous siRNAs of the large-sized class. Therefore, it is likely to be involved in systemic silencing (Xie et al., 2004). Although the function of DCL4 needs to be determined, it could be responsible for the processing of long dsRNAs (Wang and Metzlaff, 2005).

Methylation in PTGS and TGS

PTGS and TGS are both associated with methylation of DNA. However, 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., 2001b). Jones et al. (1999) used virus-induced gene silencing (VIGS) of GFP in transgenic *N. benthamiana* carrying 35S-GFP inserts. Silencing of GFP was induced by using potato virus X vectors carrying part of the coding sequence of a GFP transgene or the transcribing 35S promoter. In both cases, silencing was associated with RNA-directed DNA methylation (RdDM), as tested with two methylation-sensitive restriction enzymes. They demonstrated that when the GFP coding sequence was targeted, methylation extended beyond the region of identity between the viral and transgene sequences, but remained restricted to the transcribed region of the gene. On the other hand, when silencing was initiated by PVX carrying the 35S promoter, the methylation was detected within and upstream of the 35S promoter but did not extend downstream into the transcribed region. In TGS systems, methylation is thought to be primarily restricted to the region of RNA-DNA sequence identity between the inducing dsRNA and its target. Wassenegger (2000) observed that upon potato spindle tuber viroid (PSTVd) infection, transgenic tobacco plants containing PSTVd cDNA showed methylation in the region of sequence identity and flanking regions. However, the spreading of methylation was limited to the region within 30 to 50 bp upstream or downstream of the PSTVd cDNA and the frequency of methylation clearly decreased with increasing distance from the viroid-specific sequence (Wassenegger, 2000). No spreading of methylation was found in a NOS promoter dsRNA-mediated TGS system in *Arabidopsis* (Aufsatz et al., 2002a) or in another PSTVd system in tobacco (Vogt et al., 2004).

RNA-directed DNA methylation

More insight in the RdDM mechanism was obtained through a genetic analysis of the RdDM pathway in *Arabidopsis* (Matzke et al., 2004). To establish and maintain DNA methylation of cytosine residues, different DNA methyltransferases (DMTases) are required. Enzymes responsible for *de novo* methylation in plants are the Domains Rearranged Methyltransferases, DRM1 and DRM2. When a particular sequence acquires methylation, it is possible to maintain this modification at CGs and CNGs (where N is not G) during subsequent rounds of DNA replication through the activity of maintenance DMTases. These enzymes recognize methylated Cs in the template strand and catalyze methylation of the opposite C in the newly synthesized strand. Maintainance of methylation through subsequent rounds of DNA replication is performed by methyltransferase 1 (MET1) at CG dinuleotides whilst chromomethylase3 (CMT3) can maintain methylation in CNG trinucleotides. No maintainance activity for asymmetrical CNN nucleotide groups is known. Therefore, methylation of CNN can be taken as a measure of ongoing *de novo* methylation. In absence of the RNA trigger, CNN methylation is lost. Recently, it was demonstrated that in response to RNA signals, MET1 can also catalyze *de novo* methylation at CG dinucleotides (Matzke et al., 2004). Based on this finding, a new model is proposed in which MET1 and DRM1/DRM2 act together in response to RNA signals to catalyze *de novo* methylation at CGs and nonCGs, respectively (Matzke et al., 2004). Once *de novo* methylation has reached a level of 30-50 % at most Cs within the region of RNA-DNA sequence identity, HDA6 is recruited. HDA6 is a histone deacetylase, which can reinforce DNA methylation through the attraction of more MET1 and CMT3. This step

leads to reinforcement of C(N)G methylation which is apparently necessary to lock in the silent state, probably in conjunction with additional histone modifications (Aufsatz et al., 2002b).

RdDM can be induced within a DNA target as small as 30 bp as shown by Wassenegger (2000). It is still not known how RNA signals interact with homologous DNA regions. Proteins associated with RNA signals are thought to play a role in this process. Possible candidates are members of the Argonaute family (Matzke et al., 2004).

Starch synthesis in potato

Potato, *Solanum tuberosum* L. belongs to the family of *Solanaceae*. Potato is a heterozygous vegetatively propagated tetraploid species with multiple allelism (van de Wal et al., 2001). It is currently, globally, the fifth most important food crop after sugar cane, wheat, rice and maize (http://faostat.fao.org). In the Netherlands, potatoes are grown for consumption, the production of seed potatoes and the manufacture of potato starch. Potato starch and its derivatives are used in several food and non-food applications such as paper, textile, chemical and pharmaceutical industry.

Potato starch comprises two different glucose polymers; amylose and amylopectin. Amylose is composed of linear chains of $\alpha(1\text{-}4)$ linked glucose residues with very few $\alpha(1\text{-}6)$ branches. Amylopectin, on the other hand, is a highly branched glucan with a specific clustered distribution of $\alpha(1\text{-}4)$ and $\alpha(1\text{-}6)$ linkages (Hizikuru, 1986; Manners, 1989). In wild type potatoes, the amylose content varies from 18 to 23 % and amylopectin from 77 to 82 % (Shannon and Garwood, 1984).

Amylopectin is extremely soluble in water whilst amylose has a strong tendency to recrystallise after dispersion into water. The amylose crystallisation is an undesired effect for many applications and therefore chemical or physical adaptations are required. These adaptations are expensive, time consuming and because of the used chemicals damaging to the environment. Therefore, modification of starch inside the plant by reducing the activity of starch synthesizing enzymes, could be a good alternative (Bruinenberg et al., 1995).

Amylose production in potato has been shown to be completely dependent on the presence of Granule-Bound Starch Synthase I (GBSSI), which is encoded by a single copy gene. Three independently isolated complete GBSSI sequences have been reported while nine sequences of the GBSSI promoter region were published (van de Wal et al., 2001). On the basis of Southern blot hybridization results, these sequences were divided in four classes; A1, A2, A3 and A4. GBSSI alleles belonging to these four classes are highly homologous in the coding region but vary in promoter sequences (van de Wal et al., 2001).

Inhibition of GBSSI activity in potato

Different strategies have been applied to alter the amylose content within the potato plant. An amylose-free (amf) potato mutant was obtained after irradiation of a monohaploid with X-rays. No amylose is present in this amf mutant that lacks GBSSI protein and GBSSI activity (Hovenkamp-Hermelink et al., 1987). Since potato is a tetraploid crop, four copies of a mutant GBSSI allele are required in a cultivar to obtain amylose-free starch. Therefore, introduction of an antisense gene that can act as a dominant suppressor of endogenous genes is a faster approach to obtain amylose-free cultivars. In potato, inhibition of GBSSI has been achieved by transformation with antisense and sense GBSSI constructs. For antisense inhibition, a positive correlation between the number of T-DNA integrations and

the effect on silencing was found (Kuipers et al., 1995). This correlation was not found for the sense transformants (Wolters and Visser, 2000). In the case of antisense inhibition, the percentage of transformants showing strong silencing ranged from less than 1 percent to 23 percent, depending on the cultivar used (Heeres et al., 2002). For practical plant breeding purposes it is instrumental to maximise the occurrence and frequency of gene silencing. If the percentage of transformants showing strong silencing can be increased through the design of efficient constructs, the number of transformants and thus costs will be reduced. As known from other model species, the introduction of inverted repeat constructs targeting cDNA sequences, greatly enhanced silencing efficiency.

Outline of the thesis

The goal of this thesis research was to study the mechanism of gene silencing in potato and to improve the efficiency. We focussed our attention on potato because it is an important crop that can be used as a production crop for novel compounds. Besides, an efficient transformation system was available allowing the generation of large numbers of transformants. Silencing of GBSSI was used as a model system since the silencing effect can be easily monitored by staining starch granules with an iodine solution. We designed several GBSSI cDNA inverted repeat constructs and tested them in potato by analyzing large numbers of transformants. In studies with antisense GBSSI constructs, Kuipers et al. (1995) showed that the 3' end of the GBSSI coding region was important for GBSSI inhibition. To address whether sequence differences would be important in inverted repeat constructs as well, inverted repeat constructs harbouring different regions of the potato GBSSI cDNA were tested. Furthermore, we addressed whether the orientation of inverted repeat constructs influenced silencing efficiency. Different effects of size of the inverted repeat on silencing efficiency have been reported in literature. Therefore, we also tested the influence of the size of the cDNA GBSSI inverted repeat on silencing efficiency (Chapter 2).

Some of the GBSSI cDNA inverted repeat constructs were studied in more detail and compared with new constructs to address the importance of the presence of introns in the inverted repeat or spacer region (Chapter 3). In Chapter 4, we demonstrate that transitive silencing occurs in potato. Two approaches were used. In the first approach, transitive silencing of GBSSI was achieved through a construct harbouring a NOS terminator inverted repeat preceded by a GBSSI cDNA sequence. In the second approach, we studied whether the production of GBSSI siRNAs could spread in sequences upstream and downstream of the endogenous GBSSI sequence that was initially targeted through an inverted repeat construct (Chapter 4). Attempts were made to induce TGS of GBSSI by testing inverted repeats harbouring different sequences of the GBSSI promoter. Differences in silencing efficiency were observed and methylation of the transgene and endogenous genes was studied for two constructs (Chapter 5). A general discussion is given in Chapter 6.

Chapter 2

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

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Submitted

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 in 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. No effect of inverted repeat orientation on silencing efficiency was observed. 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 orientation of the inverted repeat did not influence silencing efficiency but size and sequence did.

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, 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 centre of the guide siRNA. Finally, the cleaved RNA is degraded by exoribonucleases (Ceruti, 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, different constructs for down-regulation of Granule-Bound Starch Synthase have been tested. Granule-Bound Starch Synthase I (GBSSI) catalyzes the synthesis of amylose in amyloplasts. Starch comprises two different glucose polymers; amylose and amylopectin. 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 (Hizikuru, 1986; Manners, 1989). In potato, inhibition of GBSSI has been achieved by transformation with antisense and sense GBSSI constructs. Silencing of GBSSI was most efficient with antisense 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 11 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 GBSS cDNA was important for GBSSI inhibition since constructs lacking this sequence resulted in substantial lower silencing efficiencies. The most efficient silencing was obtained with pKGBA50 harbouring 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 percent to 23 percent (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 were made and tested in potato. By doing so, the effect of size, sequence and inverted repeat orientation on silencing of GBSSI could be determined. No effect of inverted repeat orientation on silencing efficiency was observed 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.

Experimental procedures

Plant material and growth conditions

Potato cultivar Karnico 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., 1989) 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 (Fig. 2.1). In a similar way, the 1.3-kb 3' region of the GBSSI cDNA was cloned from pWx1.3 (Visser et al., 1989) 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 (Fig. 2.1).

To design the six small inverted repeat constructs, primers were designed on three different regions of the GBSSI cDNA (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 and 5R1 (674 bp), 5F2 and 5R1 (548 bp), 5F1 and 5R2 (519 bp), MF1 and MR1 (761 bp), MF2 and MR1 (618 bp), MF1 and MR2 (488 bp), 3F1 and 3R1 (680 bp), 3F2 and 3R1 (548 bp) and finally 3F1 and 3R2 (504 bp). Sequences of these primers are given in Table 2.1. 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/*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. 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.

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).

Table 2.1. List of primers used for the design of the small IR constructs

Primer	Sequence (5'→ 3')
5F1	cagaattccattcgttgtttgtcatc
5F2	acgaattctaggactcaggaaccata
5R1	caggatccatagatttttgaaccagt
5R2	caggatccgcatctttgtattggtca
MF1	gcaagcttatctggacaatgaactta
MF2	cgaagctttctcattccttgctactt
MR1	ctggatccttctgctcctcaagtctg
MR2	ctggatccttcacaatcccagttatg
3F1	ctgaattctttgagcaggagattgaa
3F2	cagaattcccttgtggtctcattcag
3R1	caggatccccccttacctacaaaatcat
3R2	taggatccagggagtggctacatttt

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 minutes in a 16h-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 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 CCC (chlorocholine chloride). Transformants were then incubated at 18 °C in the dark. After 4 to 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 axillairy 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 to 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. The transformants with granules with varying sizes of cores were classified as medium silenced. 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 0.5-2.0 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 hours at 30 V and subsequently vacuum blotted (Pharmacia) onto Hybond (N+) membranes (Amersham) in 0.4 N NaOH. A 722-bp fragment amplified with the *npt*II primers npt3 (5' TCGGCTATGACTGGGCACAACAG A 3') and npt 4 (5' AAGAAGGCGATAGAAGGCGATGCG 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 NOSt F (5'ATGAGATGGGTTTTTATGAT 3') and NOSt R (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 hours. The blots were rinsed twice with 2x SSC, 1 % SDS, followed by a rinse with 1x SSC, 1 % SDS.

Northern analysis

Total RNA was isolated from microtubers using Trizol agent (Sigma). RNA concentrations were measured using Ribogreen dye (Jones-Laurie 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).

Small RNA analysis

Total RNA was isolated from microtubers using Trizol agent (Sigma). From every sample, 20 µg of total RNA was electrophoresed on a 15 % polyacrylamide gel for 1.5 hours at 100 V using a vertical gel system (Biorad). RNA was then transferred onto Hybond N by overnight electro blotting at 25 V (Biorad). As probes, cDNA fragments of GBSSI were

used. Labelling and hybridisation experiments were performed as described for Southern analysis using a hybridisation temperature of 50 °C.

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= number of strongly silenced transformants/ total number of transformants
P^t=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 1.3 A-S containing an inverted repeat of the 3' half of the GBSSI cDNA in antisense-sense orientation and IR 1.1S-A harbouring an inverted repeat of the 5' half of the GBSSI cDNA in sense-antisense orientation. Construct IR 1.3A-S contained the 5' half of the GBSSI cDNA as a spacer, whereas in construct IR 1.1S-A the 3' half of the cDNA functioned as a spacer (Fig. 2.1). 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).
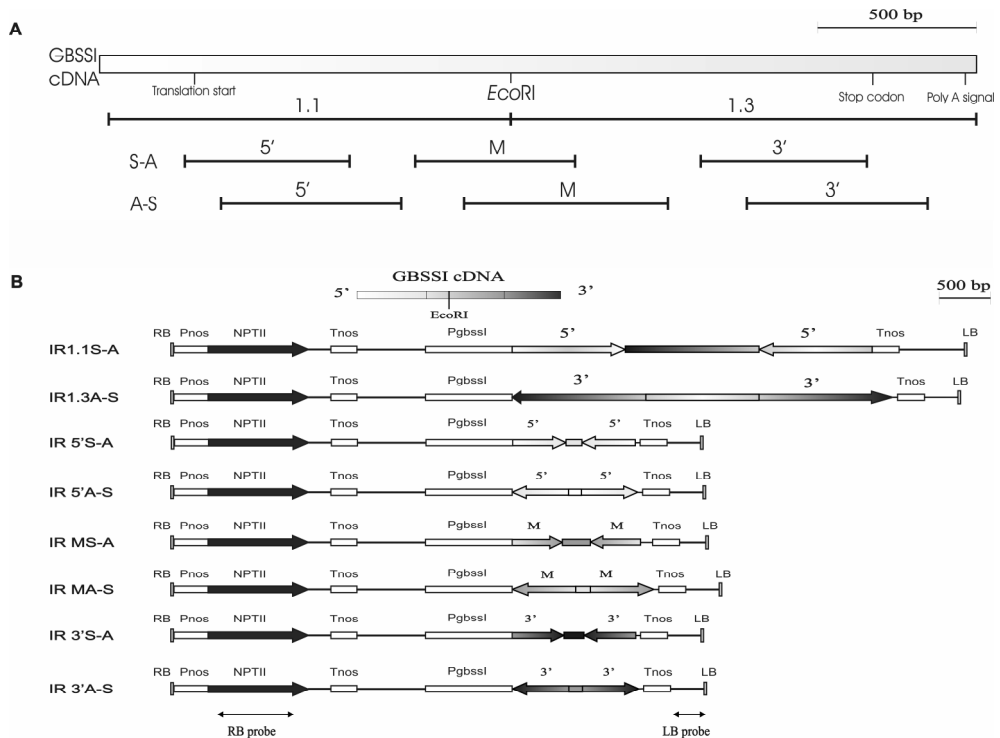
Figure 2.1. A) Origin of potato GBSSI sequences used for the construction of the inverted repeats. S-A= sense-antisense. A-S=antisense-sense. **B)** Inverted repeat constructs of potato GBSSI cDNA. Inverted repeat constructs were cloned behind the potato GBSSI promoter in binary vector pPGB-1s. RB, right 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.

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. When compared with the efficiency of the antisense construct, both inverted repeat constructs resulted in an almost 2-fold increase in frequency of silenced transformants (Fig. 2.2). 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.
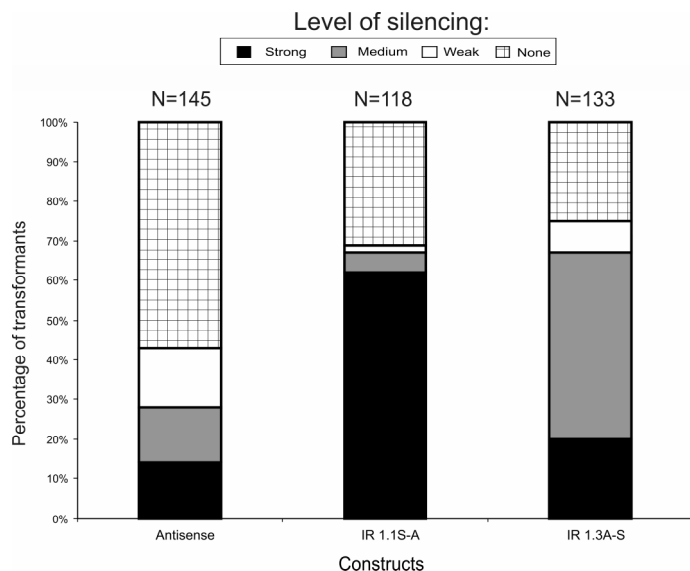
Figure 2.2. Silencing of GBSSI in Karnico transformants by GBSSI antisense and large cDNA inverted repeat constructs. N=number of transformants

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 (Fig. 2.1). The six constructs were tested in two independent transformation experiments. 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 2.3. If we consider the percentage of transformants showing silencing (Fig. 2.3), 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 analyzed 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 %.
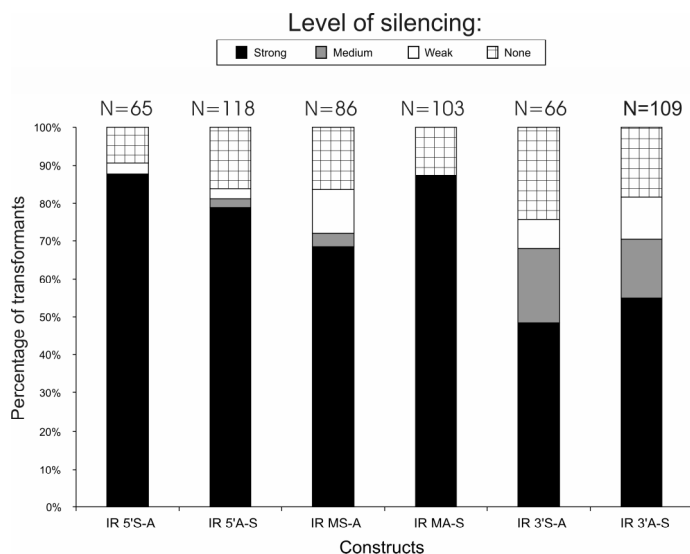

Figure 2.3. Silencing of GBSSI in Karnico transformants by small cDNA inverted repeat constructs. N=number of transformants

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. No effect of inverted repeat orientation on silencing efficiency was observed. 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 analyzed 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 hybridized with an RB probe (Fig. 2.1b) containing the *npt*II sequence. The distribution of T-DNA integration number is shown in Figure 2.4a. All eight constructs gave similar distributions of the number of T-DNA integrations. In Table 2.2, the relation between T-DNA integration number and the number of transformants showing a silencing effect is shown for all IR constructs.

Table 2.2. Number of T-DNA integrations in relation to silencing effect of transformants harbouring different inverted repeat constructs.

construct	number 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[*]	5	nt	4	nt	11	nt
IR1.3 A-S	20[*]	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
* for these constructs, only silenced transformants were tested for the number of T-DNA integrations

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 2.4b.
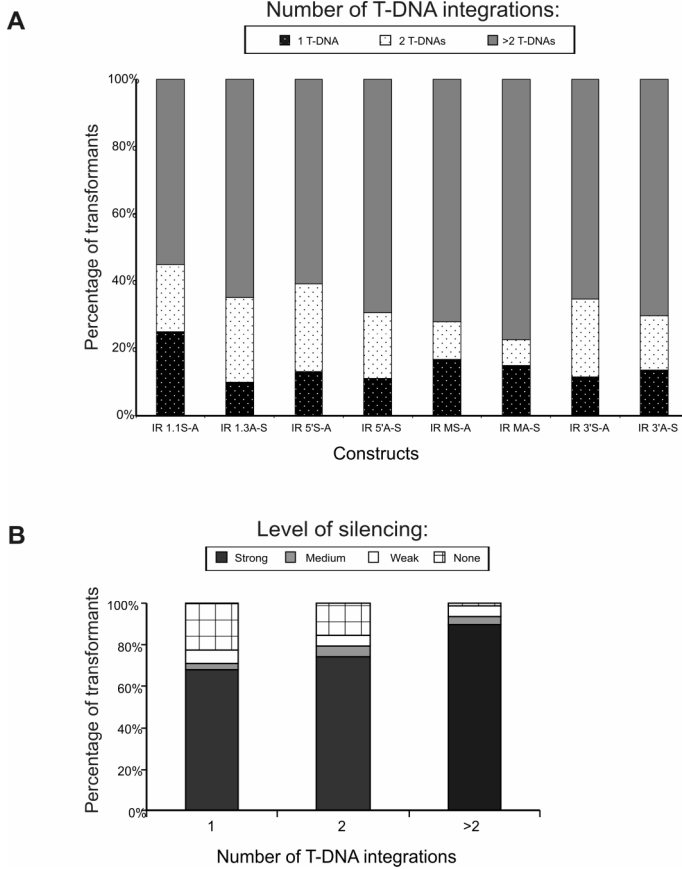
Figure 2.4. A) Distribution of T-DNA integration numbers among transformants of different inverted repeat constructs. **B)** Relation between number of T-DNA integrations and level of silencing in all IR transformants

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 (Fig. 2.1b). Out of nine non-silenced transformants, seven did not hybridize with the LB probe. Four out of 35 transformants that did show silencing did not hybridize to the LB probe.

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 a GBSSI cDNA probe. Equal RNA loading was verified by hybridisation with a 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 2.5a, the transcript level in seven transformants, harbouring inverted repeats of the middle GBSSI region, is shown. Even though GBSSI is still present in the silenced transformants, a reduction in relation to the level in wild type is visible. From Figure 2.5a, 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 other strongly silenced transformants.

To address whether the reduction in GBSSI mRNA level was accompanied by the production 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 2.5b. Only transformants showing silencing contained siRNAs. However, no clear correlation between the level of siRNAs and the level of silencing was found.
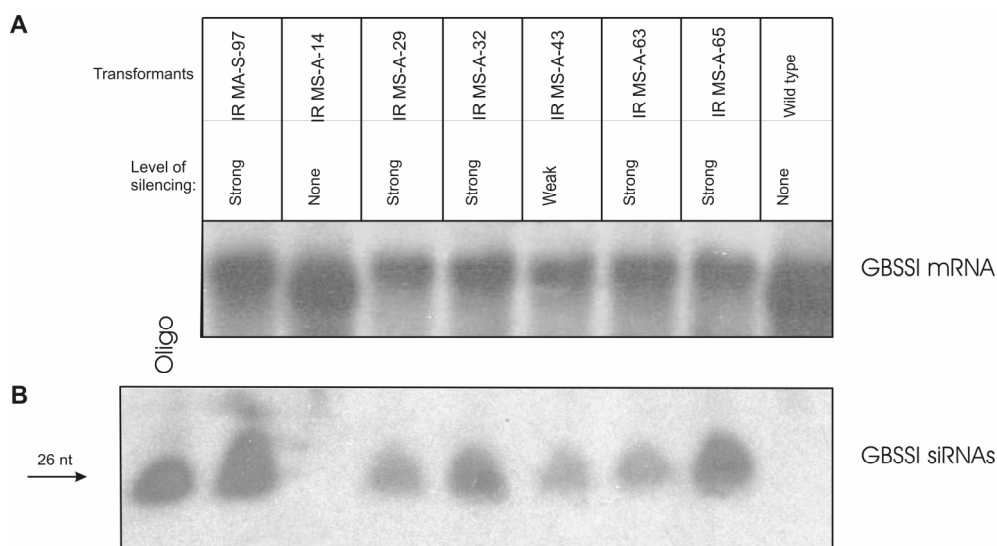

Figure 2.5. **A**) Northern blot analysis showing the reduction of GBSSI mRNA steady state level in transformants harbouring inverted repeat constructs of the middle region of the GBSSI cDNA. Total RNA was hybridised with a GBSSI cDNA probe.
B) Production of small RNAs in transformants harbouring constructs of the middle region of the GBSSI cDNA. As oligo, a 26 nt primer was used. Membranes were hybridised with a fragment derived from the middle region of the GBSSI cDNA.

Discussion

Using inverted repeat constructs of the GBSSI cDNA, we were able to induce highly efficient silencing. The level of silencing depends 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 analyzed 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 a 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 2.2, Figure 2.4b). 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 2.4a, 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 did not hybridize 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 2.1b) and thereby affects the production of dsRNA resulting in a loss of silencing. From the 35 silenced transformants, four did not hybridize to the LB-specific sequence. In this case, small deletions at the LB that do not affect the production of dsRNA probably occurred.

Orientation of inverted repeat does not affect silencing efficiency
Although it was originally believed that the orientation of the inverted repeat was of importance (Elbashir et al., 2001), we have shown that this is not the case for silencing of GBSSI in potato. The results obtained with the six small cDNA inverted repeat constructs showed that the orientation of the inverted repeat does not affect the silencing efficiency. Therefore, we think that the difference in silencing efficiency between IR 1.1S-A and IR 1.3 A-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 differences in silencing efficiency were 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 first step in which dsRNA is cleaved by Dicer determines which siRNAs are formed. Attributes of the 3'end structure of the dsRNA hairpin molecule, including overhang length and sequence composition, determine the position and efficiency of Dicer cleavage. Different substrates with similar terminal structures can exhibit slight 1-2 nt shifts in preferred cleavage positions. These single nucleotide shifts have been shown to dramatically alter the thermodynamic stability of siRNA termini (Vermeulen et al., 2005). That the structure and thermodynamic stability of siRNAs are important for their functionality was shown by Schwarz et al. (2003) and Khvorova et al. (2003). 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.

The presence of functional siRNAs does not automatically lead to efficient silencing since the accessibility of the target RNA is also important. Schubert et al. (2005) and Overhoff et al. (2005) suggest that there is a correlation between the extent of silencing and the local free energy in the target region. If the target region forms a secondary structure with a relatively low local free energy, base-pairing with the guide strand of the siRNA is prevented.

The observation that 3' IR constructs were the least efficient silencing inducers contrasts with the results obtained with the GBSSI antisense construct 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.

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).

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 ß-1,3-glucanase (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 cotransformation 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 et al. (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. On the other hand, very long inverted repeats increased the chance of recombination in bacterial host strains. Therefore, they recommend a fragment length of between 300 and 600 bp as a suitable size to maximize the efficiency of silencing.

Sijen et al. (2001b) 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. These findings could imply that, also in this viral reporter system, the 5' fragments are actually more efficient silencing inducers than the 3' fragments.

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 represents 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 is clear that the size of the spacer does not influence silencing efficiency of GBSSI in potato. In contrast, Ai-Sheng et al. (2005) demonstrated 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. However, it should be pointed out that the 1002 bp spacer is twice as large as the size of the inverted repeat sequence which might influence the stability of the dsRNA. Their results would have been more convincing if another spacer size in between these two extreme sizes would have been tested.

Concluding remarks

It is clear 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. We also demonstrated 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 an internal spacer of about 150 bp were very efficient silencing inducers.

Acknowledgements

Chapter 3

Presence of introns in inverted repeat constructs has no effect on efficiency of post-transcriptional gene silencing of GBSSI in potato

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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 efficiency 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.

Introduction

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

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 is not 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* (Ceruti, 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; Luehrsen and Walbot, 1991; Maas et al., 1991; Mascarenhas et al., 1990; Rethmeier et al., 1997), but also in *Petunia* (Dean et al., 1989) and *Arabidopsis* (Rose, 2002; Wang et al., 2002). Smith et al. (2000) 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.

Experimental procedures

Plant material and growth conditions
Potato cultivar Karnico 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}.

cDNA synthesis
Total RNA was isolated from microtubers of potato cultivar Karnico using Trizol agent (Sigma). 2 µg RNA was treated with 5 U 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 synthesized on 500 ng RNA using Superscript II Reverse Transcriptase according to the manufacturer's protocol (Invitrogen). Two µl was used for PCR amplification in a volume of 50 µl using region-specific primers (see Chapter 2). PCR fragments were cloned in pGEM-Teasy (Promega) and then sequenced.

DNA constructs
The design of constructs IR 5'A-S, IR MA-S and IR MS-A is described in Chapter 2. 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. Construct IR MS-A 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
All constructs were transformed to potato cultivar Karnico as described in Chapter 2.

In vitro tuberisation
Microtubers were induced on *in vitro* grown stem segments containing axillairy buds. These were placed on petridishes with MS medium containing 80 g/l 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 to 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 as described in Chapter 2.

Small RNA analysis
Total RNA was isolated from microtubers using Trizol agent (Sigma). 20 µg of total RNA was electrophoresed on a 15 % polyacrylamide gel for 1.5 hours at 100V using a vertical gel system (Biorad). RNA was then transferred onto Hybond N by overnight electro blotting at 25 V (Biorad). Two identical blots were made. One was hybridized with an intron-specific probe amplified with primers Infor (5'GTAACATAAGATTTTTCCAACT CC 3') and Inrev (5' CCATGACCTGTATAGAGATTTTG 3') while the other was hybridized with a spacer- specific probe amplified with primers MF1 (5'GCAAGCTTATCTGGACAATGAACTTA3') and MR3 (5'CAAGTAGCAAGGAAT GAGAGC 3'). Labelling and hybridisation experiments were performed as described in Chapter 2.

Statistical analysis
To test whether the silencing effects in constructs were significantly different, a binomial test was used whereby
P^t=number of silenced transformants/total number of transformants
P^s=number of strongly 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 and Discussion

Effect of an intron in an inverted repeat sequence on silencing efficiency
The effect of inverted repeat constructs harbouring different GBSSI cDNA sequences was described in Chapter 2. The GBSSI cDNA (Visser et al., 1989) was subdivided in three regions: the 5', the middle and the 3' region. Fragments containing these regions were sequenced and the 5' and middle fragments proved to contain introns two and nine, 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 (Fig. 3.1). 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.
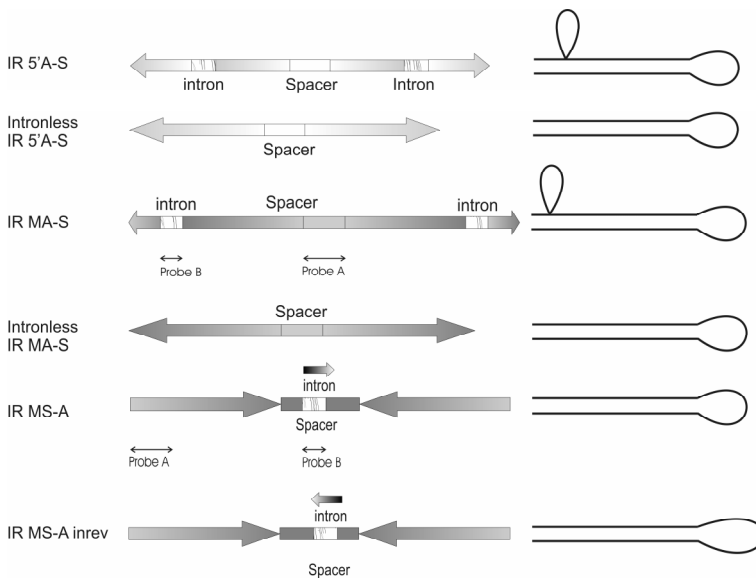

Figure 3.1. Construct composition and predicted dsRNA structures when correct splicing of the introns in sense orientation has been achieved. In constructs IR 5'A-S and IR MA-S, the intron is located in the inverted repeat. In construct IR MS-A, the intron is present in the spacer. Probes used for the detection of siRNAs are indicated.

All four constructs were tested at the same time in one transformation experiment. Per construct, 31 to 45 independent transformants were obtained. Microtubers were induced and analysed for the level of silencing by iodine staining followed by microscopic observation. Figure 3.2 shows the percentage of transformants showing different levels of silencing for the different constructs.
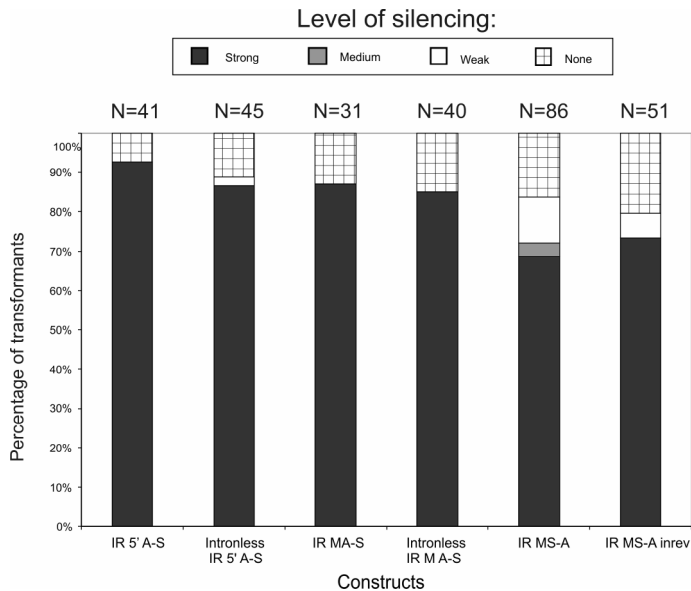
Figure 3.2. Effect of presence or absence of introns in inverted repeat or in spacer sequence on silencing of GBSSI in potato transformants. N= number of independent transformants

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. Moreover, Dicer can also handle miRNAs that are processed from their precursor stem-loop structures which also form 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 whilst an almost similar binding energy of -980.7 kcal/mol was predicted for the intronless IR 5'A-S. Binding energies of -1105.80 and -1100.9 kcal/mol 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.

After concluding that the presence of the GBSSI introns did not inhibit silencing , we raised the question whether the introns can enhance silencing like they can enhance expression in

natural situations (Rose, 2002). This is not the case since no significant differences between the inverted repeat constructs with and without intron were observed.

Effect of an intron in the spacer sequence on silencing efficiency

The intron derived from the M part of the GBSSI cDNA is located in the spacer in the IRM S-A construct. In order to determine the importance of a spliceable intron in the spacer, a second construct in which the intron was in the reverse, non-splicing, orientation was made (Fig.3.1; IR MS-A inrev). The effect on silencing efficiency of both constructs is shown in Figure 3.2. When the construct with the non-spliceable intron was transformed into potato, 71 % of the transformants showed strong silencing. Since the construct 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 hybridized with probe A, representing the spacer region in IR MA-S (Fig.3.1). Figure 3.3 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 (Chapter 2), it was known that all these transformants showed accumulation of GBSSI siRNAs. Therefore, the lack of hybridisation with the spacer probe in transformants of IR MA-S can be subscribed to the absence of spacer-derived siRNAs. This confirms that no siRNAs of spacer sequences are formed.
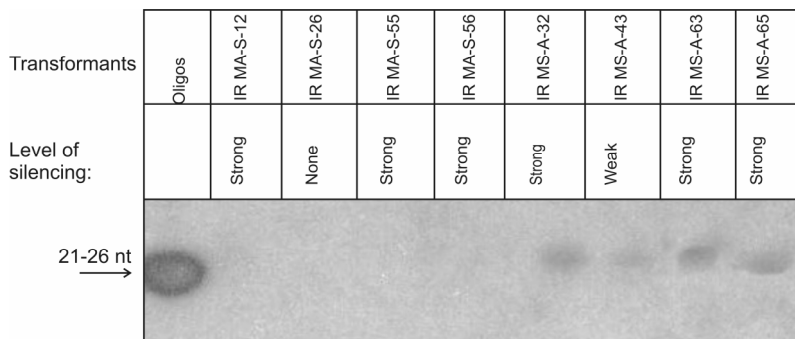
Transformants	Oligos	IR MA-S-12	IR MA-S-26	IR MA-S-55	IR MA-S-56	IR MS-A-32	IR MS-A-43	IR MS-A-63	IR MS-A-65
Level of silencing:		Strong	None	Strong	Strong	Strong	Weak	Strong	Strong
21-26 nt →									

Figure 3.3. Detection of siRNAs in IR MA-S and IR MS-A transformants by hybridisation with probe A, representing the spacer region in IR M A-S. All transformants, exept the non-silenced IRM A-S-26, showed accumulation of siRNAs using a M GBSSI specific probe (data not shown).

To demonstrate that the intron was spliced in dsRNA, siRNAs from both IR MA-S and IR MS-A transformants were hybridized with probe B, representing the intron region (Figure 3.1). No signal was observed in transformants of both constructs (data not shown) indicating that the intron is indeed spliced in IR MA-S. Since the surroundings of this intron are exactly the same when the intron is located in the spacer of IR MS-A , we assume that this intron is spliced in the spacer as well.

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 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 ssRNA 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 intron orientations gave similar silencing efficiencies implying that the splicing of the intron does not enhance silencing efficiency.

Intron-enhanced expression or silencing

From the results presented here, we can conclude that inclusion of an intron in GBSSI inverted repeat constructs neither inhibits nor enhances silencing efficiency. We also found that the presence of a spliceable intron in the spacer did not enhance silencing efficiency of GBSSI in potato. If the intron-enhanced silencing 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*. She demonstrated that five *Arabidopsis* introns varied 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 by Rose (2002), two had little or no effect on PAT1: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 PAT1: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 not the features that determine the degree to which an intron will stimulate expression. A combination of these features and other unknown factors are probably 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 do enhance RNA accumulation whereas others do not. If the intron-enhanced silencing is based on a similar mechanism, the potential of an intron to enhance silencing might explain why some introns do 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) (Nakayashiki et al., 2005). These differences in silencing efficiency indicate that not all introns contribute to enhancement of silencing in the same extent. The GBSSI introns two and nine 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 two or nine in dsRNA does not affect gene silencing efficiency. Intron nine was also tested in the spacer 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.

Acknowledgements

Chapter 4

Non-targeted endogenous sequences participate in transitive silencing in potato

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Abstract

Transitive silencing of the granule-bound starch synthase gene (GBSSI) in potato was induced by two approaches. In the first approach, silencing of GBSSI was achieved through a construct harbouring a NOS terminator inverted repeat preceded by a GBSSI cDNA sequence and transcribed by a GBSSI promoter. Obtained results provided the evidence that transitive silencing does occur in potato. To address whether the endogenous transcript could function as a template for the synthesis of new dsRNA, transformants harbouring inverted repeats of different regions of the GBSSI cDNA were analyzed for the presence of siRNAs derived from non-targeted regions. Secondary siRNAs were detected in regions 5' to the targeted regions but not in the regions 3' to the targeted regions. This is the first example of the use of endogenous transcript as a template for RdRP in transitive silencing in plants.

Introduction

Post-transcriptional gene silencing (PTGS) can be achieved by antisense or sense constructs, but the frequency of transformants showing an effect is often low. After the discovery that double-stranded RNA (dsRNA) is actually the trigger of PTGS, enormous improvement has been made in the development of inverted repeat constructs that induce highly efficient silencing.

A fascinating aspect of PTGS is its extreme efficiency; a few trigger dsRNA molecules can inactivate a continuously transcribed target mRNA for long periods of time. Therefore, an additional mechanism must be present to explain the potency and self sustaining nature of RNA interference (RNAi) (Nishikura, 2001). A model for this mechanism was proposed by Sijen et al. (2001a) and it assumes that small interfering RNAs (siRNAs) are incorporated into the RNA-induced silencing complex (RISC), after which the complex is guided to the target mRNA through conventional base-pairing interactions of the antisense strand of the siRNA. Subsequently, the target mRNA can be degraded, or amplified through the action of RNA-dependent RNA polymerase (RdRP). The RdRP-synthesized dsRNA will be recognized by an RNaseIII Dicer-like enzyme and degraded to secondary siRNAs. These secondary siRNAs cause spreading of the target site, which is also described as transitive silencing.

Evidence for this mechanism was presented by Lipardi et al. (2001) who showed that, in a *Drosophila* embryo extract, dsRNA-derived GFP siRNAs and synthetic 21-nucleotide duplex GFP siRNAs, act as mRNA-specific primers to transform the target mRNA into dsRNA. The synthesised dsRNA is then cleaved by the RNase III-related enzyme Dicer generating new siRNAs. Only the antisense strand of the siRNAs is thought to act as primer and as a consequence dsRNA synthesis takes place in the 3' to 5' direction.

Evidence for transitive silencing was also provided by Sijen et al. (2001a), who analysed the siRNAs produced during RNAi in *C.elegans*. They demonstrated that secondary siRNAs, which were not derived directly from input dsRNA but from regions upstream of the targeted mRNAs, were formed. Their analyses showed a loss of transitivity and secondary siRNA signals at distances greater than several hundred base pairs from the

original trigger. Alder et al. (2003) , who also studied transitive silencing in *C.elegans*, observed only short-range effects, fewer than 100 or 180 bases.

Schiebel et al. (1998) were the first to isolate an RNA-directed RNA polymerase-specific cDNA clone from tomato. By DNA gel blot hybridization and/or PCR amplification experiments, they showed that homologs exist in potato, tobacco, wheat, *Arabidopsis* and *Petunia*. The tomato RdRP can perform primed as well as unprimed polymerase activity. Other RdRP homologs were found in *Neurospora* (Cogoni and Macino, 1999), *C.elegans* (Sijen et al., 2001a; Smardon et al., 2000), *Arabidopsis* (Dalmay et al., 2000; Mourrain et al., 2000) and *Dictyostelium discoideum* (Martens et al., 2002). Surprisingly, so far no RdRP homolog has been found in *Drosophila*.

Himber et al. (2003) studied spreading of RNA silencing in *Arabidopsis* GFP transgenes. Two transgene lines were used; one expressed GFP in a wild type background whilst the other expressed GFP in a *sde1* mutant background. SDE1 is an RNA-dependent RNA polymerase required for transgene silencing in *Arabidopsis* (Dalmay et al., 2000). Upon introduction of an inverted repeat harbouring GFP sequences driven by a phloem-specific promoter, silencing was established in both GFP transformants in the phloem. However, silencing in the *sde1* mutant background did not expand beyond 10-15 cells outside of the phloem region whilst silencing in the wild type background spread over the whole leaf. This spreading of silencing was correlated with transitivity since siRNAs located 3' from the GFP initiator region accumulated in the GFP transgene with wild type background. These siRNAs were not detected in the line carrying the SDE1 null mutation where no extensive spreading of silencing was observed. Two constructs to silence the endogenous *Arabidopsis* RbcS and sulphur mRNAs could not induce extensive spreading indicating that transitivity of an endogenous gene could not be induced (Himber et al., 2003).

Similar results were found by Vaistij et al. (2002) who used virus induced gene silencing (VIGS) in *Arabidopsis* GFP transgenes. Also in this system, SDE1 was required for spreading of silencing.

By using VIGS in *N.benthamina*, Vaistij et al. (2002) demonstrated that the target sites and the production of siRNA can spread within the transcribed region of the GFP transgene from the initiator region in both 3' and 5' directions. A requirement for this spreading to occur is the transcription of the target GFP transgene; if no transcription occurred, no transitive silencing occurred. This spreading seems to be transgene-specific since no spreading of target site and production of siRNAs was observed for the endogenous PDS and Rubisco genes (Vaistij et al., 2002).

Brummell et al. (2003) made use of the existence of transitivity by designing a construct in which an inverted repeat of the NOS terminator of *Agrobacterium* was placed behind the polygalacturonase (PG) transgene. In this way, they induced transitive silencing of the endogenous PG gene. Not only were they able to silence the tomato PG gene, but also two plant transcription factors from *Arabidopsis* could be silenced effectively. Apparently, the siRNAs produced by the NOS inverted repeat can use the dsRNA as template for the synthesis of new dsRNA. In this example, endogenous genes are silenced through transitive silencing but whether these endogenous genes actively participate in this process by providing a template for RdRP is not clear.

Since it was shown that potato has an RdRP homolog, we have reason to believe that transitivity might occur in potato. To test this hypothesis, two approaches were used. First of all, a construct harbouring a NOS terminator inverted repeat preceded by the middle region of the GBSSI cDNA and transcribed by a GBSSI promoter, was tested for silencing efficiency of GBSSI. Silencing was established indicating that transitive silencing in a 3' to 5' direction does occur in potato. In the second approach, transformants harbouring GBSSI cDNA constructs were analyzed for the accumulation of secondary siRNAs derived from regions adjacent to the targeted regions. Secondary siRNAs were detected from those non-targeted regions. Since the secondary siRNAs can only be produced when the endogenous transcript functions as a template for the synthesis of new dsRNA, we here demonstrate that non-targeted endogenous GBSSI sequences actively participate in transitive silencing in potato.

Experimental procedures

Plant material and growth conditions

Potato cultivar Karnico 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

To design construct MGBSS NOSIR, a 267-bp NOS terminator was excised from pBI101 (Jefferson et al., 1987) by digestion with *Eco*RI and *Sac*I. Cloning of this fragment in pUC28 (Benes et al., 1993) resulted in pUC28NOS. A 406-bp fragment containing the NOS terminator and part of a BAR gene was isolated from pDE110 (Denecke et al., 1989) by digestion with *Sac*II and *Xba*I. This fragment was then cloned in pUC28NOS resulting in a NOS inverted repeat in which part of the BAR gene (139 bp) functioned as a spacer. The NOS IR was then cloned into pPGB1-s (Kuipers et al., 1995) through the *Xba*I and *Eco*RI sites. By using this approach, the original NOS terminator was replaced by the new NOS terminator inverted repeat resulting in binary vector pPGB1-sNOSIR.

The 761-bp middle fragment of GBSSI cDNA was isolated from the vector that harboured the PCR product obtained with primers MF1 and MR1 (Chapter 2) by restriction with *Hin*dIII and *Bam*HI. This fragment was then subcloned in pBluescript (Stratagene). The fragment was excised with *Sal*I and *Xba*I after which it was cloned in pPGB1-sNOSIR. In this way, the middle fragment was placed between the GBSSI promoter and the NOS IR. This construct was named MGBSS NOSIR (Fig.4.1).
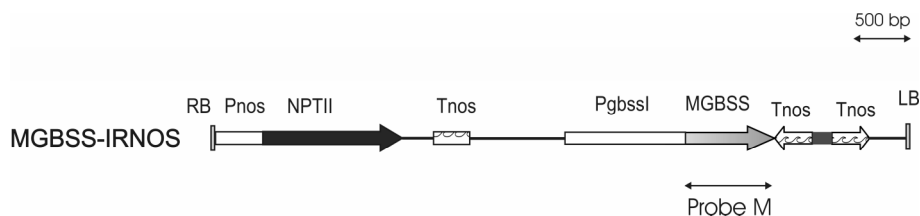
Figure 4.1. Construct containing an inverted repeat of the NOS terminator preceded by the middle region of the GBSSI cDNA (MGBSS). RB, right border; LB, left border; [P]NOS, promoter of the nopaline synthase gene; NPTII, kanamycin resistance gene; [T]NOS, terminator of the nopaline synthase gene; [P]GBSSI, promoter of GBSSI. The probe (M) used for hybridisation with siRNAs is indicated.

All subclonings were transformed into *E.coli* DH5α (Invitrogen, Breda, The Netherlands). MGBSS NOS IR was transformed into *A. tumefaciens* strain AGL0 (Lazo et al., 1991) by electroporation (Takken et al., 2000).

Transformation and regeneration
All constructs were transformed to potato cultivar Karnico as described in Chapter 2.

***In vitro* tuberisation**
Microtubers were induced on *in vitro* grown stem segments containing axillairy buds. These were placed on petridishes with MS medium containing 80 g/l 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 to 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 as described in Chapter 2.

Small RNA analysis
Total RNA was isolated from microtubers using Trizol agent (Sigma). 20 µg of total RNA was electrophoresed on a 15 % polyacrylamide gel for 1.5 hours at 100V using a vertical gelsystem (Biorad). RNA was then transferred onto Hybond N by overnight electroblotting at 25 V (Biorad). To detect accumulation of GBSSI siRNAs in MGBSS-IRNOS transformants, the M probe (Fig. 4.1) was used. Probes used to hybridize the membranes containing siRNAs of transformants harbouring inverted repeat constructs are indicated in Figure 4.4. Labelling and hybridisation experiments were performed as described in Chapter 2.

Statistical analysis
To test whether the silencing effects in constructs were significantly different, a binomial test was used whereby
P^s= number of strongly silenced transformants/ total number of transformants
P^t=number of silenced transformants/total number of transformants

The null hypothesis of no difference between proportions of (strongly) silenced transformants was rejected at an error of 0.05.

Results

Inducing transitive silencing through an inverted repeat of the NOS gene

The MGBSS-IRNOS construct harbouring a NOS terminator inverted repeat preceded by the middle region of the GBSSI cDNA and transcribed by a GBSSI promoter is shown in Figure 4.1. This construct was transformed to potato cultivar Karnico, after which independent transformants were analyzed for the level of GBSSI silencing by iodine staining of starch granules derived from microtubers. Depending on the size of the blue core in starch granules, transformants were classified into four silencing groups: strong, medium, weak or none (See Chapter 2). Silencing of the GBSSI gene was tested in 35 MGBSS-IRNOS transformants. Out of fifteen transformants that showed a silencing effect, ten were strongly silenced. The effects were compared with the effect of sense (Flipse et al., 1996), antisense (Heeres et al., 2002) and an inverted repeat GBSSI construct (IR MS-A, Chapter 2) in potato cultivar Karnico (Fig. 4.2).
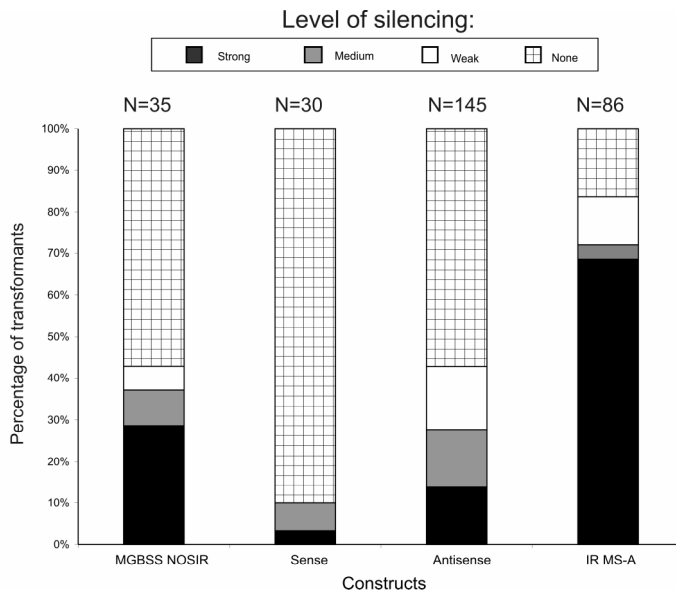

Figure 4.2. Silencing of GBSSI in Karnico transformants by different silencing inducing constructs. N=number of transformants.

Compared to the silencing efficiency of the sense GBSSI construct, the MGBSS-IRNOS construct revealed a significantly higher percentage of transformants showing silencing. For the antisense construct and the MGBSS-IRNOS construct, similar percentages of

transformants showing a silencing effect were found. However, the percentage of transformants showing strong silencing was significantly higher for the MGBSS-NOSIR construct. The inverted repeat construct (IR MS-A) harbours an inverted repeat of the same middle fragment of GBSSI cDNA as represented in the MGBSS-IRNOS construct. In 69 % of the IR MS-A transformants, strong silencing was observed whilst in the MGBSS-IRNOS transformants this percentage was 28%. These results indicate that silencing of GBSSI can be achieved by the MGBSS-IRNOS construct. Although the silencing efficiency is lower than the silencing efficiency obtained with the inverted repeat construct targeting the same GBSSI region, the proportion of strongly silenced transformants is higher than those observed with the sense and antisense constructs.

Examination of small RNAs of four transformants revealed the presence of GBSSI siRNAs in silenced transformants (Fig. 4.3). No siRNAs were detected in a non-silenced transformant whilst the most intense signal was present in the strongly silenced transformant.
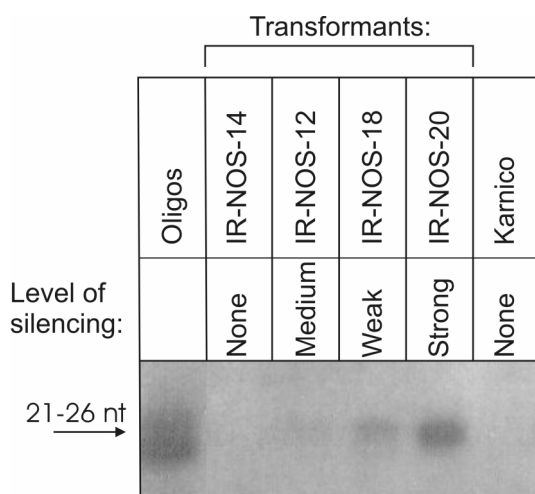

Figure 4.3. Detection of siRNAs in MGBSS-IRNOS transformants. RNA was hybridised with the probe M indicated in Figure 4.1.

Transitive silencing in transformants harbouring GBSSI inverted repeat constructs

To address whether transitive silencing occurs in transformants harbouring GBSSI cDNA inverted repeat constructs, small RNAs were analyzed for the accumulation of secondary siRNAs derived from regions adjacent to the targeted regions. The regions that were targeted for silencing are indicated in Figure 4.4.
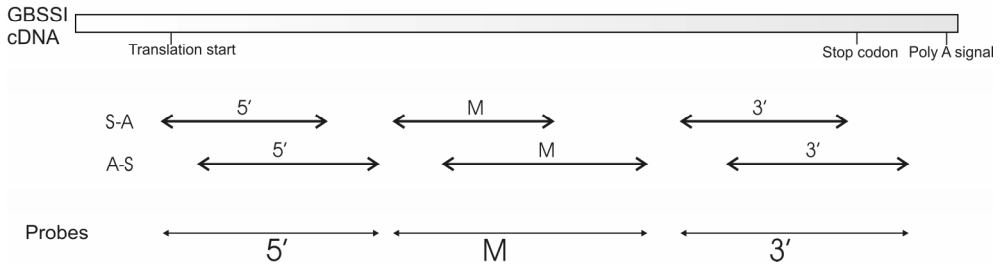
Figure 4.4. Overview of GBSSI sequences of which inverted repeat constructs were made (see Chapter 2).S-A= sense-antisense, A-S= antisense-sense. Probes used for the detection of siRNAs are indicated

High silencing efficiencies were obtained with the six GBSSI cDNA inverted repeat constructs but the percentage of strongly silenced transformants was significantly lower in 3' IR transformants than in the 5' IR and IR MA-S transformants (Chapter 2). Accumulation of siRNAs in silenced transformants was detected by hybridisation with a probe corresponding to the region that was initially targeted (Chapter 2).

If transitivity occurs in these transformants, dsRNA of adjacent sequences would be formed resulting in the formation of secondary GBSSI siRNAs. These secondary siRNAs can easily be monitored by hybridising the siRNAs with a probe that hybridises with the region adjacent to the region that was targeted for silencing. In Figure 4.5a, four transformants harbouring middle region inverted repeat constructs (lane 2-5) show strong accumulation of middle region-specific siRNAs. Although the signal is weaker, the transformants harbouring 3' inverted repeat constructs (lane 6-10) also show accumulation of middle region-specific siRNAs indicating that transitivity does occur. After the membrane was stripped and checked for the absence of signal, it was re-probed with a 5'-specific probe. All four transformants of the middle inverted repeat construct showed accumulation of 5'-specific siRNAs whilst two out of five transformants harbouring 3' inverted repeat constructs also showed accumulation of 5'-specific siRNAs (data not shown). The latter finding indicates that transitive silencing can travel over long distances. The distance between the 3' region and the 5' region is at least 761 bp.

To address whether transitive silencing occurs in the 5' to 3' direction as well, siRNAs of four transformants harbouring inverted repeats of the middle region of GBSSI were hybridized with a 3'-specific probe. Figure 4.5b shows that no signal could be detected whilst 3' siRNAs did accumulate in transformants harbouring inverted repeats of the 3' region of GBSSI. Since no signal was detected in the middle region-derived transformants after a longer exposure, we assume that indeed no siRNAs of the 3' end can be detected in middle region-derived IR transformants. To illustrate the direction of transitivity, schematic representations of the spreading of siRNAs are shown in Figure 4.5a and 4.5b.
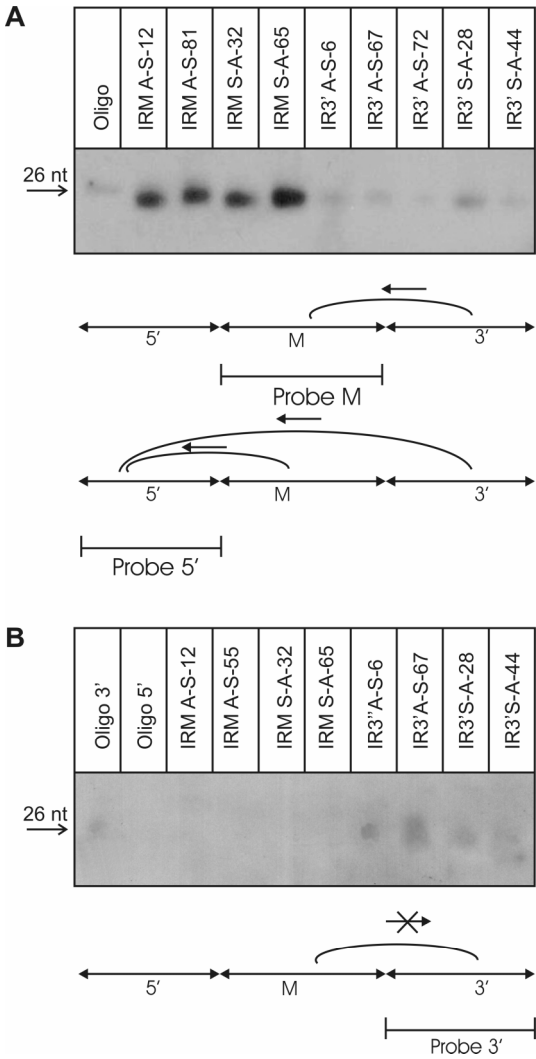
Figure 4.5. Detection of siRNAs in strongly silenced M and 3' IR transformants **A**) Accumulation of siRNAs after hybridisation with a M-specific probe. Schematic representations of spreading of siRNAs from 3' to middle region and from 3' to middle and 5' region. **B**) Accumulation of siRNAs after hybridisation with an 3' specific probe. Schematic representation of non-spreading of siRNAs from middle to 3' region. Probes used for hybridisations are indicated in all schematic representations.

We here demonstrated that transitivity occurs in a 3' to 5' direction in transformants harbouring GBSSI cDNA inverted repeat constructs.

Discussion

Inverted repeat of the NOS polyadenylation sequence causes PTGS of GBSSI

We were able to induce transitive silencing of GBSSI by using an inverted repeat construct of a NOS terminator preceded by a part of the GBSSI cDNA. This indicates that transitive silencing occurs in the 3' to 5' direction and causes the observed effect. The possibility that the observed silencing is caused by the sense M fragment is unlikely since it is known that silencing of GBSSI through sense technology is very inefficient. This is also shown in Figure 4.2. Compared to the results described by Brummell et al. (2003), the silencing efficiency that we obtained is still quite low. In 91 % of their tomato transformants, highly effective post-transcriptional gene silencing of the PG gene was obtained. However, for the two *Arabidopsis* transcription factors, the silencing efficiency was much lower. The reason which they provided for the lower silencing efficiency was that complete silencing of these transcription factors is lethal and the actual frequency of PTGS was much higher. Compared to the number of tomato transformants (56), the number of the *Arabidopsis* transformants harbouring transcription factors (8 and 4) is very low. Therefore, it is difficult to compare efficiencies. If more transformants would be available, a more reliable comparison could be made.

The observation that we could silence the GBSSI gene by attaching an inverted repeat of a NOS terminator at the 3' end of the middle fragment of GBSSI cDNA provides opportunities for silencing other genes. By cloning other sequences in between the GBSSI promoter and the NOSIR, silencing of these sequences can be induced through transitive silencing. When the construction of inverted repeats of interesting genes is too laborious or costly, this method could be a good alternative for functional analysis of genes.

Transitive silencing in GBSSI inverted repeat constructs occurs in a 3' to 5' direction

We also demonstrated the occurrence of transitive silencing in transformants harbouring GBSSI cDNA inverted repeat constructs. Besides accumulation of siRNAs corresponding to the target region, accumulation of siRNAs originating from the region located 5' from the target region was observed. This reflects that transitivity occurs in the 3' to 5' direction. No siRNAs corresponding to the region located 3' from the target region were detected indicating that transitivity in the 5' to 3' direction does not exist or can not be detected by this method. At least, it is clear that, if transitivity in the 5' to 3' direction occurs, it happens at a much lower frequency than the transitivity in the 3' to 5' direction. Spreading of transgene-derived siRNAs in plants has been described in *N.benthamiana* through virus induced gene silencing and in *Arabidopsis* through stable transformation (Vaistij et al., 2002; Himber et al., 2003). In both systems, no spreading of silencing was observed for endogenous genes. Brummel et al. (2003) showed spreading of silencing for endogenous genes but did not show whether the endogenous transcript was used as a template for RdRP. Our data provide the first example in plants that the spreading of silencing is caused by the use of endogenous transcript as a template for RdRP. Hamilton et al. (1998) observed silencing of the related ACO2 gene when the ACO1 transgene with a repeated 5'UTR was used for transformation of tomato. Since there is little homology between the 5'UTRs of ACO1 and ACO2, the trans-acting silencing signal should be derived from sequences external to the 5'UTR repeat. Whether these sequences are derived from the

transgene or the endogenous gene is not known. In our experiments, we know that the transgene only covers a third part of the GBSSI gene. The observation that siRNAs accumulate that do not correspond to the targeted GBSSI sequence implies that the endogenous GBSSI transcript must have functioned as a template for the synthesis of new dsRNA. The observed spreading in the 3' to 5' direction can most likely be ascribed to siRNA-primed RdRP-directed synthesis on an endogenous sense RNA template. That we do not observe spreading of silencing in the 5' to 3' direction can have different reasons. First of all, the frequency of transitivity in 5' to 3' direction might be below the detection limit of the method used to detect siRNAs. Secondly, the potato RdRP homolog might not be able to perform unprimed RdRP-activity on the sense mRNA template.

GBSSI siRNAs can spread over long distances
We demonstrated that 3' to 5' transitivity occurs from 3' to middle region of GBSSI, from the middle to the 5' region of GBSSI and even from the 3' to the 5' region of GBSSI. The latter finding was observed for two out of five samples indicating that transitivity is related to distance. We assume that the signal of secondary siRNAs decreases when the distance from the original trigger is larger. The distance between the 3' region and the 5' region is at least 761 bp, which indicates that siRNAs can spread over long distances. Vaistij et al. (2002) found that spreading of RNA targeting in a VIGS system can extend at least through 332 bp. In *C. elegans*, transitivity was lost at distances greater than several hundred basepairs from the original trigger (Sijen et al., 2001a). Later on, Alder et al. (2003) found that the effects of transitive silencing did not extend over a distance of 100 or 180 bp. Spreading of siRNAs was also found in transgenic tomato lines where an inverted repeat of the 5'UTR ACO1 gene was introduced upstream of the sense ACO1 transgene. siRNAs of both the 5'UTR ACO1 gene as well as from the 158 nt region immediately downstream of the repeats accumulated. This reflects transitive silencing in the 5' to 3' direction over a distance of at least 158 bp (Han and Grierson, 2002).

Direction of spreading in several systems
In potato, we found evidence for transitivity in the 3' to 5' direction. Transitivity in animals proceeds in the same direction (Lipardi et al., 2001; Sijen et al., 2001a) and is probably caused by primer-mediated spreading. In plants however, it is less clear in which direction spreading of silencing occurs. Transitivity in the 5' to 3' direction was found in *Arabidopsis* transgenes (Himber et al., 2003), tomato transgenes (Han and Grierson, 2002), virus induced silenced *N.benthamiana* and *Arabidopsis* plants (Vaistij et al., 2002) and virus induced silenced *N.benthamiana* plants (Braunstein et al., 2002). Evidence for spreading of transitivity in the 3' to 5' direction was found in virus induced silenced *N.benthamiana* (Vaistij et al., 2002)and in *N.tabacum* transgenes (Van Houdt et al., 2003). Whether spreading of transitivity in the 3' to 5' direction occurred in virus induced silenced *Arabidopsis* was not checked (Vaistij et al., 2002). Also, in *Arabidopsis* transgenes, Himber et al. (2003) did not check transitivity in the 3' to 5' direction (Himber et al., 2003). Examples of transgene RNA silencing that do not exhibit spreading also exist. No spreading of transgene RNA silencing was found in tobacco plants harbouring a GUS:viral RNA chimeric transgene. Only siRNAs corresponding to the viral part of the transgene sequence were found indicating that no spreading occurred (Wang et al., 2001).

All these examples are based on silencing of transgenes. No spreading of target site and production of siRNAs was observed for the endogenous PDS and Rubisco genes in the VIGS system in *N.benthamiana* (Vaistij et al., 2002). Neither could transitivity of the endogenous RbcS and sulphur genes be established in *Arabidopsis* transgenes (Himber et al., 2003).

Concluding remarks

Our data provide the first example in plants that the spreading of silencing is caused by the use of endogenous transcript as a template for RdRP-mediated synthesis of new dsRNA. Spreading of GBSSI siRNA production occurred in the 3' to 5' direction. We also showed that the transitivity could extend over a distance of 761 bp which, to our knowledge, is the largest distance reported so far.

Chapter 5

Inducing transcriptional gene silencing in potato; targeting of different GBSSI promoter regions leads to different silencing efficiencies

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Anne-Marie A. Wolters and Richard G.F. Visser.

Abstract

Transcriptional gene silencing of the GBSSI promoter in potato was induced by inverted repeat constructs containing different regions of the GBSSI promoter. Clear differences in silencing efficiency between the different regions were observed.

Targeting the sequences from -766 to -168 bp, relative to the transcription initiation site (TIS) induced only weak silencing effects in 57 to 60 % of the 35SGBP-IR transformants whilst the full promoter inverted repeat construct containing the sequences from -766 to +194 bp relative to the TIS induced very strong silencing in 49 % of the FP-IR transformants. In these strongly silenced transformants, no mRNA could be detected by Northern blot analysis. This was accompanied by the accumulation of promoter-specific siRNAs. 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 cause 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.

Introduction

Post-transcriptional gene silencing (PTGS) involves sequence-specific RNA degradation and can be induced by expressing double-stranded RNA (dsRNA) of coding sequences. When dsRNA of promoter sequences is expressed, 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., 2001b). The methylation is induced by RNA signals and is therefore described as RNA-directed DNA methylation (RdDM). RdDM leads to *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 siRNAs 21-26 nt in length (Matzke et al., 2004). It is not known whether the siRNAs or dsRNA guide methylation of homologous DNA sequences although there are indications that the longer class of siRNAs, 24-26 nt in length, are 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 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 can 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. (2001b) demonstrated that transcriptional and post-transcriptional gene silencing are initiated by a similar dsRNA pathway. They showed that the 35S promoter could be

silenced in *Petunia* by inverted repeat constructs harbouring 35S promoter sequences. 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., 2001b).

So far, TGS of transgene promoters has been described in *Petunia*, tobacco and *Arabidopsis*. Silencing efficiency does not only depend on the region of the promoter that is targeted but also on the sensitivity of the promoter to methylation (Matzke et al., 2004). 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., 2001b).

In potato, the granule-bound starch synthase I (GBSSI) gene has been efficiently silenced by post-transcriptional gene silencing. 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). In order to obtain a transformant with no GBSSI mRNA, transcription has to be prevented. Therefore, transcriptional gene silencing 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 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 GBSSI can be transcriptionally silenced through 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 complete promoter sequence.

Experimental procedures

Plant material and growth conditions

Potato cultivars Karnico and Ponto 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'-CTCCGTTTTGTTCATTACTT-3') and behpromr1 (5'-ATTCACGGCTGGACTTCAAC -3') were used to amplify a 599-bp product (see fig 5.1a; primers P1 and P4) from pWAM10, a pUC18-based plasmid containing the class A1 potato GBSSI promoter (van der Leij et al., 1991). This product was subcloned 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 5.2).

By inserting a 2x35S promoter in the GBP-IR construct through the *Cla*I and *Hin*dIII restriction sites, a new binary vector, 35SGBP-IR, was created (Fig 5.2). The 2x35S 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 DNA isolated from a dihaploid potato line (90-027-6) harbouring two A2 GBSSI alleles using the PCR conditions and cdf1 and cdf2 primers (P2 and P3 in figure 5.1b) as described by van de Wal et al. (2001). The primers were modified by introducing a *Nco*I and *Pst*I site at the 5'part of the cdf1 primer whereas a *Spe*I and *Sac*II site were introduced at the 5'part of the cdf2 primer. The resulting product, ASP, was subcloned 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'-CCGGAATTCAAAGTT GCGCGGAGGAGTTGTGTT -3') and spacerrev (5'-CGGGGTACCGTCGGGGTCGGCG TCGTG-3'). The ampiflied 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, correct orientation was checked by restriction analysis. The allele-specific promoter inverted repeat construct (ASP-IR) is shown in Figure 5.2.

To make the full promoter inverted repeat construct, Gateway technology was used. A 960-bp PCR product was amplified using a modified behpromf1and the mwpr4 primer (P1 and P5 in figure 5.1) (van de Wal et al., 2001). 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 pTOPO 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 5.2) was checked by restriction analysis.
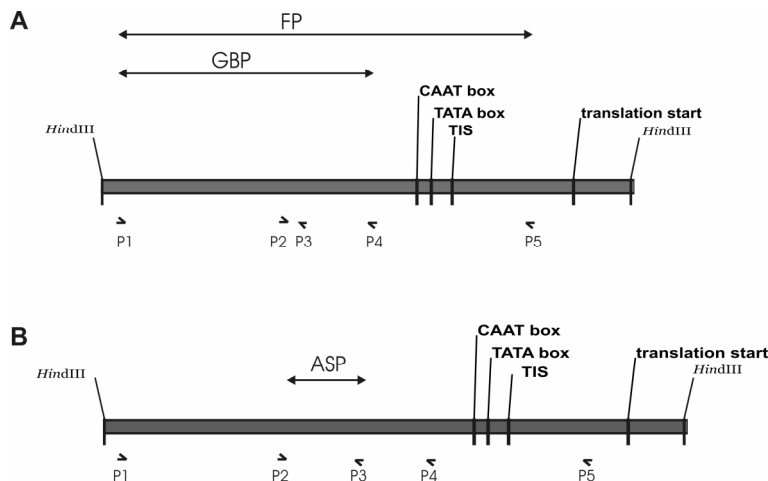

Figure 5.1. A) Schematic overview of the promoter of endogenous GBSSI allele A1 in which the GBP and FP regions are indicated. **B)** Schematic overview of the promoter of endogenous GBSSI allele A2 with the ASP region indicated. Primers used to amplify promoter regions are indicated. TIS=Transcription initiation site. P1= behpromf1, P2=cdf1, P3=cdf2, P4=behpromr1, P5=mwpr4.
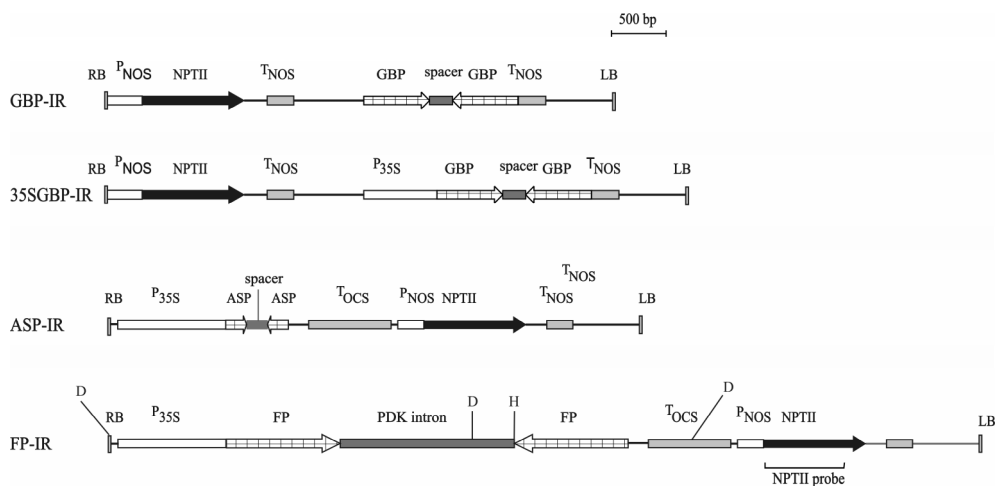

Figure 5.2. DNA constructs designed to produce GBSSI promoter dsRNA. GBP-IR: GBSSI promoter inverted repeat without a transcribing promoter. 35S-GBP-IR: GBSSI promoter inverted repeat driven by the 35S promoter. ASP-IR: Allele-specific GBSSI promoter inverted repeat harbouring 200 bp of the GBSSI A2 allele. FP-IR: Full promoter inverted repeat. NPTII probe used for Southern hybridization is indicated. H=*Hin*dIII, D=*Dra*I. 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; ^{P}35S, 35S promoter.

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 streptomycin (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 streptomycin (100 mg/l) as a selectable marker. All constructs were transformed to *A.tumefaciens* strain AGL0 (Lazo et al., 1991) by electroporation (Takken et al., 2000).

Transformation and regeneration
All constructs were transformed to potato cultivar Karnico or Ponto as described in Chapter 2.

***In vitro* tuberisation**
Microtubers were induced on *in vitro* grown stem segments containing axillairy buds. These were placed on petridishes with MS medium containing 80 g/l 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 to 3 weeks.

Starch staining
Staining of starch granules was performed as described in Chapter 2. 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 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 0.5-2.0 µg 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 hours at 30 V and subsequently blotted (Pharmacia) onto Hybond (N+) membranes (Amersham) in 10x SSC. A 722-bp fragment amplified with the *npt*II primers npt3 (5' TCGGCTATGACTGGGCACAACAG A 3') and npt 4 (5' AAGAAGGCGATAGAAGGCGATGCG 3') was used as probe (NPTII) to check for integration of T-DNA sequences near the LB (see figure 5.2).

Northern analysis
RNA isolation and Northern analysis was performed as described in Chapter 2. RNA concentrations were measured spectrophotometrically.

RT-PCR
Total RNA was isolated from microtubers using Trizol agent (Sigma). 3 µg RNA was treated with 7 U RNAse-free DNAse (Amersham) for 10 minutes at 37 °C after which DNAse was inactivated through incubation for 15 minutes at 65 °C in 0.0025 M EDTA. cDNA was synthesized 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'-GTCAGGCCCAATTACGAAGA-3') and Ubirev (5'-AAGTTCCAGCACCGCACTC-3') (Tm=55°C, 40 cycles**)**. To detect premature mRNA, intron-specific primers GBSS4 (5'-CAGGAATAGGCAAAATAAAG ATGA-3') and GBSS11 (5'-GTTCCCTTACATTTCCTGATTC-3') were used (Tm=55°C). For the detection of mature RNA, exon-specific primers MF1 (5'-GCAAGCTTATCTGGA CAATGAACTTA-3') and MR1 (5'-CTGGATCCTTCTGCTCCTCAAGTCTG-3') were used (Tm=55°C). For both primer combinations, 35 and 45 amplification cycles were performed. To visualize PCR products, 10 µl was electrophoresed on a 1 % agarose gel. To verify the effectiveness of 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 8000) and sodium chloride to final concentrations of 5 % and 500 mM, respectively. After incubation on ice for 30 minutes, 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. Electrophoresis and detection of small RNAs was performed as described in Chapter 2. As a probe, the GBP fragment (Fig 5.1a) was used.

Methylation of the GBSSI promoter

Genomic DNA of *in vitro* grown transformants was isolated from 0.5-2.0 µg of leaves, as described by Chen et al.(1992). DNA isolated from 35S GBP-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, 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, one third with *Hae*III and the remaining third was used as a control. As a reference, DNA of the construct FP-IR and of wild type Karnico were subjected to the same treatments.

After precipitation, samples were electrophoresed on a 0.8 % agarose gel for 16 hours at 30 V and subsequently vacuum blotted (Pharmacia) onto Hybond (N+) membranes (Amersham) in 0.4 N NaOH. For the 35S GBP-IR transformants, the GBP fragment (Fig 5.1a)(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 5.1a) (a 960-bp PCR fragment obtained with the primers behpromf1 and mwpr4) was 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 hours. The blots were rinsed two times with 2x SSC, 1 % SDS, followed by a rinse with 1x SSC, 1 % SDS.

Inhibition of methylation by 5-aza-cytidine
5-aza-cytidine (Aza-dC), a nucleotide analog that inhibits cytosine methylation, was added to the microtuber induction medium in concentrations of 12.5 or 25 mg/l. Microtubers were induced on this medium as described in *in vitro* tuberisation.

Results

Silencing effects of partial and full promoter inverted repeat constructs
To test whether TGS of the endogenous GBSSI gene could be induced by dsRNA, a 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 5.1a). The GBP-IR was placed under control of a 35S promoter resulting in a second construct named 35SGBP-IR (Fig 5.2).
Since endogenous GBSSI alleles are highly homologous in their coding sequences but show variability in their promoter sequences (van de Wal et al., 2001), it was hypothesized 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 5.1b: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 5.2). 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 potato cultivar Karnico whilst 35SGBP-IR and ASP-IR were transferred to the potato cultivars Karnico and Ponto. These cultivars differ in 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 an 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 5.3 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. The GBP-IR construct which has the same sequences but does not have a transcribing promoter, resulted in 2 % of 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 different potato cultivars.
The 35SGBP-IR construct targeted the region from -766 to -168 bp relative to the transcription initiation site (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 an 35S-driven full promoter inverted repeat construct (FP-IR) (Fig 5.1a and 5.2). The effect of this construct is shown in Figure 5.3.
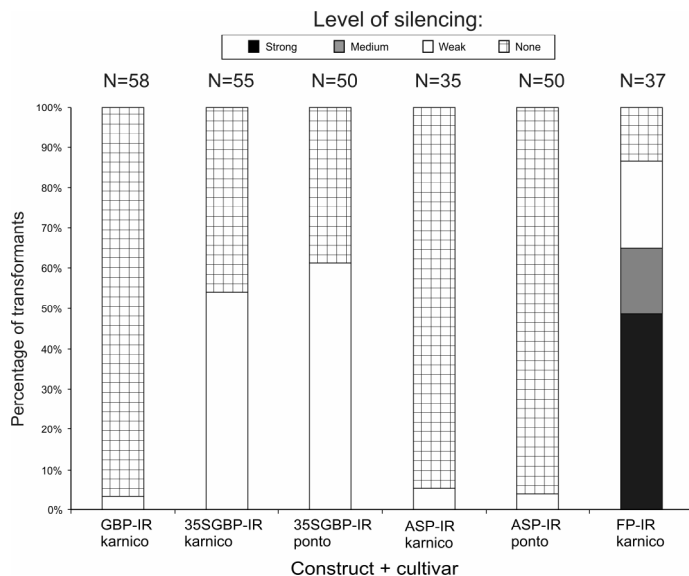

Figure 5.3. Silencing of GBSSI in Karnico and Ponto transformants by promoter inverted repeat constructs. N= total number of transformants per construct/genotype combination.

Strong silencing was observed in 49 % of the FP-IR 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 microtuber-derived starch. Using this analysis, low amylose contents are overestimated. Even in the amylose-free control, an amylose content of 2.3 % was measured. Depending on 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 5.1 shows the phenotypic observations as well as the amylose contents of 12 transformants that are discussed in more detail in this Chapter. 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 an *nptII* probe (see Fig. 5.2). Three transformants harbouring a single T-DNA integration were detected. Two of these transformants showed strong silencing.

Table 5.1. Level of silencing in relation to amylose content and number of T-DNA integrations in FP-IR transformants. When percentages are indicated, these represent the percentages of starch granules showing different levels of silencing within a microtuber of a single transformant. Nt=not tested.

Transformant	Level of silencing	% amylose in starch	# T-DNA Integrations
Fp-1	Strong	1.8	9
Fp-2	Weak	14.5	Nt
Fp-3	Strong	2.0	2
Fp-4	55 % strong 3 % medium 31 % weak 11 % none	3.2	Nt
Fp-6	93% strong 5% medium 2 % weak	2.3	Nt
Fp-8	99 % strong 1 % weak	2.1	1
Fp-13	Strong	2.2	4
Fp-16	Strong	1.9	Nt
Fp-24	95 % strong 1 % medium 4 % weak	2.7	1
Fp-25	Weak	13.6	1
Fp-33	Strong	Nt	3
Fp-39	Strong	2.2	3
Amylose-free control (amf mutant)	Strong	2.3	-
Wild-type (Karnico)	None	16.4	-

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 Northern blot analysis. Total RNA was hybridized with a GBSSI cDNA probe. Results are shown in Figure 5.4.
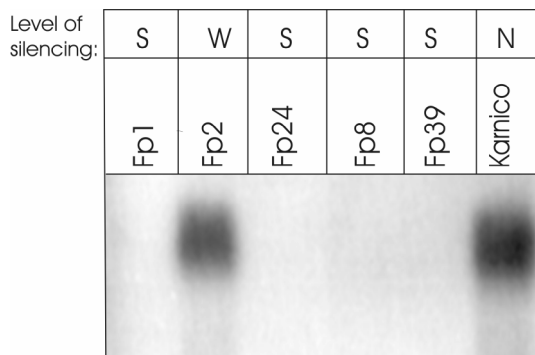

Figure 5.4. Northern blot analysis demonstrating the absence of GBSSI mRNA in strongly silenced FP transformants after hybridization with a GBSSI cDNA probe. N=non-silenced; S=strongly silenced;W=weakly silenced.

No transcript could be detected in transformants showing strong silencing whereas transcript was present in the weakly silenced transformant 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 PTGS transformant. The accumulation of mature and premature GBSSI was determined by using GBSSI intron and exon primers (see Figure 5.5). 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).
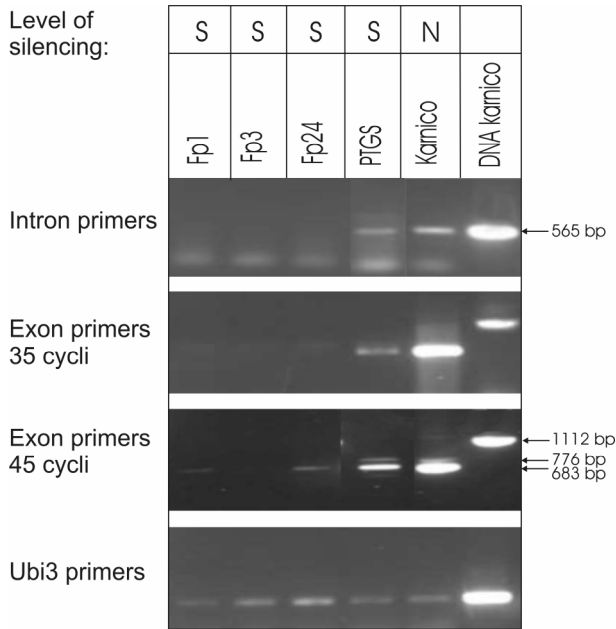
Figure 5.5. RT-PCR analysis on fp1, fp3, fp24, a PTGS transformant and wild type Karnico RNA. As a reference, wild type Karnico DNA was included. Amplification with intron primers gbss4 and gbss11 (565 bp) was used to detect premature RNA. Exon primers MF1 and MR1 (683 bp) were used to demonstrate the presence of mature RNA. The 776 bp fragment is most likely derived from mature RNA plus intron 9. Amplification of the genomic GBSSI DNA with the exon primers MF1 and MR1 resulted in a fragment of 1112 bp. The presence of cDNA was demonstrated by amplification with Ubi3 primers. N=non-silenced; S=strongly silenced.

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 was found (Figure 5.5). 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 analyzed for the presence of GBSSI promoter-derived siRNAs. For this purpose, total RNA was enriched for siRNAs. Figure 5.6 shows that GBSSI promoter-derived siRNAs accumulate in silenced FP transformants but not in wild type Karnico.
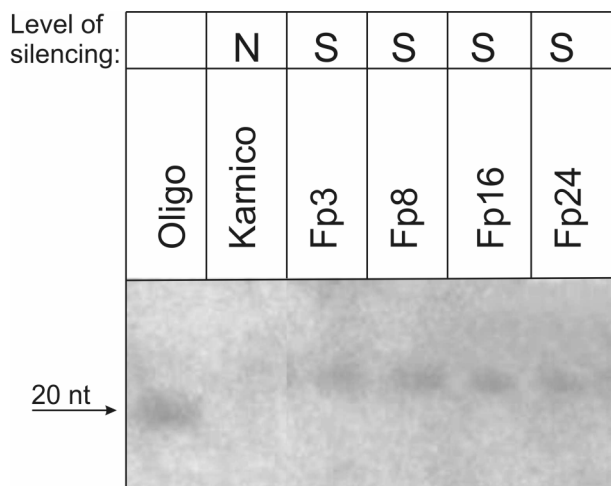
Figure 5.6. Production of GBSSI promoter-derived siRNAs in silenced FP transformants. siRNAs were detected by hybridisation with the GBP probe. As oligo, the 20-nt CDF1 primer was used. N=non-silenced; S=strongly silenced

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 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 1202 and 1346 bp (see M in fig 5.7a and 5.7b). The two endogenous fragments are derived from different alleles. The promoter of the GBSSI A1 allele gives rise to the 1202-bp fragment whilst the promoter of the other alleles gives rise to a 1346-bp fragment. In figure 5.7c, 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 construct. Since Ponto has 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 3 A1 alleles, the intensity of the 1202-bp fragment was higher than that of the 1346-bp fragment supporting the 3:1 ratio in intensity (data not shown). *Hpa*II and *Msp*I both cleave at the recognition site 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 would be 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 of the 883-bp fragment (see Fig 5.7b M*). Apparently, this *Hpa*II site is not methylated. This indicates that the methylation is restricted to the targeted sequences. In Figure 5.7c, transformant 35SGBP-72 shows the accumulation of an additional fragment of about 582 bp in the *Hpa*II or *Msp*I-digested fragments indicating partial methylation of the *Hpa*II site. In total, 22 35SGBP-IR transformants were tested of which 14 showed complete methylation and 8 showed partial methylation. No difference between the patterns of *Msp*I and *Hpa*II were observed indicating that the outer C in the CCGG sequence is always methylated, otherwise *Msp*I would be able to cut. No correlation between silencing level and 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.
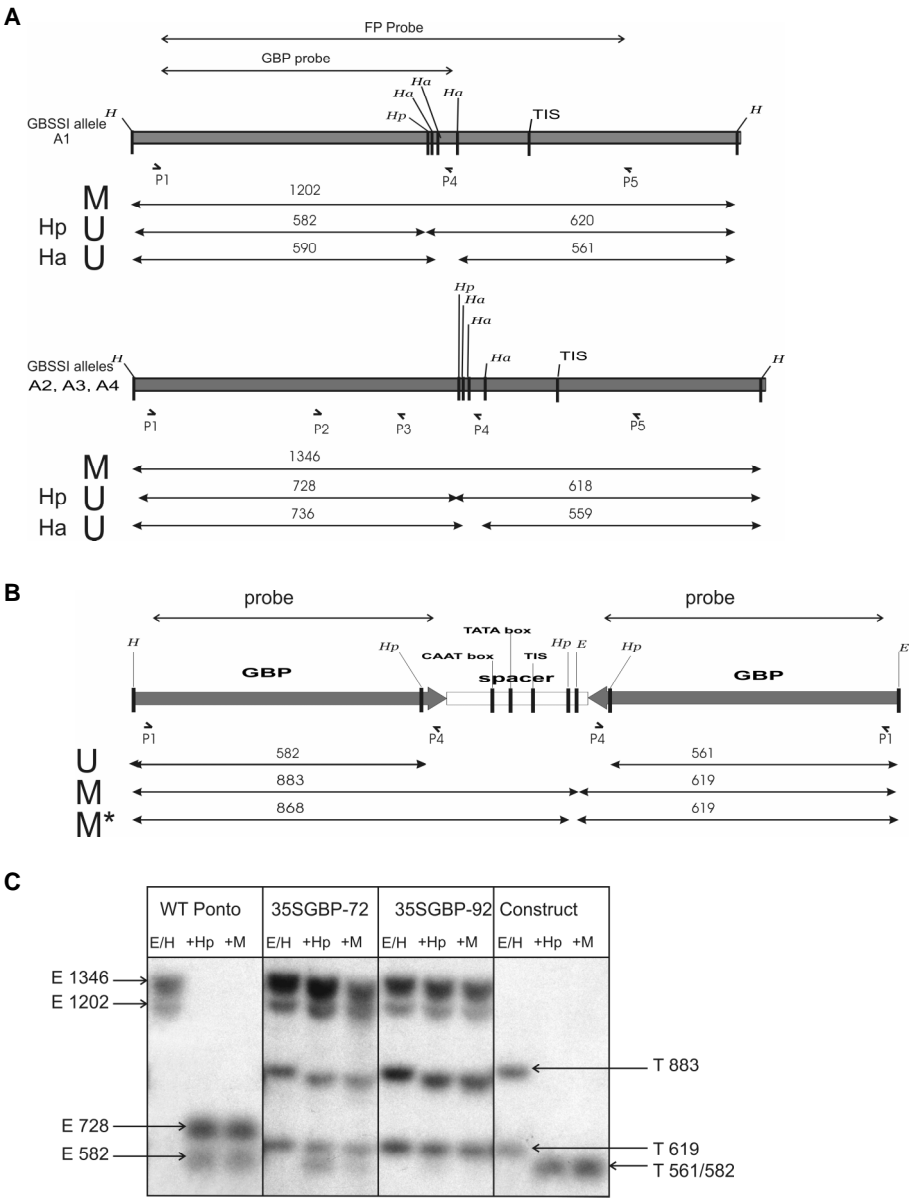
Figure 5.7. DNA methylation analysis in 35SGBP-IR transformants. **A)** Expected hybridizing endogenous fragments after digestion with *Hin*dIII and *Hpa*II or *Hae*III. Fragments derived from the A1 allele as well as for the A2, A3 or A4 allele are indicated. M: methylated. U: unmethylated. Probes are indicated. **B)** Expected transgene-derived fragments after digestion of 35SGBP-IR transformants with *Hin*dIII, *Eco*RI and *Hpa*II. U: unmethylated M: all *Hpa*II sites methylated., M*: no methylation of *Hpa*II site in spacer. **C)** Southern blot analysis showing methylation of *Hpa*II and *Msp*I site in 35S GBP-IR transformants. First lane of each panel shows a *Hin*dIII-*Eco*RI double digest. The second lane represents the triple digests with *Hin*dIII/*Eco*RI plus *Hpa*II and in the third lane, the results of the triple digests with *Hin*dIII/*Eco*RI plus *Msp*I 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 and construct are shown in the first and last panel.

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

DNA of FP transformants was digested with *Hin*dIII and *Xba*I followed by hybridisation with the FP probe (Fig 5.1a). This gave rise to transgene-derived fragments of 1043 bp and > 4189 bp while fragments of 1202 and 1346 bp were obtained for the endogenous GBSSI sequences. 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 *Hpa*II to analyse CG methylation in the FP-IR transformants. For the detection of CNN methylation, the *Hae*III site was used. In Figure 5.7a and 5.8a, the expected sizes of the *Hpa*II and *Hae*III-digested fragments are shown for the endogenous and the transgene-derived sequences, respectively. In Figure 5.8b, the results of the Southern blot are shown. Similar to the analysis of the 35GBP-IR transformants, fragments of 1202 and 1346 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 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 1202-bp A1 fragment. However, the intensity is much lower than the intensity of the endogenous 1202-bp fragment indicating that the A1 endogenous fragment is not or only partially methylated. It is clear that the endogenous A4 fragment is not methylated at the *Hae*III site. Disappearance of the *Hae*III-digested endogenous fragments corresponds with the accumulation of smaller fragments. The intense signal observed at the height of 570-620 bp most likely represents non-methylated endogenous as well as transgene-derived fragments. In transformants fp33 and fp13, the presence of non-methylated transgene-derived products indicates that methylation of transgene fragments at *Hae*III sites is partial. The 1043-bp transgene fragment does not disappear after digestion with *Hae*III. However, the size of the fragment slightly decreases. Analysis of the *Hae*III restriction sites revealed that one *Hae*III site (GGCC) in the antisense strand (indicated with an asterisk in Figure 5.8a) is followed by a G implicating that this site can still be digested by *Hae*III even if the C is methylated (New England Biolabs, Catalogue 2004-2005). Observation of the slightly decreased transgene fragment thus indicates that methylation of the transgene does occur. The same phenomenon was found for the *Hae*III site that is followed by a G in the sense FP strand (also indicated by an asterisk in Figure 5.8a). Digestion of FP-IR transformants with *Hae*III resulted in a 1831-bp fragment which indicates that the *Hae*III sites within the sense strand could not be digested but the site followed by a G, could (data not shown). In Figure 5.8a, the fragments are indicated (Ha M*). Six 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.
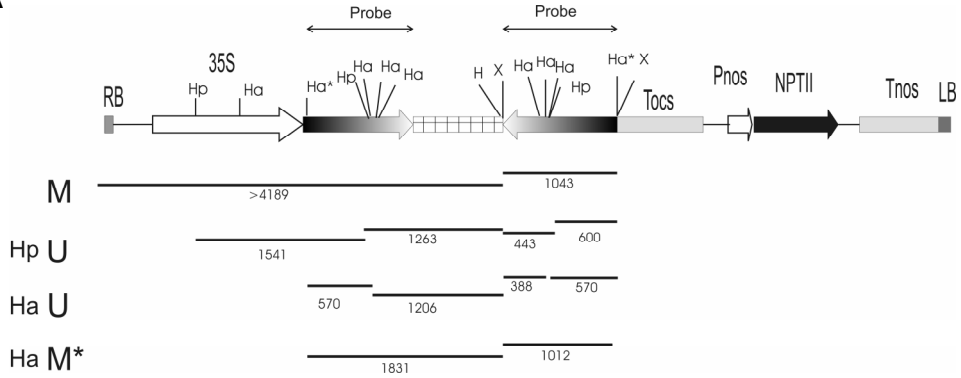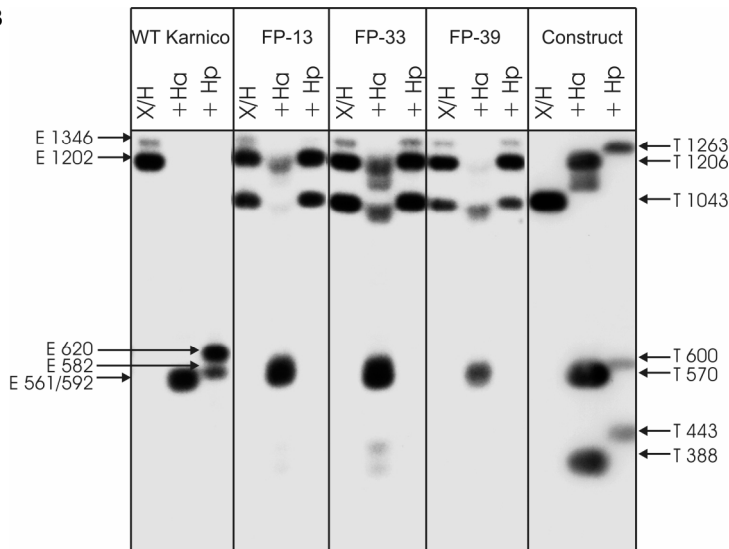
Figure 5.8. A) DNA methylation analysis in FP-IR transformants. Expected transgene-derived fragments for FP-IR transformants after digestion with *Xba*I/*Hin*dIII plus *Hae*III or *Hpa*II. Only relevant *Hae*III and *Hpa*II sites are indicated. M: all *Hpa*II or *Hae*III sites methylated. U: unmethylated. Ha M*: All *Hae*III sites methylated but Ha* can be digested **B)** Southern blot analysis showing complete or partial methylation of *Hpa*II and *Hae*III in FP-IR transformants. First lane of each panel shows a *Xba*I/*Hin*dIII double digest. The second lane represents the triple digests with *Xba*I/*Hin*dIII plus *Hae*III and in the third lane, the results of the triple digest with *Xba*I/*Hin*dIII plus *Hpa*II 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-derived fragments. T fragments: transgene-derived fragments. Expected endogenous fragments are shown in Figure 5.7a.

From these data, it seems that CG methylation is complete whilst methylation at non CG sites is only partial. To address whether methylation could spread outside the region that was initially targeted, 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 targeted region. Four transformants varying

in T-DNA integration number were subjected to these treatments together with wild type and construct DNA. Again, partial methylation was observed in all transformants. However, in FP1, spreading of methylation beyond the targeted region was detected. No spreading of methylation was found in the other three transformants (data not shown).

Effect of methylation inhibitor on silencing level in FP-transformants

To determine whether the methylation status of the FP-transformants could be changed and consequently lead to a change in silencing level, microtubers were induced on microtuber induction medium to which 5-aza-cytidine (aza-dC) was added. Aza-dC is a nucleotide analog that inhibits cytosine methylation. The effect of aza-dC was tested in wild type Karnico, a PTGS transformant, a 35SGBP transformant and in FP transformant fp3. No effect of aza-dC was observed in the wild type, the PTGS transformant, and the 35SGBP-IR transformant. However, microtubers of transformant fp3 did show a change in phenotype after treatment with 25 mg/l aza-dC; the percentage of granules showing strong silencing decreased from 100 to 94 %. Aza-dC apparently does affect the methylation status and therefore the silencing level. In a later stage, Aza-dC was added to the propagation medium and omitted from the microtuber induction medium. This did not result in a clear change in silencing levels. Therefore, it seems that when the methylation inhibitors are removed, *de novo* methylation by dsRNA can be re-initiated.

The fact that no effect was observed in the weakly silenced transformant might indicate that changing the methylation status of the region -766 to -168 bp relative to the TIS, does not influence the silencing.

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 can not 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. It is not known what the role of the allele-specific 140-bp sequence in the A2, A3 and A4 allele is 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, methylation of this sequence 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.

A more efficient silencing was obtained with the 35SGBP-IR construct targeting the region from -766 to -168 bp relative to the transcription initiation site. 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 think 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 transcription initiation site was still functional. This indicates that this sequence or part of this sequence is important for functionality of the GBSSI promoter. If this sequence is not silenced, it is likely that the promoter can 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 transcriptional gene silencing of the NOS promoter by expressing NOS promoter dsRNA through an inverted repeat. The region used in this inverted repeat comprised the region -264 to +34, relative to the transcription initiation site. 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. 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 transcription initiation site is included in the inverted repeat. Sijen et al. (2001b) 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) did only lead to partial silencing of the 35S promoter in three out of five transformants. The 35S-full sequence (-614 to +36, relative to the transcription initiation site) does contain a transcription initiation site whilst the 35S-enh sequence (-614 to -65, relative to the transcription site) 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 this 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 transformants.

Sijen et al. (2001b) describe silencing of the endogenous *dfrA* promoter in *Petunia*. For this purpose, they used the promoter sequences from position -1823 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, pers.comm.) 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, pers.comm.). 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 think 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 (Qin et al., 2003; Vaucheret et al., 1998). A similar observation was done by Sijen et al. (2001b) 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 Figure 5.7c where an additional fragment of about 582 bp in size accumulates in transformant 35SGBP-72 (panel 2). However, the intensity of the endogenous (1202 and 1346 bp) and transgene-derived (883 and 619 bp) fragments was similar in *Hpa*II-treated and non-treated DNA in all 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. (2001b). 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 transformants. A clear difference between CG and CNN methylation was found in the FP 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 with *Dde*I also revealed that methylation could spread outside the targeted 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 adjacent DNA sequences (Aufsatz et al., 2002a; Vogt et al., 2004; Wassenegger, 2000). In transformant fp1, we detected spreading of CNN methylation within the T-DNA sequence in the 5' direction over a distance of 1 kb. This transformant harboured 9 T-DNA integrations. No spreading of methylation was found in three other FP transformants harbouring 1, 2 or 3 T-DNA integrations. The spreading of CNN methylation in the FP1 transformant could also be a

consequence of the high T-DNA integration number. A complex locus, for example, might be responsible for the induction of methylation.

Concluding remarks

We showed that an endogenous promoter in potato can be transcriptionally silenced in transformants harbouring promoter inverted repeat constructs. The full promoter inverted repeat construct containing the 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 showed that it is important to include sequences in the vicinity of the TIS. No GBSSI transcript could be detected in strongly silenced FP-IR transformants by Northern blot analysis indicating that silencing is very effective.

Acknowledgements

Chapter 6

General discussion

The experiments described in this thesis were performed to study the mechanism of gene silencing and improve its efficiency in potato. As a model system, we used the GBSSI gene to study gene silencing. The influence of several factors on PTGS of GBSSI are described in Chapters 2 and 3. New insights in the mechanism of PTGS in potato are described in Chapter 4 where it was demonstrated that transitivity of this endogenous gene occurs. Furthermore, we were able to induce TGS of GBSSI in potato (Chapter 5).

Induction of PTGS by inverted repeat constructs harbouring GBSSI coding sequences
In Chapter 2, we showed that, as compared to GBSSI antisense constructs, the use of GBSSI inverted repeat constructs greatly enhanced the silencing efficiency of GBSSI in potato. Besides this, we showed that strong silencing could be obtained with a single T-DNA integration of an inverted repeat. Differences in silencing efficiency between the inverted repeat constructs could be ascribed to sequence differences, where the 3' sequences showed the least efficient silencing. Since this was found for both the small as well as for the large inverted repeat constructs, the observed differences in effect seem to be sequence-specific.

The lower silencing efficiency induced by the 3' GBSSI cDNA sequences as compared to the other GBSSI cDNA sequences, could not be explained by the presence of 3'UTR sequences or the presence of putative polyadenylation signals (Chapter 2). Other as yet unknown factors might be involved.

We tested the effect of different orientations of the repeat sequences on the silencing efficiency of GBSSI (Chapter 2). Based on data from an *in vitro* system in *Drosophila*, Elbashir et al (2001) proposed that when Dicer generates an siRNA from dsRNA, only the strand with its 3' terminus at the processed end enters RISC (RNA-induced silencing complex) and is able to guide cleavage of the complementary sequence. They showed that the introduction of dsRNA with 17-20 nt overhanging 3' ends blocked dsRNA processing. A block at the 3' end of a sense strand will only permit dsRNA processing from opposing 3' end of the antisense strand. This generates siRNAs in which only the antisense strand is able to guide sense target RNA cleavage. (Elbashir et al., 2001). According to their model, the direction of dsRNA processing could be determined by the introduction of a block at the 3' end of either sense or antisense strand. If we assume that in our GBSSI Inverted Repeat transformants, the presence of the spacer blocks dsRNA processing, dsRNA processing will preferentially take place at the end where no spacer is present. Based on this assumption, we expected to find a more efficient silencing with the sense-antisense constructs than with the antisense-sense constructs. However, we did not find a difference in silencing efficiency between the two inverted repeat orientations. This implies that either the model of Elbashir et al. (2001) does not apply to our system or the processing of dsRNA is not hindered by the presence of a spacer and therefore occurs from both sides of the dsRNA.

During the different steps in the dsRNA processing pathways, several factors can influence the silencing efficiency. Cleavage by Dicer is influenced by attributes of the 3' end structure of the dsRNA hairpin molecule. Small shifts in preferred cleavage position have been shown to dramatically alter the thermodynamic stability of siRNA termini (Vermeulen et al., 2005). The thermodynamic stability of siRNAs determines their functionality as shown by the analysis of siRNAs in *Drosophila* (Schwarz et al., 2003), *C.elegans*, mouse, human and *N. benthamiana* (Khvorova et al., 2003) They propose that the siRNA strand

with the less-tightly base-paired 5' end is usually incorporated in RISC. There are indications that dsRNA-derived siRNAs in plants also obey to the thermodynamic asymmetry rules suggesting that these properties are more important in determining which strand is incorporated in the RISC than the direction of dsRNA processing (Sontheimer, 2005). These factors might play also a role in our system, but we did not study this.

Length dependence in PTGS

In our system, susceptibility to silencing did not increase with the size of the fragments in the inverted repeats. Rather in our case the small inverted repeat constructs induced a higher silencing efficiency than the large inverted repeat constructs (Chapter 2). In an *in vitro Drosophila* system, Tuschl et al. (1999) tested the effects of dsRNA varying in size from 29 to 504 bp and found that the longer dsRNA were more efficient silencing inducers than the short dsRNAs. However, they only tested one dsRNA of 504 bp and the sizes of the other dsRNA varied between 29 and 151 bp. Akashi et al. (2001) found no differences in silencing efficiency in tobacco BY2 cells when a 300 bp and a 500 bp luciferase dsRNA expression plasmid were co-transformed with a luciferase gene construct. In *Neurospora crassa*, an increase in repeat length from 200 to 600 bp produced a substantial increase in silencing efficiency. However, this length-dependent effect was not observed for the 600-bp and 900-bp repeats since similar silencing frequencies were obtained for these two constructs (Goldoni et al., 2004) (Chapter 2). Apparently, susceptibility to silencing does increase with the size of the dsRNA until a length of dsRNA of about 300 bp. In our system, the repeat size of 500-600 bp was sufficient to induce highly efficient silencing whilst an increase in size to 1.1 or 1.3 kb reduced the silencing efficiency.

Also the length of the spacer might be a reason for difference in efficiency. It varies for the large and small inverted repeat constructs used in this research, but the difference in silencing efficiency between IR1.1 S-A and IR1.3 A-S can not be ascribed to the length of the spacer since both constructs contain large (> 1 kb) spacers.

Level of dsRNA and silencing

The level of dsRNA is important to induce silencing effects (Akashi et al., 2001; Parrish et al., 2000). Although we did not check the level of dsRNA in the transformants containing the inverted repeat constructs, we tested a large number of transformants and all inverted repeats were transcribed by the same GBSSI promoter. Therefore, the variation in dsRNA levels due to position effects is supposed to be similar for all constructs and as a consequence the percentage of transformants with high dsRNA levels should be equal in all constructs. To our opinion, the most important parameters in silencing of GBSSI are the sequence specificity and the length of the dsRNA.

Effect of introns on PTGS of GBSSI

In Chapter 3, we demonstrated that the presence of introns in dsRNA and spacer sequence do not influence the level of GBSSI silencing. This is in contrast with the general belief that the presence of introns in spacer sequences increases silencing efficiency. Smith et al. (2000) demonstrated that the presence of a Pdk intron from *Flaveria* in the spacer of the PVY inverted repeat construct enhanced silencing efficiency. The same effect was found for the *Arabidopsis* Fad2 intron whose presence in the spacer enhanced silencing efficiency of the delta-12 desaturase gene in *Arabidopsis*. Based on these findings, Wesley et al.

(2001) made a generic intron spliced hpRNA vector and tested this for different genes and crops. In general, the intron spliced hpRNA vectors resulted in high silencing efficiencies. However, a direct comparision between hpRNA vectors with and without introns was only made for the two genes described by Smith et al. (2000). Stoutjesdijk et al. (2002) compared the efficiency of an ihpRNA and an hpRNA vector for the Arabidopsis FAD2 gene and confirmed that the ihpRNA vector gave the highest degree of silencing. However, they compared different sequences and different orientations. Therefore, it is not fair to attribute the improved silencing efficiency solely to the intron.

Based on our finding, that the use of a spliceable intron in the spacer does not result in enhancement of silencing, we presume that intron-enhanced silencing is not a general phenomenon, but rather is intron-dependent. This was also shown by Nakayashiki et al. (2005) who demonstrated that the extent to which introns contribute to enhancement of silencing varies per intron. As in intron-mediated enhancement of gene expression (Rose, 2002), we think that some introns do have an effect on silencing efficiency whereas others do not or to a lesser degree show this effect.

Inducing transitivity in potato

We demonstrated that endogenous GBSSI mRNA can be used as a template for the synthesis of new dsRNA. To our knowledge, this is the first example of the active participation of an endogenous plant gene in transitive silencing (Chapter 4). As in animals (Lipardi et al., 2001; Sijen et al., 2001a), the transitivity of GBSSI proceeds in the 3' to 5' direction. This spreading is most likely caused by siRNA primed RdRP-directed synthesis on an endogenous sense GBSSI mRNA template. The transitivity of GBSSI could travel over a distance of 761 bp which is longer than any other example of transitive silencing (Chapter 4).

We also demonstrated that transitive silencing can be exploited in a general silencing construct in potato. We designed a construct in which an inverted repeat of a NOS gene was placed behind a GBSSI fragment. Through transitivity in the 3' to 5' direction, siRNAs of the GBSSI fragment were produced resulting in silencing of GBSSI. By replacing the GBSSI fragment with sequences of other genes, these genes can probably be silenced as well. This approach could provide a good alternative for the functional analysis of genes, especially if the construction of inverted repeats is too laborious or costly.

The process of transitivity results in an increased production of siRNAs of the target mRNA which makes PTGS extremely efficient. On the other hand, the production of siRNAs adjacent to the target mRNA might hamper applications such as allele-specific silencing or silencing of individual genes from a gene family.

An example of specific silencing of genes of a gene family was described by Ifuku et al.(2003). These authours demonstrated that the expression of *psb*P genes 1A and 5B could be specifically suppressed by using an inverted repeat of the 3'UTR of the *psb*P gene 1A in *N.tabacum*. The expression of other *psb*P genes, 2AF and 3F was not affected.

The coding regions of GBSSI are highly homologous for the different GBSSI alleles (van de Wal et al., 2001). Since the GBSSI alleles show variation in the 3'UTR sequences, these 3'UTR sequences might be used to induce allele-specific silencing of the GBSSI gene. However, if secondary siRNAs of the coding region are produced through transitivity, these secondary siRNAs will lead to the degradation of mRNA encoded by all four classes of GBSSI alleles.

Importance of promoter sequences in the induction of TGS

Another approach to induce allele-specific silencing is described in Chapter 5. A promoter inverted repeat of an allele-specific promoter sequence was used to induce TGS. Unfortunately, this approach was not successful since only weak silencing effects were observed in a low number of transformants

We did demonstrate that is it possible to induce TGS of GBSSI by using promoter inverted repeat constructs. A clear difference in silencing efficiency was observed between the 35SGBP-IR (partial promoter) and the FP-IR (full promoter) constructs. The 35SGBP-IR construct only resulted in weak silencing effects whereas very strong silencing was obtained in FP-IR transformants. The inclusion of important promoter boxes or TIS-surrounding sequences in the FP-IR apparently is important for the induction of strong silencing. Mette et al. (2000) and Sijen et al. (2001b) showed that TGS of a NOS promoter and a 35S promoter respectively, could be induced with or without inclusion of the transcription initiation site (TIS) in the dsRNA. In our FP-IR construct, the TIS was included. We did not test whether it was the TIS or surrounding sequences that caused the strong silencing effect. However, when we compare the effects of the 35SGBP-IR and the FP-IR construct, it seems that inclusion of the sequences downstream of -168 bp relative to the TIS is required in order to obtain strong silencing.

In the FP transformants, the sequences -766 bp until +194 bp relative to the TIS were targeted. The inclusion of 194 bp of the 5'UTR in this construct might lead to the induction of PTGS. However, from the observed phenotypes, the absence of mRNA and the accumulation of promoter-specific siRNAs, we have a strong indication that we deal with TGS.

Complete silencing can be induced by TGS

Starch granules from strongly silenced PTGS transformants always show a small blue core after staining with iodine. In the strongly silenced TGS transformants, this core is absent or definitively much smaller. This suggests that the silencing is stronger than in the PTGS transformants. This was also confirmed by Northern blot analysis where GBSSI transcript was detected in PTGS transformants, but not in TGS transformants. By using RT-PCR, no transcript could be detected when 35 cycli were used. However, when 45 cycli were used, a PCR fragment could be detected for two out of three transformants. For the transformant showing the strongest silencing, no fragment was observed indicating a complete inhibition of transcription of the endogenous GBSSI gene.

Since the silencing effect in TGS transformants is more severe than the effect in PTGS transformants, this approach could be an alternative to PTGS. It needs however, to be mentioned that the percentages of strongly silenced transformants are lower for the FP-IR transformants as compared to the transformants obtained with the most efficient cDNA IR construct. This could be due to the transcribing promoter since the FP-IR was cloned behind the 35S promoter in pHELLSGATE8 whilst the cDNA IR constructs were driven by the GBSSI promoter. To address whether the transcribing promoter or construct would influence silencing efficiency, the middle region of the GBSSI cDNA was cloned in the pHELLSGATE8 behind the 35S promoter and tested in potato. Of the transformants obtained with this construct, 39 % showed strong silencing. This percentage is lower than the percentage obtained with the cDNA IR construct harbouring the same sequence behind

the GBSSI promoter (87 %), which may indicate that the lower percentage of FP-IR is partially caused by the different transcribing promoter. A similar observation was done by Kuipers et al. (1995) who found that GBSSI promoter-driven antisense constructs resulted in a more pronounced inhibition of GBSSI gene expression than 35S-promoter driven antisense constructs. Based on this, these authors suggest that the effectiveness of inhibition in potato tubers is influenced by the transcribing promoter. Other evidence for this difference was provided by Visser et al. (1991) who demonstrated that the expression level in tuber tissue of the potato GBSSI promoter is three to tenfold higher than that of the 35S promoter.

We think the percentage of transformants showing strong silencing can be increased by cloning the promoter inverted repeat sequences behind a transcribing promoter that is more active in potato tubers than the 35S promoter, e.g. the patatin promoter. In this way, higher percentages of transformants in which transcription of GBSSI is prevented could be obtained.

Stability of expression in TGS transformants

In some of the FP transformants, different silencing levels were found within a microtuber of a single transformant. We assume 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 (Qin et al., 2003;Vaucheret et al., 1998). A similar observation was done by Sijen et al. (2001b) who found that *Petunia* transformants in which the *dfr*A promoter was silenced had flowers containing sectors of white and light purple cells.

Transformants of which microtubers showed 100 % strong silencing, were also strongly silenced in greenhouse tubers. However, transformants that showed variation within microtubers showed even larger variation in greenhouse-grown tubers. This indicates that the expression of these transformants is not stable. Kuipers et al. (1991) tested 35S-promoter driven antisense GBSSI transformants in greenhouse-grown tubers as well as in field-grown tubers. In one transformant, the strong suppression of GBSSI gene expression was reproducible during two successive generations of field-grown tubers. However, in transformants that showed red- as well as blue-staining tubers in greenhouse-grown tubers, large variation in inhibition level was found in the field (Kuipers et al., 1991). The size of the blue core in starch granules of these transgenes also varied within tubers (Kuipers et al., 1994). Even plants that originated from blue- staining or red-staining greenhouse grown tubers produced a mixture of tubers with blue, red or mixed staining starch (Kuipers et al., 1991). These findings suggest that in the red-staining greenhouse-tuber, the level of dsRNA is sufficient to induce silencing. However, a small fluctuation in this level might already result in a reduced silencing effect. Therefore, the inhibition in these transformants is not very stable. We think that a similar phenomenon occurred in our some of TGS transformants that showed variation in the level of inhibition within microtubers. Therefore, only those TGS transformants with 100 % silencing should be selected.

Final remarks

The approach to induce TGS of GBSSI has resulted in transformants that were completely silenced and in which no mRNA could be detected. Although in PTGS transformants, mRNA can still be detected, the reduction of amylose in PTGS transformants is sufficient for industrial applications. However, the TGS transformants could be a good alternative to obtain very strongly silenced transformants in which no GBSSI mRNA is produced. The effect of TGS depends on factors such as the methylation status and the activity of the transcribing promoter. As opposed to PTGS where the silencing trigger can be increased by means of transitivity, TGS is dependent on the original level of dsRNA synthesis. A strong transcribing promoter is therefore required to obtain a silencing effect.We believe it is worthwile to study whether the silencing effect in TGS transformants is stable during storage of tubers and in field experiments.

References

Ai-Sheng, X., Y. Quan-Hong, P. Ri-He, L. Xian, H. Pei-Lai, and F. Hui-Qin. 2005. Different effects of ACC oxidase gene silencing triggered by RNAi in transgenic tomato. Plant Cell Reports 23:639-646.

Akashi, H., M. Miyagishi, and K. Taira. 2001. Suppression of gene expression by RNA interference in cultured plant cells. Antisense Nucleic Acid Drug Dev 11:359-367.

Alder, M.N., S. Dames, J. Gaudet, and S.E. Mango. 2003. Gene silencing in Caenorhabditis elegans by transitive RNA interference. Rna 9:25-32.

Angell, S.M., and D.C. Baulcombe. 1997. Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. Embo J 16:3675-3684.

Aufsatz, W., M.F. Mette, J. van der Winden, A.J. Matzke, and M. Matzke. 2002a. RNA-directed DNA methylation in Arabidopsis. Proc Natl Acad Sci U S A 99 Suppl 4:16499-16506.

Aufsatz, W., M.F. Mette, J. van der Winden, M. Matzke, and A.J. Matzke. 2002b. HDA6, a putative histone deacetylase needed to enhance DNA methylation induced by double-stranded RNA. Embo J 21:6832-6841.

Benes, V., Z. Hostomsky, L. Arnold, and V. Paces. 1993. M13 and pUC vectors with new unique restriction sites for cloning. Gene 130:151-152.

Bernard, P., and M. Couturier. 1992. Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. Journal of Molecular Biology 226:735-745.

Braunstein, T.H., B. Moury, M. Johannessen, and M. Albrechtsen. 2002. Specific degradation of 3' regions of GUS mRNA in posttranscriptionally silenced tobacco lines may be related to 5'-3' spreading of silencing. Rna 8:1034-1044.

Bruinenberg, P.M., E. Jacobsen, and R.G.F. Visser. 1995. Starch from genetically engineered crops. Chemistry & Industry 6:881-884.

Brummell, D.A., P.J. Balint-Kurti, M.H. Harpster, J.M. Palys, P.W. Oeller, and N. Gutterson. 2003. Inverted repeat of a heterologous 3'-untranslated region for high-efficiency, high-throughput gene silencing. Plant J 33:793-800.

Callis, J., M. Fromm, and V. Walbot. 1987. Introns increase gene expression in cultured maize cells. Genes Dev 1:1183-1200.

Ceruti, H. 2003. RNA interference:traveling in the cell and gaining functions? Trends in genetics 19:39-46.

Chambers, S.P., S.E. Prior, D.A. Barstow, and N.P. Minton. 1988. The pMTL nic-cloning vectors. I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. Gene 68:139-149.

Chen, J., I.M. Greenblatt, and S.L. Dellaporta. 1992. Molecular analysis of Ac transposition and DNA replication. Genetics 130:665-676.

Cogoni, C., J.T. Irelan, M. Schumacher, T.J. Schmidhauser, E.U. Selker, and G. Macino. 1996. Transgene silencing of the al-1 gene in vegetative cells of Neurospora is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. Embo J 15:3153-3163.

Cogoni, C., and G. Macino. 1999. Gene silencing in Neurospora crassa requires a protein homologous to RNA-dependent RNA polymerase. Nature 399:166-169.

Dalmay, T., A. Hamilton, S. Rudd, S. Angell, and D.C. Baulcombe. 2000. An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell 101:543-553.

Dean, C., M. Favreau, D. Bond-Nutter, J. Bedbrook, and P. Dunsmuir. 1989. Sequences downstream of translation start regulate quantitative expression of two petunia rbcS genes. Plant Cell 1:203-208.

Denecke, J., V. Gossele, J. Botterman, and M. Cornelissen. 1989. Quantitative analysis of transiently expressed genes in plant cells. Meth.Mol.Cell.Biol. 1:19-27.

de Vetten, N., A.M.A. Wolters, K. Raemakers, I. van der Meer, R. ter Stege, E. Heeres, P. Heeres, and R.G.F. Visser. 2003. A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. Nat Biotechnol 21:439-442.

Elbashir, S.M., W. Lendeckel, and T. Tuschl. 2001. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 15:188-200.

English, J.J., E. Mueller, and D.C. Baulcombe. 1996. Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. Plant Cell 8:179-188.

Fire, A., S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, and C.C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391:806-811.

Flipse, E., C.J.A.M. Keetels, E. Jacobsen, and R.G.F. Visser. 1996. The dosage effect of the wildtype GBSS allele is linear for GBSS activity but not for amylose content; absence of amylose has a distinct influence on the physico-chemical properties of starch. TAG 92:121-127.

Gleave, A.P. 1992. A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Mol Biol 20:1203-1207.

Goldoni, M., G. Azzalin, G. Macino, and C. Cogoni. 2004. Efficient gene silencing by expression of double stranded RNA in Neurospora crassa. Fungal Genet Biol 41:1016-1024.

Hamilton, A., S. Brown, H. Yuanhai, I. Masakatsu, A. Lowe, S. Angel-Gabriel Alpuche, and D. Grierson. 1998. A transgene with repeated DNA causes high frequency, post-transcriptional suppression of ACC-oxidase gene expression in tomato. The Plant Journal 15:737-746.

Hamilton, A.J., and D.C. Baulcombe. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286:950-952.

Hamilton, A., O. Voinnet, L. Chappell, and D. Baulcombe. 2002. Two classes of short interfering RNA in RNA silencing. Embo J 21:4671-4679.

Hammond, A.J., E. Bernstein, D. Beach, and G.J. Hannon. 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 404:293-296.

Han, Y., and D. Grierson. 2002. The influence of inverted repeats on the production of small antisense RNAs involved in gene silencing. Mol Genet Genomics 267:629-635.

Heeres, P., M. Schippers-Rozenboom, E. Jacobsen, and R.G.F. Visser. 2002. Transformation of a large number of potato varieties: genotype-dependent variation in efficiency and somaclonal variability. Euphytica 124:13-22.

Helliwell, C.A., S.V. Wesley, A.J. Wielopolska, and P.M. Waterhouse. 2002. High-throughput vectors for efficient gene silencing in plants. Funct.Plant Biol. 29:1217-1225.

Helliwell, C., and P. Waterhouse. 2003. Constructs and methods for high-throughput gene silencing in plants. Methods 30:289-295.

Hendriks, T., D. Vreugdenhil, and W.J. Stiekema. 1991. Patatin and four serine proteinase inhibitor genes are differentially expressed during potato tuber development. Plant Mol Biol 17:385-394.

Himber, C., P. Dunoyer, G. Moissiard, C. Ritzenthaler, and O. Voinnet. 2003. Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. Embo J 22:4523-4533.

Hiraguri, A., R. Itoh, N. Kondo, Y. Nomura, D. Aizawa, Y. Murai, H. Koiwa, M. Seki, K. Shinozaki, and T. Fukuhara. 2005. Specific interactions between Dicer-like proteins and HYL1/DRB-family dsRNA-binding proteins in Arabidopsis thaliana. Plant Mol Biol 57:173-188.

Hizikuru, S. 1986. Polymodal distribution of the chain lengths of amylopectins, and its significance. Carbohydrate Research 147:342-347.

Hofacker, I.L. 2003. Vienna RNA secondary structure server. Nucleic Acids Res 31:3429-3431.

Hofvander, P., P.T. Persson, P.T. Tallberg, and O. Wikstroem. 1992. Genetically engineered modification of potato from amylopectin-type starch. International Patent Application WO 92/11376.

Hovenkamp-Hermelink, J.H.M., E. Jacobsen, A.S. Ponstein, R.G.F. Visser, G.H. Vos-Scheperkeuter, E.W. Bijmolt, J.N. De Vries, B. Witholt, and W.J. Feenstra. 1987. Isolation of an amylose-free starch mutant of the potato (*Solanum tuberosu*m L.). Theor Appl Genet 75:217-221.

Hovenkamp-Hermelink, J.H.M., J.N. de Vries, P. Adamse, E. Jacobsen, B. Witholt, and W.J. Feenstra. 1988. Rapid estimation of the amylose/amylopectin ratio in small amounts of tuber and leaf tissue in potato. Potato Research 31:241-246.

Ifuku, K., Y. Yamamoto, and F. Sato. 2003. Specific RNA interference in psbP genes encoded by a multigene family in Nicotiana tabacum with a short 3'-untranslated sequence. Biosci Biotechnol Biochem 67:107-113.

Jacobs, J.J., M. Sanders, M. Bots, M. Andriessen, G.J. Van Eldik, K. Litiere, M. Van Montagu, and M. Cornelissen. 1999. Sequences throughout the basic beta-1,3-glucanase mRNA coding region are targets for homology dependent post-transcriptional gene silencing. Plant J 20:143-152.

Jakowitsch, J., I. Papp, E.A. Moscone, J. van der Winden, M. Matzke, and A.J. Matzke. 1999. Molecular and cytogenetic characterization of a transgene locus that induces silencing and methylation of homologous promoters in trans. Plant J 17:131-140.

Jeddeloh, J.A., and E.J. Richards. 1996. mCCG methylation in angiosperms. Plant J 9:579-586.

Jefferson, R.A., T.A. Kavanagh, and M.W. Bevan. 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. Embo J 6:3901-3907.

Ji, Q., J.P. Vincken, L.C. Suurs, and R.G.F. Visser. 2003. Microbial starch-binding domains as a tool for targeting proteins to granules during starch biosynthesis. Plant Mol Biol 51:789-801.

Jones, L., A.J. Hamilton, O. Voinnet, C.L. Thomas, A.J. Maule, and D.C. Baulcombe. 1999. RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. Plant Cell 11:2291-2301.

Jones-Laurie, J., T. Yue-Stephen, Y. Cheung-Ching, and L.S.O. Singer-Victoria. 1998. RNA quantitation by fluorescence-based solution assay: RiboGreen reagent characterization. Analytical-Biochemistry 265:368-374.

Khvorova, A., A. Reynolds, and S.D. Jayasena. 2003. Functional siRNAs and miRNAs exhibit strand bias. Cell 115:209-216.

Kuipers, A.G.J., J.T.M. Vreem, H. Meyer, E. Jacobsen, W.J. Feenstra, and R.G.F. Visser. 1991. Field evaluation of antisense RNA mediated inhibition of GBSS expression in potato. Euphytica 59:83-91

Kuipers, A.G.J., E. Jacobsen, and R.G.F. Visser. 1994. Formation and deposition of amylose in the potato tuber starch granule are affected by the reduction of granule-bound starch synthase gene expression. The Plant Cell 6:43-52.

Kuipers, A.G.J., W.J.J. Soppe, E. Jacobsen, and R.G.F. Visser. 1995. Factors affecting the inhibition of granule-bound starch synthase gene expression in potato via antisense RNA. Mol Genet Genomics 246:745-755.

Landsmann, J., and H. Uhrig. 1985. Somaclonal variation in *Solanum tuberosum* detected at the molecular level. Theoretical Applied Genetics 71:500-505.

Lazo, G., P. Stein, and R. Ludwig. 1991. DNA transformation -competent Arabidopsis genomic library in Agrobacterium. Bio/Technology 9:963-967.

Lindbo, J.A., L. Silva-Rosales, W.M. Proebsting, and W.G. Dougherty. 1993. Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance. Plant Cell 5:1749-1759.

Lipardi, C., Q. Wei, and B.M. Paterson. 2001. RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. Cell 107:297-307.

Luehrsen, K.R., and V. Walbot. 1991. Intron enhancement of gene expression and the splicing efficiency of introns in maize cells. Mol Gen Genet 225:81-93.

Maas, C., J. Laufs, S. Grant, C. Korfhage, and W. Werr. 1991. The combination of a novel stimulatory element in the first exon of the maize Shrunken-1 gene with the following intron 1 enhances reporter gene expression up to 1000-fold. Plant Mol Biol 16:199-207.

Manners, D.J. 1989. Recent developments in our understanding of amylopectin structure. Carbohydrate Polymer 11:87-112.

Marano, M.R., and D.C. Baulcombe. 1998. Pathogen-derived resistance targeted against the negative-strand RNA of tobacco mosaic virus: RNA-strand-specific gene silencing? Plant Journal 13:537-546.

Martens, H., J. Novotny, J. Oberstrass, T.L. Steck, P. Postlethwait, and W. Nellen. 2002. RNAi in Dictyostelium: the role of RNA-directed RNA polymerases and double-stranded RNase. Mol Biol Cell 13:445-453.

Mascarenhas, D., I.J. Mettler, D.A. Pierce, and H.W. Lowe. 1990. Intron-mediated enhancement of heterologous gene expression in maize. Plant Mol Biol 15:913-920.

Matzke, A.J., F. Neuhuber, Y.D. Park, P.F. Ambros, and M.A. Matzke. 1994. Homology-dependent gene silencing in transgenic plants: epistatic silencing loci contain multiple copies of methylated transgenes. Mol Gen Genet 244:219-229.

Matzke, M., M.F. Mette, T. Kanno, W. Aufsatz, and A.J.M. Matzke. 2003. Regulation of the genome by double-stranded RNA., p.43-63, *In* G. J. Hannon, ed. RNAi, a guide to gene silencing. John Inglis, New York.

Matzke, M., W. Aufsatz, T. Kanno, L. Daxinger, I. Papp, M.F. Mette, and A.J. Matzke. 2004. Genetic analysis of RNA-mediated transcriptional gene silencing. Biochim Biophys Acta 1677:129-141.

Mette, M.F., J. van der Winden, M.A. Matzke, and A.J. Matzke. 1999. Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters in trans. Embo J 18:241-248.

Mette, M.F., W. Aufsatz, J. van der Winden, M.A. Matzke, and A.J. Matzke. 2000. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. Embo J 19:5194-5201.

Mourrain, P., C. Beclin, T. Elmayan, F. Feuerbach, C. Godon, J.B. Morel, D. Jouette, A.M. Lacombe, S. Nikic, N. Picault, K. Remoue, M. Sanial, T.A. Vo, and H. Vaucheret. 2000. Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell 101:533-542.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473-497.

Nakayashiki, H., S. Hanada, B.Q. Nguyen, N. Kadotani, Y. Tosa, and S. Mayama. 2005. RNA silencing as a tool for exploring gene function in ascomycete fungi. Fungal Genet Biol 42:275-283.

Napoli, C., C. Lemieux, and R. Jorgensen. 1990. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. Plant Cell 2:279-289.

Nishikura, K. 2001. A short primer on RNAi: RNA-directed RNA polymerase acts as a key catalyst. Cell 107:415-418.

Overhoff, M., M. Alken, R.K. Far, M. Lemaitre, B. Lebleu, G. Sczakiel, and I. Robbins. 2005. Local RNA target structure influences siRNA efficacy: a systematic global analysis. J Mol Biol 348:871-881.

Parrish, S., J. Fleenor, S. Xu, C. Mello, and A. Fire. 2000. Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. Mol Cell 6:1077-1087.

Qin, H., Y. Dong, and A.G. von Arnim. 2003. Epigenetic interactions between Arabidopsis transgenes: characterization in light of transgene integration sites. Plant Mol Biol 52:217-231.

Rethmeier, N., J. Seurinck, M. Van Montagu, and M. Cornelissen. 1997. Intron-mediated enhancement of transgene expression in maize is a nuclear, gene-dependent process. Plant J 12:895-899.

Rose, A.B. 2002. Requirements for intron-mediated enhancement of gene expression in Arabidopsis. Rna 8:1444-1453.

Rothnie, H.M. 1996. Plant mRNA 3'-end formation. Plant Mol Biol 32:43-61.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A laboratory Manual, 2nd edition Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schiebel, W., T. Pelissier, L. Riedel, S. Thalmeir, R. Schiebel, D. Kempe, F. Lottspeich, H.L. Sanger, and M. Wassenegger. 1998. Isolation of an RNA-directed RNA polymerase-specific cDNA clone from tomato. Plant Cell 10:2087-2101.

Schubert, S., A. Grunweller, V.A. Erdmann, and J. Kurreck. 2005. Local RNA target structure influences siRNA efficacy: systematic analysis of intentionally designed binding regions. J Mol Biol 348:883-893.

Schwarz, D.S., G. Hutvagner, T. Du, Z. Xu, N. Aronin, and P.D. Zamore. 2003. Asymmetry in the assembly of the RNAi enzyme complex. Cell 115:199-208.

Shannon, J., and D. Garwood. 1984. Genetics and physiology of starch development., p. 25-86, In B. J. Whister RL, Paschall EF, ed. Starch: chemistry and technology., 2 ed. Academic Press, Orlando.

Sijen, T., J. Wellink, J.B. Hiriart, and A. Van Kammen. 1996. RNA-Mediated Virus Resistance: Role of Repeated Transgenes and Delineation of Targeted Regions. Plant Cell 8:2277-2294.

Sijen, T., and J.M. Kooter. 2000. Post-transcriptional gene-silencing: RNAs on the attack or on the defense? Bioessays 22:520-531.

Sijen, T., J. Fleenor, F. Simmer, K.L. Thijssen, S. Parrish, L. Timmons, R.H. Plasterk, and A. Fire. 2001a. On the role of RNA amplification in dsRNA-triggered gene silencing. Cell 107:465-476.

Sijen, T., I. Vijn, A. Rebocho, R. van Blokland, D. Roelofs, J.N. Mol, and J.M. Kooter. 2001b. Transcriptional and posttranscriptional gene silencing are mechanistically related. Curr Biol 11:436-440.

Smardon, A., J.M. Spoerke, S.C. Stacey, M.E. Klein, N. Mackin, and E.M. Maine. 2000. EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in C. elegans. Curr Biol 10:169-178.

Smith, N.A., S.P. Singh, M.B. Wang, P.A. Stoutjesdijk, A.G. Green, and P.M. Waterhouse. 2000. Total silencing by intron-spliced hairpin RNAs. Nature 407:319-320.

Sonoda, S., M. Mori, and M. Nishiguchi. 1999. Homology-dependent virus resistance in transgenic plants with the coat protein gene of sweet feathery mottle potyvirus: Target specificity and transgene methylation. Virology 89:385-391.

Sontheimer, E.J. 2005. Assembly and function of RNA silencing complexes. Nat Rev Mol Cell Biol 6:127-138.

Stoutjesdijk, P.A., S.P. Singh, Q. Liu, C.J. Hurlstone, P.A. Waterhouse, and A.G. Green. 2002. hpRNA-mediated targeting of the Arabidopsis FAD2 gene gives highly efficient and stable silencing. Plant Physiol 129:1723-1731.

Takken, F.L.W., R. Luderer, S.H.E.J. Gabriëls, N. Westerink, R. Lu, P.J.G.M. de Wit, and M.H.A.J. Joosten. 2000. A functional cloning strategy, based on a binary PVX-expression vector, to isolate HR-inducing cDNAs of plant pathogens. The Plant Journal 24:275-283.

Tanksley D.S., M.W. Ganal., J.P. Prince, M.C. de Vicente, M.W. Bonierbale, P. Broun, T.M. Fulton, J.J. Giovannoni, S. Grandillo, G.B. Martin, R. Messeguer, J.C. Miller, L. Miller, A.H. Paterson, O. Pineda, Ms. Röer, R.A. Wing, W. Wu, N.D. Young. 1992. High density molecular linkage maps of the tomato and potato genomes. Genetics 132:1141-1160.

Tijsterman, M., R.F. Ketting, and R.H. Plasterk. 2002. The genetics of RNA silencing. Annu Rev Genet 36:489-519.

Tomari, Y., and P.D. Zamore. 2005. Perspective: machines for RNAi. Genes Dev 19:517-529.

Tuschl, T., P.D. Zamore, R. Lehmann, D.P. Bartel, and P.A. Sharp. 1999. Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev 13:3191-3197.

Vaistij, F.E., L. Jones, and D.C. Baulcombe. 2002. Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. Plant Cell 14:857-867.

van de Wal, M.H.B.J., E. Jacobsen, and R.G.F. Visser. 2001. Multiple allelism as a control mechanism in metabolic pathways: GBSSI allelic composition affects the activity of granule-bound starch synthase I and starch composition in potato. Mol Genet Genomics 265:1011-1021.

van der Krol, A.R., L.A. Mur, M. Beld, J.N. Mol, and A.R. Stuitje. 1990a. Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell 2:291-299.

van der Krol, A.R., L.A. Mur, P. de Lange, J.N.M. Mol, and A.R. Stuitje 1990b. Inhibition of flower pigmentation by antisense CHS genes: promoter and minimal sequence requirements for the antisense effects. Plant Molecular Biology 14:457-466.

van der Leij, F.R., R.G.F. Visser, A.S. Ponstein, E. Jacobsen, and W.J. Feenstra. 1991. Sequence of the structural gene for granule-bound starch synthase of potato (*Solanum tuberosum* L.) and evidence for a single point deletion in the *amf* allele. Mol Genet Genomics 228:240-248.

van der Steege, G., M. Nieboer, J. Swaving, and M.J. Tempelaar. 1992. Potato granule-bound starch synthase promoter-controlled GUS expression: regulation of expression after transient and stable transformation. Plant Mol Biol 20:19-30.

Van Houdt, H., A. Bleys, and A. Depicker. 2003. RNA target sequences promote spreading of RNA silencing. Plant Physiol 131:245-253.

Vaucheret, H., and M. Fagard. 2001. Transcriptional gene silencing in plants: targets, inducers and regulators. Trends Genet 17:29-35.

Vaucheret, H., T. Elmayan, P. Mourrain, and J.C. Palauqui. 1996. Analysis of a tobacco transgene locus that triggers both transcriptional and post-transcriptional silencing, p. 403-414, *In* V. E. Russo, et al., eds. Epigenetic Mechanisms of Gene Regulation. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Vaucheret, H., C. Beclin, T. Elmayan, F. Feuerbach, C. Godon, J.B. Morel, P. Mourrain, J.C. Palauqui, and S. Vernhettes. 1998. Transgene-induced gene silencing in plants. Plant J 16:651-659.

Vermeulen, A., L. Behlen, A. Reynolds, A. Wolfson, W.S. Marshall, J. Karpilow, and A. Khvorova. 2005. The contributions of dsRNA structure to Dicer specificity and efficiency. Rna 11:674-682.

Visser, R.G.F., M. Hergersberg, F.R. van der Leij, E. Jacobsen, B. Witholt, and W.J. Feenstra. 1989. Molecular cloning and partial characterization of the gene for granule-bound starch synthase from a wildtype and an amylose-free potato (Solanum tuberosum L.). Plant Science 64:185-192.

Visser, R.G.F., I. Somhorst, G.J. Kuipers, N.J. Ruys, W.J. Feenstra, and E. Jacobsen. 1991. Inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs. Mol Genet Genomics 225:289-296.

Vogt, U., T. Pelissier, A. Putz, F. Razvi, R. Fischer, and M. Wassenegger. 2004. Viroid-induced RNA silencing of GFP-viroid fusion transgenes does not induce extensive spreading of methylation or transitive silencing. Plant J 38:107-118.

Wang, H., M.M. Lee, and J.W. Schiefelbein. 2002. Regulation of the cell expansion gene RHD3 during Arabidopsis development. Plant Physiol 129:638-649.

Wang, M.B., S. Wesley, E. Finnegan, N. Smith, and P. Waterhouse. 2001. Replicating satellite RNA induces sequence-specific DNA methylation and truncated transcripts in plants. RNA 7:16-28.

Wang, M.B., and M. Metzlaff. 2005. RNA silencing and antiviral defense in plants. Curr Opin Plant Biol 8:216-222.

Wassenegger, M., S. Heimes, L. Riedel, and H.L. Sänger. 1994. RNA-directed de novo methylation of genomic sequences in plants. Cell 76:567-576.

Wassenegger, M. 2000. RNA-directed DNA methylation. Plant Mol Biol 43:203-219.

Waterhouse, P.M., M.W. Graham, and M.B. Wang. 1998. Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc Natl Acad Sci U S A 95:13959-13964.

Waterhouse, P.M., M.B. Wang, and T. Lough. 2001. Gene silencing as an adaptive defence against viruses. Nature 411:834-842.

Wesley, S.V., C.A. Helliwel, N.A. Smith, M. Wang, D.T. Rouse, Q. Liu, P.S. Gooding, S.P. Singh, D. Abbott, P.A. Stoutjesdijk, S.P. Robinson, A.P. Gleave, A.G. Green, and P.M. Waterhouse. 2001. Construct design for efficient, effective and high-throughput gene silencing in plants. The Plant Journal 27:581-590.

Wolters, A.M., and R.G. Visser. 2000. Gene silencing in potato: allelic differences and effect of ploidy. Plant Mol Biol 43:377-386.

Xie, Z., L.K. Johansen, A.M. Gustafson, K.D. Kasschau, A.D. Lellis, D. Zilberman, S.E. Jacobsen, and J.C. Carrington. 2004. Genetic and functional diversification of small RNA pathways in plants. PLoS Biol 2:0642-0652.

Zhang, H., F.A. Kolb, V. Brondani, E. Billy, and W. Filipowicz. 2002. Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. Embo J 21:5875-5885.

Summary

In the past, antisense RNA technology was used to modify the composition of potato tuber starch. Potato starch comprises amylose and amylopectin, polymers of glucose. Amylose production in potato is completely dependent on the presence of granule-bound starch synthase I (GBSSI). Inhibition of GBSSI has been achieved by transformation with antisense and sense GBSSI constructs. However, the percentages of transformants showing strong silencing were relatively low which implicated that large numbers of transformants needed to be generated in order to obtain sufficient transformants showing strong silencing. This was an undesirable situation since in practical breeding many transformants would have to be made, making the effort too time consuming and costly. Therefore, an approach that would enhance the silencing efficiency was desired.

This thesis describes the application of inverted repeat constructs for the modification of potato tuber starch. Transcription of inverted repeat constructs results in the formation of double stranded RNA (dsRNA). These dsRNA molecules are cleaved in small interfering RNAs (siRNAs) by a dsRNA-specific nuclease named Dicer. Subsequently, one strand of the siRNA is incorporated into the RNA Induced Silencing Complex (RISC) which is guided to the target mRNA through conventional base-pairing interactions. The target mRNA is then cleaved opposite the centre of the guide siRNA and finally, the cleaved mRNA is degraded.

The antisense strand of the siRNA can also act as a primer on target mRNA after which new dsRNA is synthesized by the action of a cellular RNA-directed RNA polymerase (RdRP). The RdRP-synthesized dsRNA will be recognized by Dicer and degraded to secondary siRNAs. These secondary siRNAs can be derived from regions upstream of the targeted regions and can thus induce RNA silencing of sequences that were not initially targeted. This phenomenon is called transitive RNA silencing.

First, an extensive study on the Post-Transcriptional Gene Silencing (PTGS) effects of eight different cDNA inverted repeat constructs was performed. These cDNA inverted repeat constructs were designed in such a way that the effect of size, orientation and sequence could be determined. The orientation of the inverted repeat did not affect silencing efficiency but the size and sequence did. Small inverted repeat constructs with a repeat size of 500-600 bp were more efficient silencing inducers than the large inverted repeat constructs with a repeat size of 1.1 or 1.3 kb. The two large inverted repeat constructs comprised the 5' and the 3' half of the GBSSI cDNA whilst for the construction of the small inverted repeat constructs the GBSSI cDNA was divided in three regions; the 5', the middle and the 3' region. In both instances, the 3' sequences induced the least efficient silencing implying that sequence does influence silencing efficiency. The transformants showing the highest percentages of strong silencing were obtained with two small inverted repeat constructs derived from the 5' or middle region of the GBSSI cDNA. In both cases, 87 % of transformants showed strong silencing. Silencing was accompanied by a reduction

in GBSSI mRNA levels but no relation between the amount of transcript and the level of silencing could be demonstrated. A similar phenomenon was observed for the accumulation of siRNAs. Although there was a relation between the presence of siRNAs and silencing, no relation between the level of siRNAs and the level of silencing was found.

Another factor we tested was the influence of introns in the cDNA sequences represented in the small inverted repeat constructs. To test the effect of GBSSI intron number two, two constructs were made; one with the intron in the cDNA and one without intron number two in the cDNA. The same approach was used to address the importance of GBSSI intron number nine, which was tested in two inverted repeat constructs harbouring sequences from the middle region of the GBSSI cDNA. For both introns, no effect on silencing efficiency was found. This indicates that the introns neither enhance nor inhibit silencing.

Many general silencing vectors are designed with a spliceable intron in the spacer because of a general belief that the splicing of this intron enhances gene silencing. To address whether this would apply to our experimental system, we compared constructs that contained intron number nine in a spliceable or a non-spliceable orientation in the spacer sequence. Both constructs gave rise to similar silencing efficiencies. It is likely that intron number nine was spliced in the construct in which it was in the spliceable orientation, because we demonstrated that this intron was spliced in another construct where it was located in exactly the same surrounding sequences.

Since we found no differences in silencing efficiency, we postulate that it is not a general rule that inclusion of a spliceable intron in the spacer of an inverted repeat construct enhances silencing efficiency. We think that the extent to which the presence of an intron in the spacer sequence enhances silencing efficiency might be different for each intron.

In plants and *C.elegans* examples of transitive silencing, *i.e.* silencing of sequences that were not initially targeted, have been described. Since it was shown that potato has an RdRP homolog, we investigated whether transitive silencing occurred in potato. Two approaches were used to induce transitive silencing. A construct harbouring a NOS terminator inverted repeat preceded by the middle region of the GBSSI cDNA and transcribed by a GBSSI promoter, was tested for its ability to induce silencing of GBSSI. Silencing of GBSSI could be induced indicating that transitivity occurs in potato in a 3' to 5' direction. To address whether the endogenous GBSSI mRNA can function as a template for the synthesis of new dsRNA, transformants harbouring GBSSI cDNA IR constructs were analysed for the accumulation of secondary siRNAs derived from regions adjacent to the targeted regions. Secondary siRNAs upstream of the targeted region were detected indicating that, in potato, the endogenous GBSSI transcript can function as a template for the synthesis of new dsRNA. No transitivity in the 5' to 3' direction could be detected. In plants, transitive silencing has been observed in several transgenic systems but very few examples of transitive silencing of endogenous genes exist. In these examples, it was not investigated whether the endogenous or transgene mRNA was used as a template. To our knowledge, we are first to show that endogenous mRNA is used as a template in transitive silencing of endogenous genes in plants. We also showed that the transitivity could extend over a distance of 761 bp, which is the largest distance reported until now.

When an inverted repeat construct contains promoter sequences, transcriptional gene silencing (TGS) can be induced. In this case, alterations at the DNA or chromatin level prevent transcription of the targeted genes. An alteration at the DNA level is methylation of cytosine residues. This can be induced by RNA signals and is described as RNA-directed

DNA methylation. Even though transformants obtained through the use of GBSSI cDNA inverted repeat constructs show strong silencing of GBSSI, there is still production of GBSSI mRNA. To obtain transformants in which the production of GBSSI mRNA was completely inhibited, we induced TGS of GBSSI by constructing GBSSI promoter inverted repeats. Three different regions of the GBSSI promoter were tested in inverted repeat constructs.

In potato, four classes of GBSSI alleles are known. These four classes of alleles are highly homologous in the coding region, but vary in promoter sequences. The variation in promoter sequences was exploited to make an 'allele-specific' promoter inverted repeat construct. This construct contained the sequences from -531 to -330 bp relative to the transcription initiation site (TIS) in the GBSSI A2 allele. It was hypothesized that this construct would inhibit the expression of the A2, A3 and A4 GBSSI alleles, but not of the A1 GBSSI allele. However, the percentages of weakly silenced transformants obtained with this construct were too low to distinguish the effect between two potato cultivars that varied in allele composition. Therefore, this approach could not be used to induce allele-specific silencing.

Weak silencing effects were also observed in 57-60 % of the transformants harbouring the inverted repeat construct with the promoter sequences from -766 to -168 bp relative to the TIS. We demonstrated that within this sequence, CG methylation had occurred, but apparently this was not sufficient to obtain strong silencing.

The most efficient silencing efficiency was induced with the inverted repeat construct containing the promoter sequences from -766 to +194 bp, relative to the TIS. Very strongly silenced transformants were obtained in which the transcription of GBSSI was completely or almost completely inhibited. This was accompanied by the accumulation of promoter-specific siRNAs and CG and CNN methylation.

Here, we showed that transcriptional silencing of the endogenous GBSSI promoter can be induced through the use of promoter inverted repeats. However, in order to obtain strong silencing, it is important to include sequences in the vicinity of the TIS. As compared to the PTGS approach, the TGS approach could be a good alternative to obtain very strongly silenced transformants that show a complete or almost complete inhibition of GBSSI transcription. Therefore, we think it is worthwhile to study whether the silencing effect in TGS transformants is stable during storage of tubers and in field experiments.

Samenvatting

Bij eerdere pogingen om de zetmeelsamenstelling van aardappel tijdens de knolvorming te modificeren werd de zogenaamde antisense inhibitie methode gebruikt. Aardappelzetmeel bestaat uit de glucosepolymeren amylose en amylopectine. De productie van amylose in aardappel is volledig afhankelijk van de aanwezigheid van het korrel gebonden zetmeel synthase (KGZ). Inhibitie van KGZ is bereikt door antisense en sense KGZ constructen te transformeren naar aardappel. De percentages van transformanten met een sterke silencing (d.w.z. volledige afwezigheid van amylose) waren echter relatief laag zodat grote aantallen transformanten gegenereerd moesten worden om voldoende transformanten met het beoogde sterke silencing effect te verkrijgen. Dit zou betekenenen dat in de praktische veredeling vele honderden tot wellicht duizenden transformanten gemaakt zouden moeten worden, hetgeen veel tijd zou vergen en kostbaar zou zijn. Daarom was er behoefte aan een benadering die de silencing efficiëntie zou verhogen.

In dit proefschrift wordt de toepassing van inverted repat constructen beschreven om die verbetering van efficiëntie te realiseren. De transcriptie van inverted repeat constructen leidt tot de vorming van dubbelstrengs RNA (dsRNA). Door een dsRNA-specifieke nuclease, genaamd Dicer, worden deze dsRNA moleculen omgezet in kleinere zogenaamde small interfering RNA's (siRNA's). Vervolgens wordt één streng van een siRNA opgenomen in het RNA Induced Silencing Complex (RISC). Interactie tussen de complementaire baseparen van de siRNA streng en het endogene mRNA zorgt ervoor dat het hele complex naar het endogene uit te schakelen mRNA wordt gestuurd. Het uit te schakelen mRNA wordt dan geknipt op de plek tegenover het midden van het siRNA. Uiteindelijk wordt het geknipte mRNA afgebroken.

De antisense streng van het siRNA kan ook als primer op het uit te schakelen mRNA fungeren. Onder invloed van het cellulaire RNA-directed RNA polymerase (RdRP) kan er dan nieuw dsRNA gemaakt worden. Dit RdRP-gesynthetiseerde dsRNA wordt vervolgens door Dicer afgebroken tot secundaire siRNA's. Deze secundaire siRNA's kunnen afkomstig zijn van gebieden die buiten het uit te schakelen gebied liggen en kunnen als zodanig RNA silencing van sequenties waarvan het niet de bedoeling was om ze uit te schakelen, veroorzaken. Dit fenomeen wordt transitieve RNA silencing genoemd.

In eerste instantie is er een grondige studie verricht naar de effecten van acht verschillende cDNA inverted repeat constructen op Post-Transcriptionele Gene Silencing (PTGS) van het KGZ gen. Het ontwerp van deze cDNA inverted repeat constructen was zodanig dat het effect van grootte, oriëntatie en sequentie kon worden bepaald. De oriëntatie van het inverted repeat had geen invloed op de silencing efficiëntie, maar de grootte en de sequentie wel. Kleine inverted repeat constructen met een repeat grootte van 500-600 bp induceerden een efficiëntere silencing dan de grote inverted repeat constructen met een repeat grootte van 1.1 of 1.3 kb. Voor de twee grote inverted repeat constructen werden de 5' en de 3' helft van het KGZ cDNA gebruikt terwijl voor de kleine inverted repeat constructen het KGZ cDNA in drie delen werd opgedeeld; het 5', het midden en het 3' gedeelte. In beide

gevallen leidden de 3' sequenties tot de minst efficiënte silencing hetgeen impliceert dat er invloed is van de sequentie op de silencing efficiëntie. De hoogste percentages van transformanten met een sterke silencing werden bereikt met twee kleine inverted repeat constructen afkomstig van het 5'- of het middengedeelte van het KGZ cDNA. Beide constructen resulteerden in een percentage van 87 % van transformanten die sterke silencing vertoonden. Silencing ging gepaard met een reductie in KGZ mRNA maar een relatie tussen de hoeveelheid transcript en het niveau van silencing kon niet worden aangetoond. Een vergelijkbaar fenomeen werd waargenomen voor de accumulatie van siRNA's. Hoewel er een relatie tussen de aanwezigheid van siRNA's en silencing was, werd er geen relatie tussen het niveau van siRNA's en het niveau van silencing gevonden.

De invloed van introns in the cDNA sequenties van de kleine inverted repeat constructen was een andere factor die getest werd. Om het effect van KGZ intron nummer twee te bepalen werden twee constructen gemaakt; één met het intron in het cDNA en één zonder intron nummer twee in het cDNA. Een gelijksoortige benadering werd gebruikt om het belang van KGZ intron nummer negen te bepalen in twee inverted repeat constructen die sequenties van het middengedeelte van het KGZ cDNA bevatten. Voor beide introns werd geen effect op de silencing efficiëntie gevonden. Dit duidt erop dat de aanwezigheid van introns de silencing niet verhoogt of verlaagt.

Vele algemene silencing vectoren zijn ontworpen met een intron in de spacer omdat men gelooft dat het uitknippen van dit intron de silencing efficiëntie verhoogt. Om na te gaan of dit ook van toepassing was in ons experimentele systeem hebben we constructen vergeleken die verschilden in de oriëntatie van intron nummer negen in de spacer. Het construct waarbij het intron niet uitgeknipt kon worden en het construct waarbij het intron wel uitgeknipt kon worden gaven vergelijkbare silencing efficiënties. Het is zeer waarschijnlijk dat intron nummer negen uitgeknipt werd omdat we aan konden tonen dat dit intron uitgeknipt werd in transformanten verkregen met een ander construct waarin het in exact dezelfde aangrenzende sequenties lag.

Aangezien we geen verschillen in silencing efficiëntie hebben gevonden, nemen we aan dat het geen algemene regel is dat de aanwezigheid van een uitknipbaar intron in de spacer van een inverted repeat construct de silencing efficiëntie verhoogt. Wij denken dat de mate waarin de aanwezigheid van een intron in de spacer de silencing efficiëntie verhoogt, verschillend kan zijn voor elk intron.

In planten en *C.elegans* zijn voorbeelden van transitieve silencing, d.w.z. silencing van sequenties die buiten het uit te schakelen gebied liggen, beschreven. Omdat er is aangetoond dat aardappel een RdRP homoloog heeft, hebben wij onderzocht of transitieve silencing ook in aardappel voorkwam. Twee benaderingen werden hiervoor gebruikt. Een construct met een NOS terminator inverted repeat voorafgegaan door het middengedeelte van het KGZ cDNA en afgeschreven door een KGZ promoter werd getest voor zijn vermogen om silencing van KGZ te induceren. Silencing van KGZ kon inderdaad worden geïnduceerd hetgeen er op duidt dat transitiviteit voorkomt in aardappel en zich beweegt in de richting van 3' naar 5'.

Om te bepalen of endogeen KGZ mRNA kan functioneren als een template voor de synthese van nieuw dsRNA werden transformanten met de KGZ cDNA IR constructen nader geanalyseerd. Hierbij werd gekeken of secundaire siRNA's afkomstig van gebieden buiten het uit te schakelen gebied voorkwamen. Deze kwamen inderdaad voor in het gebied voorafgaand aan het uit te schakelen gebied. Dit duidt erop dat het endogene KGZ

transcript in aardappel kan functioneren als een template voor de synthese van nieuw dsRNA. Verspreiding van 5' naar 3' kon niet worden gedetecteerd. In planten komt transitieve silencing voor in diverse transgene systemen maar er zijn maar een paar voorbeelden waarbij transitieve silencing van endogene genen is aangetoond. Van deze voorbeelden is niet bekend of het endogene of transgene mRNA als template werd gebruikt. Voor zover ons bekend is, zijn wij de eersten die aantonen dat endogeen mRNA wordt gebruikt als template in transitieve silencing van endogene genen in planten. Daarnaast hebben we aangetoond dat de transitiviteit zich over een afstand van 761 bp kan verspreiden. Dit is de grootste afstand die tot dusver is beschreven.

Wanneer een inverted repeat construct promoter sequenties bevat kan transcriptionele gene silencing (TGS) worden geïnduceerd. Veranderingen op DNA of chromatine niveau voorkomen in dit geval de transcriptie van het uit te schakelen gen. Een verandering op DNA niveau is de methylatie van cytosine residuen. Dit kan worden geïnduceerd door RNA signalen en wordt beschreven als RNA-directed DNA methylation.

Ondanks dat transformanten, die zijn verkregen met behulp van KGZ cDNA inverted repeat constructen, een sterke silencing van KGZ vertonen is er nog steeds KGZ mRNA aanwezig. Teneinde transformanten te verkrijgen waarin de productie van KGZ mRNA compleet geremd werd hebben we TGS van KGZ geïnduceerd. Drie verschillende gebieden van de KGZ promoter werden getest in inverted repeat constructen.

In aardappel kunnen vier klassen van KGZ allelen worden onderscheiden. Deze vier klassen van allelen vertonen een hoge homologie in de coderende sequentie maar variëren in promoter sequenties. Van deze variatie in promoter sequentie hebben we gebruik gemaakt door een 'allel-specifiek' promoter inverted repeat construct te maken. Dit construct bevatte de sequenties van -531 tot -330 bp t.o.v. de transcription initiation site (TIS) in het KGZ A2 allel. De verwachting was dat dit construct de expressie van de A2, A3 en A4 KGZ allelen zou remmen, maar geen invloed zou hebben op de expressie van het A1 allel. De percentages van zwak gesilencende transformanten verkregen met dit construct waren echter te laag om onderscheid te kunnen maken tussen het effect in twee aardappelrassen die varieerden in allelsamenstelling.

Zwakke silencing effecten werden ook waargenomen in 57-60 % van de transformanten die het inverted repeat construct met de promoter sequenties van -766 tot -168 bp t.o.v. de TIS bevatten. We hebben aangetoond dat er binnen deze sequentie CG methylatie had plaatsgevonden, maar dit was blijkbaar niet voldoende om sterke silencing te induceren. De meest efficiënte silencing werd verkregen met het inverted repeat construct dat de promoter sequenties van -766 tot +194 bp t.o.v. de TIS bevatte. Zeer sterk gesilencende transformanten werden verkregen waarin de transcriptie van KGZ volledig of bijna volledig was geremd. Dit ging gepaard met de accumulatie van promoter-specifieke siRNA's en CG en CNN methylatie.

We hebben hier laten zien dat transcriptionele silencing van de endogene KGZ promoter kan worden geïnduceerd door het gebruik van promoter inverted repeats. Om sterke silencing te verkrijgen is het echter belangrijk om sequenties in de buurt van de TIS op te nemen in de promoter inverted repeat. Ten opzichte van de PTGS benadering kan de TGS benadering een goed alternatief zijn om zeer sterk gesilencende transformanten met een volledige of bijna volledige remming van KGZ transcriptie te verkrijgen. Daarom denken we dat het zeer de moeite waard is om te bestuderen of het silencing effect in TGS transformanten stabiel is gedurende veldexperimenten en bij bewaring van knollen.

Nawoord

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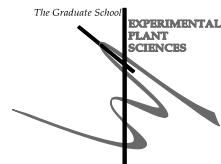
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Curriculum vitae

Berlinda Heilersig werd geboren op 6 september 1972 te Markelo. In 1990 behaalde zij haar VWO diploma, waarna zij de opleiding Laboratoriumtechniek met als specialisatie plantenbiotechnologie aan de Internationale Hogeschool Larenstein volgde. Haar stage en afstudeervak verrichte ze bij Mogen International NV. In 1994 werd de studie afgerond en begon ze met de studie Plantenveredeling en Gewasbescherming aan de Wageningen Universiteit. Voor een afstudeervak deed ze celbiologisch onderzoek bij het Queensland Agricultural Biotechnology Centre in Australië. Tijdens een afstudeervak bij de vakgroep Fytopathologie van de Wageningen Universiteit deed ze ervaring op in het moleculair-biologisch onderzoek. In 1997 werd de studie afgerond en werd ze aangesteld bij Seminis Vegetable Seeds te Enkhuizen. Hier werkte ze aan protoplastenfusies in Brassica, eerst als analist en later als onderzoekster. Eind 1999 heeft ze de overstap naar de moleculaire kant van het vakgebied gemaakt door bij Plant Research International aan de AFLP techniek in aardappel te gaan werken. In mei 2001 kreeg ze de kans om zelfstandig onderzoek te verrichten bij het Laboratorium voor Plantenveredeling binnen het door de EU gefinancierde project 'Improvement of transgenic expression and gene silencing in transgenic plants'. Dit onderzoek heeft uiteindelijk geleid tot dit proefschrift. Vanaf 1 januari 2006 zal zij als toegevoegd onderzoeker werkzaam zijn bij Keygene N.V.

Education Statement of

the Graduate School

Experimental Plant Sciences

The Graduate School **EXPERIMENTAL PLANT SCIENCES**

1) Start-up phase	*date*
► **First presentation of your project**	
Gene silencing in potato;alleles and inverted repeat constructs	28th of September 2001
Subtotal Start-up phase	*1.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	2001 through 2005
► **EPS theme symposia (Theme 4; Genome Plasticity)**	2001 through 2005
► **Attendance research discussion of the laboratory**	2001 through 2005
► **National meetings**	
ALW Lunteren (Netherlands)	2001 through 2005
► **Seminars (series), workshops and symposia**	
Plant Breeding Seminar Series	2001 through 2005
Frontiers in Plant Developments Seminar Series	2002 through 2005
Workshop RNA Interference (DGS, VU, Amsterdam)	27th of May 2004
► **International symposia and conferences**	
Contextualizing the Genome, Ghent (Belgium)	25-28 Nov 2001
Epigenetics, annual meeting (GGS, Germany)	26-29 Sep 2003
EU project meetings	2001 through 2003
► **Presentations**	
Oral presentation at ALW Lunteren (Netherlands) (one time)	5th of April 2005
Oral presentations at EU project meetings (three times)	2001 through 2003
Subtotal Scientific Exposure	*19.7 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD course**	
Summerschool "Functional Genomics" (Utrecht, Netherlands)	25-28 Aug 2003
► **Individual research training**	
Subtotal In-Depth Studies	*1.5 credits**

4) Personal development	*date*
► **Skill training courses**	
Afstudeervak organiseren en begeleiden	5-6 June 2002
Masterclass "License to Life Sciences"	23-26 Nov 2003
Scientific writing	Sep-Nov 2003
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4 credits**

TOTAL NUMBER OF CREDIT POINTS*	**26,7**

* A credit represents a normative study load of 28 hours of study