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β-1,3-Glucanase and chitinase transgenes in hybrids show distinctive and independent patterns of posttranscriptional gene silencing

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Abstract. *Nicotiana sylvestris* Speg. & Comes transformed with a tobacco class-I β-1,3-glucanase (*GLU I*) cDNA driven by CaMV 35S RNA expression signals exhibits posttranscriptional gene silencing (PTGS) which is triggered between the cotyledon and two-leaf stages of seedling development and is postmeiotically reset to the high-expressing state during seed development. The incidence of *GLU I* PTGS in sibling plants differed for the two different transformants tested and increased with the number of T-DNA loci. Comparison of host class-I and class-II β-1,3-glucanase gene expression suggests that a similarity of 60–70% in the coding-region is required for PTGS of the homologous host genes. The *GLU I* transformants exhibited a spatial gradient in PTGS, in which expression of the silent phenotype gradually increased in successive leaves toward the bottom of the plant. In contrast, transformants carrying an unrelated tobacco class I chitinase (*CHN I*) cDNA in the same expression vector exhibited discontinuous patterns of PTGS with adjacent high-expressing and silent leaves. The *GLU I*- and *CHN I*-specific patterns were maintained in hybrids homozygous for both T-DNA's indicating that two different transgenes present in the same genome can exhibit independent and distinctive patterns of PTGS. This implies that the nature of the transgene rather than a general pre-pattern of competence for PTGS or propagation of the silent state are important for pattern determination.

Key words: Chitinase – Gene co-suppression – β-1,3-Glucanase – *Nicotiana* (gene silencing) – Plant defense – Posttranscriptional gene silencing

Introduction

Posttranscriptional gene silencing (PTGS) is now recognized to be a general phenomenon reported for many plant species transformed with a variety of chimeric transgenes and has strong similarities to RNA interference in animals (for reviews, see Fire 1999; Kooter et al. 1999; Meins 2000). The mechanism for PTGS is not known. Most current models include an autoregulatory component to account for stability, production of special RNAs for sequence specificity, mobile signals involved in systemic spread, and a mechanism for increased degradation of target RNAs.

Extensive evidence suggests that PTGS is strongly influenced by environmental factors, the developmental stage of the plant, the level of target gene transcription, and T-DNA structure (for review, see Vaucheret et al. 1998; Kooter et al. 1999). To better understand the critical parameters involved, we compared PTGS in *Nicotiana sylvestris* transformed with structurally unrelated tobacco class-I chitinase (*CHN I*) and β-1,3-glucanase (*GLU I*) cDNAs in otherwise identical expression vectors.

Nicotiana sylvestris plants transformed with tobacco *CHN I* and *GLU I* cDNAs regulated by cauliflower mosaic virus (CaMV) 35S RNA expression signals show a high incidence of PTGS (Kunz et al. 1996; Holtorf et al. 1999). Earlier we characterized genetic and epigenetic factors important for *CHN I* PTGS (Hart et al. 1992; Kunz et al. 1996). Here we characterize the factors important for *GLU I* PTGS and examine the incidence and spatial patterns of PTGS in transformants homozygous for two unrelated transgenes.

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Abbreviations: CHN I = class-I chitinase; DAP = days after pollination; GLU I = class-I β-1,3-glucanase; GLU II = class-II β-1,3-glucanase; Km^R = kanamycin resistant; PTGS = posttranscriptional gene silencing

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Materials and methods

Plant materials and growth conditions

The *Nicotiana sylvestris* Speg & Comes plants and their transgenic derivatives as well as procedures for promoting silencing, sampling leaves and performing genetic crosses have been described previously (Neuhaus et al. 1992; Kunz et al. 1996). In brief, transgenic plants were obtained by *Agrobacterium tumefaciens*-mediated Ti-plasmid transformation with cDNAs representing the tobacco *CHN I* gene *CHN48* and the tobacco *GLU I* gene *GLA* regulated by CaMV 35S RNA expression signals and carrying the Tn5 *nptII* gene with nopaline synthase expression signals to provide the kanamycin–resistant (Km^R) phenotype as a plant-selectable marker. The independent GLU I transformants SSG7.1 and SSG7.2 carry a transgene structurally unrelated to the transgene carried by the CHN I transformant SSC2.3 except for a short 14-bp sequence in the 5′-UTR derived from *CHN48*. SAG2.3 is a GLU I antisense transformant and SCIB2 is an empty-vector transformant. All transformants used were homozygous for a single-copy transgene locus. Plants obtained by selfing primary transformants are designated the S_1 generation; subsequent selfed generations are numbered sequentially.

Analysis of DNA, RNA and protein

The DNA and total RNA were isolated from leaf tissues and Southern blot analyses and RNA blot hybridization were performed as described previously (Kunz et al. 1996; Holtorf et al. 1999). The probes used were the 1.2-kb *Hind*III-*Bam*HI insert of pGL43, (Shinshi et al. 1988). Exposure and quantitation of signals normalized for the 18S rRNA loading standard was done using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, Calif., USA). The procedures for preparing tissue extracts, immuno-blot analysis, and dot-blot assays that detect both tobacco and *N. sylvestris* CHN I, GLU I and class-II β-1,3-glucanase (GLU II) antigens have been described (Neuhaus et al. 1992; Kunz et al. 1996). The GLU I antigen content of extracts was measured by "rocket" immunoelectroassay and is expressed in μg tobacco GLU I equivalents per g FW (Felix and Meins 1985; Shinshi et al. 1987). Transformants were judged to express the silent phenotype if the antigen content of at least one leaf was the same or lower than comparable tissues of untransformed or control-vector pCIB200-transformed plants in the same experiment.

Agroinfiltration

The procedures for infiltrating *Agrobacterium tumefaciens* strain LBA 4404 carrying pCIB200 into *N. sylvestris* leaves and sampling of infiltrated tissues have already been described (Schöb et al. 1997).

Results

Transgene factors affecting GLU I PTGS

Two independent GLU I transformants, SSG7.1 and SSG7.2, which show PTGS (Holtorf et al. 1999) were studied in detail. The segregation of the Km^R marker in crosses indicates that the T-DNAs in both transformants are inherited as unlinked, dominant, single-locus, Mendelian traits (Table 1). The *GLU I* T-DNA has a single *Hin*dIII site and two *Eco*RI sites flanking the chimeric *GLA* transgene (Kunz et al. 1996). Southern-blot analyses of DNA digested with *Hin*dIII or *Eco*RI and probed for *GLA* cDNA sequences confirmed that SSG7.1 and SSG7.2 are independent transformants and suggested that there are two complete copies of T-DNA at each transgene locus (data not shown). To find out if the incidence of silencing depends on the number of T-DNA loci and T-DNA insertion site, we crossed homozygous SSG7.1 and SSG7.2 transformants carrying unlinked T-DNA loci with each other and with wild-type plants. Table 1 shows that in the SSG7.1 line, no hemizygous plants and only 5.5% of homozygous plants exhibited silencing. In contrast, 95% of hemizygous SSG7.2 plants and all of the homozygous SSG7.2 plants exhibited a silent phenotype. There was a high incidence of silencing, comparable to the homozygous SSG7.2 cross, in the F_1 hybrid obtained by crossing SSG7.2 and SSG7.1. These results show that the independent transformants differ markedly in incidence of silencing; that the incidence of silencing increases with the number of transgene loci; and, that the SSG7.2 locus is epistatic to the SSG7.1 locus.

Table 1. Gene dose effects on PTGS in independent GLU I transformants of *Nicotiana sylvestris*

Cross[a]	Model[b]	% Km^S [c]	% Silent[d]
SSG7.1×	0:4	0 (30)	5.5 (36)
SSG7.1 × Wt[e]	0:4	0 (49)	0 (65)
Wt × SSG7.1	0:4	0 (40)	ND[f]
(SSG7.1 × Wt)×	1:3	21 (92)	ND
(Wt × SSG7.1)×	1:3	22 (248)	ND
SSG7.2×	0:4	0 (30)	100 (60)
SSG7.2 × Wt	0:4	0 (59)	95 (39)
Wt × SSG7.2	0:4	0 (50)	
(SSG7.2 × Wt)×	1:3	25 (296)	ND
SSG7.1 × SSG7.2	0:4	ND	97 (30)
SSG7.2 × SSG7.1	0:4	ND	
(SSG7.1 × SSG7.2)×	1:15	4 (544)	ND
(SSG7.1 × SSC2.3)×	1:15	5.7 (1798)	ND
(SSG7.2 × SSC2.3)×	1:15	6.0 (718)	ND

[a] Crosses of homozygous single-locus β-1,3-glucanase transformants SSG7.1 and SSG7.2 and the homozygous single-locus chitinase transformant SSC2.3; female parent in cross is on the left and the male parent is not shown in self crosses
[b] Theoretical segregation ratios of the Kanamycin-sensitive (Km^S) phenotype in crosses for dominant, single-locus independent traits
[c] Frequency of Km^S found for the number of progeny tested indicated in parenthesis; none of the frequencies were significantly different at the 5% level (test of Binomial Proportions) from the frequencies predicted by the model
[d] Frequency of mature plants in a homogeneous population showing the silent β-1,3-glucanase phenotype in at least one leaf as judged by immunoassay or immunoblot measurements. Data for reciprocal crosses are pooled; number of plants assayed are indicated in parenthesis
[e] Wild type
[f] Not determined

Class-II β-1,3-glucanase host genes are not silenced by a class I-transgene

To find out if *GLU II* genes, which show about 60–70% sequence identity to *GLU I* genes (Meins et al. 1992), are

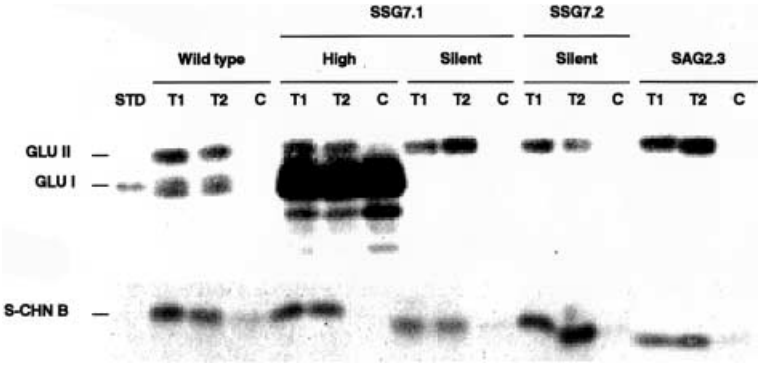

Fig. 1. Immunoblot analyses of β-1,3-glucanases and class I chitinases in extracts prepared from *A. tumefaciens*-infiltrated leaves of *N. sylvestris*. Extracts were prepared from infiltrated areas of replicate leaves 6 d after infiltration with suspensions of bacteria (*T1* and *T2*) and with buffer alone (*C*). Immunoblots were stained with antibodies that recognize tobacco and *N. sylvestris* GLU I and GLU II, and *N. sylvestris* CHN I (*S-CHN B*). Equal volumes of extracts (20 µl) were applied to each lane: *STD*, authentic tobacco GLU I; *High*, leaves showing high-level GLU I expression; *Silent*, leaves showing the silent phenotype

silenced in GLU I transformants, we infiltrated leaves of mature plants with *Agrobacterium tumefaciens*, which is known to induce GLU I, GLU II and CHN expression (Schöb et al. 1997). Figure 1 shows immunoblot analyses of extracts from leaves 6 d after infiltration with *A. tumefaciens* and buffer. Wild-type plants and the *N. sylvestris* antisense GLU I transformant SAG2.3, which shows dramatic inhibition of GLU I but not GLU II expression (Neuhaus et al. 1992), were used as controls. Wild-type leaves infiltrated with *A. tumefaciens* gave strong signals for the ca. 36-kDa GLU II, 33-kDa GLU I, and the host 32-kDa CHN, S-CHNB. Induction of GLU II and S-CHNB was also evident in high-GLU I-expressing leaves of SSG7.1 and leaves of SAG2.3. Although GLU I was not detected in silent leaves of SSG7.1 and SSG7.2 transformants or in leaves of the SAG2.3 transformant, GLU II as well as S-CHNB were strongly induced by infection. These results show that expression of GLU II induced by *A. tumefaciens* infection is not silenced in plants exhibiting GLU I silencing.

Spatial patterns of GLU I silencing

Table 2 shows the incidence of silencing in single leaves near the top (upper leaf) and bottom (lower leaf) of hemizygous and homozygous SSG7.1 and SSG7.2 GLU I transformants. Expression of host-gene encoded GLU I was approx. 5-fold higher in lower leaves than in upper leaves of empty-vector transformed SCIB2 plants as reported earlier for wild-type *N. sylvestris* (Neuhaus et al. 1992). The GLU I contents of upper and lower leaves from high-expressing SSG7.1 and SSG7.2 plants were

Table 2. The GLU I content of leaves of S_1 generation SCIB2, SSG7.1 and SSG7.2 transformants of *N. sylvestris*

Line	Genotype	Phenotype[a]	N[b]	GLU [µg (g FW)$^{-1}$]		
				Upper leaf	Lower leaf	Upper leaf/Lower leaf
SCIB2	Homozygous	Wild type	10	0.9 ± 0.58[c]	20.9 ± 5.8	0.18 ± 0.11[d]
SSG7.1	Hemizygous	High	17	78.1 ± 6.1	98.4 ± 9.1	0.86 ± 0.08
		Silent	0			
	Homozygous	High	2	99.3 ± 15.3	154 ± 3.0	0.64 ± 0.09
		Silent	1	13.3	< 0.01	
SSG7.2	Hemizygous	High	4	94.2 ± 4.5	50 ± 9.4	2.1 ± 0.32
		Silent	8	88.9 ± 15.2	15.2 ± 1.8	7.0 ± 1.5
	Homozygous	High	0			
		Silent 1	7	< 0.01	< 0.01	
		Silent 2	1	78.6	9.9	7.9

[a] Plants with GLU contents in any two leaves lower than that of comparable SCIB2 vector-control leaves were designated silent. Two types of silent plant were found: plants with no detectable GLU (*silent 1*) and a plant in which one leaf exhibited a silent phenotype (*silent 2*)

[b] Number of sibling S_1 plants of the indicated phenotype scored

[c] The mean GLU content ± SE for individual measurements of the number of plants (*N*) indicated. A pair of individual upper and lower leaves was assayed from each plant. Upper and lower leaves are defined as leaves numbered 10 to 12 and 2 to 4, respectively, counting from the bottom of the mature, nonflowering plant. The GLU content was measured by rocket immunoelectrophoresis with a limit of detection of 0.01 µg (g FW)$^{-1}$

[d] The mean of the ratio of GLU content ± SE for the number of plants (*N*) indicated. The mean of the ratios is not equal to the ratio obtained by dividing mean values for GLU content in upper and lower leaves

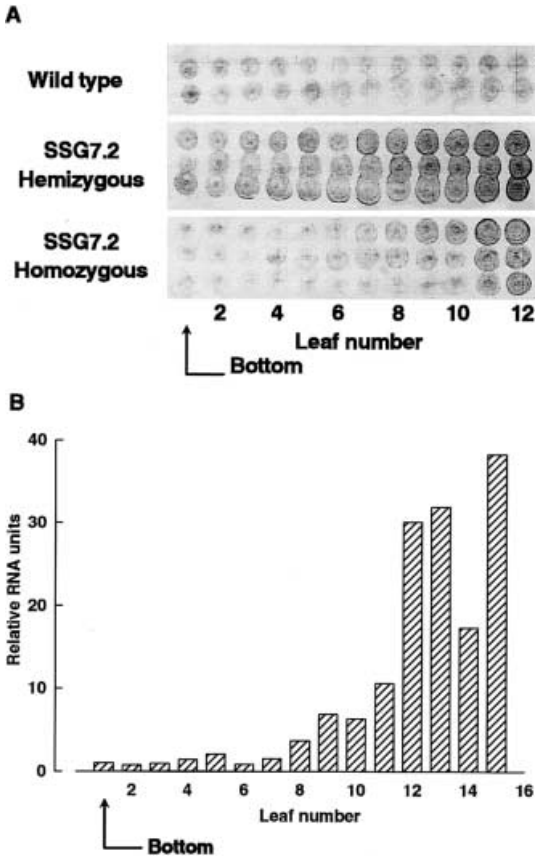

Fig. 2A,B. Spatial patterns of GLU I expression in leaves of the SSG7.2 transformant of *N. Sylvestris*. **A** Dot-blot detection of GLU I antigen in equal aliquots of extracts prepared from the same leaf (counting from the bottom of the plant) of replicate wild-type, hemizygous SSG7.2, and homozygous SSG7.2 plants. **B** Quantitation by RNA-blot hybridization of *GLU I* mRNA in leaves of a homozygous SSG7.2 transformant. Leaves are numbered counting from the bottom of the plant

roughly equal and up to 100-fold higher than in upper leaves from SCIB2 plants. Far lower concentrations of GLU I were found in both the upper and lower leaves of the single homozygous silent SSG7.1 transformant. Sibling SSG7.2 plants exhibited two patterns of GLU I expression. In seven of eight homozygotes, upper and lower leaves exhibited the silent phenotype. In one homozygote and in the eight hemizygotes, silencing was detected in lower leaves but not in upper leaves. Although the incidence of plants showing these patterns varied from experiment to experiment, the results indicate that individual leaves of the same SSG7.2 plant can differ in silencing and that silencing is most effective in lower leaves.

The pattern of GLU I expression in SSG7.2 was studied in greater detail. Figure 2A shows that the GLU I content decreases in successive leaves toward the bottom of the plant. The similarity in silencing pattern observed with small leaf discs from three replicate plants suggests that silencing changes gradually from leaf to leaf but is uniform within individual leaves at the same stage of development. The RNA-blot hybridizations confirmed for a homozygous plant that there is a gradient of GLU I mRNA steady-state levels in different leaves as well (Fig. 2B). Taken together, these results indicate that some SSG7.2 transformants exhibit a gradient of silencing, with relatively higher GLU I expression in upper leaves than in lower leaves, and that this gradient is more pronounced in homozygous plants than in hemizygous plants.

Developmental regulation of silencing

We established when silencing is initiated by measuring the GLU I content of 20 wild-type, SSG7.1 and SSG7.2 plants during seedling development. Silencing was first detected in 10% of the homozygous SSG7.1 plants at the five to six leaf stage 48 d after germination and in 90–100% of the homozygous SSG7.2 plants at the two-leaf stage 15 d after germination. Figure 3 shows that the GLU I content of wild-type plants increased from the cotyledon stage (6 d) to the two-leaf stage (15 d), remained at a constant high level in hemizygous SSG7.2 plants, and decreased from high levels to levels below those of wild-type plants in homozygous SSG7.2 plants. Thus, silencing occurs in homozygous SSG7.2 between the cotyledon and two-leaf stages of development during a period in which host GLU I expression in wild-type plants increases. Because the incidence of silencing in the SSG7.1 plants was very low, the exact onset of silencing could not be reliably determined, but it was before or at the five to six leaf stage of development.

Immunoblot analyses of silent plants indicated that floral tissues where sporogenesis occurs (i.e., anthers and ovaries) exhibit a silent phenotype, whereas mature seeds

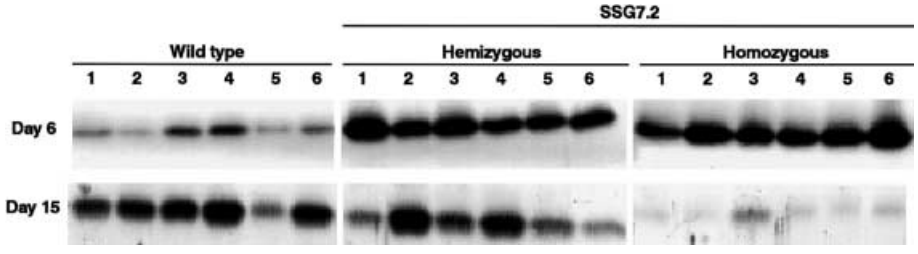

Fig. 3. The GLU I content of wild-type, hemizygous SSG7.2, and homozygous SSG7.2 seedlings of *N. sylvestris* 6 and 15 d after germination. Immunoblots were stained with antibody recognizing tobacco and *N. sylvestris* GLU I. Aliquots (20 µl) of extracts prepared from six individual seedlings (1–6) were applied to each lane. Day-6 seedlings were at the cotyledon stage and day-15 seedlings were at the two-leaf stage

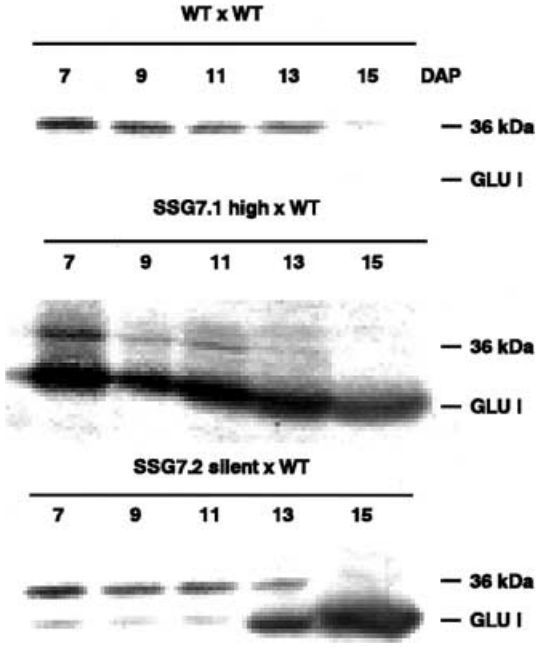

Fig. 4. Resetting of *GLU I* PTGS during development of *N. sylvestris* seeds. Developing seeds obtained by self-pollination of wild-type flowers (*WT* × *WT*), by pollination of high-expressing SSG7.1 flowers with wild-type pollen (*SSG7.1 high* × *WT*), and by pollination of silent SSG7.2 flowers with wild-type pollen (*SSG7.2 silent* × *WT*) were sampled on the indicated days after pollination (*DAP*). Immunoblots with 20 μg of protein in each lane were stained with antibody recognizing tobacco and *N. sylvestris* GLU I and GLU II. The positions of 33-kDa GLU I and 36 kDa are indicated

exhibit a high-expressing phenotype (data not shown). This suggests that silencing persists in haploid gametophytic tissue and that resetting occurs later during seed development. To establish when resetting occurs, immunoblot analyses were done with extracts prepared from developing seeds dissected from flowers 7–15 d after pollination (DAP). Figure 4 shows that 33-kDa GLU I was not detectable in developing wild-type seeds. Hemizygous seed from high-expressing homozygous SSG7.1 plants fertilized with wild-type pollen showed high expression of GLU I from 7 to 15 d DAP. In contrast, hemizygous seed from uniformly silent homozygous SSG7.2 plants fertilized with wild-type pollen showed very low levels of transgene-encoded GLU I at 7-11 DAP and high levels at 13 and 15 DAP. Independent of plant genotype, seed extracts contained a 36-kDa GLU antigen of the size expected for class-II isoforms (Beffa et al. 1993), which decreased during seed maturation. Although the exact timing of GLU I expression in seeds of silent plants varied somewhat from experiment to experiment, the results consistently showed that the silent state persists through early stages of seed formation and is then reset to the high-expressing state at 11–13 DAP.

PTGS of GLU I and CHN I genes in the same genome

We compared the incidence and spatial pattern of PTGS in hybrids homozygous for the *GLU I* T-DNA SSG7.1 and the *CHN I* T-DNA SSC2.3, which are inherited as

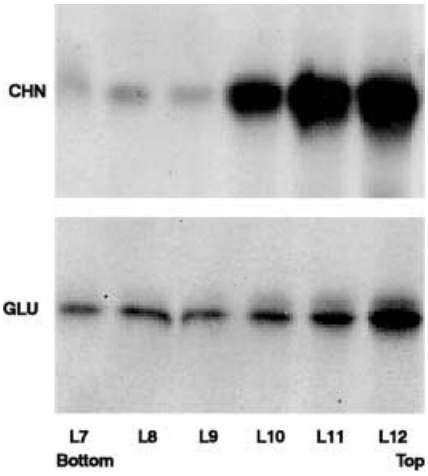

Fig. 5. Expression of GLU I and CHN I in leaves of a hybrid *N. sylvestris* plant homozygous for the SSG7.1 *GLU I* and SSC2.3 *CHN I* transgene loci. Immunoblots were stained with antibody recognizing tobacco and *N. sylvestris* GLU I and CHN I. Equal aliquots (20 μl) of extract were applied to each lane. Leaves are numbered counting from the bottom of the plant

independent traits and exhibit stochastic silencing (Table 1). The immunoblots in Fig. 5 show that in the hybrid plant, chitinase expression changes abruptly between leaf L9 and L10 counting from the bottom of the plants. In contrast, GLU I expression increased gradually in leaves from the bottom to the top of the plants. Comparison of these results with those obtained with homozygous SSG7.1 transformants (data not shown) and homozygous SSC2.3 transformants (Kunz et al. 1996) leads to two important conclusions: (i) *GLU I* and *CHN I* transgenes, which differ in transcribed sequence, can undergo PTGS in the same plant; and (ii) the abrupt changes between adjacent leaves characteristic of *CHN I* PTGS and the gradual changes in leaves

Table 3. Incidence of *GLU I* and *CHN I* PTGS in plants *N. sylvestris* homozygous for the SSG7.1 and SSC2.3 transgenes

Leaf tested[a]	Phenotype[b]			Independence[c]
	CHN I	GLU1		
		N high	N silent	
Upper leaf	N high	22 (20.1)	0 (2.0)	Association ($P = 0.030$)
	N silent	8 (9.9)	3 (1.0)	
Lower leaf	N high	1 (0.1)	1 (1.9)	No association ($P = 0.117$)
	N silent	1 (1.9)	30 (29.1)	

[a] Individual upper leaves and lower leaves from plants homozygous for both the SSG7.1 and the SSC2.3 transgenes. Upper and lower leaves are defined as indicated in Table 2

[b] Number of leaves (*N*), each from a different plant, exhibiting a high-expressing (high) or silent phenotype for *GLU I* and *CHN I* as indicated. Values in parenthesis are the numbers expected if silencing of *GLU I* and *CHN I* expression are independent events

[c] Fisher Exact Probability test for the hypothesis that expression of *GLU I* and *CHN I* is independent; 33 plants assayed

from the top to the bottom of the plant characteristic of *GLU I* PTGS are retained in the homozygous SSG7.1 × SSC2.3 hybrid.

To detect interactions between *GLU I* and *CHN I* PTGS, we compared the incidence of silencing in upper leaves and lower leaves of hybrids homozygous for both T-DNAs. If PTGS of the two transgenes are independent events, then the frequency of plants exhibiting silencing for both transgenes should equal the product of the frequencies of plants silent for one of the transgenes. Table 3 shows that in lower leaves, no significant association between the incidence of *GLU I* and *CHN I* PTGS was found at the 5% level (Fisher Exact Probability Test, $P = 0.117$); whereas, in upper leaves there was a slight bias for classes of plants showing either PTGS of both transgenes or PTGS of neither transgene ($P = 0.03$).

Discussion

The *GLU I* transformants of *N. sylvestris* provide a model system showing many features of PTGS described in other systems: the incidence and pattern of PTGS depend on gene dose and transformation event (e.g. Palauqui and Vaucheret 1995); roughly 60–70% sequence similarity of related genes is required to trigger PTGS (Angenent et al. 1993; De Carvalho Niebel et al. 1995; Kunz et al. 1996); and, at least for transgenes regulated by the 35S promoter, PTGS is triggered during a phenocritical period early in seedling growth and reset post-meiotically during seed development (Dehio and Schell 1994; De Carvalho Niebel et al. 1995; Vaucheret et al. 1995; Kunz et al. 1996; Balandin and Castresana 1997).

Assuming that the termination signals of the cDNAs are used, the transcribed sequences of the *GLU I* and *CHN I* transgenes are unrelated except for a short 14-nucleotide sequence of *CHN48* origin in the 5′-UTR regions of both transgenes. Earlier we showed for single-transgene transformants that these transgenes undergo independent silencing in response to their homologous host genes (Kunz et al. 1996). The present experiments show, for the first time, that unrelated transgenes present in the same genome can undergo independent PTGS. Comparison of transformants with the transgenes alone and or in combination provided insight into how PTGS is propagated and maintained. The *GLU I* and *CHN I* transformants show distinctive spatial patterns of PTGS. Based on studies with other PTGS systems, these differences could reflect the nature of the transgene, the structure of the transgene locus, the insertion site, and the transcriptional levels of homologous host genes (e.g. Palauqui and Vaucheret 1995; Palauqui et al. 1996; Que et al. 1997; Vaucheret et al. 1997). Our present and earlier studies (Kunz et al. 1996; Kunz 1997) show that replicate, independent *GLU I* and *CHN I* transformants with two T-DNA copies at each particular locus and inserted at different sites exhibit similar patterns. Moreover, the host homologues of these transgenes show coordinated developmental regulation (Shinshi et al. 1987). Taken together, this suggests that the nature of the transgene may be a critical parameter in PTGS pattern determination. Similar conclusions have been drawn from comparison of tobacco transformants carrying nitrate reductase or nitrite reductase transgenes (Palauqui et al. 1996).

Analysis of spatial patterns of silencing (Jorgensen 1995; Kunz et al. 1996; Palauqui et al. 1996; Que et al. 1998), grafting experiments (Palauqui et al. 1997), and studies of systemic silencing triggered by a local signal (Voinnet et al. 1998) provide compelling evidence that sequence-specific silencing signals can move from cell to cell via plasmodesmata and systemically via the phloem. A simple signal-based model is difficult to reconcile with the stable patterns reported here and for certain other silencing systems (Jorgensen 1995; Kunz et al. 1996; Jones et al. 1998; Que et al. 1998). One possible explanation is that pre-patterns are established that influence production, transmission or reception of signals or have more general effects on competence for silencing. This is an attractive hypothesis because trafficking via plasmodesmata and the phloem is known to be physiologically and developmentally regulated (Oparka et al. 1999; Thompson and Schultz 1999); and, in some cases, responsiveness to silencing signals depends on the developmental state of the tissue (Palauqui et al. 1996).

We did not detect significant interactions in the incidence of *GLU I*- and *CHN I*-PTGS in lower leaves of hybrid transformants. This indicates that PTGS of a transgene does not result in increased competence for PTGS of a second, unrelated transgene. Moreover, the sequence-specific patterns generated by *CHN I* and *GLU I* PTGS are maintained in the same hybrid plant. Therefore, these patterns are not specified by a pre-pattern of competence for PTGS or propagation of the silent state. If pre-patterns exist, then their perception appears to be transgene specific.

These conclusions and the dependence of PTGS on target-gene expression (Vaucheret et al. 1998) are consistent with biochemical switch models for PTGS, which emphasize the importance of sequence-specific host-gene and transgene expression in silencing (Meins and Kunz 1995; Meins 2000). According to these models, phenocritical periods affecting PTGS, transgene-specific recognition of pre-patterns, as well as the extreme sensitivity of PTGS to environmental influences could reflect regulation of target genes by developmental and environmental cues. When the system is near a critical threshold for triggering PTGS, transient fluctuations in the content of target RNAs or mobile signals could generate stochastic patterns of silencing. On the other hand, stable developmental regulation could result in more predictable patterns of silencing and nonstochastic resetting.

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