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Post-Transcriptional Cosuppression of β-1,3-Glucanase Genes Does Not Affect Accumulation of Transgene Nuclear mRNA

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Silencing of a *Nicotiana plumbaginifolia* β-1,3-glucanase (*gn1*) transgene in tobacco line T17 occurs in homozygous and in haploid plants with one transgene locus dosage per chromosome set. We have previously shown that the silent state is manifested by a reduced *gn1* steady state mRNA level and results from a post-transcriptional process that is under developmental control in homozygous T17 plants. In this study, we show that specific endogenous β-1,3-glucanase genes are cosuppressed with *gn1* in homozygous T17 plants. We also demonstrate that the developmental timing of cosuppression depends on environmental conditions and that once silencing is established it is stably maintained during plant development. Analyses of additional transgenic tobacco lines revealed that *gn1* silencing is not restricted to the T17 line and showed that silencing can also take place in R_0 plants containing independent loci in hemizygous states. Furthermore, silencing can also be obtained in progeny plants in which expressing loci have been combined. Importantly, cosuppression occurs via a post-transcriptional mechanism that does not interfere with the accumulation of transgene nuclear mRNA. These results strongly suggest that the silencing mechanism operates at RNA transport and/or RNA stability levels.

INTRODUCTION

In recent years it has been demonstrated that in plants, sense transgenes can cause gene silencing. Several types of gene suppression are currently recognized: the mutual suppression of transgenes, the unidirectional silencing of transgenes or endogenous genes, and the cosuppression of both transgenes and homologous resident genes (reviewed by Jorgensen, 1992; Kooter and Mol, 1993; Matzke and Matzke, 1993; Flavell, 1994).

Typically, silencing involves genes that contain regions with high sequence similarity and is manifested by reduced steady state mRNA levels of the affected genes. The silent state may be the result of the transcriptional inactivation of the genes (Brusslan et al., 1993; Meyer et al., 1993; Neuhuber et al., 1994) or may be caused by post-transcriptional processes (Mol et al., 1991; de Carvalho et al., 1992; Lindbo et al., 1993; Dehio and Schell, 1994; Smith et al., 1994).

In most cases, when a chimeric transgene is introduced, only some of the resulting transgenic plants exhibit gene silencing. Thus, silencing is not a direct consequence of the transgene(s) itself; rather, it requires additional factors. It is believed that properties of the transgene locus, such as the number of copies, the structure, and/or the genomic site of integration, are important determinants of silencing. In this respect, several groups have demonstrated that silencing occurs more often in plants that contain multiple transgene copies per locus (Hobbs et al., 1990; Linn et al., 1990; Mittelsten Scheid et al., 1991; Assaad et al., 1993). The transgene copy number may be important for the silencing strength of specific loci (Matzke et al., 1994). Furthermore, Hobbs et al. (1993) demonstrated that a high copy number may result in suppression of β-glucuronidase (*gus*) transgenes in tobacco only when the transgene locus contains two linked copies as inverted repeats. However, increased copy number and specific configurations are not absolute requirements because silencing is also observed with single transgene inserts (Linn et al., 1990; van der Krol, 1990; Assaad et al., 1993; Seymour et al., 1993). Nevertheless, these parameters may promote the silencing effect.

The chromosomal context into which the transgenes are inserted may determine the competence of one locus to induce silencing and/or to become silenced. This position effect may explain the silencing behavior of the unique transgenic plants described by Meyer et al. (1993) and Vaucheret (1993). Meyer et al. (1993) described crosses with a transgenic petunia line in which an inactive transgene behaved paramutagenically to the active allele. Instead, Vaucheret (1993) reported that an inactive transgene locus is able to suppress any newly introduced gene copies that contain similar cauliflower mosaic virus (CaMV) 35S and CaMV 19S promoter sequences. In addition, Matzke et al. (1994) suggested that the strong silencing activity of a specific locus in tobacco is probably influenced by its telomeric location, which is often associated with gene silencing in other eukaryotic systems. Furthermore, the relative

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position of two genes in the nucleus may determine whether unidirectional or mutual inactivation occurs (Jorgensen, 1992). However, relative nuclear position also seems insufficient to evoke silencing because in some cases silencing had occurred in all transgenic plants examined (Goring et al., 1991; Lindbo et al., 1993; Seymour et al., 1993). In addition, the strong silencer H_2 locus described by Matzke et al. (1994) inactivated target transgenes, regardless of their position in the genome. This suggests that the genomic position might be an important parameter in some but not all cases.

Silencing of transgenes has often been correlated with increased DNA methylation (Matzke et al., 1989, 1993, 1994; Hobbs et al., 1990, 1993; Linn et al., 1990; Matzke and Matzke, 1991; Kilby et al., 1992; Meyer et al., 1992, 1993; Assaad et al., 1993; Vaucheret, 1993). An open issue is whether the involvement of DNA methylation is strictly associated only with transgene silencing or whether it also applies to endogenous plant genes. In the only cosuppression case analyzed, no correlation between cosuppression and methylation was found (Hart et al., 1992).

Environmental conditions can also influence the occurrence of silencing. High light intensities positively affected the incidence (Elkind et al., 1990; van der Krol et al., 1990; Meyer et al., 1992) or timing (Dorlhac de Borne et al., 1994) of suppression, whereas Hart et al. (1992) demonstrated that cosuppression of chitinase genes occurred only when seeds were germinated in vitro before transfer to the greenhouse.

The diverse examples of gene silencing in plants may be the result of different underlying mechanisms. Thus, a detailed analysis of a number of cases is required to evaluate the potential mechanism(s) of gene silencing.

We are studying a silencing phenomenon that was observed in a single line, named T17, of a series of tobacco transformants containing a *Nicotiana plumbaginifolia* β-1,3-glucanase (*gn1*) transgene. Previously, we reported that *gn1* was silenced exclusively when present in a homozygous condition (homozygous T17) or in a haploid background (de Carvalho et al., 1992). Here we demonstrate that a specific class of endogenous β-1,3-glucanase genes is cosuppressed with *gn1* in homozygous T17 plants. Furthermore, we present data suggesting that silencing is a dose-dependent phenomenon that requires specific loci. We also demonstrate that the time point at which silencing initiates is influenced by developmental and environmental conditions. We provide additional evidence that the silencing mechanism operates mainly at later stages of the gene expression pathway by affecting nucleocytoplasmic transport or stability of the RNAs.

RESULTS

Cosuppression of Endogenous β-1,3-Glucanase Genes

One of the first results showing that sense transgenes can become silenced in plants was reported by Napoli et al. (1990) and van der Krol et al. (1990). In these cases, chalcone synthase (*chs*) and dihydroflavonol reductase (*dfr*) transgenes were simultaneously silenced with their corresponding homologous host *chs* and *dfr* genes; this phenomenon is referred to as cosuppression. Based on these observations, we decided to determine whether endogenous β-1,3-glucanase genes were also affected by a "cosuppression-like" mechanism in T17 tobacco plants homozygous for *gn1* (homozygous T17). Preliminary results showed no significant differences in the β-1,3-glucanase activity levels between young leaves of wild-type tobacco plants and those of suppressed, homozygous T17 plants (de Carvalho et al., 1992), probably because of low enzyme quantities. Therefore, we looked for possible differences in β-1,3-glucanase levels in stressed plants in which endogenous β-1,3-glucanase levels were enhanced (for review, see Cutt and Klessig, 1992).

Wild-type, homozygous, and hemizygous T17 plants were treated with known inducers of β-1,3-glucanases; these treatments included spraying with 5 mM of salicylic acid and bacterial infection (*Pseudomonas syringae* pv *syringae*) of plant leaves. Two and 7 days after the treatments, proteins were extracted from leaves, and the different isoforms were visualized by isoelectric focusing β-1,3-glucanase assays. Both treatments gave similar results. As shown for salicylic acid–treated plants in Figure 1, the endogenous acidic β-1,3-glucanase activity (pI of <4.6) was similar in all plant extracts. A high activity corresponding to GN1 (pI of ~9.6) was found only in extracts from hemizygous T17 plants. Furthermore, the endogenous basic β-1,3-glucanase activity (pI of ~8.0) detected in both hemizygous

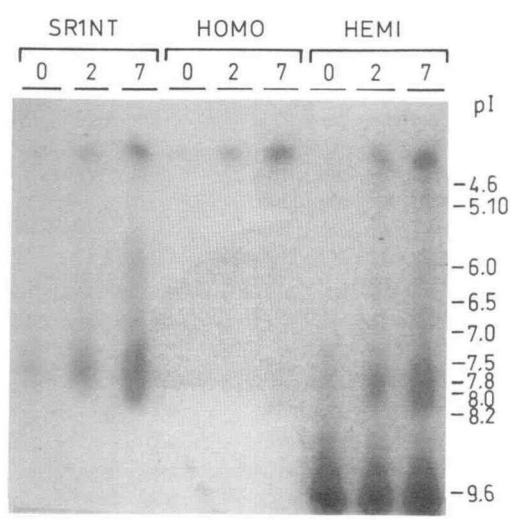

Figure 1. Direct Assay for β-1,3-Glucanase Activity on 7.5% Isoelectric Focusing Gel.

SR1NT, HOMO, and HEMI correspond to total proteins extracted from leaves of wild-type and homozygous and hemizygous T17 tobacco plants, respectively, before (0) and after (2 and 7 days) spraying with 5 mM of salicylic acid.

T17 and wild-type plant extracts was absent in extracts of homozygous T17 plants. RNA gel blot analysis demonstrated that the absence of endogenous basic β-1,3-glucanase activity in homozygous T17 plants correlates with strongly reduced levels of tobacco class I basic β-1,3-glucanase gene transcripts, as shown in Figure 2.

Taken together, these data imply that *gn1* and endogenous class I basic β-1,3-glucanases are cosuppressed in homozygous T17 plants and that the stress-induced acidic β-1,3-glucanase genes are not affected. This selective suppression may occur as a consequence of the higher degree of DNA sequence identity between the coding sequences of *gn1* and class I basic isoform genes (~81%) than between the coding sequences of *gn1* and the acidic class II and class III β-1,3-glucanases (~63%).

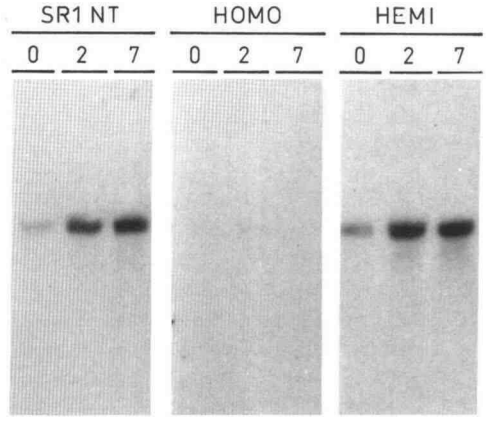

Figure 2. RNA Gel Blot Analysis of Total RNA Extracted from Leaf Tissues of Wild-Type Tobacco and Homozygous and Hemizygous T17 Plants.

Leaves were harvested before (0) and after (2 and 7 days) spraying with 5 mM of salicylic acid. RNA gel blots were hybridized with riboprobes homologous to class I β-1,3-glucanases. Abbreviations are as given in the legend to Figure 1.

Environmental Conditions Affect the Timing of Cosuppression during Plant Development

Our previous results indicated that *gn1* silencing occurs during plant development because the GN1 protein was present in plant extracts from homozygous T17 plants during the first weeks of development and then disappeared gradually after 6 to 7 weeks of development (de Carvalho et al., 1992). To estimate more accurately the timing of *gn1* silencing during plant development, *gn1* RNA accumulation was examined in leaf tissues of homozygous and hemizygous T17 plants at different weeks after seed germination in soil (greenhouse 1). As shown in Figure 3, at all developmental stages, hemizygous T17 plants specified similar levels of *gn1* mRNA. In contrast, in homozygous T17 plants, *gn1* mRNA was abundant during the first weeks of plant development but disappeared after 6 to 7 weeks of development. The endogenous class I genes were suppressed together with *gn1* during plant development (data not shown). In the next experiment, we analyzed in which tissues *gn1* silencing took place. To this end, RNA was extracted from upper, middle, and lower leaves as well as from roots and flowers of mature, homozygous T17 plants. Accumulation of *gn1* mRNA was detected in none of the assayed tissues (data not shown). This implies that *gn1* silencing occurred throughout the plant. However, because RNA of complete tissues was analyzed, it is possible that *gn1* was not silenced in specific cells.

The time point at which *gn1* silencing initiates was, in independent experiments, always between 6 and 7 weeks after seed germination. Because the experiments were conducted in the same greenhouse under identical circumstances, we tested whether the timing of *gn1* silencing was affected by changing the growth conditions. Homozygous T17 seeds were germinated at 25°C in a greenhouse with a light intensity of 55 μmol m^{-2} sec^{-1} and a photoperiod of 16 hr light/8 hr dark and in a greenhouse with a light intensity of 70 μmol m^{-2} sec^{-1} and a photoperiod of 14 hr light/10 hr dark. Pooled leaf samples for RNA analysis were taken at weekly intervals. Typically, the *gn1* RNA level in leaves of homozygous T17 plants grown in greenhouse 1 was reduced strongly between weeks 6 and 7 after seed germination (data not shown; Figure 3). Instead, the *gn1* mRNA level in leaves of homozygous T17 plants grown in greenhouse 2 started to diminish 4 weeks after seed germination and was undetectable 1 week later (data not shown). This implies that initiation of *gn1* silencing was indeed affected by environmental conditions. Because tobacco plants developed faster in greenhouse 2 than in greenhouse 1, initiation of *gn1* silencing may have coincided with a certain stage of development. Alternatively, environmental conditions such as light intensity could have been directly responsible for the timing of silencing. Furthermore, because pooled leaf samples were analyzed, it remains to be determined how silencing initiates temporally and spatially in the developing homozygous T17 plants.

Cosuppressed *gn1* and Endogenous β-1,3-Glucanase Genes Are Transcriptionally Active

Previously, we demonstrated that *gn1* silencing in homozygous T17 plants occurs post-transcriptionally (de Carvalho et al., 1992). Because endogenous β-1,3-glucanase genes are cosuppressed, we decided to investigate how the β-1,3-glucanase mRNA levels in hemizygous and homozygous T17 plants correlate with the transcriptional activities of the endogenous basic β-1,3-glucanase genes. To this end, runoff transcription analyses were performed with nuclei isolated from hemizygous and suppressed homozygous T17 plants. Figure 4A shows that the ratio between the transcriptional activities of the *gn1* and the class I basic β-1,3-glucanase genes relative to that of the endogenous gene encoding the small subunit of ribulose-1,5-

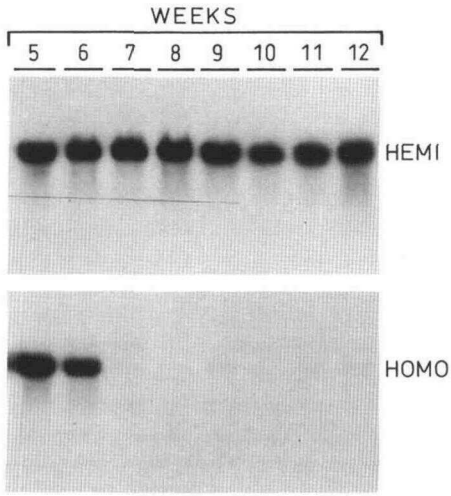

Figure 3. RNA Gel Blot Analysis of Total RNA Extracted from Leaf Tissues of Hemizygous and Homozygous T17 Plants.

Leaves from plants were harvested at the indicated number of weeks after seed germination. RNA gel blots were hybridized with riboprobes homologous to the *gn1* cDNA. HEMI, hemizygous; HOMO, homozygous.

bisphosphate carboxylase is similar in nuclei of homozygous and hemizygous T17 plants. This result indicates that reduced RNA accumulation in silenced homozygous plants is due to a post-transcriptional mechanism specifically affecting both *gn1* and endogenous basic class I β-1,3-glucanase transcripts.

Antisense Transcription of *gn1* Does Not Seem To Be Required for Cosuppression of β-1,3-Glucanase Genes

It has been previously proposed that the unexpected production of antisense RNA from a converging promoter, either within or near the inserted T-DNA, could be responsible for the down-regulation of sense RNA production by transgenes (Grierson et al., 1991; Mol et al., 1991). If this were the case for β-1,3-glucanase gene silencing, promoter sequences in the neighboring plant DNA should transcribe the chimeric gene encoding neomycin phosphotransferase II (NPTII) present downstream of the chimeric *gn1* gene in the antisense orientation (de Carvalho et al., 1992). Similarly, we cannot exclude the possibility that promoter sequences of plant origin got inserted downstream of the chimeric *gn1* gene during the T-DNA integration in T17, which would generate RNA molecules exclusively complementary to *gn1* RNA. To test whether the silenced *gn1* gene is transcribed in an antisense direction, in vitro–generated sense and antisense RNAs were probed with labeled nuclear RNA from homozygous T17 plants. As shown in Figure 4B, antisense transcription detected for the *gn1*, the *nptII*, and the endogenous ribulose-1,5-bisphosphate carboxylase small subunit gene sequences was at similar, low background levels. This suggests that antisense transcription is not involved in this silencing event.

Post-Transcriptional Silencing Does Not Affect Mature *gn1* RNA Production

Our results demonstrated that both *gn1* and endogenous β-1,3-glucanase genes are normally transcribed in suppressed plants, even though cytoplasmic mRNA accumulation of these genes is strongly reduced. To understand at which post-transcriptional level the silencing mechanism operates, we compared the accumulation of mature *gn1* RNA in the nucleus of hemizygous and suppressed, homozygous T17 plants. To this end, a semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) was performed with nuclear RNAs prepared from wild-type SR1 and from homozygous and hemizygous T17 plants. First-strand cDNA synthesis reactions primed with oligo(dT) were amplified by PCR using primers specific for *gn1* and actin as described in Methods. As shown in Figure 5, a 0.5-kb RT-PCR product corresponding to mature *gn1* RNA was detected in nuclei from both homozygous and hemizygous T17 plants. In both reactions, a 0.7-kb PCR product was also obtained. This 0.7-kb product corresponds with the unprocessed form of *gn1* RNA containing intron 2, which was confirmed by probing the filter with *gn1* intron 2 (data not shown). Polyadenylated precursor *gn1* RNA containing intron 1 was not detected (Figure 5 and Methods). This RNA species was also not detected in RT-PCRs using primers directly flanking intron 1 (data not shown). This suggests that either the cDNA synthesis of polyadenylated full-length precursor

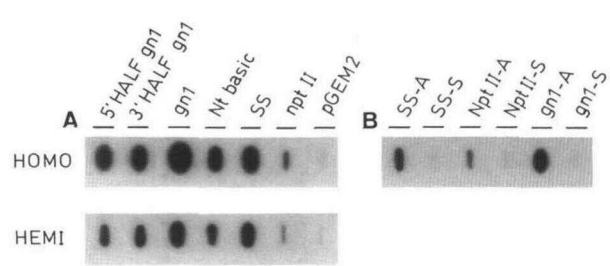

Figure 4. Nuclear Runoff Transcription Analysis of Homozygous and Hemizygous T17 Plants.

(A) Labeled nuclear RNAs hybridized to plasmid DNA–containing gene-specific probes.

(B) Labeled nuclear RNA of suppressed homozygous T17 plants hybridized to sense (S) and antisense (A) in vitro–generated RNAs.

Abbreviations refer to the transcription activity detected: 5′ HALF gn1, nucleotides 65 to 595 of the *gn1* cDNA; 3′ HALF gn1, nucleotides 596 to 1065 of the *gn1* cDNA; gn1, *gn1* β-1,3-glucanase cDNA sequence; Nt basic, endogenous basic β-1,3-glucanase gene; SS, small subunit of the ribulose-1,5-bisphosphate carboxylase gene; npt II, neomycin phosphotransferase gene; pGEM2, vector DNA (negative control). HOMO, homozygous; HEMI, hemizygous.

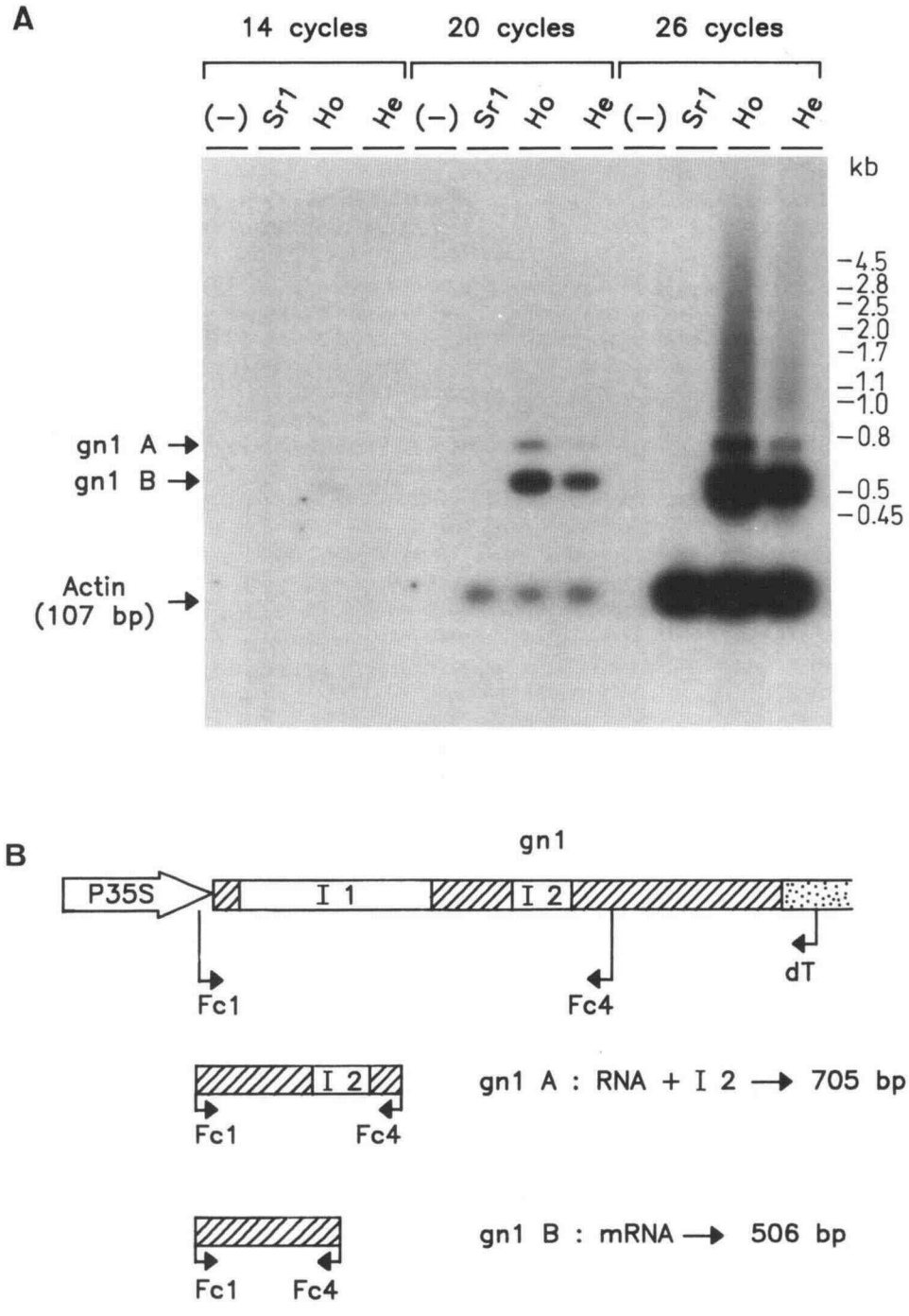

Figure 5. RT-PCR Analysis of Nuclear RNAs from Wild-Type, Homozygous, and Hemizygous Plants.

The negative control (–) and number of PCR cycles are indicated. Sr1, wild type; Ho, homozygous; He, hemizygous.

(A) DNA gel blot analysis of RT-PCR products hybridized with *gn1* and actin-encoding DNA probes (see Methods). Length markers are given at right in kilobases.

(B) Schematic drawing of the P35S–*gn1* gene sequences. *gn1* exons are represented as hatched boxes, intron sequences consist of intron 1 (I1) and intron 2 (I2), and the *gn1* 3′ untranslated region is represented as a dotted box. First-strand cDNA synthesis reactions were performed with a 3′ oligo(dT) primer (dT). For the PCRs, a 5′ primer located in the P35S leader sequences (Fc1) and a 3′ primer located at the third exon of *gn1* (Fc4) were used. The *gn1* PCR amplification products shown in **(A)** are indicated by A and B.

gn1 transcripts was incomplete or intron 1 is removed from most *gn1* RNAs before addition of the poly(A) tail.

As shown in Figure 5, the abundance of the actin PCR product was similar using cDNA reactions with SR1 and hemizygous and homozygous T17 nuclei. This implies that the relative abundance of the *gn1* PCR products has an informative value. First, it seems that the intron 2–containing polyadenylated transcript was more abundant in nuclei of suppressed, homozygous T17 than in hemizygous T17 plants. This observation is not inconsistent with a normal gene dose response. Furthermore, the level of mature *gn1* RNA present in nuclei of suppressed, homozygous T17 plants appeared similar to, or maybe slightly higher than, that in hemizygous T17 plants.

Taken together, these results show that mature nuclear *gn1* mRNA accumulates in silenced T17 plants and that the reduced cytoplasmic *gn1* mRNA level in silenced plants is not a consequence of a proportionally reduced accumulation of nuclear *gn1* mRNA. The data thus support the idea that *gn1* silencing results from an increased nuclear or cytoplasmic instability of the spliced and polyadenylated transcripts or from a reduced nucleocytoplasmic *gn1* RNA flow, possibly coupled to an inefficient production of mature mRNA.

β-1,3-Glucanase Gene Silencing Is Not Restricted to T17

To test whether *gn1* silencing is unique to line T17, new transformants were generated containing the P35S–*gn1* gene. The T-DNA introduced into the plants contained the chimeric genes in the same orientation as in the T-DNA present in T17 plants, except that the *nptII* marker gene was replaced by the hygromycin phosphotransferase (*hpt*) gene. As shown in Figure 6, among 12 independent R_0 transformants examined, two plants, S1 and S11, which contained multiple (at least seven) T-DNA copies, showed cosuppression of both the *gn1* and the endogenous β-1,3-glucanase genes. This result demonstrates that β-1,3-glucanase gene silencing is a more general phenomenon not restricted to T17 plants. Furthermore, because cosuppression was seen in primary transformants, it reinforces our previous conclusions (de Carvalho et al., 1992) that interaction between allelic copies is not required. On the basis of the lines analyzed, we cannot yet establish a relationship between copy number and suppression (data not shown).

To evaluate whether suppression could be achieved by combining different loci derived from plants that express *gn1* at high levels, the two R_0 plants, S8 and S9, that carry two and three T-DNA loci, respectively, were crossed with homozygous T17 plants. In the antibiotic-resistant progeny, all plants should contain the T17 locus in a hemizygous state together with one to several of the loci provided by either the S8 or the S9 parent. The hemizygous T17 locus should direct a normal *gn1* mRNA level, unless the interaction with loci from S8 or S9 plants caused silencing.

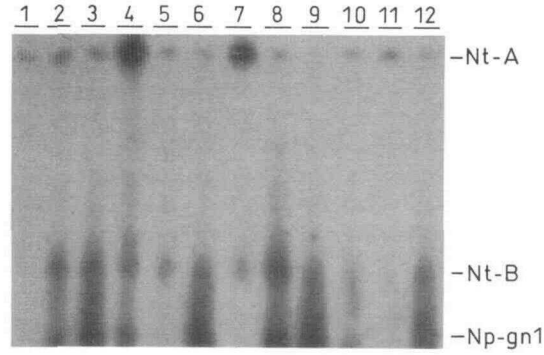

Figure 6. Direct Assay for β-1,3-Glucanase Activity on a 7.5% Isoelectric Focusing Gel.

Total proteins (1 to 12) were extracted from leaves of 12 R_0 plants. The activities corresponding to acidic (Nt-A) and basic (Nt-B) endogenous β-1,3-glucanases and *gn1* (Np-gn1) are indicated.

As shown in Figure 7 and Table 1, five of 11 plants examined from the cross between S9 and T17 showed the silencing phenotype. In these plants, *gn1* silencing correlated with the presence of the S9.1 locus, which contains two transgenes. The remaining six plants expressed *gn1* and carried the S9.2 locus (one transgene), the S9.3 locus (one transgene), or both loci. Importantly, suppressed plants containing the T17 and S9.1 loci and nonsuppressed plants containing the T17, S9.2, and S9.3 loci have a similar number of transgenes. This implies that *gn1* suppression is activated by the combination of certain loci (T17 and S9.1) rather than by an increased number of transgene copies.

In the progeny of the cross between S8 and T17 plants, the presence of the S8.1 locus, which contains two transgenes, correlated in seven of 13 cases with a silenced phenotype (data not shown). However, one plant carrying the S8.1 locus had directed a just slightly reduced *gn1* mRNA level. Plants containing only the S8.2 locus (one transgene) showed either a high (three of 13) or a suppressed (two of 13) level of *gn1* mRNA. Thus, in this instance, no strong correlation can be established between silencing and the presence of specific loci. This suggests that for some loci, physiological conditions may determine whether the locus is expressed or silenced.

DISCUSSION

Specific Transgene Loci Are Required for the Dose-Dependent β-1,3-Glucanase Gene Silencing

We have shown that the introduction of a chimeric *N. plumbaginifolia gn1* gene in tobacco can result in silencing of both

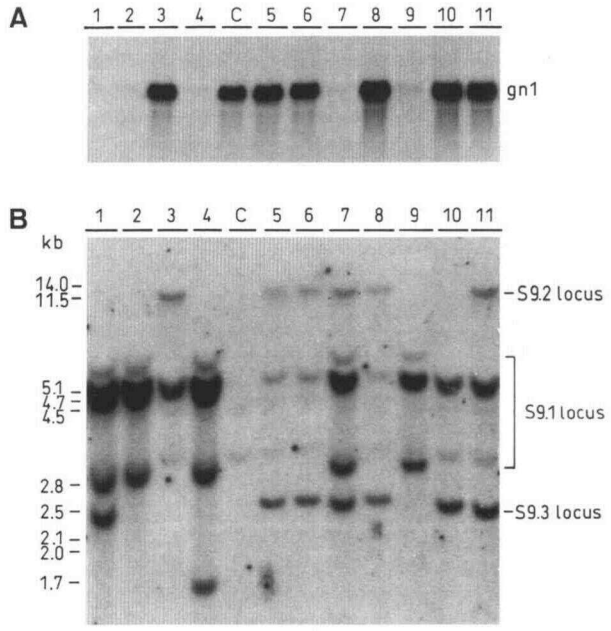

Figure 7. DNA and RNA Gel Blot Analyses of Hygromycin-Resistant and Kanamycin-Resistant Progeny Plants Derived from Crosses between the S9 R_0 Plant and Homozygous T17 Plants.

The plant numbers are provided above each gel.
(A) RNA gel blot analysis of total RNA extracted from plant leaf tissues. RNA gel blots were hybridized with riboprobes homologous to the *gn1* cDNA. C corresponds to a control hemizygous T17 sample.
(B) DNA gel blot analysis of total DNA extracted from plant leaf tissues and digested with EcoRI. The T-DNA has two EcoRI sites: one before the CaMV 35S promoter and one inside the *hpt* gene. Hybridization with an *hpt* probe shows one common 5.3-kb band containing P35S–*gn1*–P*nos* and part of the *hpt* gene. The other hybridizing band(s) represents the 3′ part of *hpt* genes plus plant-flanking sequences and reveals the number of T-DNA copies. Molecular length markers are indicated at left in kilobases. The bands that distinguish the S9 plant loci S9.1, S9.2, and S9.3 are indicated at right. C, corresponds to a control hemizygous T17 sample.

the transgene and endogenous homologous β-1,3-glucanase genes. In the T17 plant line, cosuppression of β-1,3-glucanase genes is associated with a transgene locus dose response. Cosuppression of chitinase genes was observed only in plants homozygous for the transgene locus (Hart et al., 1992). In situations in which transgenes were silenced when hemizygous, homozygosity at the suppressed loci has been correlated with enhanced suppression effects (Elkind et al., 1990; Angenent et al., 1993; Brusslan et al., 1993; Matzke et al., 1993; Dehio and Schell, 1994; Neuhuber et al., 1994). Thus, in some of these cases, gene dose effects may be important, as has also been shown in our work.

By studying haploid T17 tobacco plants, we previously found that silencing does not require allelic interactions between T-DNA copies but rather is correlated with the transgene dose in the plant genome (de Carvalho et al., 1992). In this work, we show that in a second series of transformants, β-1,3-glucanase genes were silenced in the primary S1 and S11 transformants as well as in the progeny of S8 and S9 plants crossed with homozygous T17 plants. This implies that suppression is not restricted to the T17 line and that nonallelic interactions can result in silencing. Thus, suppression in homozygous T17 plants is likely to occur as a consequence of increased amounts of transgene copies or increased levels of transgene expression relative to hemizygous plants. However, increased transgene copy number alone is not sufficient to activate silencing because similar copy numbers can be found in silenced S1 and S11 plants and in nonsilenced S4 primary transformants (data not shown), as well as in silenced plants containing the T17 and the S9.1 loci and in nonsilenced plants containing the T17, S9.2, and S9.3 loci. Hobbs et al. (1993) demonstrated for several transgenic tobacco lines that the presence of two *gus* genes at a single locus correlated with relatively low GUS activity and with the ability to silence high-expressing *gus* loci.

These observations presume the existence of loci with different abilities to promote silencing because of transgene configurations, position, and/or expression levels. Hobbs et al. (1993) demonstrated that the T-DNA configuration of specific loci containing two linked copies was associated with reduced gene activity. However, transgene configuration is clearly not an absolute requirement because in a few cases, suppression was obtained with plants containing single-copy transgenes (Linn et al., 1990; van der Krol et al., 1990; Meyer et al., 1993; Seymour et al., 1993). Transgenic plants showing β-1,3-glucanase gene silencing always contained at least one locus with multiple transgene copies. This suggests that transgene configuration may be important for β-1,3-glucanase gene suppression.

Locus-dependent silencing may also depend on the positions of the T17 and different S9 loci relative to each other in the nucleus, in a manner similar to that proposed by Jorgensen (1992). In this proposal, the transgene loci that are able to generate suppression, such as T17 and S9.1 as well as the endogenous homologous β-1,3-glucanase genes, should be

Table 1. Segregation Analysis of the *gn1* Silencing Phenotype in the Kanamycin-Resistant and Hygromycin-Resistant Progeny of Homozygous T17 Plants Crossed with the S9 R_0 Transformant

	Plant Number										
Locus	1*	2*	3	4*	5	6	7*	8	9*	10	11
T17	+	+	+	+	+	+	+	+	+	+	+
S9.1	+	+	−	+	−	−	+	−	+	−	−
S9.2	−	−	+	?	+	+	+	+	−	−	+
S9.3	+	−	−	?	+	+	+	+	−	+	+

*, *gn1* silencing; +, presence; −, absence; ?, not understood.

located in compatible nuclear domains for silencing. This would imply that plants containing the T17 locus and the S9.2 and S9.3 loci are not suppressed because these S9 loci are located in nuclear domains incompatible with T17 and endogenous genes. However, plants containing the S9.1, S9.2, S9.3, and T17 loci also showed the silenced state. One possible explanation is that the reduced *gn1* mRNA levels in these plants are due to the suppression of the T17 and S9.1 loci only and that S9.2 and S9.3 are not suppressed but specify very low *gn1* mRNA levels. Alternatively, S9.2 and S9.3 may become silenced when T17 and S9.1 are also present. In this view, the initiation of silencing would be triggered in limited areas of the nucleus, and then the mechanism itself would affect other homologous gene sequences at other nuclear positions.

Another explanation for the locus-dependent silencing is that the level and constitutive character of expression of individual loci are major determinants of silencing. In such a scenario, the transgene configuration as well as the position of the T-DNA in the genome would be indirectly involved in gene silencing by dictating the stability as well as the amount of gene activity for each locus. Dose-dependent β-1,3-glucanase suppression would then be activated as a consequence of a (combined) high level of expression produced by specific transgene loci. This assumption favors the biochemical switch model proposed by F. Meins, Jr. (see Jorgensen, 1992) to explain silencing in plants. The model invokes a mechanism sensitive to a certain threshold level of gene expression. Based on this model, the S9.1 locus should express a higher level of *gn1* than the S9.2 and S9.3 loci, because only the S9.1 locus, and not the S9.2 and S9.3 loci, leads to suppression when combined with the T17 locus. This hypothesis can be tested by comparing *gn1* expression levels of individual S9.1, S9.2, and S9.3 loci.

Sequence-Specific Cosuppression of Host β-1,3-Glucanase Genes

Silencing of *gn1* correlates specifically with suppression of class I β-1,3-glucanase genes that encode members of the same group of basic isoforms that are located in the plant cell vacuoles (for review, see Meins et al., 1992). This selective suppression is most likely due to the higher degree of sequence identity that exists between the coding sequences of *gn1* and class I genes (~82%) than between the coding sequences of *gn1* and class II and III members (~63%). Similarly, Angenent et al. (1993) reported that the petunia homeotic gene encoding the floral binding protein (FBP1) can suppress only its homolog, whereas members with ~30% sequence divergence are not affected.

We looked for sequences in *gn1* present in class I basic β-1,3-glucanases and absent in acidic β-1,3-glucanases. On average, regions common to *gn1* and class I β-1,3-glucanases are not longer than 9 bp, except for the 3′ part of the coding region, where ~50- to 70-bp sequences are unique to basic β-1,3-glucanases. The specificity of β-1,3-glucanase cosuppression thus requires, at least in part, sequence information present in short sequences common to the transcripts of the *gn1* and class I basic β-1,3-glucanase genes and absent in their acidic counterparts. In this respect, it would be interesting to perform genetic deletion analysis to identify the region(s) important for class I β-1,3-glucanase gene silencing.

β-1,3-Glucanase Gene Silencing: Possible Mechanisms

There are at least two silencing mechanisms in plants; these mechanisms operate at either the transcriptional or post-transcriptional level. Transcriptional silencing was reported for homologous P35S–*dfr* transgenes (Meyer et al., 1993), endogenous chlorophyll *a/b* binding protein (*cab*) genes with homology restricted to the promoter and 14 nucleotides of the leader sequence (Brusslan et al., 1993), and chimeric transgenes with the *cab* promoter transcriptionally fused to the tumor morphology shoot gene (*Pcab–tms2*). It has also been described for the unidirectional suppression of chimeric transgenes having the CaMV 35S promoter as a region of homology (Neuhuber et al., 1994). Post-transcriptional silencing was reported for genes in which the similar region was in the coding region (*chs*, Mol et al., 1991; tobacco etch virus [TEV] coat protein, Lindbo et al., 1993; basic β-1,3-glucanases, this work) or for P35S–*rolB* transgenes (Dehio and Schell, 1994). Except for the instances of silencing described by Meyer et al. (1993) and Dehio and Schell (1994), in which similarity was for entire transgenes, transcriptional or post-transcriptional silencing occurs when the genes involved have homology with the promoter or coding regions, respectively. Analysis of other instances of silencing is necessary to elucidate a correlation between the type of silencing control (transcriptional versus post-transcriptional) and the location of the homologous regions (promoter versus coding region).

Several parameters were identified that are important for cosuppression of β-1,3-glucanase genes in tobacco. Silencing depends on transgene dosage, but this applies only to loci competent to promote silencing. We believe that this competence to silence depends mainly on the level and continuity of expression of specific loci. However, T-DNA configuration and position of integration may also influence this competence. Furthermore, resetting of *gn1* expression occurs in young seedlings, and silencing is again established during development in mitotically dividing cells. The timing of *gn1* silencing appears to depend on environmental conditions, as was also observed for cosuppression of the nitrate reductase gene in tobacco (Dorlhac de Borne et al., 1994). Silencing of the β-1,3-glucanase and nitrate reductase genes in tobacco, and of the *rolB* gene in Arabidopsis (Dehio and Schell, 1994; Dorlhac de Borne et al., 1994), is developmentally controlled, suggesting similar control points in the suppression process. It is still not known which are the developmental signals that switch on the silencing mechanism, but once silencing is settled, it is maintained. This observation suggests that maintenance of silencing requires

a constant process of signaling or imprinting. Because β-1,3-glucanase silencing appears to be a post-transcriptionally regulated process, maintenance of silencing might require a constant transcription rate for the affected genes in the cell nuclei. However, for all models it is crucial to explain how mature, polyadenylated *gn1* mRNA accumulates in the nucleus of homozygous T17 plants. A question is whether regulation of splicing is involved in β-1,3-glucanase silencing. It is possible that an alternative splicing mode is initiated and maintained in a dose-dependent fashion, as has been described for the determination of the sexes in Drosophila (Baker, 1989). We observed that intron 2–containing *gn1* RNA accumulates in nuclei of suppressed and nonsuppressed plants. It cannot be excluded that this unspliced RNA positively influences initiation of silencing. However, the endogenous β-1,3-glucanase genes that are also cosuppressed contain only one intron sequence; this sequence corresponds to the spliced intron 1 of *gn1* (for review, see Meins et al., 1992). Furthermore, most of the cosuppression instances reported involve transgenes containing cDNA sequences (for review, see Flavell, 1994). Thus, we expect that the unspliced intron 2–containing *gn1* RNA is not involved in silencing activation.

The absence of a significant cytoplasmic steady state level of *gn1* mRNA in the suppressed plant cells suggests that silencing is due to a block of *gn1* mRNA transport from the nucleus to the cytoplasm and/or an increased turnover of transcripts either before or after transport. Transport of mRNA from the nucleus to the cytoplasm involves RNA–protein complexes. Furthermore, transport of different classes of RNA is mediated by specific protein factors. These processes are energy requiring and saturable by increased concentrations of RNA (Dargemont and Kühn, 1992; Jarmolowski et al., 1994). It can be envisaged that *gn1* mRNA accumulating in the nucleus can be the trigger event to block its own transport. Alternatively, a dose-dependent control could be operating similarly to the manner proposed by Dehio and Schell (1994) for the silencing of P35S–*rolB* transgenes in Arabidopsis. Their model presupposes that suppression is activated when a certain mRNA threshold level is reached and titrates an mRNA-stabilizing factor. A cytoplasmic-silencing process has been suggested to explain why certain transgenic tobacco lines expressing either TEV- or potato virus Y–derived sequences show resistance to the cytoplasmically replicating TEV and potato virus Y viruses, respectively (Lindbo et al., 1993; Smith et al., 1994).

Similar control mechanisms could be envisaged for other post-transcriptional cases of gene silencing. However, one has to assume that for each gene, specific interactions with a protein factor(s) would be required for silencing to occur. Such precise control could explain the high degree of specificity that exists for each silencing event, because only specific homologous transgenes and endogenous genes are suppressed.

The recent findings that RNAs might induce and direct sequence-specific de novo methylation of their corresponding genes may have some implications for models of transgene-induced gene silencing in plants (Wassenegger et al., 1994). These authors demonstrated that viroid sequences integrated in the tobacco genome become methylated only after replication of autonomous viroid RNA has taken place. Thus, it has been proposed that sequence-specific de novo methylation of genes might be induced by the encoded RNA.

Any tentative model to explain silencing in plants must address the unresolved contradiction that exists between the specificity of silencing and the general occurrence of silencing with a variety of genes in different plant systems. Future experiments should be designed to determine whether silencing requires other factors besides nucleic acids. It is also crucial to define in which cellular compartment the destruction of the cosuppressed gene transcripts occurs.

METHODS

Transgene Construction and Plant Transformation

A chimeric cauliflower mosaic virus (CaMV) 35S promoter–*gn1* gene contained in a 4.5-kb blunt-ended EcoRI-HindIII DNA fragment was inserted into the blunt-ended XbaI and HindIII sites of the binary vector pGSC1704, a derivative of pGSC1700 (Cornelissen and Vandewiele, 1989) which carries a chimeric P*nos*–*hpt* selectable gene in the polylinker. The resulting plasmid, pDEG2, had the following T-DNA configuration: RB–P35S–*gn1*–P*nos*–*hpt*–LB. RB and LB represent the right and left borders of the T-DNA; *gn1*, the *Nicotiana plumbaginifolia* β-1,3-glucanase transgene; P*nos*, the nopaline synthase promoter; and *hpt*, the hygromycin phosphotransferase gene.

The T-DNA of pDEG2 was introduced into *N. tabacum* cv Petit Havana SR1 by *Agrobacterium tumefaciens*–mediated transformation of leaf discs as described by de Carvalho et al. (1992). Transformed shoots were selected on hygromycin-containing medium (30 mg liter^{-1}) and regenerated as described by De Block et al. (1984).

Plant Growth Conditions

Primary tobacco transformants (R_0) were transferred to greenhouse 1 (25°C, 55 μmol m^{-2} sec^{-1} light intensity, 16-hr-light/8-hr-dark photoperiod), and leaf material was collected from upper leaves of R_0 plants 8 weeks after transfer to soil. The S8 and S9 R_0 plants, which contained the pDEG2 T-DNA (*hpt* marker gene), were crossed with homozygous T17 plants containing the pDEG1 T-DNA carrying the neomycin phosphotransferase II (*nptII*) marker gene (de Carvalho et al., 1992), and progeny seeds were sown in medium containing kanamycin (100 mg liter^{-1}) and hygromycin (30 mg liter^{-1}). Resistant seedlings were transferred to soil (greenhouse 1) and analyzed, as were the R_0 plants.

Analyses of the plants during development were done in greenhouse 1 and greenhouse 2 (25°C, 70 μmol m^{-2} sec^{-1} light intensity, 14-hr-light/10-hr-dark photoperiod). Homogeneous seed stocks of homozygous and hemizygous T17 plants and untransformed SR1 plants were germinated in soil, and leaf material was collected from a pool of five to 10 plants, at intervals of 1 week.

Stress Treatments of Plants

Leaf material was collected from 8- to 10-week-old mature plants before and after the following treatments. For salicylic acid treatment, plant leaves were sprayed with a 5-mM solution. *Pseudomonas syringae* pv *syringae* bacterial infection of the plant leaves was performed essentially as described by Castresana et al. (1990). No visual differences in symptoms between homozygous and hemizygous T17 plants were observed.

β-1,3-Glucanase Enzyme Assays

Direct β-1,3-glucanase assays on isoelectric focusing gels were performed as previously described (de Carvalho et al., 1992). β-1,3-Glucanase activity was determined by hydrolysis of the substrate laminarin. For the assays, crude protein extracts (20 to 50 μg) were used.

cDNA Synthesis and Polymerase Chain Reaction Amplification

RNA was extracted from isolated nuclei of T17 and wild-type plants as described by Roop et al. (1978). One microgram of DNA-free nuclear RNAs was used in a first-strand cDNA synthesis reaction primed with 0.5 μg of oligo(dT). Reverse transcription was performed by using the Superscript preamplification system kit (Bethesda Research Laboratories). A parallel reaction without RNA was performed and served as a negative control in the next polymerase chain reactions (PCRs) using a Biomed 100 Thermocycler (B. Braun). PCRs were performed in a total volume of 100 μL consisting of synthesis buffer (Bethesda Research Laboratories), 100 μM deoxynucleotide triphosphates, 30 pmol of each primer, and 2 U of Taq polymerase (Boehringer Mannheim). PCR mixtures contained primers to amplify *gn1* and actin cDNA products simultaneously. Specific primers for the CaMV 35S leader (Fc1, 5′-TGAAATCACCAGTCTCTCTCT-3′) and for the *gn1* exon 3 (Fc4, 5′-GAAGAAATCGCATTCCGAATGTTCCT-3′) were used to amplify the chimeric *gn1* RNA. For the amplification of actin RNA, primers corresponding to internal sequences of a tobacco actin cDNA were used (R. van Aarssen and M. Cornelissen, unpublished results). Aliquots (18 μL) were collected after 8, 14, 20, 26, and 32 cycles of 94°C for 1 min, 66°C for 2 min, and 72°C for 3 min. Reverse transcriptase–PCR (RT-PCR) products were then separated on agarose gels as described in the following section.

DNA Isolation and Analysis

Total DNA was prepared from leaves using the method described by Dellaporta et al. (1983). Plant DNA samples were digested with restriction enzymes, electrophoresed in a 0.8% agarose gel, and blotted according to Sambrook et al. (1989). RT-PCR products were electrophoresed in a 1.3% agarose gel and blotted according to Sambrook et al. (1989).

Radioactively labeled probes were generated by the random primed method (Boehringer Mannheim). The probes used for detection of the *gn1*, actin, and *hpt* gene sequences and for the *gn1* intron 2 sequence were a 1-kb SstI-PstI DNA fragment corresponding to part of the *gn1* cDNA (De Loose et al., 1988), a 1-kb BamHI-PstI DNA fragment isolated from the plasmid pRVA12 (R. van Aarssen and M. Cornelissen, unpublished results) containing cDNA sequences of a tobacco actin gene, a 0.8-kb BamHI-SacII fragment isolated from the plasmid pGV1503 containing the coding sequences of the *hpt* gene (Ingelbrecht et al., 1989), and a 0.26-kb EcoRI-HindIII DNA fragment corresponding to the *gn1* intron 2 sequences, respectively. Blots were hybridized for 24 hr at 65°C in 3 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.25% nonfat milk powder, 0.5% SDS, 20 μg mL^{-1} denatured sheared herring sperm DNA. Filters were washed twice in 3 × SSC, 0.1% SDS and twice in 1 × SSC, 0.1% SDS at 65°C.

RNA Isolation and Analysis

RNA was extracted from leaf, root, and floral tissues as described by Jones et al. (1985). Total RNA (6 μg per lane) was electrophoresed in a 1.5% agarose–formaldehyde gel according to Sambrook et al. (1989). To verify RNA quality and quantity, ethidium bromide (0.25 μg per RNA sample) was added to samples before denaturing at 65°C. After electrophoresis, gels were photographed before blotting. A plasmid containing the *gn1* cDNA sequences cloned in the pGEM-2 vector (Promega) was used to prepare *gn1* riboprobes by using the Boehringer Mannheim transcription kit. Riboprobes to detect tobacco class I β-1,3-glucanase transcripts were generated from a pGEM-2 derivative containing the 3.5-kb HindIII DNA fragment of the class I β-1,3-glucanase gene *gn2* (*N. plumbaginifolia*) (Gheysen et al., 1990).

Blots were hybridized overnight at 65°C in 50% formamide, 3 × SSC, 0.25% nonfat milk powder, 0.5% SDS, and 20 μg mL^{-1} denatured sheared herring sperm DNA. Filters were washed twice in 3 × SSC, 0.1% SDS at 68°C, and twice in 1 × SSC, 0.1% SDS at 68°C.

Isolation of Nuclei and Nuclear Runoff Transcription Assays

Nuclei isolation, reactions to label nascent RNA chains, and isolation of labeled RNA transcripts were conducted essentially as described by Ingelbrecht and de Carvalho (1992), except that the labeled RNAs were purified by Bio-Spin column centrifugation (Bio-Rad) rather than by trichloroacetic acid precipitation. Slot blotting of linearized plasmids (100 ng per slot) and of single-stranded in vitro–generated RNAs (300 ng per slot) was conducted as described by Sambrook et al. (1989); hybridization was with equal amounts of labeled transcripts from wild-type tobacco and homozygous and hemizygous T17 plants.

The linearized plasmids contained the *gn1* cDNA, 5′ half of the *gn1* cDNA, 3′ half of the *gn1* cDNA, the *N. plumbaginifolia gn2* class I β-1,3-glucanase gene, and the *nptII* gene sequences, which were all cloned in the pGEM-2 vector. A genomic clone of the small subunit of tobacco ribulose-1,5-bisphosphate carboxylase (NtSS23; Mazur and Chui, 1985) cloned in the same vector was used as a positive control. The vector pGEM-2 was included as a negative control.

In vitro–generated sense and antisense RNA transcripts corresponding to *gn1*, *NtSS23*, and *nptII* sequences were prepared using the Boehringer Mannheim transcription kit and subsequently purified by Bio-Spin column centrifugation before slot blotting.

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