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Posttranscriptional gene silencing of *gn1* in tobacco triggers accumulation of truncated *gn1*-derived RNA species

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ABSTRACT

Posttranscriptional silencing of basic β-1,3-glucanase genes in the tobacco line T17 is manifested by reduced transcript levels of the *gn1* transgene and homologous, endogenous basic β-1,3-glucanase genes. An RNA ligation-mediated rapid amplification of cDNA ends (RLM-RACE) technique was used to compare the 3′ termini of *gn1* RNAs present in expressing (hemizygous and young homozygous) and silenced (mature homozygous) T17 plants. Full-length, polyadenylated *gn1* transcripts primarily accumulated in expressing plants, whereas in silenced T17 plants, mainly 3′-truncated, nonpolyadenylated *gn1* RNAs were detected. The relative abundance of these 3′-truncated *gn1* RNA species gradually increased during the establishment of silencing in homozygous T17 plants. Similar 3′-truncated, nonpolyadenylated *gn1* RNA products were observed in an independent case of β-1,3-glucanase posttranscriptional gene silencing. This suggests that these 3′-truncated *gn1* RNAs are a general feature of tobacco plants showing posttranscriptional silencing of the *gn1* transgene.

Keywords: β-1,3-glucanase; cosuppression; polyadenylation; PTGS; RLM-RACE

INTRODUCTION

Gene silencing is a homology-based phenomenon that is frequently observed in transgenic plants containing multiple copies of a particular transgene. Gene silencing may be limited to the transgenes, or may affect both transgenes and homologous endogenous genes. The mechanisms underlying gene silencing are not yet well understood. However, it is clear that in some cases the reduced level of specific mRNAs is a result of promoter inactivation, whereas in other cases it results from posttranscriptional mRNA degradation. Transcriptional gene silencing due to promoter inactivation is usually observed if the sequence homology is in the promoter region, whereas posttranscriptional mRNA turnover is typically associated with homology in the transcribed region (for reviews, see Baulcombe & English, 1996; Meyer & Saedler, 1996; Depicker & Van Montagu, 1997; Stam et al., 1997; Vaucheret et al., 1998).

One of the hallmarks of posttranscriptional gene silencing (PTGS) is its sequence specificity. All models explaining PTGS propose a leading role for antisense RNA to account for this feature (Dougherty & Parks, 1995; Baulcombe & English, 1996; Depicker & Van Montagu, 1997; Stam et al., 1997). The antisense RNA would base pair with the complementary mRNA, thus generating a substrate for RNA degradation by dsRNA-dependent RNases. However, despite a number of efforts to test this model (van Blokland et al., 1994; de Carvalho Niebel et al., 1995; Kunz et al., 1996), direct evidence for the involvement of antisense RNA in PTGS has not been reported so far.

Another common theme in most current models is the involvement of "aberrant" RNA. This RNA would in some way be derived from a silenced transgene or from a homologous endogenous gene and would be different from the expected full-length mRNA and might or might not be present in nonsilenced plants (Metzlaff et al., 1997). This aberrant RNA could originate from various cellular processes (premature termination of transcription, read-through transcription, abnormal processing, or degradation of the messenger), and could serve as a template for antisense RNA production by a plant-encoded RNA-dependent RNA polymerase

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(RdRp; Lindbo et al., 1993; Schiebel et al., 1993, 1998; Wassenegger & Pélissier, 1998; Cogoni & Macino, 1999). As proposed by Lindbo et al. (1993), this antisense RNA would then base pair with the complementary mRNAs and this interaction would result in RNA degradation.

Silencing-related low-molecular-weight RNAs have been observed in several cases of posttranscriptional gene silencing (Smith et al., 1990; Goodwin et al., 1996; Jacobs et al., 1997; Lee et al., 1997; Metzlaff et al., 1997; Tanzer et al., 1997; van Eldik et al., 1998). Metzlaff et al. (1997) proposed that a degradation product from the 3′ end of the endogenous chalcone synthase gene (*chsA*) is involved in posttranscriptional silencing of the chalcone synthase genes. Independently, English et al. (1996) and Sijen et al. (1996) have observed that the 3′ end of the mRNA is the predominant target for the silencing-related degradation mechanism. Moreover, it has been observed in several cases that the 5′ ends of the mRNAs from genes that are posttranscriptionally silenced are more stable than the 3′ ends of these mRNAs (Lee et al., 1997; Tanzer et al., 1997). Taken together, these observations point to a prominent role for the 3′ end of the genes and/or mRNAs in posttranscriptional gene silencing.

We are studying silencing of a basic β-1,3-glucanase (*gn1*) transgene and endogenous homologs in tobacco line T17. Silencing is posttranscriptional (de Carvalho et al., 1992; de Carvalho Niebel et al., 1995) and results, at least partially, from an increased turnover of the *gn1* and the endogenous, basic, β-1,3-glucanase mRNAs (Jacobs et al., 1997).

Our current research focuses on identifying *gn1*-related RNA species that are degradation intermediates or that are involved, directly or indirectly, in the triggering of *gn1* silencing. For this purpose, a PCR-based amplification technique was adopted that is poly (A) tail independent, which allowed us to analyze all different 3′ ends of *gn1* mRNAs. We show that a quantitatively and qualitatively different pattern of *gn1*-derived RNA species correlates with silencing. Furthermore, it is shown that 3′-truncated, nonpolyadenylated *gn1* RNA species are the predominant type in silenced plants of two independent transgenic tobacco lines. The possible origin of these aberrant *gn1* RNA species and their putative role in silencing are discussed.

RESULTS

A changed pattern of *gn1*-derived RNA species coincides with β-1,3-glucanase gene silencing in the transgenic T17 line

In this study, a search was undertaken for aberrant *gn1* RNA species that are associated with *gn1* silencing in homozygous T17 plants. For several posttranscriptionally silenced genes, it has been observed that sequences corresponding to the 3′ end of the encoded mRNAs are more susceptible to silencing (English et al., 1996; Sijen et al., 1996; Metzlaff et al., 1997; Tanzer et al., 1997). We therefore characterized the 3′ ends of the *gn1* mRNAs in silenced versus expressing T17 plants first. To identify the 3′ ends of the *gn1* RNAs, an RNA ligation-mediated rapid amplification of cDNA ends (RLM-RACE) technique was used. This technique allows us to enrich for gene-specific RNAs independent of whether they are polyadenylated. In brief, an arbitrary DNA oligo (oligo 1) was ligated to RNA species with an 3′ OH using T4 RNA ligase, followed by cDNA synthesis with the complementary oligo (oligo 2) and a selective amplification using a gene-specific primer, located in the 3′ region of the coding sequence (oligo 3) and oligo 2 (Liu & Gorovsky, 1993; Fig. 1).

First, we tested whether the RLM-RACE technique was sensitive enough to detect the low level of *gn1* mRNA present in the cells of silenced plants. We previously determined by RNA gel-blot analysis that the *gn1* mRNA level decreases gradually during the establishment of *gn1* silencing in homozygous T17 plants and that the overall reduction in *gn1* mRNA level is about 30–40-fold in mature plants (data not shown). Based on this 30–40-fold difference in *gn1* mRNA level, a reconstruction experiment was carried out. The RLM-RACE technique was applied on RNA from 10-week-old hemizygous plants and a 40-fold dilution of this RNA in total mRNA from a wild-type SR1 tobacco plant. *gn1*-specific sequences were amplified in a PCR reac-

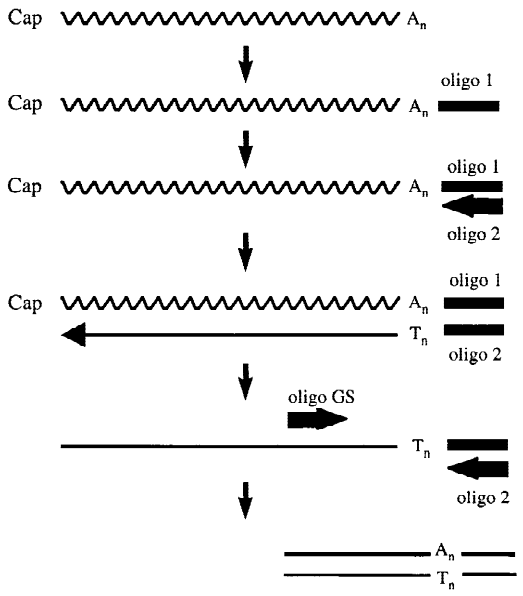

FIGURE 1. Strategy for RLM-RACE of 3′ termini of RNA molecules. Wavy lines represent RNA, straight lines represent DNA, and arrows represent primers. A_n represents a poly(A) tail of n ($n \geq 0$) adenosine residues. Total RNAs are ligated to DNA oligo 1 using T4 RNA ligase. The complementary oligonucleotide (oligo 2) is used to prime cDNA synthesis. Oligo 2 and oligo GS (gene specific) are used in a PCR reaction to amplify the 3′ end of the RNA molecules.

tion with oligo KL28 (see Materials and Methods) and a *gn1*-specific primer located 200 bp upstream of the translation stop codon (oligo KL36; see Materials and Methods). The PCR products were analyzed via DNA gel-blot hybridization using a probe corresponding to the *gn1* cDNA sequence. Both samples showed similar patterns of the *gn1*-specific PCR products (Fig. 2B, lanes 2 and 4). The total amount of radioactivity per lane was quantified by phosphorimaging and was approximately 40-fold less in the 40-fold diluted sample (data not shown). This implies that the technique is sufficiently sensitive to detect low amounts of *gn1* RNA species quantitatively.

Second, the reproducibility of the technique was tested through amplification of cDNAs from a control gene. For this purpose, the RLM-RACE technique was applied on RNA from 10-week-old hemizygous and homozygous T17 plants and untransformed SR1 plants. After cDNA synthesis, the 3′ ends of tobacco actin transcripts were amplified by using an actin-specific oligo (Tob 71; Moniz de Sa & Drouin, 1996) and oligo KL28. Figure 2A shows that for untransformed SR1 plants as well as for the expressing (hemizygous) and silenced (homozygous) T17 plants, a similar pattern of PCR products was obtained. This result shows that the technique reproducibly amplifies a set of RNA species into a distinct pattern of PCR products.

Next, the RLM-RACE technique was applied to investigate whether *gn1* RNA species in silenced T17 plants differ qualitatively and/or quantitatively from those in expressing T17 plants. The same first strand cDNAs used to amplify the actin sequences were used as a template in a PCR reaction in which the 3′ part of *gn1* transcript was amplified using KL36 and KL28. Figure 2B (lanes 2 and 3) shows that the patterns of amplified PCR products differed considerably between the samples obtained from the expressing and silenced T17 plants, and that in the negative control sample

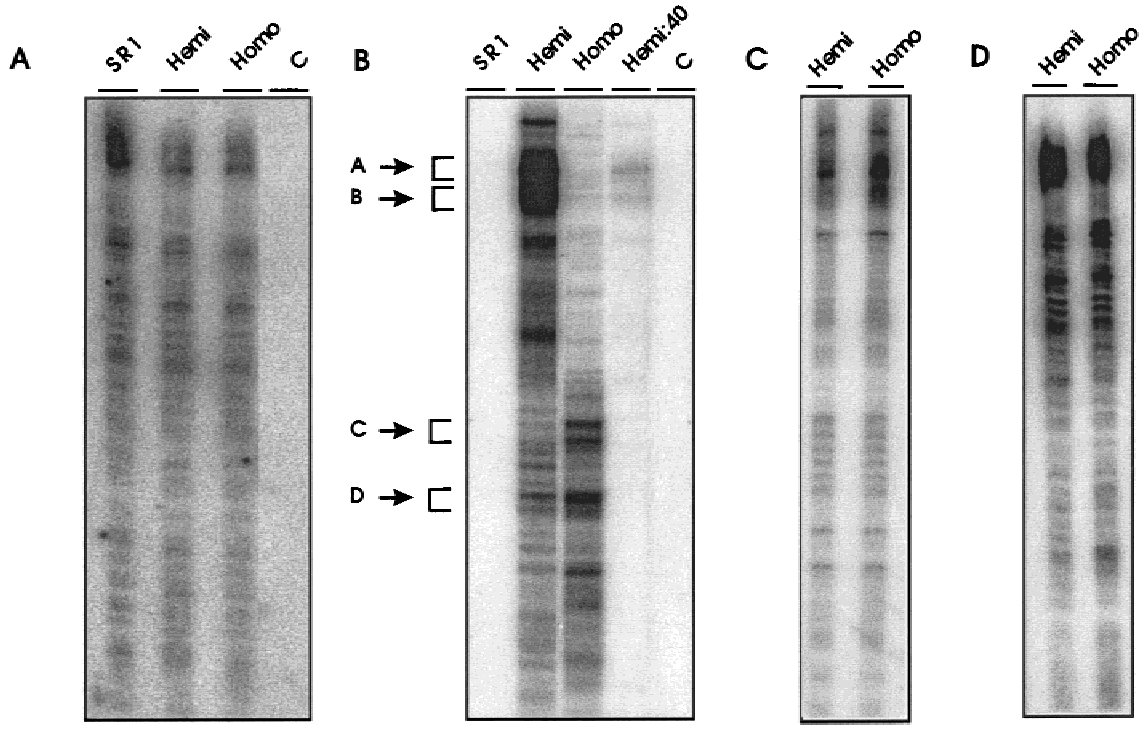

FIGURE 2. DNA gel-blot analysis of the *gn1* RLM-RACE products. **A**: 10 μg of total RNA from wild-type (SR1), hemizygous (Hemi), and silenced homozygous (Homo) T17 plants were ligated to DNA oligo KL29 (oligo 1 in Fig. 1) using T4 RNA ligase. An oligo complementary to KL29 (oligo KL28; oligo 2 in Fig. 1) was used to prime cDNA synthesis. Oligo KL28 and an actin gene-specific oligo were used in a PCR reaction to amplify the 3′ ends of actin mRNAs. As a negative control for the RLM-RACE technique, 10 μg of total RNA from hemizygous T17 plants was ligated to oligo KL29 without prior DNase treatment and in the subsequent RT reaction, reverse transcriptase was omitted (**C**). **B**: The same first-strand cDNAs as in **A** were used as a template in a PCR reaction to amplify the 3′ ends of the *gn1* RNA molecules. In addition, a 40-fold dilution of RNA from hemizygous T17 plants in 10 μg RNA from SR1 tobacco plants (Hemi:40) was ligated to DNA oligo KL29 using T4 RNA ligase. Oligo KL28 was used to prime cDNA synthesis and the cDNA was used as a template in a PCR reaction to amplify the 3′ ends of the *gn1* RNA molecules. Arrows indicate the regions A, B, C, and D. **C**: 10 μg of total RNA from 14-day-old expressing hemizygous (Hemi), and expressing homozygous (Homo) T17 plants were ligated to DNA oligo KL29 using T4 RNA ligase. The complementary oligonucleotide (oligo KL28) was used to prime cDNA synthesis. Oligo KL28 and the gene specific oligo KL36 were used in a PCR reaction to amplify the 3′ ends of the *gn1* RNA molecules. **D**: The same first-strand cDNAs as in **C** were used as template in a PCR reaction with oligo KL28 and an actin-specific primer to amplify the 3′ ends of actin mRNA. PCR products were separated on denaturing polyacrylamide gels, transferred to nylon membrane, and hybridized to an *actin* DNA probe (**A** and **D**) or a *gn1* DNA probe (**B** and **C**).

SR1 no products were generated. In the samples of expressing plants, PCR products of approximately 520 bp and 450 bp in regions A and B, respectively, predominated, whereas the amount of these products was highly reduced in the samples of the silenced plants. In the *gn1* gene, a poly(A) addition site is located 380 bp downstream of the *gn1*-specific oligo KL36 used in the RLM-RACE technique (De Loose et al., 1988). Therefore, PCR products corresponding to full-length *gn1* transcripts lacking a poly(A) tail should be in the size range of 400 bp, as the ligated oligo extends the messenger by 21 nt. This implies that the observed PCR products in the 450- and 520-bp regions (the ligated oligo included) most likely correspond to *gn1* transcripts with a poly(A) tail of about 50 and 120 nt, respectively.

Smaller products of approximately 200 and 260 bp in regions C and D, respectively, predominated in the sample of the silenced T17 plants (Fig. 2B, lane 3). These products were only present in relatively small amounts in the sample of expressing hemizygous plants and in the 40-fold diluted control sample of expressing hemizygous plants (Fig. 2B, lanes 2 and 4). This indicates that the RNAs corresponding to these products are relatively more abundant in samples of silenced plants compared to expressing plants. These smaller PCR products could correspond to RNA species originating from aberrant processing events such as premature 3′ end formation, premature termination of transcription, and cryptic splicing, or they could represent *gn1* mRNA degradation intermediates resulting from endonuclease and/or 3′-to-5′ exonuclease activity.

In addition to the major products in regions A, B, C, and D, a significant number of less abundant PCR products were consistently present in samples of expressing and silenced T17 plants in independent experiments. These products could reflect less abundant members of the normal *gn1* RNA population present in the cell or they could result from inefficient PCR amplification. Furthermore, a number of bands were observed that did not consistently occur in all experiments. This probably reflects variations in the intensity of the *gn1* gene silencing between different experiments, which in turn could be due to slight differences in environmental conditions between experiments. It has been shown before that environmental conditions can affect the outcome of silencing (Napoli et al., 1990; Hart et al., 1992; Flavell et al., 1998). A PCR reaction with an oligo located 205 bp upstream from the first gene-specific primer (oligo KL36) yielded products that were approximately 200 bp larger than the predominant species present in expressing and silenced T17 plants (data not shown). This shows that the RLM-RACE products truly represent *gn1*-derived RNA species and excludes the possibility that the predominant RLM-RACE products are due to technical PCR artefacts.

Taken together these results show that a changed pattern of *gn1*-derived RNA species coincides with β-1,3-glucanase gene silencing in T17 tobacco.

The changed pattern of *gn1* RNA species correlates with the establishment of silencing in homozygous T17 plants

The observation that there is a difference in the RLM-RACE product pattern between plants that express the basic β-1,3-glucanase genes and plants that are silenced suggests that this feature is associated with the silencing phenomenon. However, the difference could also be typical for the genotype of the plants, that is, the homozygous versus hemizygous state of the *gn1* transgene. The fact that silencing of β-1,3-glucanase genes in homozygous T17 plants is typical for mature plants and is not observed in young seedlings allowed us to discriminate between these two possibilities.

The 3′ ends of *gn1* RNA species present in 14-day-old hemizygous and homozygous T17 plants, both expressing the β-1,3-glucanases, were amplified using the RLM-RACE method described above. Figure 2C shows that the same pattern of *gn1*-derived PCR products was observed in both samples. Also the pattern of PCR products corresponding to actin transcripts was similar in seedlings of hemizygous and homozygous T17 plants (Fig. 2D).

This implies that the differences observed between expressing and silenced T17 plants do relate to the silencing phenomenon and are not a direct consequence of the difference in genotypes.

We subsequently investigated to what extent the pattern of PCR products, corresponding to the *gn1* RNA species with different 3′ ends, followed the changes observed for *gn1* mRNA levels in homozygous T17 plants during the establishment of silencing. RNA gel-blot analysis previously revealed that the *gn1* mRNA levels decline gradually in developing homozygous T17 plants. The establishment of β-1,3-glucanase gene silencing takes several weeks and the length of this period can vary, which makes it difficult to determine when plants are fully silenced. Although in the current experiment plants were not yet completely silenced at the end of the monitoring period, RNA gel-blot analysis showed that the *gn1* RNA levels gradually declined as the homozygous T17 plants aged (data not shown). The RLM-RACE technique was used to characterize the 3′ ends of the *gn1* RNA species in a selection of samples taken during the period when silencing was being established in homozygous T17 plants. The PCR products were analyzed via DNA gel-blot hybridization using a probe corresponding to the *gn1* cDNA sequence (Fig. 3A). Quantification of the signals by phosphorimaging showed that the total amount of radioactivity per lane gradually decreased with increasing age of the plants (Fig. 3B). The reduction in the total

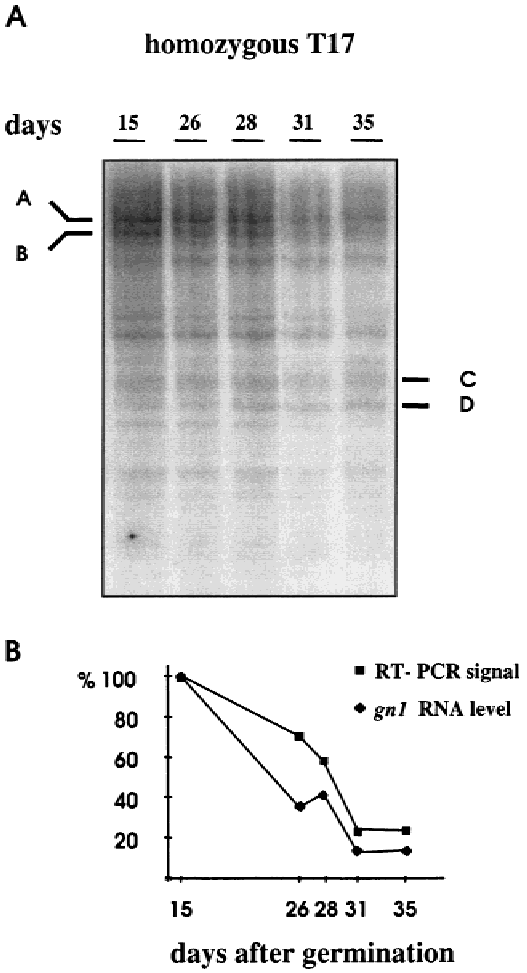

FIGURE 3. Time course analysis of *gn1*-derived RNA species from homozygous T17 plants. **A**: DNA gel-blot analysis of the *gn1* RLM-RACE products at different time points after seed germination. Regions A, B, C, and D are indicated. **B**: Comparison of the total amount of radioactivity of the different samples analyzed both by RNA gel-blot analysis and by RLM-RACE.

amount of signal in PCR products, corresponding to the *gn1* RNA species, was concurrent with the reduction in *gn1* mRNA levels observed by RNA gel-blot analysis. This indicates that the amount of RLM-RACE products faithfully represent the amount of *gn1*-derived RNA species present in the samples. Quantification of the signals corresponding to the individual bands in each lane showed that during the establishment of silencing, the products of regions A and B strongly decreased in abundance, whereas the products of regions C and D remained at a constant level. This indicates that the abundance of the *gn1* RNA species represented by the PCR products from regions A and B decreases during the establishment of silencing, whereas the abundance of the *gn1* RNA species represented by the PCR products from regions C and D remains stable. When expressed as a fraction of all *gn1* RNAs, the abundance of these latter RNA species increases during the establishment of silencing.

The changed pattern of the *gn1*-derived sense RNA species is typical for plants exhibiting PTGS of the *β*-1,3-glucanase genes

To understand whether the silencing-related *gn1* mRNA abundances observed for line T17 are typical for glucanase gene silencing and the *gn1* gene or for the T17 line per se, a second, independently generated transgenic tobacco line exhibiting PTGS of *β*-1,3-glucanase genes was analyzed. This line, S9.1, carries two T-DNA copies inserted at a single locus (de Carvalho Niebel et al., 1995). Similar to the T17 line, homozygous progeny plants of the S9.1 line become silenced during development (data not shown). We applied the RLM-RACE technique on RNA samples isolated from hemizygous and homozygous silenced T17 and S9.1 plants. Figure 4 shows that the patterns of RLM-RACE products in the samples of hemizygous T17 and S9.1 plants are comparable and that similar changes in the patterns are observed in silenced T17 and S9.1 plants. Thus, the changes in the patterns of the RLM-RACE products, which correspond to gn1 RNA species with different 3′ ends, appear to be common to two independent cases of posttranscriptional *β*-1,3-glucanase gene silencing. This suggests that the changes in the pattern reflect a general feature of posttranscriptional silencing of the *β*-1,3-glucanase genes.

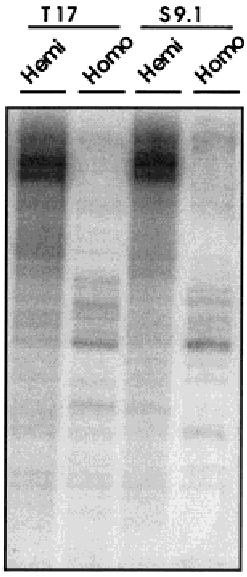

FIGURE 4. DNA gel-blot analysis of the *gn1* RLM-RACE products in two independent transgenic tobacco lines (T17 and S9.1). 10 *μ*g of total RNA from 10-week-old hemizygous (Hemi), and homozygous (Homo) plants from line T17 and S9.1 were ligated to DNA oligo KL29 using T4 RNA ligase. The complementary oligonucleotide (oligo KL28) was used to prime cDNA synthesis. Oligo KL28 and the gene-specific oligo KL36 were used in a PCR reaction to amplify the 3′ ends of the *gn1* RNA molecules. PCR products were separated on a denaturing polyacrylamide gel, transferred to nylon membrane, and hybridized to a *gn1* DNA probe.

Characterization of the 3′ ends of the *gn1* RNA species in T17 plants

To understand the biological significance of the silencing-related changes in the abundance of specific *gn1* RNA species, the predominant RLM-RACE products of four different regions were cloned. The products from regions A and B (450–520 nt) were selected for cloning because they represent RNA species that are the most predominant in plants expressing the β-1,3-glucanase genes and occur at highly reduced levels in silenced T17 plants (Fig. 2). In addition, the products from regions C and D (200–260 nt) were selected for cloning because they represent RNA species that occur at a high relative abundance in silenced T17 plants and constitute a minor fraction of the *gn1* RNAs in expressing T17 plants (Fig. 2). The products from the four regions were eluted from gel, reamplified in a PCR reaction using the same set of oligos, and cloned.

For the RLM-RACE products from hemizygous T17 samples, clones were obtained from all four regions of hemizygous plants. In contrast, for the RLM-RACE products from homozygous T17 samples, clones were obtained from regions C and D, and not from regions A and B, probably due to the very low abundance of the latter products.

Sequence analysis of 47 RLM-RACE clones from regions A and B revealed that *gn1* has at least 16 different 3′ end formation sites scattered over a region of 220 nt (Fig. 5A). The 3′ termini of 44 clones were followed by stretches of A residues and the length of the stretch ranged from 4–49 nt. The observation that in 17 out of 47 clones, the poly(A) stretch starts at nucleotide position 3867 in the *gn1* sequence (numbering according to sequence M38281 of the European Molecular Biology Laboratory (EMBL)/GenBank databases), suggests that this site is the preferred poly(A) addition site for the *gn1* transcript in *Nicotiana tabacum.* In the following sections this species will be referred to as the full-length *gn1* messenger. Interestingly, 2 of the 47 clones correspond to *gn1* RNA species that contain a poly(A) tail at position 3703, which is 164 nt upstream of the most frequently used polyadenylation site of the full-length *gn1* messenger. These clones may represent *gn1* RNA species that reflect a normal 3′ end formation event at that position. Alternatively, these RNA species may be a result of readenylation of truncated RNAs as proposed by Metzlaff et al. (1997).

Sequencing of the 3′ ends of 21 RLM-RACE clones (6 from hemizygous and 15 from homozygous) from region C and D revealed that they correspond to *gn1*

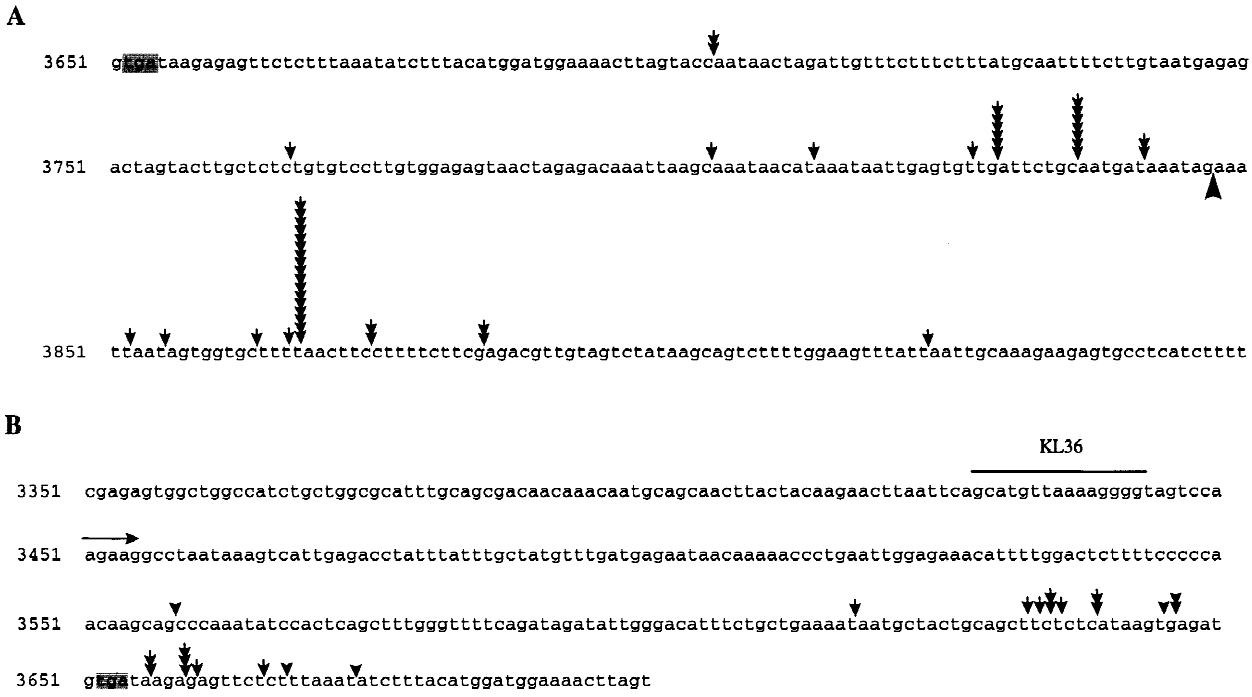

FIGURE 5. Characterization of the 3′ ends of the *gn1* transcripts in hemizygous and homozygous T17 plants. **A**: Mapping of the 3′ ends of the RLM-RACE products from regions A and B from hemizygous T17 plants. Black arrows mark the polyadenylation sites as identified by RLM-RACE product cloning; multiple arrows represent the number of independent clones terminating at this site. The stop codon is marked by a shaded box. ▲ indicates the polyadenylation site from the *gn1* gene as published by De Loose et al. (1988). Numbering is according to sequence M38281 of the EMBL/Genbank databases. **B**: Mapping of the 3′ ends of the RLM-RACE products from regions C and D from hemizygous and suppressed homozygous T17 plants. Black arrows (homozygous)and arrow heads (hemizygous) mark the termination site as identified by RLM-RACE product cloning; multiple arrows represent the number of independent clones terminating at the site. The stop codon is marked by a shaded box. The sequence corresponding to the *gn1*-specific primer KL36 is indicated.

RNA species that are truncated to various positions at the 3′ end (Fig. 5B). These species lack a poly(A) tail and terminate in a region near the stop codon. The mixed distribution of 3′ end points for RNAs originating from expressing and silenced samples and the lack of a poly(A) tail in all cases indicates that similar 3′-truncated *gn1* RNAs are present in expressing hemizygous and silenced homozygous T17 plants. It is possible that the 3′-truncated *gn1* RNAs are generated through a premature termination of transcription or through endonuclease and/or exonuclease degradation at the 3′ end.

In conclusion, sequence analysis of the RLM-RACE products shows that *gn1* RNA species that are more abundant in hemizygous expressing T17 plants are polyadenylated *gn1* RNA species and that multiple poly(A) addition sites are used. On the other hand, *gn1* RNA species that are relatively more abundant in silenced homozygous T17 plants terminate at sequences near the stop codon and lack a poly(A) tail.

DISCUSSION

In this study, the 3′ ends of *gn1* RNA species present in two independent tobacco lines (T17 and S9.1) exhibiting posttranscriptional β-1,3-glucanase gene silencing were characterized using an RLM-RACE technique (Liu & Gorovsky, 1993). In *gn1*-expressing plants of both independent transgenic lines, similar full-length, polyadenylated *gn1* RNAs predominate. In contrast, in silenced plants of both lines, full-length, polyadenylated *gn1* RNAs are barely detectable, whereas similar 3′-truncated *gn1* RNAs that terminate at sites near the translation stop codon and are devoid of a poly(A) tail predominate. Furthermore, it was shown that the relative abundance of the 3′-truncated *gn1* RNA species in homozygous T17 plants gradually increases during the establishment of *gn1* silencing in these plants.

Characterization of the cDNA clones obtained from the RLM-RACE products of silenced and expressing T17 plants revealed several features of *gn1* transgene expression and silencing. Sequencing of the 47 clones from the RLM-RACE products from the regions that are most abundant in expressing T17 plants (regions A and B) showed that the predominant *gn1* mRNA species in hemizygous T17 plants are polyadenylated and that at least 16 different polyadenylation sites are used, one of which appears to be used preferentially. This is not unique, as different poly(A) addition sites have also been observed in *chsA* mRNA transcripts from petunia and in *cab* and *rbcS* mRNA transcripts from petunia and tobacco (Dean et al., 1986; Koes et al., 1986; Klahre et al., 1995; Rothnie, 1996). None of the currently identified sites for the polyadenylation of the *gn1* mRNAs in *N. tabacum* corresponds to the previously identified site for polyadenylation in *Nicotiana plumbaginifolia* (De Loose et al., 1988). Possibly, *N. tabacum* uses different poly(A) addition sites than *N. plumbaginifolia,* the species from which the *gn1* β-1,3-glucanase gene was originally isolated. This assumption is consistent with the observation that wheat *rbcS* genes showed a deviant behavior in relation to polyadenylation when expressed in tobacco cells (Keith & Chua, 1986).

The most striking observation was that truncated, nonpolyadenylated *gn1* RNAs terminating at positions around the translation stop codon (regions C and D) are relatively abundant in silenced T17 plants whereas they constitute a minor fraction in expressing T17 plants (hemizygous and young homozygous T17 plants). In several cases of posttranscriptional gene silencing, small, polyadenylated and nonpolyadenylated RNAs corresponding to proximal or distal parts of the mature mRNA have been identified (Smith et al., 1990; Goodwin et al., 1996; Lee et al., 1997; Metzlaff et al. 1997; Tanzer et al., 1997). In most cases it was proposed that these RNAs originate from cytoplasmic degradation of the mature mRNA. In only one case was the complete sequence of such truncated species determined (Metzlaff et al. 1997). Several scenarios may explain the presence of the 3′-truncated *gn1* RNAs terminating at various positions near the translation stop codon that were identified in this study. They could be produced as alternative cleavage intermediates of the 3′ end formation and polyadenylation machinery. Consistent with this is the presence of a putative polyadenylation site (AAUAAU) 10–40 nt upstream of the 3′ termini of the clones from regions C and D. However, as none of the clones contained a poly(A) tail, one would have to assume that poly(A) addition was unsuccessful after cleavage at these sites.

Alternatively, the truncated *gn1* RNAs could be the result of a (silencing-related) premature termination of transcription. In *Ascobolus immersus*, the duplication of the 3′ part of the *met2* gene resulted in methylation of the corresponding region of the endogenous *met2* gene and in the concomitant production of truncated *met2* transcripts (Barry et al., 1993). Similarly, for several posttranscriptionally silenced genes, it was shown that the 3′ region of the mRNA molecules is a target of PTGS. Importantly, also in these cases, the corresponding regions of the genes were C-methylated (English et al., 1996; Sijen et al., 1996).

A third possibility is that the 3′-truncated *gn1* RNA species are silencing-related *gn1* RNA degradation intermediates. The 3′-truncated *gn1* RNA species could be generated through 3′-to-5′ exonuclease or endonuclease activity, or a combination of both. Pausing of the 3′-to-5′ exonuclease enzyme because of inhibitory secondary structures within the RNA molecule or because of interference by pausing ribosomes (Bjornsson & Isaksson, 1996) could explain why the 3′ ends of the cloned *gn1* RNA species all map within a 116-nt region around the stop codon of the *gn1* transcript. Alternatively, this 116-nt region could contain a hot spot(s) for

endonuclease activity. This might be the case, for example, if during translation, ribosomes would pause near the stop codon, leaving *gn1* RNA sequences downstream of the ribosomes unprotected (Wolin & Walter, 1988).

Importantly, the 3′-truncated, nonpolyadenylated *gn1* RNA species are also present in hemizygous T17 plants, albeit at lower levels than in homozygous, silenced T17 plants. This implies that the RNA degradation pathway generating these 3′-truncated *gn1* RNAs is also active in *gn1*-expressing T17 plants, and possibly also in nontransgenic tobacco plants. The higher relative abundance of these species in silenced T17 plants should then be attributed to a higher activity of this *gn1* RNA degrading pathway. This would agree with the observed eightfold reduced half-life of *gn1* mRNAs in silenced T17 plants (Jacobs et al., 1997). An increased rate of turnover of glucanase mRNA in posttranscriptional gene silencing has also been suggested by Holtorf et al. (1999). Similarly, Metzlaff et al. (1997) showed that the introduction of a *chsA* gene in petunia plants upregulates an already operating *chsA* specific posttranscriptional control mechanism.

Importantly, independently generated transgenic *gn1* lines (T17 and S9.1) show similar patterns of *gn1* RLM-PCR products. Yet, for the T17 line independent comparisons show some qualitative differences (see Figs. 2, 3, and 4). This variation was so far only observed for silenced plants grown in different greenhouses, indicating that environmental factors somehow affect the accumulation of specific silencing-related mRNAs.

With the RLM-RACE method employed here, it was not possible to identify the 5′ ends of the 3′-truncated *gn1* RNA species. However, using a conventional 5′ RACE approach, it was shown that polyadenylated *gn1* RNAs truncated at their 5′ ends are present in silenced T17 plants (van Eldik et al., 1998). Therefore, it appears that the above described degrading activities at the 3′ end of the truncated *gn1* RNAs concur with the action of endo- and/or exonucleases in the proximal region of *gn1* RNAs.

MATERIALS AND METHODS

Plant growth conditions

Homogeneous seed stocks of homozygous and hemizygous T17 and S9.1 plants and untransformed SR1 plants were germinated in soil. Plants were grown in different greenhouses during different seasons of the year. Samples were taken at 2 and 10 weeks after germination.

RNA ligation-mediated rapid amplification of cDNA ends (RLM-RACE)

An arbitrary deoxyribonucleotide oligo (20-mer) 5′-ATCTCA GAGGCTGCTCATCAC-3′ (KL29) was synthesized and kinated using polynucleotide kinase (New England Biolabs) as described by the manufacturer. To prevent concatenation of the 5′-P, 3′-OH donor molecules (oligo KL29), we have used Terminal Deoxynucleotidyl Transferase (Amersham Life Science) to efficiently add a terminal dideoxyadenosine to the 3′-OH end of the donor oligo.

Prior to ligation, RNA samples were treated with RQ1 DNase (Promega) as described by the manufacturer. Standard ligation assays were carried out according to Liu and Gorovsky (1993). In brief, 80 pmol of the kinated and blocked oligo and 10 μg of total RNA (from T17 and S9.1 hemizygous and homozygous plants and from SR1 plants) were mixed and heated at 90 °C for 2 min. Ligation was done in 100 μL containing 50 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 10% dimethyl sulfoxide (DMSO), 10 μg/mL RNasin (Promega), 240 pmol of adenosine 5′-triphosphate (ATP), and 100 U of ligase at 16 °C for 5 h (Liu & Gorovsky, 1993). Free oligonucleotides were eliminated by centrifugal ultrafiltration (Centricon 30 microconcentrators; Amicon) with five washes using 2 mL of diethyl pyrocarbonate- (Sigma) treated H_2O. For cDNA synthesis, 2.5 μg ligated RNA were primed with oligo KL28 (0.5 μg) that is complementary to the oligo (KL29) (see Fig. 1). First-strand reverse transcription was carried out using Moloney murine leukemia virus H^- reverse transcriptase (Superscript, GIBCO-BRL) at 42 °C according to the manufacturer's instructions. Second-strand synthesis is not necessary for PCR amplification. Excess primers were removed by centrifugal ultrafiltration as described above. Control reactions contained unligated RNA samples and samples where the reverse transcriptase was omitted.

PCR amplification

Two percent (2/100 μL) of the cDNA was used in subsequent PCR amplifications using Taq DNA polymerase and PCR buffer (Boehringer). For the 3′-end amplification, oligo KL28 and an appropriate gene-specific primer corresponding to a sequence near the 3′ end of coding sequence was used (KL36 for the *gn1* transgene and an actin-specific oligo, located at position 1221–1242 [numbering according to sequence U60490 of the EMBL/GenBank databases (Tob 71; Moniz de Sa & Drouin, 1996)]. In the PCR reactions, an initial denaturation step of 5 min at 94 °C was followed by 30 cycles of the PCR reaction (1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C). Control reactions omitted cDNA or one of the two primers.

Equal amounts of the PCR reaction mixture were separated on a 4.75% sequencing gel (Severn Biotech, Ltd.). After electrophoresis, the PCR products were transferred to a nylon membrane (Genescreen^{TM}, DuPont) through capillary transfer and the DNA was subsequently cross-linked in an UV cross-linker (GS gene linker, UV chamber, BioRad) according to the manufacturer's instructions. Membranes were hybridized for 16 h in 500 mM phosphate buffer, pH 7.2, 1 mM ethylene diamine tetraacetic acid (EDTA), 7% sodium dodecyl sulfate (SDS). Membranes were twice washed in 100 mM phosphate buffer, pH 7.2 , 0.5% SDS, 1 mM EDTA and once in 50 mM phosphate buffer, pH 7.2, 0.5% SDS, 1 mM EDTA. Radiolabeled DNA probes were generated by a *Redi*prime random primer labeling kit (Amersham Life Science) using DNA fragments corresponding to the coding region of the actin gene and to the 3′ end of *gn1* as template. The DNA

fragment corresponding to the 3′ end of *gn1* was obtained by a PCR reaction using the *gn1* genomic sequence as template and using KL36 located in exon 3 at position 3434–3455 and primer FC3′ located at position 3891–3913 (numbering according to sequence M38281 of the EMBL/Genbank/DDBJ databases).

Characterization of the 3′ ends of the *gn1* RNA species

The PCR products present in regions A, B, C, and D were cut out and eluted from gel and used as a template in a new PCR reaction with the same oligos (KL36 and KL28) (see above). The PCR fragments were cloned (pGEM-T Easy vector system, Promega) and positive clones were identified by colony hybridization as described by Sambrook et al. (1989) using a DNA probe corresponding to the 3′ end *gn1* cDNA. The nucleotide sequences were determined using automated dideoxy-sequencing systems (A.L.F. DNA Sequencer, Pharmacia; 370A DNA Sequencer, Applied Biosystems).

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