5176–5181 *Nucleic Acids Research, 1998, Vol. 26, No. 22* *© 1998 Oxford University Press*

Silencing of β-1,3-glucanase genes in tobacco correlates with an increased abundance of RNA degradation intermediates

Gerben J. van Eldik, Katia Litière+, John J. M. R. Jacobs[1], Marc Van Montagu* and Marc Cornelissen[1]

Laboratorium voor Genetica, Departement Genetica, Vlaams Interuniversitair Instituut voor Biotechnologie, Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium and [1]Plant Genetic Systems N.V., J. Plateaustraat 22, B-9000 Gent, Belgium

Received July 6, 1998; Revised and Accepted October 6, 1998

ABSTRACT

Post-transcriptional gene silencing of β-1,3 glucanase genes in the transgenic tobacco line T17 is characterised by an increased turnover and, as a consequence, reduced levels of *gn1* transgene and endogenous β-1,3 glucanase mRNAs. Here, additional *gn1* RNAs, both larger and smaller than the full-length messenger, are shown to accumulate in silenced plants of the transgenic tobacco line T17. The longer-than-full-length *gn1* RNAs are the result of cryptic processing of the *gn1* messenger. The small *gn1* RNAs in silenced plants correspond to distal and proximal parts of the mature *gn1* messenger. The proximal RNA products are intact at their 5′ extremity, but terminate at different positions at the 3′-end. The distal RNA products contain a poly(A) tail and are truncated to various positions at the 5′-end. These observations indicate that degradation of the mature *gn1* transcript does not start at the 5′- or 3′-end, but rather are consistent with degradation of the *gn1* transcript starting with an endonucleolytic cleavage followed by internal exonuclease digestion. Importantly, the truncated products are more abundant in silenced plants than in expressing plants. This suggests, together with the previously reported silencing-related increased *gn1* mRNA turnover and the similar rates of *gn1* transcription in silenced and expressing T17 plants, that the predominant decay route for the *gn1* transcripts differs between silenced and expressing conditions.

INTRODUCTION

Genetic transformation of plants may lead to a coordinated suppression of the expression of the introduced transgenes and homologous endogenous genes. In several cases, the transcription of the affected genes appears normal, indicating that a post-transcriptional mechanism causes the reduction of the mRNA levels of the affected genes, a phenomenon called post-transcriptional gene silencing (PTGS) (1–4). The mechanism(s) underlying PTGS must either affect the processing, the nucleocytoplasmic transport or the stability of the messengers of the affected genes, or a combination of these processes. In the case of silenced *chsA* genes it has been proposed that the reduction in the transcript levels results from an increased rate of mRNA turnover (5,6). For PTGS of β-1,3-glucanase genes it was shown that the mRNA half-life was 8-fold shorter (7). Moreover, it is believed that the silencing-related RNA turnover occurs in the cytoplasm (8,9). This assumption is mainly based on the observation that transgenic plants exhibiting PTGS are also resistant to infection with cytoplasmically replicating RNA viruses that contain sequences with homology to the silenced genes (9–12). In addition, it was shown that silencing-related, low molecular weight RNAs are present in the polysomal fraction of plants exhibiting PTGS of the tobacco etch virus (TEV) coat protein gene and of ACC synthase genes (13,14).

It is becoming increasingly clear that regulation of mRNA turnover plays an important role in maintaining appropriate levels of specific cellular mRNAs and in eliminating unproductive RNAs (15–17). mRNA degradation is reported to proceed extremely rapidly once it is initiated. In higher plants, only a few cases of degradation intermediates have been characterised (18–21). For example, the degradation of the soybean *rbcS SRS4* messenger generates a distinct pattern of proximal (5′ intact) and distal (3′ intact) RNA degradation products. The presence of a 5′ cap and a 3′ poly(A) tail on the proximal and distal products, respectively, is taken as evidence that the first step in the major degradation pathway for *SRS4* mRNAs is an endonucleolytic cleavage (19).

Silencing-related RNA degradation intermediates, i.e. discrete low molecular weight RNA products corresponding to less-than-full-length messengers, have been described for tomato plants exhibiting co-suppression of polygalacturonase genes or ACC synthase genes, and in tobacco plants displaying homology dependent resistance to TEV (13,14,22,23). In the latter case, it was shown that the different products correspond to either the

*To whom correspondence should be addressed. Tel: +32 9 2645170; Fax: +32 9 2645349; Email: mamon@gengenp.rug.ac.be

+Present address: Wellcome/CRC Institute, Tennis Court Road, Cambridge CB2 1QR, UK

Downloaded from nar.oxfordjournals.org at Biomedische Bibliotheek on September 20, 2011

5′-end, the middle part or the 3′-end of the TEV RNA (22). In contrast, in tomato plants displaying PTGS of ACC synthase genes, only the 5′-end of the ACC synthase mRNA was found, suggesting that it was much more stable than the 3′ part (13). Metzlaff *et al*. (5) showed that truncated, poly(A)$^-$ *chsA* RNAs and small RNAs of 304 nucleotides, corresponding to a 3′ segment of *chsA* RNA, are present in petunia plants exhibiting PTGS of *chsA* genes. Importantly, identical RNAs were also identified in a petunia variety exhibiting natural PTGS of *chsA* genes (5). It was proposed that these truncated RNAs are generated through specific endonucleolytic cleavages in the endogenous *chsA* mRNA.

We previously demonstrated that the reduction in β-1,3-glucanase mRNA levels in homozygous, silenced T17 *Nicotiana tabacum* plants is at least partially due to an increased turnover of the β-1,3-glucanase transcripts (7). Here we show that cryptic, longer-than-full-length and truncated *gn1* mRNAs are present in silenced T17 plants. The longer-than-full-length *gn1* RNAs do not contain intron sequences or surrounding genomic sequences of the *gn1* transgene. The truncated *gn1* RNAs correspond to proximal and distal parts of the *gn1* mRNA. In both expressing and silenced T17 plants the proximal RNA products have two discrete 5′-termini and extend up to a range of positions at the 3′-end; the distal RNA products have a poly(A) tail and terminate at different sites at the 5′-end. Both types of RNA products show no sequence abnormalities. The fact that such truncated RNAs are much more abundant in silenced as compared with expressing T17 plants indicates that they are probably produced due to the enhanced activity of a silencing-related *gn1* mRNA decay mechanism, and that ‘normal’ degradation of *gn1* transcripts in non-silenced plants predominantly proceeds along a different pathway.

MATERIALS AND METHODS

Plant growth conditions

Homozygous, silenced and hemizygous, expressing T17 plants (transgenic *N.tabacum* cv SR1) (24) and wild-type *N.tabacum* SR1 plants were germinated in soil and grown in a greenhouse (25°C, 70 µmol/m^2/s light intensity, 14 h light/10 h dark period).

RNA isolation and analysis

RNA was isolated from leaf tissue as described by Jones *et al*. (25). Poly(A)$^+$ and poly(A)$^-$ RNA was isolated with the Oligotex mRNA mini kit (Qiagen Hilden, Germany). Northern blot analysis was performed with total RNA (10 µg for silenced T17 plants and 2.5 µg for expressing T17 plants), poly(A)$^-$ RNA (10 µg) and poly(A)$^+$ RNA (0.15 µg). The RNA concentration was determined spectrophotometrically. RNA was separated in a 1.5% agarose–formaldehyde gel according to Sambrook *et al*. (26). After electrophoresis, the integrity of the RNA was checked by ethidiumbromide staining, and the gels were blotted onto Genescreen membranes (NEN Research Products, Dupont Wilmington, DE) by capillary transfer in 10× SSC and the RNA was subsequently crosslinked in a UV crosslinker (GS gene linker, UV chamber, Biorad Hercules, CA) according to the manufacturer’s instructions. Radiolabelled DNA probes were generated by the *Redi*prime DNA labelling system (Amersham, UK) using DNA fragments corresponding to the *gn1* cDNA, the 35S promoter, intron 1 and 2 and the 3′-end of *gn1* genomic sequence as template. Membranes were hybridised for 16 h at 65°C in 500 mM phosphate buffer, pH 7.2, 1 mM EDTA, 7% SDS and 1% BSA. Membranes were washed three times in 100 mM phosphate buffer, pH 7.2, 1 mM EDTA and 1% SDS at 65°C. Signals were visualised with a PhosphorImager (Molecular Dynamics Sunnyvale, CA) using ImageQuant software.

Cloning and characterisation of gn1 RNA species

The Marathon cDNA Amplification kit from Clontech (Palo Alto, CA) was used to characterise the 5′-ends of the RNAs from region 3. Briefly, poly (A)$^+$ RNA was isolated from silenced T17 plants. First strand cDNA was primed with a modified lock-docking oligo-dT primer. Second strand synthesis was performed according to the method of Gubler and Hoffmann (27) with a cocktail of *Escherichia coli* DNA polymerase I, RNase H and *E.coli* DNA ligase. After making the ends blunt with T4 DNA polymerase, the Marathon cDNA adaptor was ligated to the double-stranded cDNA with T4 DNA ligase. After adaptor ligation, the *gn1*-specific sequences were amplified by PCR using the AP1 primer complementary to the ligated adaptor and the *gn1*-specific primer GE5 corresponding to nucleotides 3544–3566 (numbering according to sequence M38281 of the EMBL database).

The 3′-ends of the proximal *gn1* RNA species were characterised by the RNA ligation-mediated rapid amplification of cDNA ends (RLM-RACE) method according to Liu and Gorovsky (28). In short, total RNA was isolated from expressing and silenced T17 plants and an arbitrary DNA primer (KL29) was ligated to the 3′-OH end of the RNAs with T4 RNA ligase. Subsequently, first strand cDNA synthesis was primed with primer KL28, which is complementary to KL29. The 3′-ends of the proximal product were amplified by PCR using the first strand cDNA as a template with KL28 and a *gn1*-specific primer GE8, located in exon 2 at position 2593–2617 (numbering according to sequence M38281 of the EMBL database).

PCR fragments were cloned (pGEM-T Easy vector system; Promega Madison, WI) and positive clones were identified by colony hybridisation as described by Sambrook *et al*. (26) using a DNA probe corresponding to the 3′ part (distal fragments) or the 5′ part (proximal fragments) of the *gn1* cDNA. Nucleotide sequences were determined using automated dideoxy-sequencing systems (A.L.F. DNA Sequencer, Pharmacia Uppsala, Sweden).

Primer extension analysis

Primer extension analysis was performed by using the Primer Extension System-AMV Reverse Transcriptase (Promega). In short, primer GE10 (position 1711–1746, numbering according to sequence M38281 of the EMBL database) was labelled with [γ-^{32}P]ATP, annealed to the total RNA samples (50 µg) at 65°C and primer extension was carried out with AMV reverse transcriptase at 42°C for 30 min. The DNA marker (φX174 DNA digested with *Hinf* I) was generated by end-labelling with [γ-^{33}P]ATP. A sequence ladder was used to determine the exact length of the bands. The samples were separated on a 17 cm 8% polyacrylamide sequencing gel at 250 V for 3 h. Relative band intensities were quantified using a PhosphorImager (Molecular Dynamics) and ImageQuant software.

Downloaded from nar.oxfordjournals.org at Biomedische Bibliotheek on September 20, 2011

RESULTS

Three additional silencing-related *gn1* RNA species are present in silenced T17 plants

To gain an insight into the molecular details of silencing-related turnover of *gn1* mRNA, a search for RNA degradation products related to silencing was initiated. Jacobs *et al.* (7) showed that total RNA of homozygous, silenced T17 plants contains, besides the full-length *gn1* RNA transcript, additional RNAs. These RNAs migrate on northern blots in three different regions and are hardly detectable in samples of hemizygous, expressing T17 plants (Fig. 1B). RNA species in region 1 migrated slower than the full-length *gn1* messenger, whereas RNA species in regions 3 and 4 migrated faster (Fig. 1B). A difference between the relative intensities of the signals in the four regions is regularly observed when RNA preparations from different silenced plants, grown at different times, are used, indicating that the relative abundance of the corresponding RNAs varies. This most probably reflects variations in the intensity of the gene-silencing.

The novel silencing-related *gn1* RNA species result from at least two separate processing events

The *gn1* RNA species from region 1 were ~600 nucleotides longer than the full-length *gn1* messenger and co-purified with the poly(A)$^+$ RNA fraction, indicating that they contain a poly(A) tail (Fig. 1C). We investigated whether additional transgene-derived sequences, besides those present in the mature *gn1* mRNA sequence, are present in these large RNA species. Northern blots of leaf tissue from expressing and silenced T17 plants were hybridised to probes corresponding to the 35S promoter, intron 1 and 2 of the *gn1* gene and *gn1* genomic sequences located downstream of the published 3′-end formation site of the *gn1* mRNA (29). The 35S promoter probe did not hybridise with any of the additional *gn1* RNA species. Surprisingly, two new RNA species were identified with the 35S promoter probe both in expressing and silenced T17 plants, the abundance of which did not appear to be silencing-related (data not shown). Hybridisation with the intron and 3′-gene probes failed to generate a signal (data not shown), suggesting that the large *gn1* RNAs in region 1 do not contain either of the two introns or sequences downstream of the reported polyadenylation site of the *gn1* gene (29). Thus, it appears that the additional 600 nucleotides present in the larger-than-mature *gn1* transcripts originate either from a silencing-related sequence duplication, or from a long-range splicing event.

The RNA species from region 4 were more abundant than those from region 3 in total RNA preparations of silenced plants. Furthermore, RNA species in region 3 were more readily apparent in the poly(A)$^+$ RNA fraction, whereas RNA species in region 4 were only detected in the total RNA and poly(A)$^-$ RNA fractions (Fig. 1C). This indicated that RNA species from region 3 contain a poly(A) tail whereas those of region 4 do not. To investigate to which part of the mature *gn1* mRNA the RNA species of regions 3 and 4 correspond, northern blots containing poly(A)$^+$ and poly(A)$^-$ RNA from silenced T17 plants were probed with the 5′ and the 3′ part of the *gn1* cDNA. Figure 1D shows that both the 5′ and the 3′ probe detected the mature, polyadenylated *gn1* transcripts. In addition, the 5′ probe hybridised to *gn1* RNA species in region 4 that are poly(A)$^-$ and the 3′ probe hybridised to polyadenylated *gn1* RNAs in region 3. This implies that *gn1* RNA species from region 4 correspond to the proximal end of *gn1* messenger and that *gn1* RNA species from region 3 correspond to the polyadenylated, distal end of the *gn1* messenger.

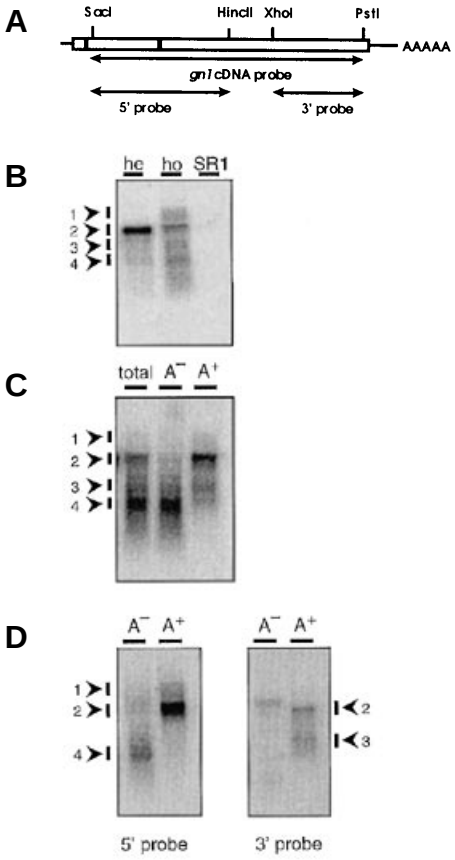

Figure 1. Northern blot analysis of *gn1*-related RNA species. (**A**) Schematic representation of the full-length *gn1* RNA, and the different probes [*gn1* cDNA, 5′ part (5′ probe) and 3′ part (3′ probe) of the *gn1* cDNA] used for hybridisation. (**B**) Total RNA isolated from hemizygous, expressing T17 plants (he), homozygous, silenced T17 plants (ho) and wild-type SR1 plants (SR1). Northern blots were hybridised with a *gn1* cDNA probe. (**C**) Total RNA, poly(A)$^+$ and poly(A)$^-$ RNA isolated from silenced T17 plants. Northern blots were hybridised with a *gn1* cDNA probe. (**D**) Northern blots containing poly(A)$^+$ and poly(A)$^-$ RNA from silenced T17 plants hybridised with probes corresponding to the 5′ region (5′ probe) and the 3′ region (3′ probe) of the *gn1* cDNA sequence, respectively. Arrows indicate the different regions that hybridise to the different probes. total, total RNA; A$^+$, poly(A)$^+$ RNA; A$^-$, poly(A)$^-$ RNA.

Mapping of the 5′-ends of full-length and proximal RNA products

The observation that the RNAs in region 4 appeared as a small smear rather than as a discrete product, suggested that these RNAs were heterogeneous in size. To determine the exact 5′-ends of RNAs containing proximal sequences of the *gn1* transgene, primer extensions were performed with a primer in exon 1 of the *gn1* transgene (Fig. 2). Two primer extension products, 125 and 103 nucleotides, were generated, both in expressing and in silenced T17 plants. In both expressing and silenced T17 plants the product of 125 nucleotides gave a 1.3-fold stronger signal than the product of 103 nucleotides. No extension products were generated with mRNA of SR1 tobacco plants indicating that the two products obtained are the result of a primer extension reaction

Downloaded from nar.oxfordjournals.org at Biomedische Bibliotheek on September 20, 2011

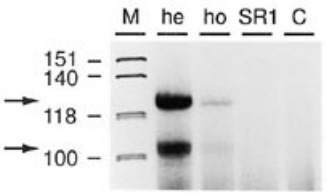

Figure 2. Primer extension analysis of full-length and 3′ truncated *gn1* transcripts. The 5′-ends of the full-length and the proximal RNAs were identified by primer extension. Primer GE10 was end-labelled and annealed to total RNA of hemizygous, expressing (he) and homozygous, silenced (ho) T17 plants and wild-type (SR1) tobacco plants. A sample without RNA was used as a control (C). The primer extension products are indicated by an arrow. The size (nucleotides) of a DNA marker is indicated at the left (M).

on the transgene *gn1* mRNA rather than on an endogenous glucanase mRNA. The product of 125 nucleotides extended exactly up to the expected transcription start site of the *gn1* transgene, indicating that this is also the predominant transcription start site in the transgenic T17 plants. The presence of the same additional product, both in expressing and in silenced T17 plants indicates that the 35S promoter has two transcription start sites in the transgenic T17 tobacco line. The results demonstrate that no extensive degradation of the *gn1* mRNA occurs at the 5′-end, most likely due to the presence of the 5′ cap.

Proximal *gn1* RNA fragments contain truncated 3′-ends

Since the 5′-end appears relatively fixed, the size heterogeneity of proximal (region 4) RNAs must reside in their 3′-ends. To determine whether this is the case, the 3′-ends of proximal *gn1* fragments were identified. For this purpose, the 3′ parts of these RNAs were cloned using an RLM-RACE technique (28,30). In total, 26 clones from RNA of silenced T17 plants and 54 clones from RNA of expressing T17 plants were obtained. None of these clones corresponded to endogenous β-1,3-glucanase transcripts.

For the expressing T17 plants, 80% of the clones (44) corresponded to the full-length *gn1* messenger (Fig. 3). In contrast, no clones corresponding to the full-length *gn1* messenger were obtained from silenced T17 plants. Figure 3 shows that all 26 clones of silenced T17 plants and 10 of 54 expressing T17 plants corresponded to *gn1* RNA species lacking parts of the 3′-end of the *gn1* messenger. For silenced T17 plants, the 3′-ends of the clones were situated between nucleotide 401 (numbering relative to the transcription start site) and nucleotide 616; in expressing T17 plants, the end points ranged from nucleotide 407 to 802. This result is consistent with a progressive degradation of the *gn1* messenger in a 3′ to 5′ direction. Sequence analysis provided no indication for sequence aberrancies in the proximal *gn1* RNAs indicating that their occurrence was not related to unfaithful transcription and that silencing-related RNA degradation is not due to abnormalities in the mRNA sequence (data not shown).

The sequence of four clones obtained from silenced T17 plants and of three clones obtained from expressing T17 plants terminated exactly at the last nucleotide of exon 2 of the *gn1* transcript. Furthermore, two clones were obtained from silenced T17 plants representing *gn1* RNAs that contain intron 2 and at the same time are truncated at the 3′-end. The presence of these two types of clones indicates that splicing of the transgene *gn1* transcript may be not efficient in the transgenic T17 plants.

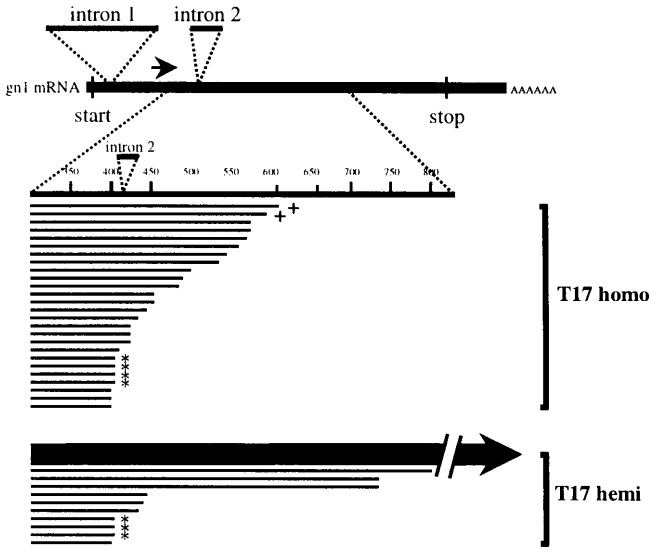

Figure 3. Characterisation of the 3′ truncated *gn1* transcripts in silenced and expressing T17 plants. The 3′-ends of the 3′ truncated *gn1* transcripts were mapped through cloning using the RLM-RACE technique. The *gn1* transcript is schematically represented and the numbers indicate the position in the cDNA sequence. The position of the introns is indicated (introns are not drawn in scale). The two cDNA clones containing intron 2 are indicated by a plus (+), whereas the seven cDNA clones which terminate at the exon 2/intron 2 boundary are indicated by an asterisks (*). The cDNA clones are represented by lines. The thick arrow indicates the 44 cDNA clones representing full-length *gn1* RNA species. The *gn1* gene-specific primer (GE8) used in the procedure is indicated by an arrowhead.

The fact that exclusively 3′ truncated cDNAs were obtained from silenced plants, whereas most clones from expressing plants corresponded to the full-length *gn1* mRNA indicates that the proximal, truncated RNAs are much more abundant in silenced T17 plants than in expressing T17 plants. This is also consistent with the northern blot analysis (Fig. 1B).

Distal *gn1* RNA fragments are truncated at the 5′-end

The truncated *gn1* RNAs from silenced T17 plants in region 3 on northern blot corresponded to a distal region of the *gn1* mRNA and contained a poly(A) tail. To determine whether truncated, polyadenylated *gn1* RNAs contained any sequence aberrancies and where these *gn1* RNA species terminate, poly(A)$^+$ *gn1* RNAs were cloned from silenced plants using the Marathon cDNA Amplification technique (Clontech) and their 5′-termini were determined. Analysis of the 16 clones obtained gave no indication for the occurrence of sequence deviations compared with the published *gn1* sequence (data not shown). Figure 4 reveals that the 16 clones from silenced T17 plants represent *gn1* RNAs that lack increasing parts of the 5′-end of the *gn1* mRNA. The longest clone terminates at nucleotide 701 and the shortest clone at nucleotide 1013 (numbering relative to the predicted transcription start site). Thus, the 5′-termini are scattered over a range of 300 nucleotides. Consistent with this observation, primer extension analyses did not yield any discrete extension products (data not shown). These results are in agreement with the idea that the *gn1*-derived distal RNAs are the result of on-going degradation by 5′ to 3′ exonucleases.

Downloaded from nar.oxfordjournals.org at Biomedische Bibliotheek on September 20, 2011

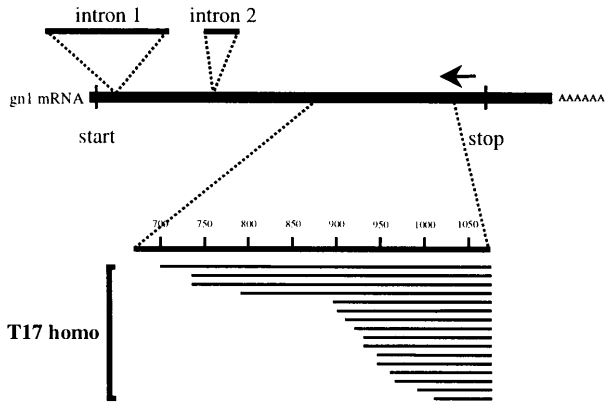

Figure 4. Characterisation of the 5′ truncated *gn1* transcripts in silenced T17 plants. The 5′-ends of the 5′ truncated *gn1* transcripts were mapped through cloning of polyadenylated *gn1* RNA species of silenced T17 plants. The *gn1* mRNA transcript is schematically represented and the numbers indicate the position in the mRNA sequence. The position of the introns is indicated (introns are not drawn in scale). The cDNA clones are represented by lines. The *gn1* gene-specific primer (GE5) used in the procedure is indicated by an arrow.

DISCUSSION

The present study focuses on the characterisation of *gn1* RNAs specifically found in silenced T17 plants. It is shown that silencing of the β-1,3-glucanase genes positively correlates with the accumulation of additional *gn1* RNA species in three size classes which result from at least two processing events. The longer-than-full-length *gn1* RNAs are polyadenylated and do not contain intronic or surrounding genomic sequences of the *gn1* transgene. The RNA sequences that makes these *gn1* RNAs longer-than-full-length also seems not to reside within the region spanned by the primers used for cDNA cloning since no cDNA clones that could correspond to these longer-than-full-length RNAs were detected. We conclude that these longer-than-full-length *gn1* RNAs must originate from a silencing-related sequence duplication, or from a long-range splicing event.

The less-than-full-length *gn1* RNA species correspond to the 5′ part (proximal fragments) and to the 3′ part (distal fragments) of the *gn1* mRNA, and are most probably silencing-related *gn1* degradation intermediates which result from an endonucleolytic cleavage followed by exonuclease digestion. Less-than-full-length RNA species have also been described for co-suppression of polygalacturonase and ACC synthase genes and for homology dependent resistance to TEV (13,14,22,23). However, this is the first report describing the cloning and sequence analysis of less-than-full-length RNA species implicated in silencing-related mRNA turnover.

The proximal and distal *gn1* RNA species were further analysed to understand whether these could be relatively stable intermediates of an on-going silencing-related RNA degradation mechanism. In the case of T17 plants expressing *gn1*, >80% of the proximal clones represented full-length, polyadenylated *gn1* transcripts. In contrast, all of the proximal cDNA clones from silenced T17 plants were truncated at the 3′-end. The absence of clones representing full-length RNA species in silenced T17 plants is consistent with the substantial (20–40-fold) reduction in the steady-state level of full-length *gn1* messengers in silenced compared with expressing T17 plants (30).

The isolation of two cDNA clones from silenced T17 plants that contain the intron 2 sequence, and at the same time are truncated at the 3′-end could indicate that the processing of pre-*gn1*-RNAs in the nucleus is disturbed in silenced plants and that the presence of intron sequences could ‘mark’ these RNA molecules for degradation in the cytoplasm. However, such aberrant splicing does not seem essential for silencing, since most of the cDNA clones obtained from silenced plants correspond to RNAs that were normally spliced over intron 2.

Importantly, cDNA cloning and sequence analysis did show that truncated proximal *gn1* RNAs are present in expressing T17 plants. However, both northern blot and sequence analysis of a large number of cDNA clones indicated that such products are much more abundant in silenced T17 plants. In principle, these products could arise from premature termination of transcription. However, nuclear-runoff transcription has previously shown that transcription proceeds normally over the entire *gn1* coding sequence (31). Therefore, we prefer the interpretation that the truncated, proximal RNA species represent intermediates of an on-going RNA degradation mechanism. It is possible that the identified *gn1* degradation intermediates are relatively stable due to the protection from RNases by interacting proteins or because they are trapped in the translation complex as described for other silencing-related RNA degradation intermediates (13,14,32).

The observation of two discrete 5′-ends, that correspond to two transcription start sites, in the proximal region of *gn1* transcripts indicates that no 5′ to 3′ exonuclease activity is occurring at the 5′-end of the *gn1* transcript. Similarly, the presence of a poly(A) tail at the truncated distal *gn1* products suggests that these RNAs are not degraded from their 3′-ends. Together, these results imply that the mature *gn1* mRNA is not degraded from the 5′ or 3′-termini, but that RNA degradation is initiated via an endonucleolytic cleavage. The range of 3′ and 5′-end points could be interpreted in several ways: one possibility is that endonucleolytic cleavage occurs at one specific internal site and is immediately followed by 5′ to 3′ and 3′ to 5′ exonuclease activity. Alternatively, endonucleolytic cleavage may occur at many relatively random internal sites of the *gn1* mRNA, followed by both 5′ to 3′ and 3′ to 5′ exonuclease activity or followed by either one of the exonuclease activities alone. Thus, silencing-related *gn1* mRNA degradation in transgenic tobacco appears not to involve decapping and deadenylation, steps that are generally assumed to be important for normal mRNA decay (16–19,33). A similar scenario was suggested previously for silencing-related degradation of *chsA* mRNA in petunia (5) and for silencing-independent degradation of *rbcS* SRS4 RNAs (19).

The observation that the proximal and distal *gn1* RNA degradation products are much more abundant in silenced T17 plants than in expressing T17 plants, could indicate that these RNAs are more stable in silenced plants. More likely, however, these products are a direct result of the silencing-related increased turn-over of *gn1* RNAs (7). The abundance of any given mRNA species is primarily determined by its rate of synthesis and its rate of decay. As the overall *gn1* transcription rate in silenced and expressing T17 plants is similar (31), and the truncated *gn1* RNA species are most likely not more stable in silenced T17 plants, the only way to explain their higher levels would be to assume that in silenced T17 plants a larger fraction of *gn1* RNAs is shuttled into a pathway that specifically generates these truncated RNAs. Therefore, we suggest that various RNA degradation pathways

Downloaded from nar.oxfordjournals.org at Biomedische Bibliotheek on September 20, 2011

are active in tobacco cells at the same time, and that during silencing, the partitioning of *gn1* RNAs over these pathways is shifted in favour of a more active, silencing-related (not necessarily silencing-specific) pathway. This would be in agreement with the results obtained by Tanzer *et al*. (14), who propose distinctive silencing-related and a silencing-independent decay mechanism for TEV messengers. It remains to be determined to what extent such a silencing-related RNA degradation pathway operating in silenced T17 plants is active in non-silenced plants. Some level of activity in non-silenced plants is expected as this would explain why some of the truncated products are also present in non-silenced T17 plants expressing the β-1,3-glucanase genes at normal levels.

ACKNOWLEDGEMENTS

We thank Raimundo Villarroel for sequence analysis. This work was supported by an EC grant in the context of the EC Network 'Control of gene expression and silencing in transgenic plants'.

REFERENCES

1 Baulcombe,D.C. (1996) *Plant Mol. Biol*., **32**, 79–88.
2 Baulcombe,D.C. and English,J.J. (1996) *Curr. Opin. Biotechnol*., **7**, 173–180.
3 Depicker,A. and Van Montagu,M. (1997) *Curr. Opin. Cell Biol*., **9**, 373–382.
4 Stam,M., Mol,J.N.M. and Kooter,J.M. (1997) *Ann. Bot*., **79**, 3–12.
5 Metzlaff,M., Odell,M., Cluster,P.D. and Flavell,R.B. (1997) *Cell*, **88**, 845–854.
6 van Blokland,R., van der Geest,N., Mol,J.N.M. and Kooter,J.M. (1994) *Plant J*., **6**, 861–877.
7 Jacobs,J.J.M.R., Litière,K., van Dijk,V., van Eldik,G.J., Van Montagu,M. and Cornelissen,M. (1997) *Plant J*., **12**, 885–893.
8 Dougherty,W.G. and Parks,T.D. (1995) *Curr. Opin. Cell Biol*., **7**, 399–405.
9 Lindbo,J.A., Silva Rosales,L., Proebsting,W.M. and Dougherty,W.G. (1993) *Plant Cell*, **5**, 1749–1759.
10 English,J.J., Mueller,E. and Baulcombe,D.C. (1996) *Plant Cell*, **8**, 179–188.
11 Mueller,E., Gilbert,J., Davenport,G., Brigneti,G. and Baulcombe,D.C. (1995) *Plant J*., **7**, 1001–1013.
12 Smith,H.A., Swaney,S.L., Parks,T.D., Wernsman,E.A. and Dougherty,W.G. (1994) *Plant Cell*, **6**, 1441–1453.
13 Lee,K.Y., Baden,C., Howie,W.J., Bedbrook,J. and Dunsmuir,P. (1997) *Plant J*., **12**, 1127–1137.
14 Tanzer,M.M., Thompson,W.F., Law,M.D., Wernsman,E.A. and Uknes,S. (1997) *Plant Cell*, **9**, 1411–1423.
15 Gera,J.F. and Baker,E.J. (1998) *Mol. Cell. Biol*., **18**, 1498–1505.
16 Decker,C.J. and Parker,R. (1994) *Trends Biochem. Sci*., **19**, 336–340.
17 Sachs,A.B. (1993) *Cell*, **74**, 413–421.
18 Higgs,D.C. and Colbert,J.T. (1994) *Plant Cell*, **6**, 1007–1019.
19 Tanzer,M.M. and Meagher,R.B. (1995) *Mol. Cell. Biol*., **5**, 6641–6652.
20 Thompson,D.M., Tanzer,M.M. and Meagher,R.B. (1992) *Plant Cell*, **4**, 47–58.
21 Wanner,L.A. and Gruissem,W. (1991) *Plant Cell*, **3**, 1289–1304.
22 Goodwin,J., Chapman,K., Swaney,S., Parks,T.D., Wernsman,E.A. and Dougherty,W.G. (1996) *Plant Cell*, **8**, 95–105.
23 Smith,C.J., Watson,C.F., Bird,C.R., Ray,J., Schuch,W. and Grierson,D. (1990) *Mol. Gen. Genet*., **224**, 477–481.
24 de Carvalho,F., Gheysen,G., Kushnir,S., Van Montagu,M., Inzé,D. and Castresana,C. (1992) *EMBO J*., **11**, 2595–2602.
25 Jones,D.G., Dunsmuir,P. and Bedbrook,J. (1985) *EMBO J*., **4**, 2411–2418.
26 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
27 Gubler,U. and Hoffman,B.J. (1983) *Gene*, **25**, 263–269.
28 Liu,X. and Gorovsky,M.A. (1993) *Nucleic Acids Res*., **21**, 4954–4960.
29 De Loose,M., Alliotte,T., Gheysen,G., Genetello,C., Gielen,J., Soetaert,P., Van Montagu,M. and Inzé,D. (1988) *Gene*, **70**, 13–23.
30 Litière,K. (1997) PhD Thesis, Universiteit Gent, Belgium.
31 de Carvalho Niebel,F., Frendo,P., Van Montagu,M. and Cornelissen,M. (1995) *Plant Cell*, **7**, 347–58.
32 Tuite,M.F. (1996) *Nature*, **382**, 577–579.
33 Abler,M.L. and Green,P.J. (1996) *Plant Mol. Biol*., **32**, 63–78.

Downloaded from nar.oxfordjournals.org at Biomedische Bibliotheek on September 20, 2011