The EMBO Journal vol.11 no.7 pp.2595 - 2602, 1992

Suppression of β-1,3-glucanase transgene expression in homozygous plants

Fernanda de Carvalho, Godelieve Gheysen, Sergei Kushnir, Marc Van Montagu, Dirk Inzé[1] and Carmen Castresana[2]

Laboratorium voor Genetica and [1]Laboratoire associé de l'INRA, Universiteit Gent, B-9000 Gent, Belgium
[2]Present address: Centro de Investigaciones Biologicas, Velázquez 144, E-28006 Madrid, Spain

Communicated by M.Van Montagu

A chimeric construct containing the *Nicotiana plumbaginifolia* β-1,3-glucanase *gn1* gene was introduced into *Nicotiana tabacum* SR1 to produce high levels of the enzyme constitutively. We determined that the GN1 protein represents a basic β-1,3-glucanase isoform which accumulates into the vacuoles of the transgenic plants. Analysis of the progeny of the transgenic plant with the highest levels of *gn1* expression revealed an unexpected phenomenon of gene suppression. Plants hemizygous for the T-DNA locus contained high levels of *gn1* mRNA and exhibited a 14-fold higher β-1,3-glucanase activity than untransformed plants. However, the expression of *gn1* was completely suppressed in the homozygous plants: no corresponding mRNA or protein could be detected. This suppression mechanism occurs at a post-transcriptional level and is under developmental control. In addition, by generating haploid plants we found that this silencing phenomenon is not dependent on allelic interaction between T-DNA copies present at the same locus of homologous chromosomes, but rather is correlated with the transgene dose in the plant genome. We postulate that high doses of GN1 protein relative to the level(s) of other still unknown plant products could trigger the cellular processes directed to suppress *gn1* expression.

Key words: gene expression/β-1,3-glucanase/*Nicotiana*/post-transcriptional regulation/suppression

Introduction

β-1,3-Glucanases constitute a family of plant hydrolytic enzymes which is thought to play a role in the defence mechanisms against microbial attack. These enzymes belong to a broader group of proteins, the so-called pathogenesis-related (PR) proteins, which rapidly accumulate in plants reacting hypersensitively to pathogen infections. PR proteins were initially identified in tobacco (Gianinazzi *et al.*, 1970; van Loon and van Kammen, 1970) where they have been extensively characterized. Subsequent studies have led to the identification of these proteins in many species including both monocotyledonous and dicotyledonous plants (for review see Bol *et al.*, 1990; Carr and Klessig, 1990).

β-1,3-Glucanases are constitutively present in roots and floral tissues of healthy, non-stressed plants (Felix and Meins, 1987; Lotan *et al.*, 1989; Memelink *et al.*, 1990; Coté *et al.*, 1991). In addition, they have been found to be induced not only after pathogen infections but also in response to different stress situations such as chemical and hormonal treatments (Mohnen *et al.*, 1985; Vögeli *et al.*, 1988; Van den Bulcke *et al.*, 1989; Memelink *et al.*, 1990). The induction of β-1,3-glucanases in the plant cell is the result of transcriptional activation of the corresponding genes (Castresana *et al.*, 1990; Hennig *et al.*, 1991; Vögeli-Lange *et al.*, 1991).

Subcellular localization and protein analysis revealed that this family of hydrolytic enzymes is made of independently encoded isoforms with acidic and basic isoelectric points. Furthermore, it has been shown that in tobacco plants the acidic isoforms are secreted to the intercellular spaces whereas the basic ones are located intracellularly in the cell vacuoles (Van den Bulcke *et al.*, 1989). Although acidic and basic isoforms display similar enzymatic activities, the corresponding genes appear to be differentially expressed (Memelink *et al.*, 1990). Additionally, the real function of distinct β-1,3-glucanase isoforms in healthy and stressed plants is still not known.

We have previously reported a detailed study on the analysis of the expression characteristics of a β-1,3-glucanase gene (*gn1*) isolated from *Nicotiana plumbaginifolia* (De Loose *et al.*, 1988; Castresana *et al.*, 1990). A role in development as well as in plant defence was postulated for the examined GN1 β-1,3-glucanase. Now, we have characterized the GN1 protein produced in transgenic tobacco plants containing the *gn1* gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter. This study has not only allowed us to discover more about some of the basic properties of this protein, but has also revealed an interesting phenomenon: dosage-dependent suppression of gene expression.

A number of cases concerning suppression of gene expression in plants has been recently reported (for a review, see Jorgensen, 1990). In some cases, the introduction of sense transgenes in plants reduces or eliminates the expression of homologous gene sequences already present in the plant genome (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). Plant genes as well as genes of bacterial origin previously integrated into the plant genome have been found to be silenced after transformation with homologous gene sequences (Matzke *et al.*, 1989; Goring *et al.*, 1991). In addition, the silencing of endogenous genes has been similarly achieved by transformation with closely related but not identical sequences (Elkind *et al.*, 1990). Furthermore, in some cases the use of truncated genes has been sufficient to reduce the expression of the corresponding endogenous genes (Smith *et al.*, 1990).

In spite of the increasing body of accumulated information, the cellular mechanisms governing the reported suppression events are still unknown. In some cases, the silencing phenomenon has been correlated with methylation in the promoter sequences which controlled the expression of the

specific transgenes (Matzke *et al.*, 1989; Matzke and Matzke, 1991). By contrast, other studies on the suppression of a chalcone synthase gene (*chs*) in petunia plants indicated that the suppressed genes are transcribed and that the silencing event occurs post-transcriptionally through the reduction of steady-state mRNA levels (Mol *et al.*, 1991). It is likely that some of the reported cases represent related, but nevertheless independent, processes and that their biological significance and the cellular mechanisms implicated in their regulation are different.

In this report, we show a new case of gene suppression, where the expression of the p35S−*gn1* chimeric construct is blocked in homozygous plants generated by self-pollination of a highly expressing transgenic plant. A detailed characterization of the phenomenon observed reveals that the suppression of *gn1* expression is developmentally regulated and occurs at a post-transcriptional level. In addition, we demonstrate that the silencing of the *gn1* gene is not due to allelic interaction between the T-DNA copies present at the same locus of homologous chromosomes, but rather is correlated with the transgene dose in the plant genome. Our results will be contrasted with the suppression events previously reported as well as with the models postulated to explain gene suppression by sense genes.

Results

Expression of the chimeric β-1,3-glucanase gene in transgenic tobacco plants

The chimeric construct diagrammed in Figure 1 was made by fusing the CaMV 35S promoter to a 3.3 kb DNA fragment containing the entire coding region and 1.35 kb 3′-flanking sequence of the *gn1* gene. This construct was introduced into SR1 tobacco and transgenic plants were regenerated on medium containing kanamycin. None of the transgenic plants showed any apparent abnormality during development when compared with untransformed tobacco plants.

The abundance of *gn1* mRNA was examined by RNA gel blot analysis using total RNA isolated from leaves of independent primary transformants (R_0). As expected, the *gn1* expression directed by the 35S promoter varied between the individual transgenic plants examined. Plants exhibiting high levels of *gn1* transcripts (Figure 2A) were selected for further studies.

Analysis of the progenies obtained by self-pollination of primary transformants revealed that in T3, T11, T15, T17 and T20, the T-DNA segregated as a single locus. The level of *gn1* expression was examined in the R_1 plants obtained from those five R_0 lines. The *gn1* RNA was found in all the kanamycin-resistant plants derived from T3, T11, T15 and T20 plants. Unexpectedly, a different pattern of gene expression was observed in the kanamycin-resistant progeny obtained from the T17 line. As shown in Figure 2B, approximately two-thirds of the plants exhibited a high level of *gn1* expression like the original T17 plant, whereas the same transcript was undetectable in the remaining plants. DNA gel blot analysis revealed that, independently of the *gn1* mRNA levels, all the kanamycin-resistant R_1 T17 plants contained a complete T-DNA (data not shown).

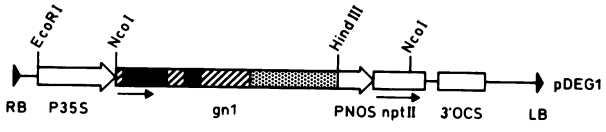

Fig. 1. Structure of the T-region of the *pDEG1* vector containing the p35S−*gn1* chimeric construct. Abbreviations used are: P35S, CaMV 35S promoter; gn1, β-1,3-glucanase *gn1* gene sequences; PNOS, nopaline synthase promoter; nptII, neomycin phosphotransferase II coding sequence; 3′OCS, octopine synthase 3′-untranslated region. RB and LB represent the right and left borders of the T-DNA, respectively. The black arrows indicate the direction of transcription. The *gn1* sequences are represented as follows: ▨, coding region; ■, introns; and ⬚, 3′-flanking region.

Suppression of gn1 expression in T17 transgenic plants is associated with the homozygous state of the transgene

To examine in more detail the phenomenon observed in the T17 line, R_1 plants expressing (RNA^+) and not expressing (RNA^-) the p35S−*gn1* gene were self-pollinated and the R_2 progenies were further characterized. R_2 plants derived from *gn1* RNA^+ transformants showed a 3:1 segregation ratio for the kanamycin-resistant phenotype. In contrast, 100% of the R_2 progenies obtained from the *gn1* RNA^- plants survived on medium containing kanamycin. These

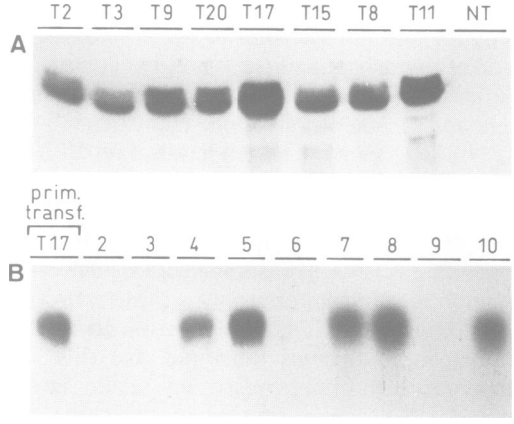

Fig. 2. RNA gel blot analysis of total leaf RNA from transgenic SR1 plants. (**A**) Total RNA from independent R_0 transformants and an untransformed tobacco plant (NT). (**B**) Total RNA from progeny plants of the primary transformant T17. Lane 1 represents total leaf RNA from the original R_0 T17 plant. RNA gel blots were hybridized with riboprobes homologous to the *gn1* cDNA.

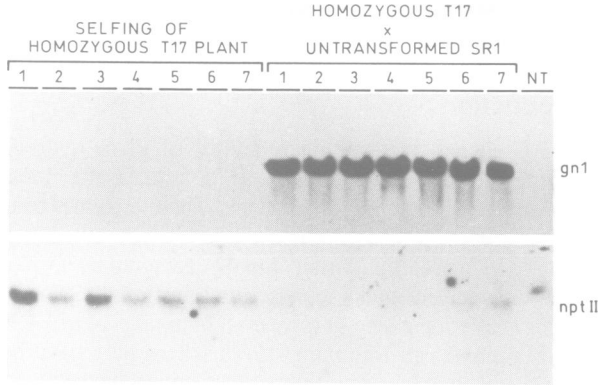

Fig. 3. RNA gel blot analysis of total leaf RNA from R_3 homozygous and F_3 hemizygous T17 plants. R_3 homozygous and F_3 hemizygous T17 plants were generated as described in Materials and methods. RNA gel blots were hybridized with riboprobes homologous to the *gn1* and *nptII* cDNAs (top and bottom panel, respectively). A RNA sample from an untransformed tobacco plant (NT) is included.

results allowed us to conclude that the *gn1* RNA$^+$ and *gn1* RNA$^-$ R_1 plants were hemizygous and homozygous for the T-DNA locus, respectively. Our data demonstrated that the lack of *gn1* expression in the plants examined corresponded to the homozygous state of the transgene.

Similarly, we analysed the expression of the p35S−*gn1* chimeric gene in the progenies obtained by self-pollination of three independent R_2 T17 homozygous lines (R_2 T17: 4, 5 and 7). In this way, the R_3 progenies obtained consisted exclusively of homozygous plants. As shown in Figure 3, the *gn1* mRNA was absent from all the R_3 plants examined, revealing that the suppression detected was stably transmitted to subsequent generations. In addition, to examine whether or not the *gn1* expression could be restored, the same R_2 T17 homozygous plants (R_2 T17: 4, 5 and 7) were back-crossed with untransformed SR1 plants producing an F_3 generation consisting entirely of hemizygous plants. Interestingly, in all the F_3 plants the expression of *gn1* was restored (Figure 3) reaching similar levels of mRNA accumulation to those detected in R_2, R_1 and R_0 hemizygous plants.

By contrast, the chimeric neomycin phosphotransferase II (*nptII*) gene in the same T-DNA was not only never suppressed but showed the normal gene dose−response since *nptII* mRNA accumulated to ~2-fold higher levels in homozygous than in hemizygous plants (Figure 3).

Characterization of the GN1 protein in the transgenic plants

Protein analyses were carried out to evaluate the presence of GN1 protein in the transgenic plants. As shown in Figure 4, analysis of Coomassie blue-stained two-dimensional non-equilibrium pH gel electrophoresis (NEPHGE) gels revealed the appearance of an abundant protein spot corresponding to GN1 β-1,3-glucanase, exclusively in the protein extracts obtained from the F_3 T17 hemizygous plants. Protein gel blot analysis using antisera against tobacco β-1,3-glucanase confirmed this observation (data not shown). The absence of GN1 protein in the homozygous plants was consistent with the results obtained in the analysis of the *gn1* mRNA accumulation.

To investigate whether the produced GN1 protein was enzymatically active, we performed β-1,3-glucanase activity assays in total protein extracts from transgenic and untransformed SR1 plants. As shown in Figure 5, two endogenous β-1,3-glucanase isoforms were detected in plant extracts from both untransformed SR1 and transgenic plants. In addition, a strongly active band corresponding to GN1 was visualized exclusively in the samples obtained from the hemizygous plants. These analyses also confirmed that GN1 is a basic isoform with an isoelectric point (pI) of ~9.6. Similarly, we estimated that the presence of GN1 produced a 14-fold increase in β-1,3-glucanase activity in hemizygous plants in comparison with the activity determined in the untransformed tobacco plants. However, homozygous plants give results similar to those of untransformed SR1 plants in β-1,3-glucanase assays. Only the two endogenous tobacco isoforms were visualized on isoelectric focusing (IEF) gels and the activity levels were close to those found in untransformed plants (data not shown).

Immunolocalization studies allowed us to demonstrate that the GN1 protein corresponded to a vacuolar isoform. As shown in Figure 6, gold-label particles were clearly visible within the plant vacuoles when root tissues from hemizygous plants were examined by transmission electron microscopy. Occasionally, gold label was also observed on the endoplasmic reticulum. No label was found in tissues derived from control plants.

Suppression of gn1 expression occurs post-transcriptionally

To determine whether the absence of *gn1* transcript in the T17 homozygous plants was caused by transcriptional inactivation of the p35S−*gn1* chimeric gene, the levels of *gn1* transcription were examined in nuclei isolated from leaf tissues of T17 homozygous and hemizygous plants. As shown in Figure 7, run-off transcription assays demonstrated that the chimeric *gn1* gene was transcribed in both homozygous and hemizygous plants, the amount of transcription being clearly higher in the homozygous plants. No hybridization signal was detected when the labelled RNA assayed against the *gn1* DNA sequence was prepared from nuclei isolated from untransformed tobacco plants (data not shown). In parallel with the level of *nptII* mRNA accumulation previously evaluated (Figure 3), the amount of transcription determined for the *nptII* gene was higher in homozygous than in hemizygous plants. In addition, we analysed the transcription corresponding to an endogenous tobacco gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase which, as expected, was transcribed similarly in all the examined plants. Similar results were obtained when probes corresponding to the first

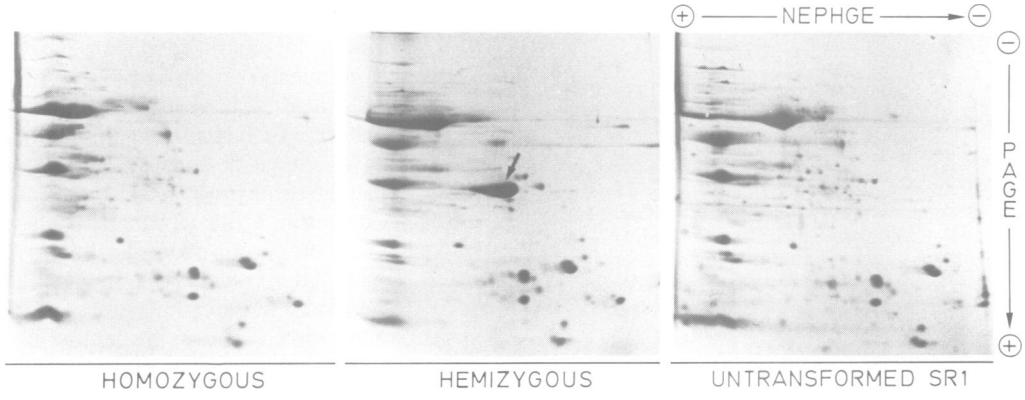

Fig. 4. Analysis of GN1 protein in transgenic plants. Coomassie blue-stained NEPHGE two-dimensional gels of total protein extracts from leaves of transgenic T17 and untransformed plants. The arrow indicates the spot corresponding to the GN1 protein.

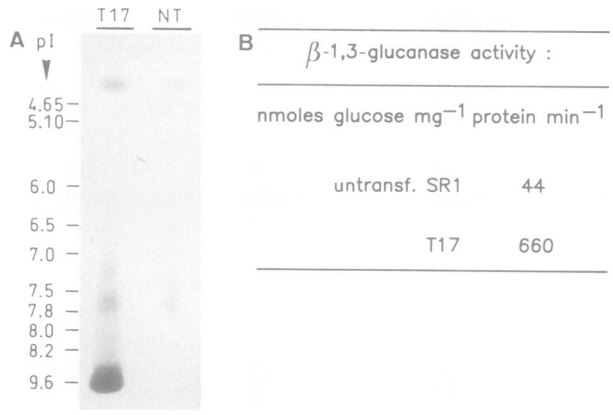

Fig. 5. Analysis of β-1,3-glucanase activity in tobacco plants. (**A**) Direct assay for β-1,3-glucanase activity on 7.5% IEF gel. T17 and NT correspond to total protein extracts from leaves of F_3 T17 hemizygous and untransformed tobacco plants, respectively. (**B**) β-1,3-glucanase activity in total protein extracts from leaves of untransformed and F_3 T17 hemizygous plants.

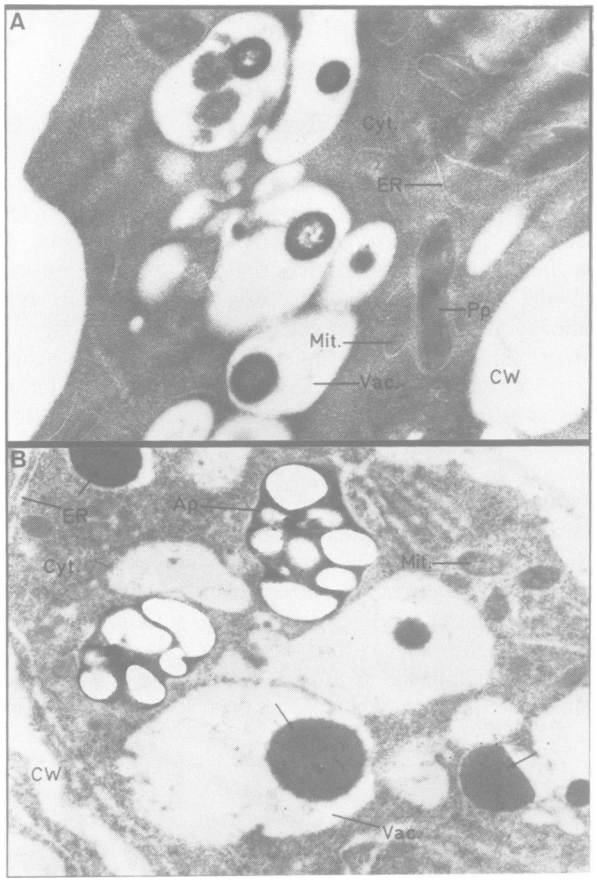

Fig. 6. Immunogold localization of GN1 β-1,3-glucanase in transgenic plants. Root sections of untransformed (**A**) and T17 F_3 hemizygous (**B**) plants labelled with anti-β-1,3-glucanase antibodies. Gold label (→) is found over protein aggregates in the vacuoles of T17. No label is detected in root sections of untransformed plants. Abbreviations are: Ap, amyloplast; Cyt, cytoplasm; CW, cell wall; ER, endoplasmic reticulum; Mit, mitochondria; Pp, proplastid; vac, vacuole.

intron and a 3′ end region of the *gn1* gene were used, demonstrating that the complete *gn1*-coding sequence was transcribed in both hemizygous and homozygous plants (data not shown). From these results we conclude that the strong differences in *gn1* mRNA steady-state levels between T17 homozygous and hemizygous plants depend on post-transcriptional processes.

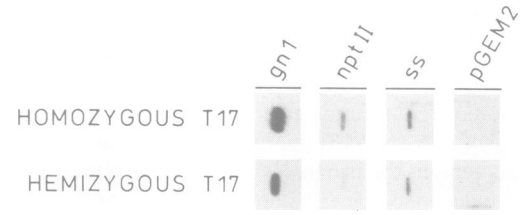

Fig. 7. Analysis of labelled run-off transcripts of homozygous and hemizygous T17 plants. Slot blots of linearized plasmids (gn1, nptII, ss and pGEM 2) were hybridized with labelled RNA transcripts from nuclei isolated from leaves of R_3 homozygous and F_3 hemizygous T17 plants as described in Materials and methods.

The suppression mechanism does not require transgene allelic interaction

The fact that the inactivation phenomenon occurred exclusively in T17 plants homozygous for the T-DNA locus led us to address the question of whether the suppression of gene expression was produced as a consequence of allelic interaction between the T-DNA copies present at the same locus of homologous chromosomes. To test this hypothesis, we prepared haploid tobacco plants from both R_3 homozygous and F_3 hemizygous plants. Kanamycin-resistant plants were selected for further studies and, as shown in Figure 8, contained the appropriate number of chromosomes for haploid tobacco. Interestingly, RNA gel blot analysis carried out to monitor the expression of the p35S−*gn1* gene revealed the absence of the *gn1* transcript in all the plants examined (Figure 8). These results demonstrated that the expression of the *gn1* chimeric gene is suppressed in haploid plants regardless of whether they were generated from homozygous or hemizygous plants. Therefore, we conclude that the suppression of *gn1* expression in T17 homozygous plants does not depend on allelic interactions between gene copies present on homologous chromosomes.

Suppression of gn1 gene expression is developmentally regulated

To evaluate if the suppression phenomenon described is stably maintained during plant development, the presence of GN1 protein was examined in plant seedlings at different stages after germination. T17 homozygous, T17 hemizygous, and untransformed SR1 seeds were germinated in soil and leaf proteins were extracted from a pool of six independent seedlings. As shown in Figure 9, the GN1 protein was present in all the extracts obtained from the T17 hemizygous seedlings. Interestingly, the GN1 protein was detected at high levels in the homozygous plants during the first 4 weeks of development. Afterwards, the amount of GN1 started to decrease gradually in the homozygous plants. By contrast, no such reduction was seen in the hemizygous plants, so the decrease in expression cannot be due merely to changes in the activity of the 35S promoter. These observations show that the suppression mechanism in homozygous plants is developmentally regulated.

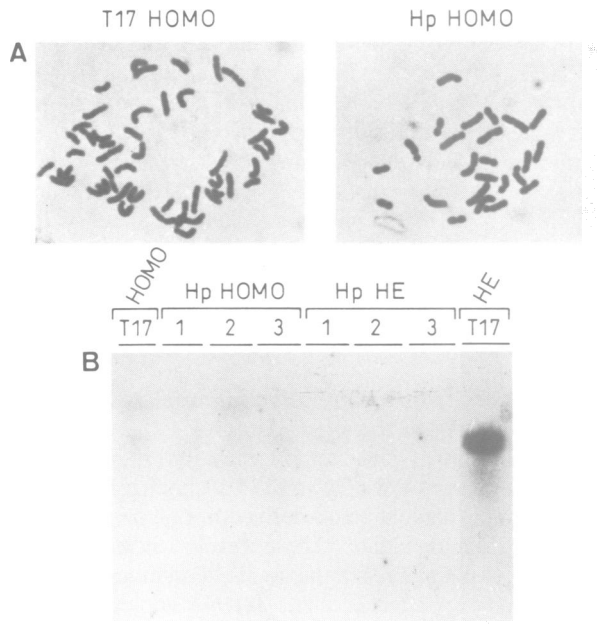

Fig. 8. Analysis of haploid plants. Haploid tobacco plants were generated as described in Materials and methods. (**A**) Chromosome number of homozygous (T17 Homo) and haploid (Hp Homo) tobacco plants. (**B**) RNA gel blot analysis of total leaf RNA isolated from independent haploid plants generated from anthers cultures of T17 homozygous (Hp Homo 1, 2 and 3) and T17 hemizygous (Hp He 1, 2 and 3) plants. RNA samples from R_3 homozygous (T17 Homo) and F_3 hemizygous (T17 He) plants are included as controls.

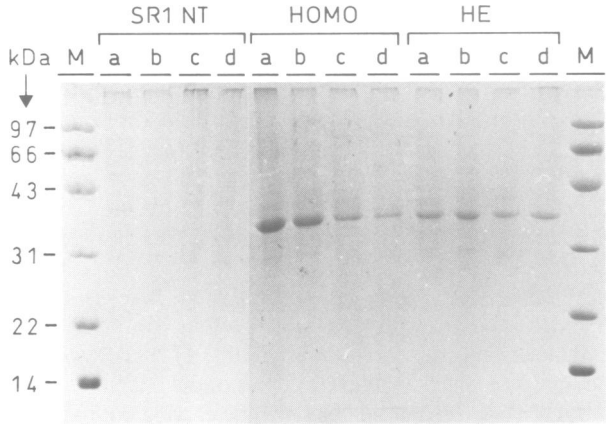

Fig. 9. Developmental pattern of GN1 β-1,3-glucanase accumulation in tobacco plants. Coomassie blue-stained SDS–PAGE gels of total protein extracts (5 μg) from leaf tissues collected at 3 (a), 4 (b), 6 (c) and 7 (d) weeks after seed germination. At stages a, b, c and d of growth the total number of leaves per plant was 5, 7, 9 and 10, respectively. Abbreviations are: He, hemizygous F_3 T17 plants; Homo, homozygous R_3 T17 plants; NT, untransformed plants. Molecular mass (M) markers (kDa) are indicated to the left.

Discussion

The *N.plumbaginifolia gn1* gene coding for a β-1,3-glucanase has been constitutively expressed in tobacco SR1 plants. Transgenic plants showing high levels of *gn1* transcripts have been extensively analysed. The GN1 protein produced has been identified, its enzymatic properties characterized and its subcellular localization determined. Detailed studies of the chimeric *gn1* gene expression in successive plant generations have allowed us to characterize an interesting phenomenon of suppression of gene expression.

GN1 corresponds to a basic β-1,3-glucanase isoform localized in the plant vacuoles

We have previously described that the *N.plumbaginifolia gn1* gene encodes a hydrolytic enzyme with β-1,3-glucanase activity (Castresana *et al.*, 1990). In this study, the expression of *gn1* under the CaMV 35S promoter has allowed us to obtain transgenic tobacco plants accumulating high levels of GN1 protein. By analysis of the protein synthesized in these plants we have confirmed that GN1 is a basic β-1,3-glucanase with a pI of ~9.6. Subcellular localization of tobacco isoforms has shown that the acidic β-1,3-glucanases are secreted into the intercellular spaces of the plant, whereas the basic isoforms are localized intracellularly in the plant vacuoles (Van den Bulcke *et al.*, 1989). Consistent with this, we have demonstrated that the basic isoform encoded by *gn1* accumulates in the plant vacuoles.

Although the levels of *gn1* mRNA and GN1 protein are similar in leaf and root tissues of the transgenic plants, GN1 was easily detected in vacuoles of root tissues but could not be detected in leaf sections from the same transgenic plants. Possibly, this difference is simply a reflection of the much greater vacuolar size in the cells of leaf tissues.

Constitutive expression of GN1 does not cause any apparent phenotypic abnormality

Previous studies in *N.plumbaginifolia* plants revealed that the endogenous *gn1* gene is expressed in root tissues and at lower levels in the older leaves of the plant (Castresana *et al.*, 1990). On the other hand, the expression of *gn1* when transcribed from the 35S promoter is constitutive and considerably higher than the normal expression levels of the *gn1* gene. In this sense, we have shown that the high level of activity determined in the T17 hemizygous plants does not have any visible effect on plant development even though a role for this β-1,3-glucanase in basic aspects of plant metabolism has been suggested (Castresana *et al.*, 1990). In addition, such levels of GN1 protein had no obvious effect on the overall pattern of proteins, as far as could be assessed by two-dimensional gel analysis (see Figure 4). Alternatively, higher doses of GN1 β-1,3-glucanase could be required to produce any obvious abnormalities.

Expression of the chimeric gn1 gene is suppressed in homozygous transgenic plants

Although the high level of constitutive β-1,3-glucanase activity does not have any visible phenotypic effect, our studies showed that the p35S–*gn1* was itself being regulated by the host. Among the examined independent primary transformants, the plant showing the highest level of *gn1* transcript, designated T17, manifested an unexpected pattern of gene expression in its progeny. Whereas plants hemizygous for the T-DNA locus contained as much *gn1* mRNA as the original T17 transformant, *gn1* expression was completely blocked in homozygous plants. There, suppression of the p35S–*gn1* gene was characterized by the absence of steady-state mRNA levels as well as lack of GN1 protein accumulation. By genetic analysis, we demonstrated that the suppression was strictly correlated with homozygosity and transmitted to subsequent generations. In

addition, the fact that the expression of the *nptII* gene present in the same T-DNA was not altered in a similar way reveals that the suppression mechanism is specific for the p35S−*gn1* gene.

Recent attempts to express some genes in plants have also led to transgene suppression (Jorgensen, 1990). In certain cases, the silencing of the transgene was associated with the inhibition of the expression of endogenous genes in the genome, suggesting a homology-based mechanism of suppression (Napoli *et al.*, 1990; van der Krol *et al.*, 1990; Goring *et al.*, 1991). By contrast, we observed that β-1,3-glucanase isoforms detected in untransformed SR1 plants (see Figure 6) are not suppressed in the T17 homozygous plants simultaneously with the GN1 protein. It is known that *N.plumbaginifolia* and *N.tabacum* are closely related species with a high level of sequence conservation (Poulsen *et al.*, 1986; Castresana *et al.*, 1990); however, the similarity between the genes encoding those particular tobacco β-1,3-glucanases and the *N.plumbaginifolia gn1* gene sequences is not known. At this point we cannot exclude the possibility that other endogenous β-1,3-glucanase isoforms, specifically induced upon different plant treatments, would be affected by a suppression-like mechanism. Further experiments to test this possibility are now under way.

Alternatively, it is possible that the mechanism controlling the suppression phenomenon reported here differs, at least partially, from most of the suppression events previously described, since in these cases gene silencing has not been shown to be correlated to the homozygous state of the transgene. Interestingly, the only exception to this has been found in transgenic *N.sylvestris* plants overexpressing a basic chitinase from tobacco, where suppression of the transgene expression has been similarly associated with the homozygous state of the transgene (Neuhaus *et al.*, 1991). The expression of both basic β-1,3-glucanases and chitinases from tobacco plants is coordinately regulated and the corresponding encoded proteins are thought to be similarly involved in plant defence mechanisms (Felix and Meins, 1986; Shinshi *et al.*, 1987; Memelink *et al.*, 1990). Therefore, the striking similarity between the suppression events affecting these closely related genes might not be a coincidence, but rather might be correlated in a still unknown manner with a natural mechanism controlling the expression of these related genes.

Suppression of gn1 expression is a post-transcriptional event

We have shown that despite the absence of *gn1* mRNA transcripts, the p35S−*gn1* gene is accurately transcribed in the T17 homozygous plants and, therefore, the suppression is the result of a post-transcriptional event. According to these data, the suppression of the chalcone synthase (*chs*) transgene in petunia plants has also been shown to be imposed post-transcriptionally (Mol *et al.*, 1991).

It has been proposed that the mechanism of down-regulation of gene expression by sense transgenes would involve an antisense-like process (Grierson *et al.*, 1991). In this way, promoter sequences driving antisense transcripts could be located near to the suppressed genes either inside or outside the T-DNA insert. However, due to the relative positions of the p35S−*gn1* and pNos−*nptII* chimeric genes in the T-DNA (see Figure 1), the synthesis of a presumed antisense RNA should similarly modify the level of *nptII* mRNA. Moreover, it is likely that if an antisense RNA were responsible for the suppression of *gn1* gene expression, then the same RNA molecule would also be synthesized in the hemizygous plants, suppressing the expression of the *gn1* gene in a similar manner. Therefore, the absence of suppression in the hemizygous plants together with the normal expression pattern of the *nptII* chimeric gene in both hemizygous and homozygous plants, strongly indicates that the suppression of *gn1* gene expression is not caused by production of an antisense RNA.

Suppression of gn1 expression is correlated with the transgene dose in the plant genome

We have shown that suppression of the p35S−*gn1* expression occurs not only in the T17 homozygous plants, but also in the haploid plants derived from both homozygous and hemizygous plants. These results indicate that the silencing of the *gn1* is not due to allelic interaction between the *gn1* genes present in allelic T-DNA copies at the same locus of homologous chromosomes. Interestingly, we observe that the ratio between the transgene dose and the genome copy is identical (one transgene/haploid genome) in all plants where the expression of the p35S−*gn1* gene has been silenced. However, this ratio is halved in the hemizygous plants, where the *gn1* chimeric gene is highly expressed. These observations indicate that the silencing phenomenon examined here is correlated with the transgene dose in the plant genome. It is possible that this correlation is unrelated to the functional properties of the GN1 protein. Alternatively, the effect observed could be the result of a natural mechanism of gene regulation triggered by the abnormally high β-1,3-glucanase activity in combination with the level of other still unknown plant products. Supporting this idea, we observe that the GN1 protein is synthesized in the T17 homozygous seedlings at early stages of development but then declines as plants mature, probably due to specific metabolic changes occurring in older plant cells.

Whether the phenomenon presented here is a plant response to the level of the GN1 protein will be addressed by subsequent transformation of both T17 homozygous and hemizygous plants with extra copies of the p35S−*gn1* gene. Additionally, further analysis will be carried out to identify potential factors which differ between the hemizygous and homozygous T17 plants and which might be involved in the control of this suppression mechanism.

At this point, we cannot exclude the possibility that the genomic site at which the T-DNA has been inserted influences the described process. The cloning of the plant sequences flanking the T-DNA in the T17 transgenic plants will be undertaken to examine this possibility.

The elucidation of this phenomenon may reveal a novel mechanism of regulation of gene expression occurring naturally in plants. Additionally, our findings could be an important clue for understanding the mystery of the advantages of increased heterozygosity in many plants, termed hybrid vigour (Fehr, 1987). In this sense, one could hypothesize that the advantage of heterosis is primarily determined by the selective expression of genes mediated by mechanisms related to the phenomenon described above.

Materials and methods

Recombinant DNA techniques

Standard DNA techniques were carried out as described by Sambrook *et al.* (1989). Restriction fragments for cloning were purified from agarose gels by centrifugal filtration (Zhu *et al.*, 1985). Plasmid vector pGEM2 was used for DNA subcloning. The *Escherichia coli* strain MC1061 was used for transformation (Casadaban and Cohen, 1980).

Construction of the chimeric p35S – gn1 gene

The cloning and sequencing of the genomic clone of the *N.plumbaginifolia* β-1,3-glucanase *gn1* gene have been previously described (De Loose *et al.*, 1988; Castresana *et al.*, 1990). A 3.3 kb *Nco*I – *Hin*dIII fragment containing the entire coding region and the 3′ end of *gn1* was cloned behind the CaMV 35S promoter by insertion into the *Nco*I – *Hin*dIII sites of pDE300 (Denecke *et al.*, submitted). The *Nco*I site overlaps the ATG initiation codon of the *gn1* gene. The chimeric p35S – *gn1* gene contained in a 4.5 kb *Eco*RI – *Hin*dIII DNA fragment was isolated and inserted into the binary vector pDE100-1 (Denecke *et al.*, submitted), previously digested with *Eco*RI and *Hin*dIII. The obtained plasmid designated pDEG1 is shown on Figure 1.

Agrobacterium-mediated transformation and analyses of transgenic plants

pDEG1 was mobilized by the helper plasmid pRK2013 of *E.coli* HB101 (Figurski and Helinski, 1979) to *Agrobacterium tumefaciens* (C58C1 Rif^R) harbouring the plasmid pGV2260 (Deblaere *et al.*, 1985). Leaf discs from *N.tabacum* cv. Petit Havana SR1 were infected with the transconjugant *Agrobacterium* strain. Transformed shoots were selected on kanamycin-containing medium (100 μg/ml) and regenerated as described by De Block *et al.* (1984). Primary transgenic plants will be referred to as the R_0 generation. Successive progenies obtained by self-fertilization were designated R_1, R_2 and R_3 generations, respectively. Plants obtained from the back-cross of R_2 plants with untransformed SR1 tobacco plants will be referred to as the F_3 generation (Potrykus *et al.*, 1985).

Transformed plants growing under a 14 h light/10 h dark photoperiod were examined 8 – 12 weeks after transfer to soil.

Total DNA was prepared from leaves using the method described by Dellaporta *et al.* (1983). DNA samples (10 μg) were digested with restriction enzymes and electrophoresed in a 0.8% (w/v) agarose gel. DNA gel blots were performed according to Sambrook *et al.* (1989). Radiolabelled probes were generated by the randomly primed method (Boehringer, Mannheim, FRG). A 1 kb *Sst*I – *Pst*I DNA fragment corresponding to part of *gn1* cDNA (De Loose *et al.*, 1988) was utilized as a probe for the detection of the *gn1* glucanase sequences. The probe used for the detection of the *nptII* gene was isolated from the plasmid pKC7 (Rao and Rogers, 1979) as a 1 kb *Bgl*II – *Sma*I fragment containing the complete coding sequence of the *nptII* gene. Blots were hybridized for 38 h at 42°C in a formamide buffer (50 mM phosphate buffer, pH 7.2, 3×SSC, 10×Denhardt's, 50% formamide, 20 μg/ml denatured sheared herring sperm DNA). Filters were washed in 1×SSC, 0.1% sodium dodecyl sulfate (SDS) at 50°C.

RNA was extracted from *N.tabacum* leaf and root tissues as described by Jones *et al.* (1985). Total RNA (12 μg per lane) was electrophoresed in a 1.5% agarose – formaldehyde gel according to Sambrook *et al.* (1989). A *Sst*I – *Pst*I fragment from the *gn1* cDNA was cloned in the pGEM2 vector (De Loose *et al.*, 1988); this vector was used to prepare riboprobes using the Boehringer (Mannheim, FRG) transcription kit. Similarly, riboprobes homologous to the *nptII* gene were generated from the plasmid pNptIIC (I.Ingelbrecht, unpublished results). Blots were hybridized overnight at 65°C in 50% formamide, 3×SSC, 0.25% non-fat milk powder, 0.5% SDS, and 20 μg/ml denatured herring sperm DNA. Hybridizations were washed at 68°C twice with 3×SSC, 0.1% SDS and twice with 1×SSC, 0.1% SDS for 20 min.

Protein extraction and electrophoresis

One dimensional SDS – PAGE. Plant proteins were extracted from leaves using a solution of 84 mM citric acid and 24 mM Na_2HPO_4 (pH 2.9). After filtration through a double Miracloth layer, the extracts were centrifuged at 100 *g* for 20 min. The supernatants were then extensively dialysed against water at 4°C. Protein concentrations were determined by the method of Bradford (1976) using the reagent supplied by Bio-Rad Laboratories. Five to ten micrograms of crude protein extracts were then separated by SDS – PAGE according to Laemmli (1970) using a 12.5% (w/v, acrylamide) separation gel overlaid by a 5% (w/v, acrylamide) stacking gel. Low molecular weight standards (Bio-Rad) were used to calibrate gels.

Two dimensional electrophoresis. Proteins from leaves of *N.tabacum* plants were extracted with phenol as described by Hurkman and Tanaka (1986). The final protein pellets were solubilized in lysis buffer (9.8 M urea, 2% Nonidet P-40, 2% ampholytes, pH 5 – 7, and 100 mM dithiothreitol). Sixty μg of total proteins were loaded for the first dimension focusing gels as described by O'Farrell (1975). Isoelectric focusing at pH range 5 – 7 or NEPHGE were performed at 400 V for 18 or 4 h, respectively. These one-dimensional gels were equilibrated for 20 min with the following buffer: 0.06 M Tris – HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol and 10% glycerol, placed on top of the second-dimension gels (O'Farrell, 1975) and run at a constant current of 30 mA. Gels were then fixed and stained with Coomassie brilliant blue R-250.

β-1,3-Glucanase enzyme assays

15 μg of crude protein extracts were used for the assays. β-1,3-Glucanase activity was measured using the method described by Somogyi (1952) and Nelson (1957). Direct assays for β-1,3-glucanase activity were performed on IEF gels as described by Pan *et al.* (1989). In both cases, β-1,3-glucanase activity was determined by detection of reducing sugars released after incubation with the substrate laminarin.

Immunolocalization of GN1 β-1,3-glucanase

Leaf and root samples from transgenic and untransformed tobacco plants were used for immunocytochemical studies. The procedure was done essentially as described by De Clercq *et al.* (1990). Anti-β-1,3-glucanase serum was used at dilutions of 1:100 and 1:500. Incubation with a rabbit 2S albumin antiserum was done in parallel as a control experiment.

Isolation of nuclei and nuclear run-off transcription assays

Nuclei were isolated from 20 g of leaf tissue as described by Walling *et al.* (1986) and stored in aliquots at −70°C. To label nascent RNA chains, 200 μl (1.7×10^7) of nuclei were used in a transcription reaction containing 100 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.3 μM phosphocreatine, 0.025 μg/μl creatine phosphokinase, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP and 250 μCi [α-^{32}P]UTP (800 Ci/mmol; 20 mCi/ml) in a final volume of 400 μl. The reaction was incubated at 30°C for 20 min. Labelled RNA transcripts were isolated as described by Sambrook *et al.* (1989) except that the NaOH treatment was omitted. Slot blots of linearized plasmids and DNA fragments (50 ng/slot), hybridization, and washing conditions were carried out as described by Sambrook *et al.* (1989).

Labelled RNA transcripts were hybridized to the linearized plasmids containing the *gn1* cDNA and the *nptII* gene in pGEM2. 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 pGEM2 was included as a negative control. In addition, RNA samples were hybridized to a 309 bp *Sst*I and a 389 bp *Pst*I – *Xba*I DNA fragment corresponding to the first intron and 3′ end region downstream from the stop codon of the *gn1* gene, respectively.

Haploid production

Unopened flower buds from R_3 homozygous and F_3 hemizygous T17 plants were collected at the proper stage as described by Nitsch and Nitsch (1969) and kept at 4°C for 3 days. The anthers were then excised and cultured *in vitro* on Murashige and Skoog medium (Murashige and Skoog, 1962) without hormones. Developing plantlets were isolated individually and transferred to kanamycin-containing medium. Chromosome counting was done essentially as described by Schwarzacher *et al.* (1980). The chromosome numbers of regenerated haploids and the original tobacco plants are 24 and 48, respectively.

Acknowledgments

The β-1,3-glucanase antibodies were kindly given by Dr Bernard Fritig (Strasbourg, France). We thank Allan Caplan for helpful discussions and critical reading; Chris Genetello and Riet De Rycke for expert technical assistance; Martine De Cock for help with the manuscript; Karel Spruyt, and Vera Vermaercke for figures and diagrams. This work was supported by grants from the 'ASLK-Kankerfonds', the Services of the Prime Minister (IUAP 12OC0187) and the 'Vlaams Actieprogramma Biotechnologie' (174KP490). F.d.C. and S.K. are indebted to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 200353/90-0) for a predoctoral fellowship and a Koerber Foundation (Hamburg, FRG) grant, respectively; D.I. is a Research Director of the Institut National de la Recherche Agronomique (France).

References

Bol,J.F., Linthorst,H.J.M. and Cornelissen,B.J.C. (1990) *Annu. Rev. Phytopathol.*, **28**, 113–138.

Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248–254.

Carr,J.P. and Klessig,D.F. (1990) In Setlow,J.K. (ed.), *Genetic Engineering, Principles and Methods*. Plenum Press, New York, Vol. 11, pp. 65–109.

Casadaban,M.J. and Cohen,S.N. (1980) *J. Mol. Biol.*, **138**, 179–207.

Castresana,C., de Carvalho,F., Gheysen,G., Habets,M., Inzé,D. and Van Montagu,M. (1990) *Plant Cell*, **2**, 1131–1143.

Coté,F., Cutt,J.R., Asselin,A. and Klessig,D.F. (1991) *Mol. Plant–Microbe Interactions*, **4**, 173–181.

Deblaere,R., Bytebier,B., De Greve,H., Deboeck,F., Schell,J., Van Montagu,M. and Leemans,J. (1985) *Nucleic Acids Res.*, **13**, 4777–4788.

De Block,M., Herrera-Estrella,L., Van Montagu,M., Schell,J. and Zambryski,P. (1984) *EMBO J.*, **3**, 1681–1689.

De Clercq,A., Vandewiele,M., De Rycke,R., Van Damme,J., Van Montagu,M., Krebbers,E. and Vandekerckhove,J. (1990) *Plant Physiol.*, **92**, 899–907.

De Loose,M., Alliotte,T., Gheysen,G., Genetello,C., Gielen,J., Soetaert,P., Van Montagu,M. and Inzé,D. (1988) *Gene*, **70**, 13–23.

Dellaporta,S.L., Wood,J. and Hicks,J.B. (1983) *Plant Mol. Biol. Rep.*, **1**, 19–21.

Elkind,Y., Edwards,R., Mavandad,M., Hedrick,S.A., Ribak,O., Dixon,R.A. and Lamb,C.J. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9057–9061.

Fehr,W.R. (1987) *Principles of Cultivar Development*, Vol. 1 (Theory and Technique). MacMillan Publishing Company, New York.

Felix,G. and Meins,F. Jr (1986) *Planta*, **167**, 206–211.

Felix,G. and Meins,F. (1987) *Planta*, **172**, 386–392.

Figurski,D.H. and Helinski,D.R. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1648–1652.

Gianinazzi,S., Martin,C. and Vallée,J.C. (1970) *C. R. Acad. Sci. Paris D*, **270**, 2383–2386.

Goring,D.R., Thomson,L. and Rothstein,S.J. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 1770–1774.

Grierson,D., Fray,R.G., Hamilton, A.J., Smith,C.J.S. and Watson,C.F. (1991) *Trends Biotechnol.*, **9**, 122–123.

Hennig,J., Dewey,R.E., Cutt,J.R. and Klessig,D.F. (1991) In Hallick,R.B. (ed.), *Program and Abstracts* (Third International Congress of Plant Molecular Biology on 'Molecular Biology of Plant Growth and Development', 6–11 October 1991, Tucson, AZ USA). University of Arizona, Tucson, #1137.

Hurkman,W.J. and Tanaka,C.K. (1986) *Plant Physiol.*, **81**, 802–806.

Jones,J.D.G., Dunsmuir,P. and Bedbrook,J. (1985) *EMBO J.*, **4**, 2411–2418.

Jorgensen,R. (1990) *Trends Biotechnol.*, **8**, 340–344.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Lotan,T., Ori,N. and Fluhr,R. (1989) *Plant Cell*, **1**, 881–887.

Matzke,M.A. and Matzke,A.J.M. (1991) *Plant Mol. Biol.*, **16**, 821–830.

Matzke,M.A., Primig,M., Trnovsky,J. and Matzke,A.J.M. (1989) *EMBO J.*, **8**, 643–649.

Mazur,B.J. and Chui,C.-F. (1985) *Nucleic Acids Res.*, **13**, 2373–2386.

Memelink,J., Linthorst,H.J.M., Schilperoort,R.A. and Hoge,J.H.C. (1990) *Plant Mol. Biol.*, **14**, 119–126.

Mohnen,D., Shinshi,H., Felix,G. and Meins,F., Jr (1985) *EMBO J.*, **4**, 1631–1635.

Mol,J., Van Blokland,R. and Kooter,J. (1991) *Trends Biotechnol.*, **9**, 182–183.

Murashige,T. and Skoog,F. (1962) *Physiol. Plant.*, **15**, 473–497.

Napoli,C., Lemieux,C. and Jorgensen,R. (1990) *Plant Cell*, **2**, 279–289.

Nelson,N.J. (1957) *Methods Enzymol.*, **3**, 85–86.

Neuhaus,J.-M., Ahl-Goy,P., Hinz,U., Flores,S., Meins,F., Jr (1991) *Plant Mol. Biol.*, **16**, 141–151.

Nitsch,J.P. and Nitsch,C. (1969) *Science*, **163**, 85–87.

O'Farrell,P.H. (1975) *J. Biol. Chem.*, **250**, 4007–4021.

Pan,S.-Q., Ye,X.-S. and Kuć,J. (1989) *Anal. Biochem.*, **182**, 136–140.

Potrykus,I., Paszkowski,J., Saul,M.W., Petruska,J. and Shillito,R.D. (1985) *Mol. Gen. Genet.*, **199**, 169–177.

Poulsen,C., Fluhr,R., Kauffman,J.M., Boutry,M. and Chua,N.-H. (1986) *Mol. Gen. Genet.*, **205**, 193–200.

Rao,R.N. and Rogers,S.G. (1979) *Gene*, **7**, 79–82.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Somogyi,M. (1952) *J. Biol. Chem.*, **195**, 19–23.

Schwarzacher,T., Ambros,P. and Schweizer,D. (1980) *Plant Syst. Evol.*, **134**, 293–297.

Shinshi,H., Mohnen,D. and Meins, F.J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 89–93.

Smith,C.J.S., Watson,C.F., Bird,C.R., Ray,J., Schuch,W. and Grierson,D. (1990) *Mol. Gen. Genet.*, **224**, 477–481.

Van den Bulcke,M., Bauw,G., Castresana,C., Van Montagu,M. and Vandekerckhove,J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 2673–2677.

van der Krol,A.R., Mur,L.A., Beld,M., Mol,J.N.M. and Stuitje,A.R. (1990) *Plant Cell*, **2**, 291–299.

van Loon,L.C. and van Kammen,A. (1970) *Virology*, **40**, 199–211.

Vögeli,U., Meins,F.Jr and Boller,T. (1988) *Planta*, **174**, 364–372.

Vögeli-Lange,R., Hart,C., Nagy,F. and Meins,F. (1991) In Hallick,R.B. (ed.), *Program and Abstracts* (Third International Congress of Plant Molecular Biology on 'Molecular Biology of Plant Growth and Development', 6–11 October 1991, Tucson, AZ USA). University of Arizona, Tucson, #288.

Walling,L., Drews,G.N. and Goldberg,R.B. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2123–2127.

Zhu,J., Kempenaers,W., Van Der Straeten,D., Contreras,R. and Fiers,W. (1985) *Bio/technology*, **3**, 1014–1016.

Received on February 17, 1992; revised on April 2, 1992