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Specific expression of the tobacco Tnt1 retrotransposon in protoplasts

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The Tnt1 transposable element of tobacco belongs to the retrotransposon family and shares the structural features of viral retroelements including two long terminal repeats (LTRs) which are known to contain promoter regions. We show that two Tnt1 RNAs of 5.2 and 6.5 kb can be found. The 5.2 kb RNA matches with the size of the Tnt1 elements so far isolated (5.3 kb), whilst the evidence suggests that the 6.5 kb RNA could be a chimaeric RNA initiated in a gene in which Tnt1 has inserted. The Tnt1 5.2 kb RNA starts in the LTR, and the LTR can promote the expression of a translational LTR-β-glucuronidase (GUS) fusion at a high level in transient expression assays. The Tnt1 5.2 kb RNA and the LTR-GUS fusion of transgenic tobacco plants are specifically expressed in leaf-derived protoplasts whereas they are not expressed in leaf tissue. The 5.2 kb RNA is also transcribed at low levels in roots. This RNA is induced after 2 h of maceration in the protoplast isolation medium, and its level declines rapidly after protoplast isolation. The induction requires only the presence of cell wall hydrolases, and is independent of wounding and plasmolysis. The induction of Tnt1 expression is not mediated by typical oligosaccharide elicitors released from the cell wall known to mediate defense gene responses. Tnt1 transcription features provide a first example of tissue culture-induced mutagenesis in plants and a molecular basis for some of the somaclonal variation events.

Key words: LTR/protoplasts/retrotransposon/RNA/tobacco

Introduction

Two major categories of transposable elements have been found in eukaryotic organisms. One class of elements transposes via a DNA intermediate, through excision or replication, and is found in both prokaryotic and eukaryotic kingdoms. The second one, so far restricted to eukaryotes and known as retroposons, has structural features required for transposition via an RNA intermediate through the reverse flow of genetic information (Weiner *et al.*, 1986). Of these, two groups can be defined. One group (retrotransposons or viral-like retroposons) is composed of elements structurally similar to integrated retroviruses (see Varmus and Brown, 1989). These elements have long terminal repeats (LTRs) at their termini and contain long open reading frame(s) resembling the gag and pol retroviral domains and encoding a potential reverse transcriptase. The retrotransposons copia from *Drosophila melanogaster* (Bingham and Zachar, 1989) and Ty from *Saccharomyces cerevisiae* (Boeke, 1989) have been well studied, and transposition via a retroviral-like mechanism has been demonstrated in the case of Ty (Boeke *et al.*, 1985). The second group (non-viral retroposons) comprises heterogeneous sequences lacking LTRs, but presenting homologies with reverse transcriptase and having a poly(A) tract at their 3′ end (Weiner *et al.*, 1986).

In higher plants, numerous transposable elements of the first class have been identified (Weil and Wessler, 1990). Only recently, plant retroposons have been described and most of them belong to the retrotransposon family (Johns *et al.*, 1985; Harberd *et al.*, 1987; Voytas and Ausubel, 1988; Smyth *et al.*, 1989; Grandbastien *et al.*, 1989).

The Tnt1 retrotransposon found in tobacco (*Nicotiana tabacum*) is the first transposable element identified in this plant species, where it is present at a high copy number (at least 100 copies per haploid genome). It was isolated after its transposition into the coding sequence of the nitrate reductase (NR) structural gene (Grandbastien *et al.*, 1989). Transposition events have been detected through *in vitro* selection of spontaneous NR-deficient mutant lines in cell cultures derived from tobacco leaf protoplasts. Two insertions of 5.3 kb have been isolated. Sequence analysis showed that Tnt1 belongs to the retrotransposon family and that it is closely related to the copia element. Typical features of retrotransposons were observed, such as two perfect 610 bp long terminal repeats and a single 3984 bp open reading frame typically organized in gag-pol functional domains. Transposition of Tnt1 into the NR gene strongly suggests that it is active. Since retrotransposon activity requires the synthesis of an RNA intermediate, the study of Tnt1 expression in tobacco thus constitutes a preliminary step in the understanding of Tnt1 activity.

A general understanding of the expression of animal and yeast retroviral elements has already emerged during the past ten years. RNA substrates for retrotransposition start in the 5′ LTR and end in the 3′ LTR (Boeke, 1989; Varmus and Brown, 1989), and therefore LTRs can be functionally defined as both promoter and terminator elements, and they can be used to direct the expression of a reporter gene.

Most well-studied retrotransposons are transcribed at relatively high levels (Flavell *et al.*, 1980; Schwartz *et al.*, 1982; Boeke, 1989). Their transcription is modulated by a number of environmental and physiological factors, such as steroid hormones (Ziarczyk *et al.*, 1989), stress, including heat shock (Strand and McDonald, 1985), cAMP level (Yun and Davis, 1989), and DNA damaging agents like UV light (Bradshaw and McEntee, 1989). Furthermore, RNAs of a number of *Drosophila* retrotransposons are also developmentally regulated (Schwartz *et al.*, 1982; Parkhurst and Corces, 1987), and Ty transcription is under mating-type

control (Boeke, 1989). This indicates that retrotransposon expression is controlled by cellular genes. In addition, transcription of most *Drosophila* retrotransposons is activated by cell culture (Bingham and Zachar, 1989). This finding is of particular interest since tissue culture is also reported to activate DNA-mediated transposition of elements in higher plants (Lee and Phillips, 1988). Cell culture activation of transposable elements is thus assumed to be a general feature.

There are no reports yet about expression of retrotransposons in higher plants. The aim of this work was to analyse Tnt1 expression in tobacco by measuring Tnt1 RNA levels, and testing the ability of the LTR to direct the expression of a reporter β-glucuronidase (GUS) gene both in transient assays and in transgenic plants containing this construct. We investigated the tissue specificity of Tnt1 expression, and we tested the inducibility of this expression by stresses such as protoplast isolation, wounding and plasmolysis.

Results

Analysis of Tnt1 RNA expression in tobacco

The presence of Tnt1 RNA was investigated in leaf tissue and protoplasts derived from both the *tl* line, from which NR-deficient mutants resulting from Tnt1 insertion were derived, and the wild-type (WT) line (Figure 1). No difference between *tl* and WT lines could be detected in the expression pattern of Tnt1 related transcripts. However, two RNA species of 6.5 kb and 5.2 kb could be observed in protoplasts. The expected size for a full length Tnt1 RNA, which can be used as a template for Tnt1 retrotransposition, should be close to 5 kb. The 5.2 kb transcript is, therefore, the only one to match with this expected size. It cannot be excluded, however, that larger copies of Tnt1-related sequences, expressing the 6.5 kb RNA, exist in the genome. Both RNAs were present at comparable levels in protoplasts, whether these were isolated in the presence or in the absence of typical hormones used for cell culture (naphthalene acetic acid and benzyladenine). In leaf tissue, however, only the 6.5 kb RNA could be detected with longer exposure time of the autoradiogram, as shown in Figure 3. This RNA species was expressed at higher levels when plants were cultivated under greenhouse conditions (Pouteau *et al.*, 1991) instead of growing them in a growth chamber with low light intensity, whereas the 5.2 kb RNA remained undetectable in all conditions. When leaf tissue was placed under protoplast isolation conditions, the 5.2 kb RNA was rapidly induced. Figure 2 shows that the 5.2 kb RNA could be detected after a 2 h maceration period, whereas no clear induction occurred in the same time for the 6.5 kb RNA. When the maceration was extended to longer periods, the 5.2 kb RNA level decreased and was hardly detectable after 3 days, whilst the 6.5 kb RNA level was maintained for 4 days.

Tnt1 RNA expression was also investigated in different tissues of WT tobacco plants (Figure 3). The 6.5 kb RNA was detectable in most tissues, but always at much lower levels than in protoplasts. The 5.2 kb RNA could only be detected at low levels in roots and was also visible in tobacco hairy roots after *Agrobacterium rhizogenes* infection (data not shown).

It can be concluded, therefore, that the 5.2 kb and 6.5 kb RNAs are differentially regulated. To explain these differences, it could be proposed that two subfamilies of Tnt1 related sequences have diverged quite early and acquired specific controls. We suggest that the 6.5 kb RNA is a chimaeric transcript, resulting from Tnt1 insertion into a gene

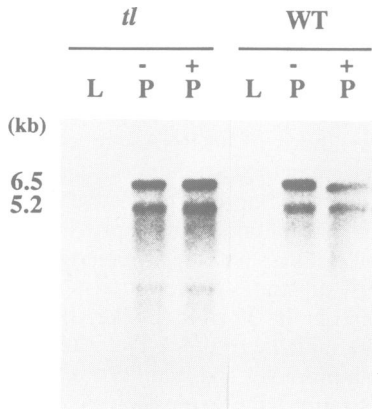

Fig. 1. Northern analysis of Tnt1 RNA in *tl* and WT tobacco lines. Total RNA (8 μg) from either leaf tissue (L) or leaf-derived protoplasts (P) prepared in the absence (−) or in the presence (+) of hormones (naphthalene acetic acid and benzyladenine) was separated in a formaldehyde−agarose (1%) gel and transferred to a Hybond N nylon membrane (Amersham). Hybridization was done with a ^{32}P-labelled *Bgl*II DNA fragment corresponding to the Tnt1 gag domain. (Hybridization with different DNA fragments of the pol domain and of the LTR gave the same results.) Equal loading of the samples in the different lanes was controlled on the basis of ethidium bromide staining of the RNAs.

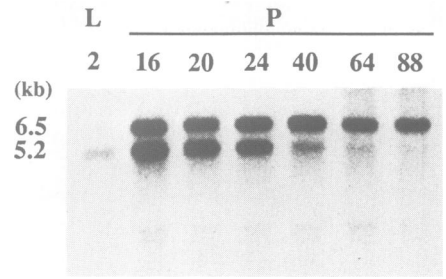

Fig. 2. Fate of Tnt1 RNAs in WT tobacco line during extended periods of protoplast maceration. Leaf tissues were macerated in standard protoplast isolation medium for the indicated periods (in hours). Total RNA (12 μg) from leaf tissue (L) and from protoplasts (P) was analysed as described in the legend to Figure 1.

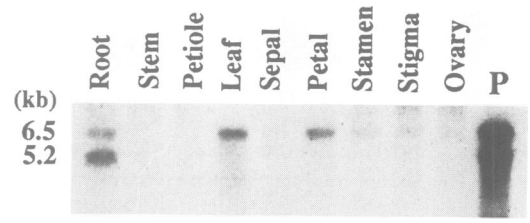

Fig. 3. Analysis of tissue specificity of Tnt1 RNAs in WT tobacco. Root tissue was collected on plants grown aeroponically. Other tissues were collected on plants cultivated in the same conditions as for protoplast isolation. Total RNA [16 μg for the different tissues and 8 μg for the protoplast control (P)] was analysed as described in the legend to Figure 1.

directing the expression of the hybrid RNA (see the Discussion).

Determination of Tnt1 RNA 5′ end(s)

Transcription starts of the Tnt1 RNA expressed in protoplasts were mapped by primer extension analysis (Figure 4). Two initiation sites were found within the LTR. A major one is located at nt 234−236 and a minor one is located at nt 467−469. The two site environments are compatible with what is known about plant promoters (Joshi, 1987) or animal and viral promoters, although no CAAT boxes were observed in Tnt1 (Breathnach and Chambon, 1981). TATA boxes could be identified at consensus distances upstream from the two sites (Figure 4b), at nt 204 for the most upstream one and nt 434 for the second one (with regard to the first T of TATA boxes).

No signal was detected with leaf tissue RNA containing the 6.5 kb RNA (data not shown). This suggests that only the 5.2 kb RNA starts in the LTR, and that the 6.5 kb RNA starts far outside of the LTR. It is also possible, however, that the 6.5 kb RNA sequence is very divergent from the sequence of the Tnt1 elements isolated so far, at least in the region of annealing of both oligonucleotides used in the primer extension analysis.

Transient expression of the LTR−GUS fusion in protoplasts

A translational fusion of the GUS reporter gene with the Tnt1 LTR was constructed. Constructs with the GUS coding sequence fused to the cauliflower mosaic virus (CaMV) 35S promoter (Jefferson *et al.*, 1987) or to its derivative containing a duplicated enhancer region, the 70S promoter (Kay *et al.*, 1987), were used as controls. It should be remembered that, in contrast with the 35S−GUS and the 70S−GUS constructs, the LTR−GUS construct results from a translational fusion of the GUS coding sequence with the LTR and the adjacent sequence of the Tnt1 central region including 25 amino acids of the N-terminal part of the polyprotein sequence. The three plasmids were electroporated in parallel into protoplasts of both WT and *tl* tobacco

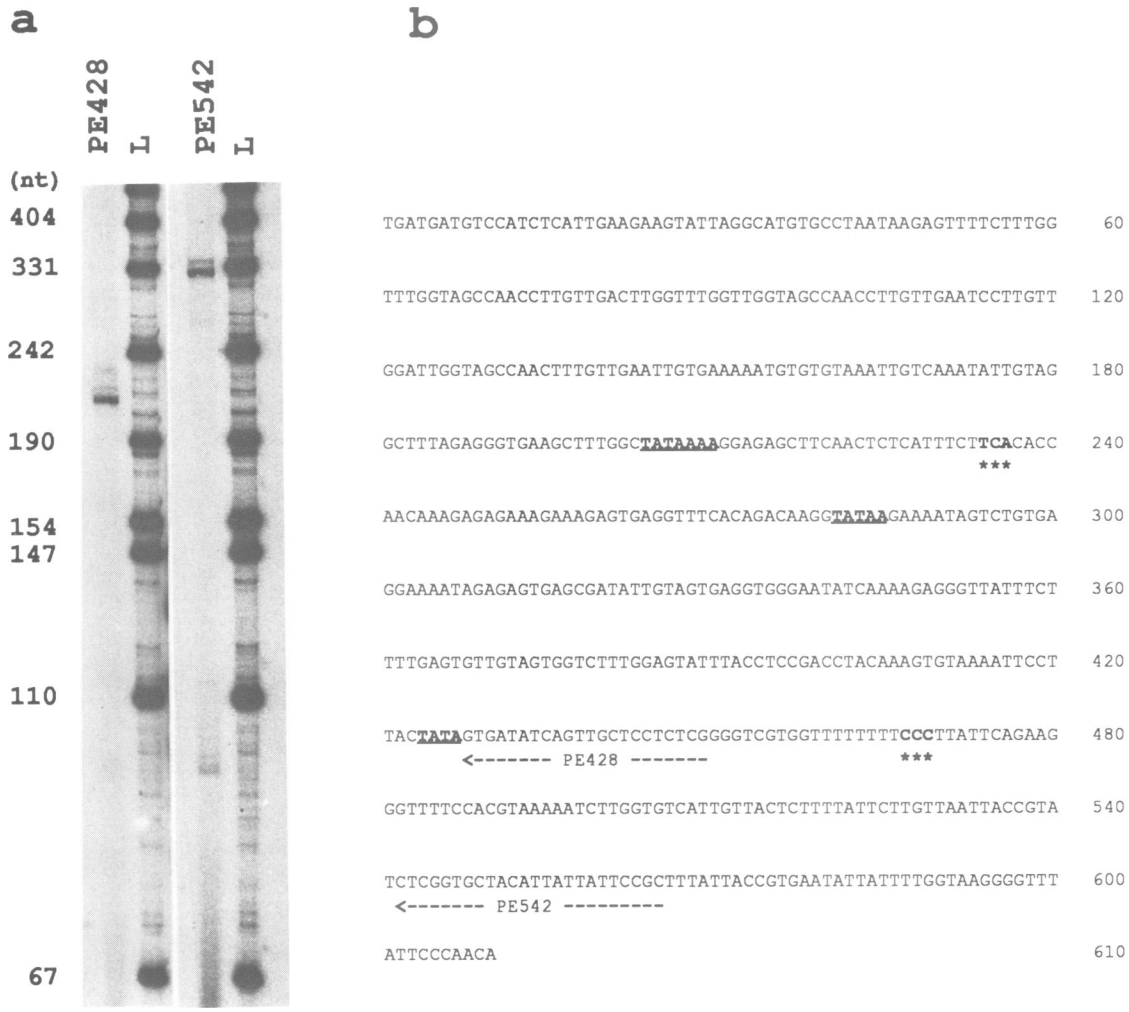

Fig. 4. Determination of the 5′ ends of Tnt1 RNAs by primer extension. (**a**) Total RNA (20 μg) from WT tobacco leaf tissue, macerated overnight in T0 medium in the presence of cell wall degrading enzymes, was annealed at 50°C for 3 h in 50 mM Tris−HCl pH 8.3, 0.3 M KCl with ^{32}P-kinased PE542 and PE428 oligonucleotides. Primer extension was carried out with 200 U of M-MLV reverse transcriptase (Bethesda Research Laboratories) following the instructions of the manufacturer. After denaturation, samples were separated on a 7 M urea 6% acrylamide gel. DNA molecular weight markers (L) were obtained by ^{32}P-labelling an *Hpa*II digest of pBluescript SK$^+$ (Stratagene). (**b**) LTR sequence of the Tnt1 element isolated from mutant h9-Nia4. The positions of the PE542 and PE428 oligonucleotides are shown by arrows. Asterisks indicate the positions of Tnt1 RNA 5′ ends. The position of the most upstream 5′ end was verified by Sanger sequencing. Corresponding TATA boxes are underlined.

lines. Table I shows the results of GUS transient expression assays.

Protoplast electroporation of the three different plasmids was much less efficient in the *tl* line than in the WT line. This can be attributed, at least partially, to the difference in protoplast morphology observed between these two lines. The *tl*-derived protoplasts were effectively much smaller than the WT-derived protoplasts, probably because they were isolated from leaf tissue from plants grown in the greenhouse instead of growing in a growth chamber under low light intensity. Despite the discrepancy of electroporation efficiency between the two lines, the ratios of GUS activities obtained with the three plasmids after a one day cultivation were similar. GUS expression was much higher under the control of the Tnt1 LTR than under the control of the 35S and 70S promoters, giving 30-fold and 20-fold lower expression in WT protoplasts, and 16-fold and 8-fold lower expression in *tl* protoplasts, respectively. GUS activity expressed by the 70S−GUS construct was about two-fold higher than GUS activity expressed by the 35S−GUS construct.

We also analysed the accumulation of GUS activity after a second day of protoplast cultivation after electroporation. Although GUS activity did not accumulate further for the three constructions in *tl* protoplasts, higher accumulation for both the 35S−GUS and 70S−GUS constructs was found in WT protoplasts. In contrast, GUS activity did not accumulate significantly in WT protoplasts when the GUS gene was expressed under the control of the Tnt1 LTR. This result correlates well with the decrease of the Tnt1 5.2 kb RNA level observed under the same conditions (data not shown).

Table I. GUS activity in electroporated WT and *tl* protoplasts cultivated for 1 or 2 days

DNA electroporated		GUS activity			
		WT		*tl*	
		GUS units	day 2/day 1	GUS units	day 2/day 1
LTR−GUS	day 1	24632		410	
	day 2	39315	1.6	677	1.7
35S−GUS	day 1	806		25	
	day 2	2002	2.5	34	1.8
70S−GUS	day 1	1207		49	
	day 2	3578	3.0	83	2.0
no DNA	day 1	12		14	

The plasmids electroporated are pBMCV 102120 for the LTR−GUS construct, pBI 121 for the 35S−GUS construct and pCH1 for the 70S−GUS construct. GUS units are expressed as pmol MU produced per min per mg protein. The ratio day 2/day 1, expressing the degree of GUS activity accumulation, was calculated after subtraction of the background level. Experiments were done three times for day 1, and twice for day 2.

Expression of LTR−GUS fusion in transgenic plants

The LTR−GUS translational fusion was transferred to both *tl* and WT × *tl* hybrid tobacco lines. Three to five insertions were detected in transgenic plants by Southern analysis using a GUS gene specific probe (data not shown).

Table II shows GUS activities detected in leaf tissue or in leaf-derived protoplasts from several primary transformants. A very low activity was detected in leaf tissue, but nearly half of the transgenic plants analysed (transformants 4−9) expressed GUS activity in freshly isolated leaf-derived protoplasts. For these plants, induction resulting from protoplastization was between 20- and 90-fold (transformant no. 7). No clear correlation between the level of expression in protoplasts and the number of copies of LTR−GUS fusions in the transgenic plants could be observed. In three transformants (transformant nos 1, 7 and 9), accumulation of GUS activity in protoplasts was tested after 24 h of culture. No significant accumulation of GUS activity was observed, as in the GUS transient expression assay. This was true even for transformants 7 and 9 which were the most inducible ones.

Further insights into Tnt1 5.2 kb RNA expression characteristics

We were interested by the factors that influence Tnt1 5.2 kb RNA expression during protoplast isolation. The protoplast isolation process constitutes at least a triple stress for the

Table II. GUS activity in leaf tissue and leaf-derived protoplasts from plants transformed with the LTR−GUS construct

Plant genotype		GUS activity		Induction factor
		In leaf tissue (L)	In protoplasts (P)	(P/L)
Non-transformed *tl*		1.0	2.6 (3.3)	2.6
tl transformant	1	6.4	39 (58)	6.1
	2	4.6	4.4	1
	3	4.5	25	5.6
	4	6.7	174	26
	5	4.0	198	50
WT × *tl* transformant	6	2.0	61	30
	7	2.1	190 (200)	90
	8	5.3	192	36
	9	2.0	115 (98)	58
	10	4.3	6.5	1.5
	11	2.7	12.4	4.6
	12	8.0	39	4.8

The plasmid pBMCV 102106, carrying this construct, was introduced into plants of both *tl* and heterozygote WT × *tl* tobacco lines via *Agrobacterium tumefaciens* transformation. GUS activity in protoplasts was measured immediately after isolation or, for the values indicated in brackets, after 24 h culture. GUS activity is expressed in pmol MU produced per min per mg of protein.

leaf cells: firstly, plasmolysis resulting from an osmotic shock; secondly, wounding and thirdly, cell wall digestion. Both wounding and cell wall digestion simulate a pathogen attack. We therefore attempted to determine which steps of the protoplast isolation process were involved in the 5.2 kb Tnt1 RNA induction. Plasmolysis, shredding and cell-wall hydrolysis, were tested either separately or together (Figure 5). Plasmolysis (with 8% mannitol and 2% sucrose), either alone or combined with shredding, did not result in detectable 5.2 kb RNA induction in leaf tissue. Neither osmotic status nor wounding seems, therefore, to be directly responsible for 5.2 kb RNA expression. Addition of cell wall hydrolases, with or without shredding, always resulted in the 5.2 kb RNA induction. Presence of these enzymes appeared to be the only requirement for the induction.

Cell wall hydrolases are a mixture of polygalacturonase activities, which are required for the cell−cell dissociation, and of cellulase activities, for degradation of the cell wall. We attempted to see whether one of the three hydrolase sources alone could induce comparable levels of the 5.2 kb RNA. The results are presented in Figure 6A. The strongest response was obtained with Onozuka, especially when combined with Macerozyme or Driselase. Driselase, alone or together with Macerozyme, did not induce 5.2 kb RNA expression, and Macerozyme alone gave a slight response. Little is known about the composition of the different hydrolase sources used in protoplast isolation. Macerozyme is essentially composed of polygalacturonase activities, whereas Onozuka and Driselase are rather crude extracts containing very high cellulase activity. Onozuka also contains some polygalacturonase activities, leading to a partial protoplast release. The other two preparations, alone or together, did not result in any protoplast release. Therefore, the pattern of Tnt1 5.2 kb RNA induction appears to be linked with the level of protoplast release, except in the case of Macerozyme alone. This observation raised the possibility that Tnt1 RNA expression could be mediated by elicitors present in the medium.

For numerous defense genes coding for wound-induced or pathogenesis related proteins, induction of the expression has been demonstrated to be due to specific oligosaccharides released from the cell wall (Albersheim and Darvill, 1985). We thus tested the presence of such oligosaccharide inducers in the maceration media by assaying the elicitor effect of these media after heat denaturation of the enzymes. Figure 6B shows that no induction of Tnt1 5.2 kb RNA could be detected by the procedure applied. This suggests that the cell wall hydrolase-dependent expression of Tnt1 is not mediated by oligosaccharide elicitors.

Discussion

A full length Tnt1 RNA is specifically expressed in protoplasts

Two RNAs homologous to a Tnt1 probe, 6.5 kb and 5.2 kb long, are expressed in tobacco. Only the 5.2 kb RNA has the expected size for a full length Tnt1 transcript, according to the size of the two tobacco Tnt1 copies isolated so far (Grandbastien *et al.*, 1989). The two RNAs are not regulated in the same way. The 6.5 kb RNA is detectable in leaf tissue and to a greater or lesser extent in other tissues. The 5.2 kb RNA can only be detected at low levels in roots, and this RNA species appears early during the process of protoplast isolation. It also declines rapidly during prolonged maceration periods. The 6.5 kb RNA is induced later than the 5.2 kb RNA and is maintained for at least 4 days. The differential expression pattern of the 6.5 kb RNA probably does not reflect the expression of a second subfamily of larger Tnt1 related sequences, but rather results from the expression of a chimaeric transcript directed by the promoter of a gene in which Tnt1 has inserted. This assumption can be supported by two facts. Firstly, such chimaeric transcripts have already been observed in the case of Tnt1 insertional mutants of the NR structural gene (Pouteau *et al.*, 1991). Secondly, retroviral transcripts initiated in the 5′-flanking region and transcribed through the viral LTR have already been reported for proviruses integrated in close proximity

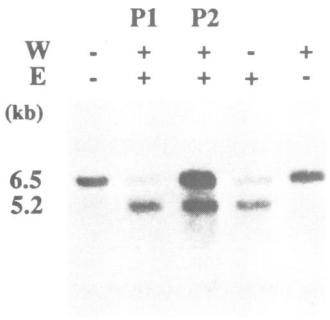

Fig. 5. Effect of the different steps of protoplast isolation on Tnt1 5.2 kb RNA induction. Leaf tissues were incubated in standard protoplast isolation conditions except that shredding (W) of the tissues and addition of the cell wall degrading enzymes (E) were done or not (+/−). Total RNA (8 μg) from leaf tissue, or from protoplasts when the complete treatment was done (P1 and P2 correspond to two independent samples), was analysed as described in the legend to Figure 1.

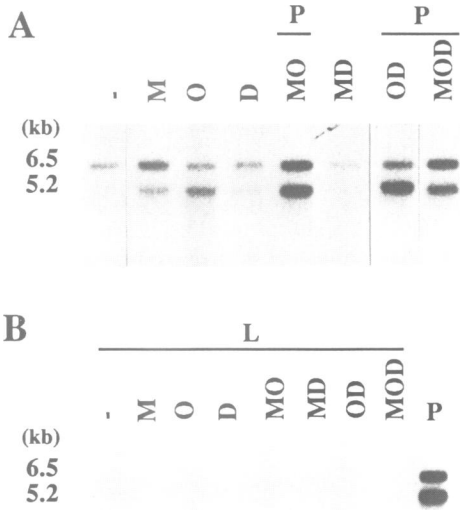

Fig. 6. Effect of the cell wall hydrolases and elicitor assay of the different cell wall hydrolases media on Tnt1 5.2 kb RNA induction. (**A**) Leaf tissues were macerated in standard protoplast isolation conditions except that the different hydrolases were added separately as indicated: M, Macerozyme; O, Onozuka; D, Driselase. (**B**) Maceration media from the experiment described in (A) were heat-denatured and re-used for overnight maceration of fresh leaf tissues. Total RNA (15 μg) from leaf tissue, or from protoplasts when indicated (P), was analysed as described in the legend to Figure 1.

to active cellular promoters (Peckham *et al.*, 1989). Further evidence is provided by the fact that, in the ancestral tobacco parent *N.sylvestris*, in which genomic Tnt1 related sequences are present at a high copy number (Tnt1 homologous sequences are almost undetectable in the other ancestral tobacco parent *N.tomentosiformis*), only the 5.2 kb RNA species, and not the 6.5 kb RNA, is detected (M.C.Criqui, personal communication). We thus consider that the 5.2 kb RNA is the only Tnt1 specific transcript.

Tnt1 RNA is initiated within the LTR

Two transcriptional initiation sites, a major one and a minor one (based on the intensity of bands) at nucleotide positions 234–236 and 467–469 respectively, are used within the LTR, demonstrating that Tnt1 elements are transcriptionally functional. A TATA box sequence is found at a consensus distance upstream from each site (nt 204 and 434 respectively). In most retroviruses, polyadenylation signals are located downstream from the initiation site of transcription within the LTR (Coffin and Moore, 1990). In some retroviruses, however, the polyadenylation signal lies upstream of the initiation site of transcription. In the LTR of Tnt1, no clear-cut AATAAA polyadenylation signals can be found downstream or even upstream of both initiation sites of transcription. The position and the length of the terminally repeated sequence that should be found at both ends of the Tnt1 RNA template for transposition, therefore, are still unknown. However, at least the most upstream start site of transcription should produce Tnt1 genomic RNAs. Currently, we are investigating whether both initiation sites of transcription could result in RNA substrates for Tnt1 retrotransposition events.

Tnt1 LTR efficiently promotes GUS expression in protoplasts

A translational fusion of Tnt1 LTR with the GUS reporter gene is transiently expressed in leaf-derived protoplasts. The expression level was much higher than the levels observed with the 35S and 70S CaMV promoters (about 20- and 10-fold higher respectively). Both CaMV promoters are known to be highly active in plants (Jefferson *et al.*, 1987; Kay *et al.*, 1987). The higher level of GUS expression observed with the LTR–GUS construct cannot be definitively attributed to a direct transcriptional effect. Support for a transcriptional effect is provided by the finding that comparisons between transcriptional and translational fusions of the GUS coding sequence with the promoters of the glutamine synthetase and the phenylalanine ammonia-lyase genes did not show any significant difference for the expression of GUS activity (Bevan *et al.*, 1989; Forde *et al.*, 1989). However, the influence of a translational enhancer on the efficiency of GUS translation has been already reported (Gallie *et al.*, 1989). Such a translational enhancer could be present in the leader sequence or interspersed within the first 25 amino acids of the Tnt1 coding sequence. The comparison between the nucleotide environment of the translation start sites of Tnt1 and GUS coding sequences and the consensus sequence of translation start sites in plants (Joshi, 1987) suggests that the Tnt1 translation start site could be used more efficiently than the GUS genuine start site. The resulting chimaeric protein could be stabilized or have modified kinetic properties due to the N-terminal addition of 25 amino acids of Tnt1.

Several LTR–GUS fusion transgenic plants have been obtained, but in none of them could GUS activity be found at significant levels in leaf tissue. However, after protoplast isolation, half of the transgenic plants showed significant induction of GUS expression. Neither in these plants nor in transient assays did GUS activity accumulate during continued protoplast culture. This protoplast specificity of expression is completely correlated with the observed RNA levels, suggesting that the LTR and the 5.2 kb RNA are identically regulated.

These findings demonstrate that the Tnt1 LTR is functional as a promoter unit, and that it is probably an efficient promoter in transient assays. Since transcription is one of the major regulation steps for Tnt1 transposition, transposition events may occur at a high rate in such conditions. We are currently addressing ourselves to the determination of the relative importance of transcription and translation for the generation of high levels of GUS activity in transient assays.

Tnt1 expression is not specifically associated with the tl somatic instability phenotype

The original aim of this work was the isolation of a transposable element thought to be responsible for the chlorophyllian somatic instability displayed by the mutant tobacco line *tl* (Deshayes, 1979). For this purpose, NR-deficient mutants were therefore selected from haploid NR monogenic *tl* lines (Grandbastien *et al.*, 1989). Only three of the insertional mutations obtained have been analysed yet, and all three result from Tnt1 insertion. Our results show that no difference between the *tl* mutant and the WT tobacco lines exists for Tnt1 expression, whether at the RNA level or in GUS transient expression assays. Since retrotransposition is at least partially controlled at the transcriptional level (Curcio *et al.*, 1990), we suggest that Tnt1 transposition activity is similar in both *tl* and WT lines. In addition, the instability of the *tl* mutation takes place in developing leaves (Deshayes, 1979), where Tnt1 is not significantly transcribed. This supports our hypothesis that the high somatic instability of the *tl* mutation is unrelated to Tnt1 transposition activity. The possibility that Tnt1 could not be responsible for the *tl* mutation is also supported by the fact that transposition and excision are independent events for retrotransposons, and that retrotransposons are known to excise at very low frequencies.

Tnt1 expression is not under the control of typical stress responses through oligosaccharide release

Separation of the different factors involved in the protoplast isolation procedure showed that Tnt1 5.2 kb RNA expression requires the presence of cell wall hydrolases. In contrast, wounding and plasmolysis did not result in substantial induction of expression. Abscissic acid (ABA), which has been shown to mediate osmotic shock response (Skriver and Mundy, 1990), did not give any response when applied on leaf tissue (data not shown). Separation of the three different hydrolase sources showed that Tnt1 5.2 kb RNA induction correlates with their ability to release protoplasts. That oligosaccharides, released from the cell wall (Albersheim and Darvill, 1985; Ryan, 1988) by the hydrolase activities might be inducers for Tnt1 expression could not be demonstrated by our elicitor assay. Oligosaccharides are also responsible for the plant wound response, and ABA can

replace the oligosaccharide signal for this response (Bowles, 1990). In our experiments, neither wounding nor potential oligosaccharides released in the medium by the cell wall hydrolases, nor ABA application (not shown) resulted in induction of Tnt1 expression. Therefore, Tnt1 expression might not be mediated by typical stress responses through oligosaccharide release. The pattern of expression of the Tnt1 5.2 kb RNA was very different from the pattern of RNA expression in protoplasts for typical stress genes (Grosset *et al.*, 1990). These genes showed a later induction of their RNA expression after protoplast isolation, and their expression was maintained for 3 days. Moreover, wounding rather than cell wall hydrolases was the essential effector for their induction. That Tnt1 5.2 kb RNA was expressed at low levels in roots from plants grown in the greenhouse provides further evidence that mechanisms other than stress can be involved in Tnt1 expression.

Conclusions

Tnt1 expression seems to be confined to very specific conditions, such as freshly isolated protoplasts and roots. This situation is different from that found with *Drosophila* retrotransposons, which are all transcribed both in somatic tissue and at higher levels, in cell culture lines (Bingham and Zachar, 1989). All *Drosophila* retrotransposons have shown extensive amplification in copy number in tissue culture cell lines (Bingham and Zachar, 1989). In addition, Junakovic *et al.* (1988) reported that in cultured *Drosophila* cells, retrotransposition is restricted to the transition from embryo to continuous cell lines. The activation of Tnt1 expression during protoplast isolation and the selection of Tnt1 insertional NR-deficient mutants through tissue culture seem tightly related to the results obtained with *Drosophila*.

In higher plants, activation of transposable elements through tissue culture has been also well documented (Peschke *et al.*, 1987, Planckaert and Walbot, 1989). This is considered to be part of the chromosomal basis of somaclonal variation (Lee and Phillips, 1988). However, it is not known yet whether a particular step of tissue culture is responsible for this activation. Tnt1 expression features thus provide, for the first time in plants, strong evidence that the very first step of tissue culture via protoplasts is involved in the activation of a transposable element.

Tissue culture constitutes one of the stress situations in which the cell genome is submitted to what McClintock (1984) called a genomic shock. This results in a traumatic resetting of the genome. During protoplast isolation, a rapid turnover of the leaf cell program, such as massive RNA degradation, occurs, initiating the resetting of the cell genome. The leaf cell program is rapidly replaced by a new one, which allows the cells to restart division cycles (Fleck *et al.*, 1979, 1980; Meyer, 1985; Grosset *et al.*, 1990). A cell program status sensor may be involved in the mechanism for the protoplast release-dependent expression of Tnt1, which detects either the loss of the leaf cell program or the set-up of the protoplast program itself. From our results, we assume that transgenic plants containing the LTR–GUS fusion will provide useful tools for a better understanding of the specificity of Tnt1 expression at the cellular level. With the eventual aim of using Tnt1 as a new tool for gene-tagging in plants, we can hope that the findings concerning its expression in protoplasts can lead to the set-up of an interesting system of transient transposition.

Materials and methods

Plant material

Plants of *N.tabacum* cv Xanthi, wild type line XHFD8 and of *N.tabacum* cv Samsun, mutant line *tl* (Deshayes, 1979) were grown in a controlled culture room under low light intensity conditions, except for the GUS transient assay with *tl* protoplasts which were from plants grown in the greenhouse. Some of the transformation experiments were done with the heterozygote WT × *tl*, showing an intermediate phenotype between the phenotypes of WT and *tl* lines.

Protoplast isolation and culture

Protoplasts were isolated from fully expanded young leaves according to Chupeau *et al.* (1974). Standard cell wall enzymatic digestions were done overnight (16 h). Longer or shorter incubation periods are indicated. The standard digestion medium was either T0 medium (Bourgin *et al.*, 1979) or GSG medium (Chupeau *et al.*, 1989). No difference was observed between these two media. The standard cell wall hydrolase concentrations were: Macerozyme R10 (Yakult Biochemicals Co., Ltd), 200 mg/l; Onozuka R10 (same origin), 1000 mg/l, and Driselase (Kyowa Hakko Kogyo Co., Ltd) 500 mg/l. Identical concentrations were used for incubations with only one or two of these enzyme sources. Except when indicated, the leaves were shredded before incubation and macerations were done in the absence of plant hormones. When present, the hormones were added at a concentration of 3 mg/l naphthalene acetic acid and 1 mg/l benzyladenine.

Protoplast culture in T0 medium was done as described by Chupeau *et al.* (1974).

RNA isolation and gel blot analysis

Total RNA was prepared according to Verwoerd *et al.* (1989). RNA fractionation by formaldehyde gel electrophoresis, transfer onto Hybond N membranes (Amersham), hybridization, and washes under stringent conditions were essentially done as described in Pouteau *et al.* (1989). An 852 bp *Bgl*II DNA fragment corresponding to the gag domain of the coding sequence of the Tnt1-94 element cloned from the h9-Nia4 mutant (Grandbastien *et al.*, 1989) was radiolabelled with the multiprime DNA labelling system (Amersham) and used as a probe.

Primer extension analysis

Since more than 100 Tnt1 copies are present in the tobacco genome (Grandbastien *et al.*, 1989), and might give a high background level in this experiment, total DNA-free RNA was prepared by the CsCl–guanidine thiocyanate gradient method, according to Galangau *et al.*, (1988). RNA was from intact WT tobacco leaves macerated overnight in the presence of cell wall hydrolases in T0 medium. The sequence and position of the two primers used are indicated in Figure 4. Primer extension experiments, with 10 and 20 μg of total RNA, were carried out essentially as described by Boorstein and Craig (1989).

Constructs and transgenic plants

An 800 bp *Sal*I–*Bgl*II fragment from Tnt1, containing the complete LTR and a sequence coding for the first 25 amino acids of the open reading frame, was cloned into the *Sal*I–*Bam*HI sites of plasmid pBI 101.2 (Jefferson *et al.*, 1987). In the resulting plasmid, named pBMCV 102106, the Tnt1 coding sequence was fused in-frame with the GUS open reading frame. This plasmid was transferred to the *Agrobacterium tumefaciens* strain LBA 4404 (Hoekema *et al.*, 1983), which was used for the transformation of both *tl* and hybrid WT × *tl* tobacco lines, as described by Horsch *et al.* (1985). The resulting transgenic plants were analysed by Southern blotting with a GUS specific probe.

For transient expression studies, the *Pst*I–*Eco*RI fragment of plasmid pBMCV 102106, containing the LTR–GUS fusion gene, was subcloned into the *Pst*I–*Eco*RI sites of the Bluescript KS$^-$ plasmid vector (Stratagene). This construct was named pBMCV 102120.

GUS transient expression and GUS assay

Protoplasts were electroporated with plasmid pBMCV 102120 essentially as described by Guerche *et al.* (1987). Plasmids pBI 121, containing a 35S–GUS construct (Jefferson *et al.*, 1987), and pCH1, containing a 70S–GUS construct (gift from C.Horlow) were used as controls. Ten micrograms of supercoiled CsCl purified plasmid DNA were added to $1-2 \times 10^6$ protoplasts in 1 ml of electroporation buffer. No carrier DNA was used. After electroporation, the protoplasts were cultured overnight or during one night and a further 24 h period, washed twice in washing buffer and then transferred to microfuge tubes. They were finally lysed in GUS buffer and the GUS activity was measured according to Jefferson *et al.*

(1987). The protein concentration in the extract was determined by standard techniques (Bradford, 1976).

For the transgenic plants, GUS activity was measured immediately after protoplast isolation, or after an overnight culture.

Elicitor assay

Shredded WT tobacco leaves were macerated overnight in T0 medium containing either no enzyme (control) or one, or two or the three cell wall hydrolase sources together. The media were then separated from leaf fragments and/or protoplasts, and autoclaved for 20 min at 120°C. Fresh shredded leaves were then macerated overnight in the autoclaved media, and afterwards analysed for their RNA content.

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