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Activation of tobacco retrotransposons during tissue culture

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Sequences of at least three new families of retrotransposons (Tto1 – Tto3) were amplified by PCR from cDNA prepared from protoplasts of an established tobacco cell line, based on the fact that certain amino acids are highly conserved in the reverse transcriptases encoded by retrotransposons. Structural analysis indicates that Tto1 is 5.5 kb long and has features typical of retrotransposons. Transcription of Tto1 starting in the long terminal repeat was active only in cultured cells. Protoplast formation enhanced the transcription. The copy number of Tto1 increased 10-fold in established cell lines; it also increased in plants regenerated from tissue cultures and in transgenic plants. These results indicate that Tto1 is activated during tissue culture. This is the first demonstration of activation of a plant retrotransposon by tissue culture. The copy number of Tto2 and a previously isolated transposon, Tnt1, also increased in established cell lines, indicating that these two retrotransposons may also be activated by tissue culture. These three retrotransposons are cryptic in normally propagated plants: no difference in the copy number was observed between individuals of the same cultivars or even between different cultivars.
Key words: LTR/retrotransposon/reverse transcriptase/tissue culture/transposition

Introduction

Transposable elements were first found in maize more than 40 years ago. Since then, many plant transposable elements have been identified by genetic analysis because they induce unstable mutations. These elements include the well characterized Ac/Ds and Spm elements of maize (Fedoroff, 1989) and Tam elements of snapdragon (Coen *et al.*, 1989). Recent studies have shown that plant genomes also have a second type of transposable elements, namely retrotransposons (Johns *et al.*, 1985; Harberd *et al.*, 1987; Voytas and Ausubel, 1988; Grandbastien *et al.*, 1989; Smyth *et al.*, 1989). Retrotransposons differ both functionally and structurally from the transposable elements of maize and snapdragon. The latter type excises from one site in the genome and reintegrates into new sites in the genome, thus inducing unstable mutations. Retrotransposons resemble the integrated copies of retroviruses and are characterized by the presence of long terminal repeats (LTRs) and an internal domain encoding proteins analogous to the group-specific antigen (*gag*) and the *pol* polyprotein of retroviruses (Bingham and Zachar, 1989; Boeke, 1989). LTRs carry the sequences required for transcription and transcribed RNA works as an intermediate for transposition through reverse transcription. Because retrotransposons generally induce stable mutations, it is not easy to distinguish retrotransposon-induced mutations from others by genetic analysis. Most plant retrotransposons have therefore been identified only recently through molecular approaches (see references in Grandbastien, 1992).

Regulation of transcription, which is the first step in the transposition of retrotransposons, has been well studied in yeast and *Drosophila* (Bingham and Zachar, 1989; Boeke, 1989; Echalier, 1989). Transcription of retrotransposons is active during the normal life cycle of these organisms. Many environmental or endogenous factors can also modulate transcription, for example heat shock (Strand and McDonald, 1985), UV light (Bradshaw and McEntee, 1989), steroid hormones (Ziarczyk *et al.*, 1989) and cAMP (Yun and Davis, 1989). It has been shown that transcription of *Drosophila* retrotransposons is both developmentally regulated and activated in cell culture (for review, see Echalier, 1989). Although recent reports have suggested that retrotransposons (Flavell *et al.*, 1992b; Voytas *et al.*, 1992; Hirochika and Hirochika, 1993) and the major transposable elements in plants are ubiquitous (Hirochika and Fukuchi, 1992; Hirochika *et al.*, 1992), tobacco Tnt1 is the only retrotransposon whose expression has been reported (Grandbastien *et al.*, 1989; Pouteau *et al.*, 1991). Transcription and transposition of Tnt1 have been shown to be activated only in protoplasts.

Extensive genetic and cytogenetic modifications are induced in *in vitro* cultures of plant cells. Such genetic variability recovered in plants regenerated from tissue culture has been termed 'somaclonal variation' (Larkin and Scowcroft, 1981). It has been reported in most plant species investigated, as reviewed by Scowcroft (1985). Although somaclonal variation has been studied extensively as a source of plant improvement, little is known about its molecular basis. One possible mechanism suggested for somaclonal variation is the activation of transposable elements (McClintock, 1984; Scowcroft, 1985). Although the activation of maize Ac/Ds and Spm elements during tissue culture has been reported (Peschke *et al.*, 1987; Peschke and Phillips, 1991), there are no reports on the behaviour of plant retrotransposons during tissue culture. It is possible that activation of retrotransposons is reponsible for somaclonal variation: most tissue culture-induced mutations are stable like the mutations induced by retrotransposons and the ubiquity of somaclonal variation may be explained by that of retrotransposons.

To examine whether plant retrotransposons are activated during tissue culture, retrotransposons were first cloned using a recently devised method employing the polymerase chain reaction (PCR) (Konieczny *et al.*, 1991; Flavell *et al.*, 1992a; Hirochika *et al.*, 1992) and their activity was

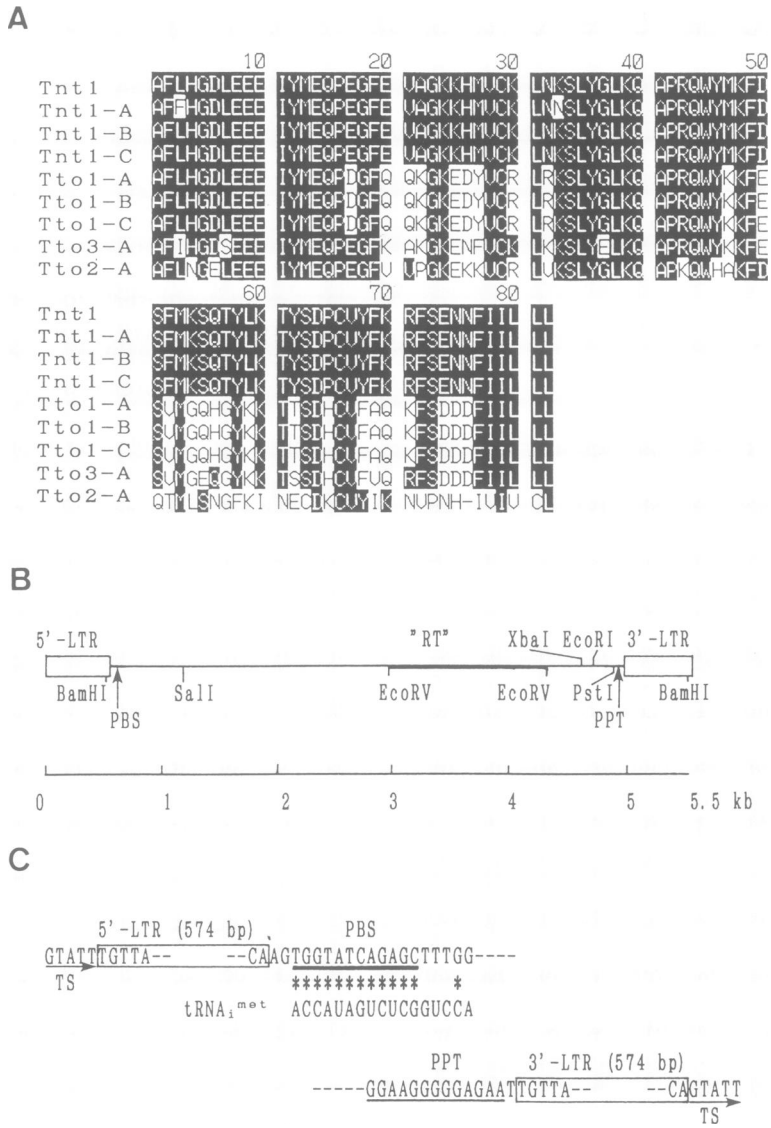

Fig. 1. Structural features of retrotransposons of tobacco. (**A**) Sequences corresponding to the reverse transcriptase domain of retrotransposons. Deduced amino acid sequences of eight clones of sequences amplified by PCR are shown. QMDVKT and YVDDM sequences at the amino- and carboxyl-termini, respectively, corresponding to the oligonucleotide primers used are not included, because they may not represent the true cDNA sequences. The sequence of the Tnt1 element (Grandbastien *et al.*, 1989) is included for comparison. Residues identical to those of Tnt1 are shown on a dark background. The Tto1–Tto3 sequences have been deposited in the DDB/EMBL/GenBank DNA databases under accession numbers D12827–D12829. (**B**) Restriction map of Tto1-1. 'RT' denotes the region hybridizing to the amplified sequence of Tto1 (Tto1-A, Figure 1A). (**C**) Nucleotide sequences flanking the LTRs of Tto1-1. LTRs are boxed and the primer binding site (PBS) and polypurine tract (PPT) are indicated by thick and thin underlining, respectively. The site of insertion target sequence (TS) and the resulting sequence duplication are indicated by arrows. The complementarity between the PBS and the 3′ end of the initiator methionine tRNA is shown by asterisks.

examined. The results obtained with three newly discovered tobacco retrotransposons and with Tnt1 are reported here.

Results

Detection of three new families of tobacco retrotransposons by PCR amplification

Retrotransposons carry the gene for reverse transcriptase, certain amino acid sequences of which are highly conserved between different retrotransposons (Doolittle *et al.*, 1989). Two oligonucleotide primers corresponding to two conserved motifs (QMDVKT and YVDDM) have been successfully utilized to amplify the reverse transcriptase domain of retrotransposons of rice from genomic DNA by polymerase chain reaction (PCR) (Hirochika *et al.*, 1992). Because all of the plant retrotransposons so far identified (apart from Tnt1) are defective or inactive (Grandbastien, 1992), it is important—especially for our purpose—selectively to clone intact, active retrotransposons. As suggested previously (Hirochika *et al.*, 1992), it should be possible selectively to amplify the active retrotransposons by applying the PCR cloning method to cDNA, instead of genomic DNA, because the active ones should be transcribed. To identify the retrotransposons whose expression is activated during *in vitro* culture, cDNA made from cultured cells was used.

Poly(A)$^+$ RNA was prepared from protoplasts of an established tobacco cell line, BY2 (Kato *et al.*, 1972), and the first strand cDNA was made using reverse transcriptase. cDNA was subjected to PCR amplification and the amplified sequences of the expected size (280 bp) were cloned into an M13 vector and sequenced. Deduced amino acid sequences of eight randomly selected clones were compared

(Figure 1A). Three clones had sequences identical or nearly identical to the published sequence of the reverse transcriptase domain of Tnt1 (Grandbastien *et al.*, 1989) and were therefore named Tnt1-A, -B and -C. It is not surprising that these clones were obtained, because Tnt1 is actively transcribed in protoplasts (Pouteau *et al.*, 1991). Three additional families of retrotransposons, which are distinct from Tnt1, were identified and named Tto1, 2 and 3, respectively. Amino acid sequence homologies between Tnt1 and Tto1 or Tto2 or Tto3 were 68, 57 and 71%, respectively. The Tto2 sequence is one amino acid shorter than the others. These three newly identified retrotransposons were characterized further. Recently, two retrotransposons of tobacco were isolated by applying the PCR cloning method to genomic DNA (Flavell *et al.*, 1992a). Sequence analysis showed that these two retrotransposons are distinct from Tto1, Tto2 and Tto3.

Structural analysis of Tto1

The genomic library constructed in phage λ EMBL3 from the cell line BY2 was screened using the cloned sequence of Tto1-A as a probe. Of 1×10^6 plaques, 1.4×10^3 positive plaques were identified. Sixteen positive plaques were purified and the insert DNAs were partially mapped. Restriction mapping and hybridization analysis showed that eight of these clones carried the entire Tto1 sequence with identical restriction maps. The restriction maps of flanking sequences differed between the eight clones, indicating that they were independent clones. One clone was analyzed further. This clone has the 5.5 kb Tto1 sequence containing two identical 574 bp LTRs (Figure 1B and C). This Tto1 sequence, named Tto1-1, was flanked by direct repeats of 5 bp (Figure 1C) which were shown by PCR analysis to be the consequence of a duplication of the target sequence during the insertion into the genome (data not shown). Two other structural features typical of retrotransposons (Bingham and Zachar, 1989; Boeke, 1989) were identified (Figure 1C): the primer binding site (PBS) and the polypurine tract (PPT). The PBS is homologous to the 3′ end of a tRNA and works as a priming site for first strand DNA synthesis. As discussed previously (Hirochika *et al.*, 1992), all of the plant retrotransposons so far sequenced have a PBS complementary to plant initiator tRNA. As expected, a PBS homologous to the plant initiator methionine tRNA (Sprinzl *et al.*, 1987) was found downstream of the 5′ LTR in Tto1-1. PPT is a polypurine tract which may serve to prime plus-strand DNA synthesis (Bingham and Zachar, 1989; Boeke, 1989). The PPT was located upstream of the 3′ LTR. Thus Tto1-1 is a typical retrotransposon.

Transcription of Tto1 is active in cell lines

The presence of Tnt1 and Tto1 RNAs, which are putative intermediates for transposition by reverse transcription, was examined in cultured cells, in protoplasts prepared from cultured cells and in leaves of tobacco (Figure 2). A 5.0 kb Tnt1 RNA corresponding to the 5.2 kb RNA (Pouteau *et al.*, 1991) was detected only in protoplasts, as reported previously (Pouteau *et al.*, 1991). The unclear bands larger than 5.0 kb in Figure 2 could be chimeric transcripts. Pouteau *et al.* (1991) detected the Tnt1 transcript in protoplasts derived from leaves; our results show that the transcription of Tnt1 is activated even in protoplasts prepared from cultured cells.

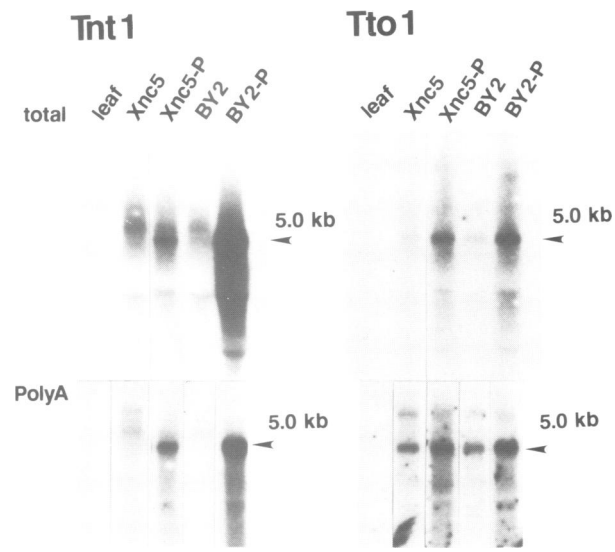

Fig. 2. Differential expression of Tnt1 and Tot1 RNAs. Total or poly(A)$^+$ RNA prepared from leaves, cultured cells (Xnc5 and BY2) and protoplasts prepared from cultured cells (Xnc5-P and BY2-P) were analysed by Northern blotting. The amplified sequences (Tnt1-A and Tto1-A; Figure 1A) corresponding to Tnt1 and Tto1 were used as probes. After hybridization with the Tnt1-A probe and autoradiography, the probe was removed from the filter and the Tto1-A probe was then used to probe the same filter. Equal loading of the samples in the different lanes was confirmed by ethidium bromide staining of RNAs.

In contrast to the Tnt1 RNA, Tto1 RNA (5.0 kb) was detected in intact cells as well as in protoplasts. The 5.0 kb RNA must be a full length transcript of Tto1 starting within the LTR. This was confirmed by determining the 5′ end of the Tto1 RNA (Figure 3). Two starting points were mapped within the LTR (nt 200 and 202) by primer extension analysis (Figure 3B) and the TATA box was identified at the expected site (Figure 3A). No signal corresponding to these two starting points was detected with leaf tissue RNA containing no detectable Tto1 RNA (Figure 3B, Figure 2). One band detected in both samples is probably an artefact. There was more Tto1 RNA in protoplasts than in intact cells (Figure 2). This difference between protoplasts and intact cells was less marked in the poly(A)$^+$ fraction of the RNA than in the total RNA. At present, the reason for this difference is unclear. Differential stabilities of RNAs may be responsible for the difference. The same mechanism may also explain the difference between protoplasts and intact cells. Alternatively, the LTR sequence of Tto1 may have two *cis*-regulatory elements, one to culture and the other to protoplast formation. The identification of these elements is under way. The 15 bp and 13 bp repeats identified upstream of the TATA box (Figure 3A) may be those regulatory elements.

The amount of Tto2 RNA was very low in any conditions and no clear bands were detected (data not shown). Although the amount of Tto3 RNA was much lower than that of Tnt1 RNA, the expression pattern was identical (data not shown).

Copy number of Tto1 is increased 10-fold in cell lines

Retrotransposons transpose via RNA through reverse transcription. Their transposition is regulated mainly at the transcription level (Boeke, 1989). The above results with the Tto1 RNA strongly suggest that the transposition of Tto1

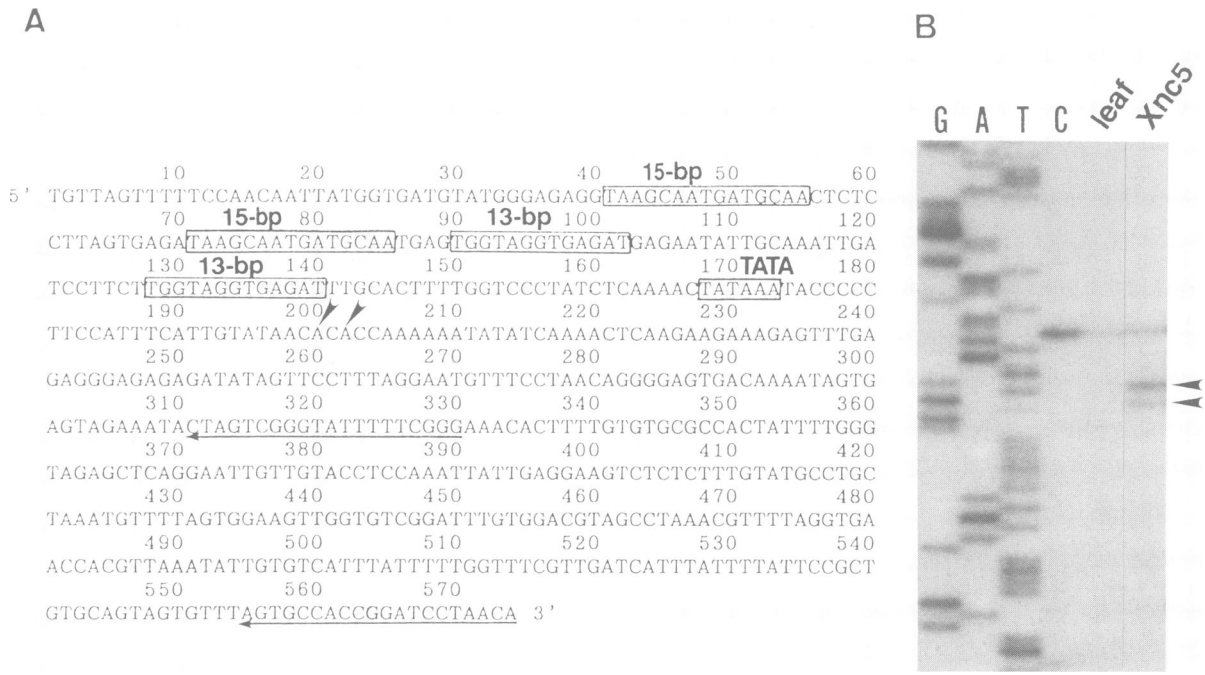

Fig. 3. Determination of the 5′ ends of Tto1 RNAs by primer extension. (**A**) Nucleotide sequence of the LTR of Tto1-1. Only one LTR sequence is shown: the sequences of the 5′ and 3′ LTRs are identical. The 5′ ends of Tto1 RNAs determined by primer extension are indicated by arrowheads. The TATA box sequence, and 15 bp and 13 bp direct repeats in the upstream region are boxed. The positions of the two oligonucleotides used for primer extension analysis are indicated by arrows. (**B**) Primer extension analysis. Ten micrograms of total RNA extracted from leaf tissue and cultured cells (Xnc5) were hybridized with a 5′ end-labelled oligonucleotide complementary to the LTR sequence of nt 311−330. The hybrids were extended with reverse transcriptase and the cDNA products were electrophoresed on a sequencing gel alongside a sequencing reaction using the same primer. Two bands (indicated by arrowheads) were observed only in the Xnc5 sample. The same results were obtained using another primer complementary to the LTR sequence of nt 555−574 (data not shown).

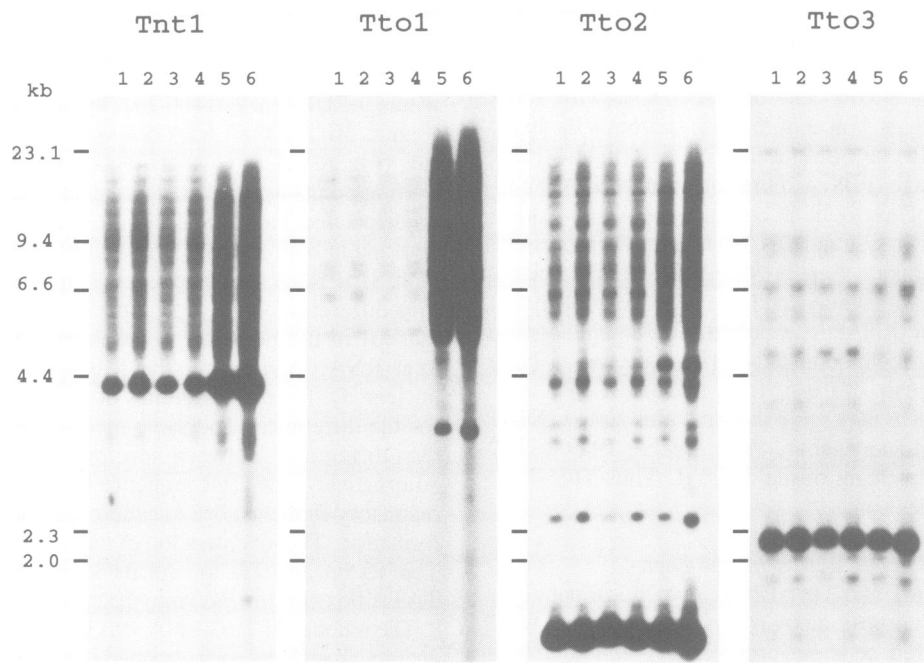

Fig. 4. An increase in the copy number of Tnt1 and Tto in the genomes of the established cell lines. DNAs of leaves or cultured cells were digested with *Xba*I and analysed by Southern blotting using ^{32}P-labelled Tnt1-A, Tto1-A, Tto2-A and Tto3-A sequences (Figure 1A). Lanes 1 and 2, leaves of SR1 strain; lanes 3 and 4, leaves of the cultivar Xanthi nc; lane 5, Xnc5 cell line; lane 6, BY2 cell line. To avoid unequal loading of samples between filters, the same filter was re-hybridized successively after removal of the previous probe.

is activated in cultured cells. As a consequence of this activation, the copy number of Tto1 may have been increased in cultured cells. This possibility was examined by genomic Southern blotting with the labelled Tto1 sequence (Figure 4). The copy number of Tto1 was nearly the same (~30 copies per haploid genome, as estimated by slot blot hybridization) in all individual plants (lanes 1−4). This was also true of different cultivars (see for example Figure 4, lanes 2 and

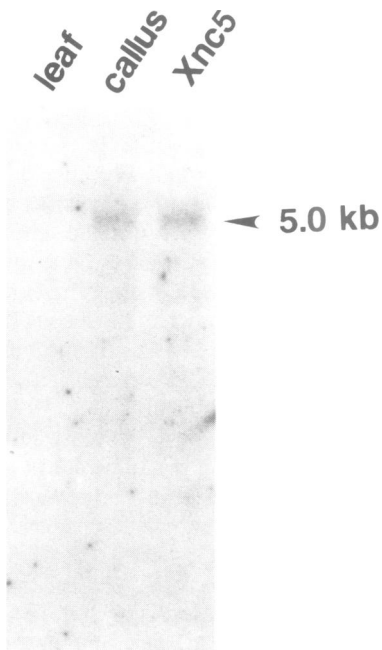

Fig. 5. Activation of transcription of Tto1 by tissue culture. Poly(A)$^+$ RNA prepared from leaf tissue, callus induced from leaf tissue and cultured cells (Xnc5) was analysed by Northern blotting. Callus was induced for 1 month as described in Materials and methods. The Tto1-A sequence (Figure 1A) was used as a probe.

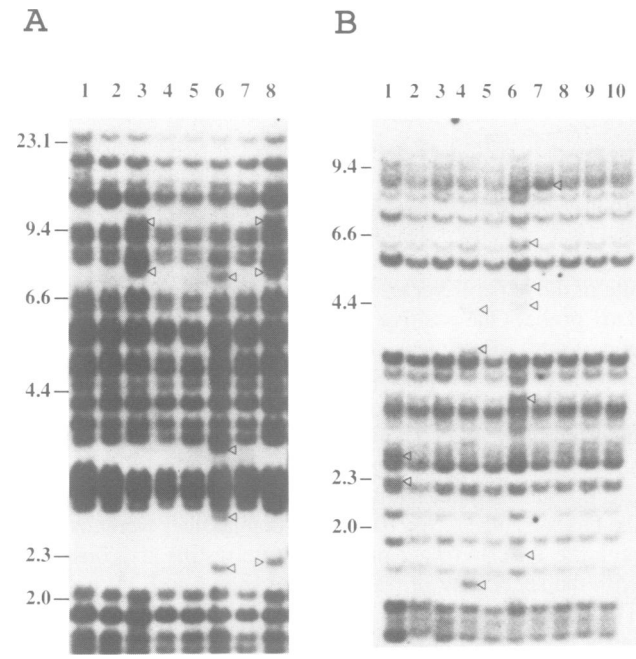

Fig. 6. The copy number of Tto1 increases in regenerated plants (**A**), in transgenic plants and in a tissue culture-induced mutant strain (**B**). DNAs were prepared from leaves and analysed by Southern blotting with a ^{32}P-labelled *Xba*I−*Pst*I fragment of Tto1-1 (Figure 1B) after digestion with *Xba*I (**A**) or *Eco*RV (**B**). These enzyme−probe combinations were chosen to obtain the high resolution of the band patterns. Arrowheads indicate the bands that are absent in the control plants. (**A**) Lane 1, the control plant Xanthi nc; lanes 2−8, plants regenerated from calli which had been induced from leaves of the control plant. (**B**) Lane 1, Petit Havana SR1; lanes 2 and 3, control plants Petit Havana and Samsun NN, respectively; lanes 4−10, independent transgenic plants obtained from Samsun NN.

3; Figure 6B, lanes 2 and 3). The copy number was dramatically increased in cultured cells (lanes 5 and 6). As expected, the Tto1-1 sequence cloned from the BY2 cell line was absent in the corresponding locus of the parental cultivar (data not shown), indicating that Tto1-1 transposed in the BY2 cells. Quantitative analysis by slot blot hybridization showed that the copy number was increased 10-fold (this does not necessarily mean that the copy number was increased 10-fold in all of the cells, because the DNA analyzed was isolated from a sub-population of the cells). The increase in the copy number in cultured cells seems to be a general phenomenon since it was found in two independent cell lines, BY2 and Xnc5 (the Xnc5 line was derived from cultivar Xanthi nc and maintained as suspension cells for 3 years).

The copy number of Tnt1 and Tto2 increased only slightly (1.8- and 1.3-fold, respectively, as estimated by slot blot hybridization) in cultured cells (Figure 4). By shorter exposure of autoradiographs, several new bands were detected with both probes over an intense hybridization signal (smear) observed only in DNAs of cultured cells (data not shown). These bands could result from a transposition induced early in the culture, and the smear background could be due to subsequent transpositions. The intensity of some bands (e.g. a 4.2 kb band detected with the Tnt1 probe and a 3.5 kb band with the Tto1 probe) was increased in DNAs of cultured cells. These fragments are probably internal *Xba*I fragments derived from retrotransposon sub-families which were activated in cultured cells. Although the smear was not observed with the Tto3 probe, at least one new band of 3.0 kb was detected in the Xnc5 line.

Tissue culture activates both transcription and transposition of Tto1

The above results show that transcription and transposition of Tto1 were strongly activated in the cell lines. Those cell lines have lost the ability to regenerate and are committed to rapid cell growth. Activation of transcription and transposition could therefore be connected to these characteristics. However, if the activation was instead triggered by tissue culture itself, activation of transcription should be observed in fresh tissue cultures having the ability to regenerate. An increase in copy number should be also observed in the plants regenerated from these tissue cultures as a consequence of transposition. These possibilities were examined as follows. Leaf tissues were cultured *in vitro* for ~1 month to induce callus formation. RNA prepared from the induced calli and DNA from the plants regenerated from these calli were analysed. The results of Northern blotting are shown in Figure 5. Tto1 RNA was detected in the calli, strongly suggesting that the transposition of Tto1 is activated in the calli. The amount of Tto1 RNA in the calli was comparable to that in the established cell line (Xnc5). DNAs of regenerated plants and normally propagated control plants were analysed by Southern blotting after digestion with *Xba*I (Figure 6A). Regenerated plants were obtained 3−4 months after culture initiation. Additional DNA bands were observed in three (lanes 3, 6 and 8) out of seven regenerated plants. Almost no changes were observed in pre-existing bands. Similar results were obtained by digestion with *Eco*RV (data not shown). Because band patterns and band intensities were exactly the same in 20 control plants examined (data not shown) and even between different cultivars, it is concluded that these changes are induced by tissue culture. Appearance of additional bands without a decrease in the intensity of pre-existing bands strongly suggests that the additional bands

result from transposition rather than from changes such as DNA methylation (Brown, 1989), point mutations (Dennis *et al.*, 1987) and chromosomal rearrangements (Scowcroft, 1985) which have been described in plants derived from tissue culture. Further evidence for activation of transposition was obtained by cloning one (the 2.2 kb band in lane 6, Figure 6A) of the additional bands found in the regenerated plants. The library was constructed by cloning 2.2 kb DNA fragments generated by digestion with *Xba*I into the *Xba*I site of the λ ZapII vector (Stratagene) and screened with the Tto1 probe. One positive clone contained 1.0 kb of the right end of the Tto1 sequence (Figure 1B) and 1.2 kb of flanking sequence. By PCR analysis using primers corresponding to the LTR and the flanking sequence, this junction was detected in the plant shown in lane 6 but not in other plants shown in Figure 6A (data not shown), indicating that the 2.2 kb band results from transposition of Tto1 rather than the DNA changes. No changes in copy number were detected with the Tnt1 and Tto2 probes. These results are consistent with the specificity of transcription shown in Figure 2. No Tto1 RNA was detected in the regenerated plants (used in lanes 6 and 8, Figure 6) in which extra Tto1 bands were observed (data not shown), indicating that the transcription of Tto1 is active only during tissue culture. This result also suggests that the transposition of Tto1 is active only during tissue culture. The copy number of Tto1 increased much less in the regenerated plants than in the established cell lines. The reason for this difference is discussed later.

Tto1 is also activated during the production of transgenic plants

Plant tissue culture is used for selection of transformed cells to produce transgenic plants (Weising *et al.*, 1988) and for induction of mutations (Larkin and Scowcroft, 1981). It is also known that mutations are frequently observed in the transgenic plants (Yoder, 1990). Therefore, it is important to examine whether Tto1 is activated during the production of transgenic or mutant plants. This was examined by analysing *Eco*RV-digested DNAs of the transgenic plants and the mutant plant isolated through tissue culture (Figure 6B). The transgenic plants analysed were obtained by *Agrobacterium*-mediated gene transfer and carry the kanamycin resistance gene (Ohshima *et al.*, 1990; Y.Ohashi, M.Ohshima and M.Ugaki, unpublished results). In three (lanes 4, 6 and 7) out of seven transgenic plants (lanes 4–10), DNA bands were found that were absent in the control plant (lane 3). The variation in the intensities of these bands may be due to the chimeric nature of the transgenic plants which is induced by transposition at different stages of regeneration or to differences in the extent of homology to the probe deriving from the heterogeneity of Tto1 members. Alternatively, the weak hybridization signal may be due to deletions induced during transposition. The streptomycin-resistant mutant Petit Havana SR1 was isolated through tissue culture (Maliga *et al.*, 1973). This mutant (lane 1) also had DNA bands that were absent in the parental strain Petit Havana (lane 2). These results show that Tto1 is also activated during the production of transgenic and mutant plants.

Discussion

Isolation of retrotransposons using cDNA-PCR

The PCR has been used to identify the sequences of plant retrotransposons from the genomic DNA of many flowering plants (Konieczny *et al.*, 1991; Flavell *et al.*, 1992a; Hirochika *et al.*, 1992). Recently, the same method has also been used to show the ubiquity of retrotransposons in plants (Flavell *et al*, 1992b; Voytas *et al.*, 1992; Hirochika and Hirochika, 1993) and to demonstrate the presence of the retrotransposon in the vertebrate (Flavell and Smith, 1992). However, most of the plant retrotransposons thus identified seem to be defective, as judged from the following criteria: (i) the copy number of each family is quite low (one to two copies in *Arabidopsis thaliana*; Konieczny *et al.*, 1991), (ii) their sequences are extremely heterogeneous (Flavell *et al.*, 1992a,b) and (iii) stop codons or frameshifts have been detected at high frequency (Flavell *et al.*, 1992a,b; Voytas *et al.*, 1992; Hirochika and Hirochika, 1993). This is in contrast to the retrotransposons of yeast and *Drosophila*, most of which have been shown to be active (Bingham and Zachar, 1989; Boeke, 1989).

In this report, three previously unknown types of retrotransposon (Tto1–Tto3) were isolated from tobacco by applying the PCR method to cDNA rather than to genomic DNA. At least two of these, Tto1 and Tto2, are active, because an increase in the copy number of these two retrotransposons was observed in cultured cells (Figure 4). Sequences of Tnt1, the activity of which in tissue culture has previously been demonstrated, were also isolated. Heterogeneous retrotransposon sequences distinct from those described in this report have been isolated from tobacco and related species by applying the PCR method to genomic DNA (Flavell *et al.*, 1992a; Hirochika and Hirochika, 1993). These results indicate that the cDNA-PCR method is very useful for isolating retrotransposons that are activated under certain conditions.

As discussed earlier, the transcription of yeast and *Drosophila* retrotransposons can be modulated by environmental or endogenous factors. Although only two factors, namely protoplast formation and tissue culture, have been shown to activate the transcription of the plant retrotransposons, it seems likely that many other factors also modulate the transcription of retrotransposons. It will be possible to use the cDNA-PCR method to identify retrotransposons that respond to other factors.

Activation of retrotransposons by tissue culture

Of the three retrotransposons identified in this report, two (Tto1 and Tto2) are active in the established cell lines studied. Although Tnt1 was reported to be active only in protoplasts (Grandbastien *et al.*, 1989; Pouteau *et al.*, 1991), the present results indicate that it is also active in cell lines (Figure 4). These three retrotransposons are cryptic in normally propagated plants: no difference in the copy number was observed between individuals of the same cultivar or even between different cultivars. This is in contrast to yeast and *Drosophila* retrotransposons, the distribution of which in the genome can differ between different stocks of the same strain (Cameron *et al.*, 1979; Strobel *et al.*, 1979). At present, it is not clear whether this difference may reflect the difference between organisms or simply that between different retrotransposon families.

The copy number of Tto1 increased as much as 10-fold in the cell lines studied, whereas that of Tto2 and Tnt1 increased less than 2-fold. The difference in the transcription level may be responsible for this difference. Although Tnt1 RNA was not detected in cell lines by Northern analysis,

there may be enough Tnt1 RNA for transposition. Another possibility is that the transcription of Tnt1 was transiently activated during the early stage of cell culture. A similar increase in the copy number of retrotransposons has been reported in *Drosophila* cell lines (Potter *et al.*, 1979). Although the active transcription was detected in these cell lines, the amplification and redistribution of retrotransposons seem to occur essentially during the very early stage of cell culture (Echalier, 1989). Further studies are needed to examine whether this is also true of plant retrotransposons.

The transcription of Tto1 was activated in the fresh calli induced for 1 month from leaf tissue (Figure 5). In the plants regenerated from these calli, no Tto1 RNA was detected (data not shown), indicating that the transcription of Tto1 is only active during tissue culture. Many factors are involved in callus induction. To understand the mechanism of activation of Tto1 transcription, it is necessary to examine what factor is responsible for this activation. Furthermore, *cis*- and *trans*-acting elements for this transcription need to be identified. As discussed earlier, the 15 bp and/or 13 bp repeats found upstream of the transcription start site (Figure 3A) may be responsible for the activation of transcription by tissue culture. We are currently examining this possibility by using transgenic plants. Preliminary data indicate that the sequence upstream of the transcription start site is sufficient for the activation by tissue culture (H.Hirochika *et al.*, unpublished results). The copy number of Tto1 was increased in the plants regenerated from the calli, probably as a consequence of activation of transcription (Figure 6). This was also found in the transgenic plants and the mutant plant obtained through tissue culture. The activation of the retrotransposons during tissue culture indicates that the mutation frequently induced during tissue culture may be the result of insertion of retrotransposons. The ubiquity of retrotransposons in plants may explain that of somaclonal variation. At present, we do not have any direct evidence correlating the insertion of retrotransposons and the specific mutation induced during tissue culture. If this correlation can be shown, it will be possible to use retrotransposons to isolate genes mutated in culture. Tissue culture techniques have become a powerful tool for breeding plants as well as studying basic problems in plant biology. However, the means to prevent the undesired mutations frequently induced during tissue culture are not available. If we understood the mechanisms of activation of retrotransposons during tissue culture, it would be possible to regulate the appearance of those mutations.

The copy number of Tto1 increased 10-fold in the established cell lines studied, but much less in regenerated plants. This difference may be connected to the duration of tissue culture (more than three years in the former case, compared with several weeks in the latter). No changes in the copy number of Tnt1 and Tto2 were detected in regenerated plants, although the copy number did increase in the cell lines. The difference in the duration of tissue culture may also explain this copy number difference. An increase in the copy number of retrotransposons with time in culture may explain the general observation that the frequency of somaclonal variation increases with prolongation of the culture period (Scowcroft, 1985).

Generally, tissue cultures lose totipotency with time in culture. As expected, the cell lines examined in this report had lost the ability to regenerate. It is possible that the genes essential for regeneration had been mutated by Tto1 transposition in these cell lines. However, other retrotransposons may be involved, because the copy number of Tnt1 and Tto2 also increased, though to be a lesser extent, and it can be seen by extrapolating from the results with the rice genome (Hirochika *et al.*, 1992; Hirochika and Fukuchi, 1992) that many other retrotransposons could exist in the tobacco genome.

We have shown that retrotransposons are activated in tobacco during tissue culture. Further studies are needed to detemine whether these conclusions also apply to other plants. Hybridization analysis has indicated that Tto1-related sequences are present in other Solanaceae, petunia (*Petunia hybrida*), potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*) (H.Hirochika *et al.*, unpublished results). Because our experimental strategy for cloning and characterizing retrotransposons is quite simple and generally applicable to any plant species, this matter could be resolved in the near future. Recently, we have found that some retrotransposons of rice are activated during tissue culture (H.Hirochika *et al.*, unpublished results).

Evolutionary importance of retrotransposons

The copy number of Tto1, Tto2 and Tnt1 increased in cultured cells. As discussed above, a similar phenomenon has been reported in *Drosophila* cell cultures. This indicates that the mechanisms regulating transposition have been conserved throughout their long evolution. It is known that many agents, including X-rays, UV and virus infection, activate transposable elements of maize (Nuffer, 1966; Mottinger *et al.*, 1984; Walbot, 1988). McClintock (1984) proposed that these agents activate transposable elements by causing chromosome breakage. Plant tissue culture, which is known to induce chromosome breakage (Scowcroft, 1985), also activates the maize Ac/Ds and Spm elements (Peschke *et al.*, 1987; Peschke and Phillips, 1991), and tobacco retrotransposons , as shown in this report. Although the mechanism of activation of transposable elements is not known, transposable elements may play an important role in the evolution by inducing mutations under traumatic conditions, as proposed by McClintock (1984). The evolutionary conservation in the manner of activation of retrotransposons supports this hypothesis.

Materials and methods

Plant materials

Tobacco cell lines, BY2 and Xnc-5, were cultured and their protoplasts were prepared as described by Nagata *et al.* (1981). Regenerated tobacco plants were obtained as follows. To induce tobacco callus, leaf sections of *Nicotiana tabacum* cultivar Xanthi nc were cultured on Murashige–Skoog (MS) medium containing naphthalene acetic acid (NAA, 2 p.p.m.) and benzyl amino purine (BA, 0.1 p.p.m.). After 1 month, induced callus was transferred onto MS medium containing NAA (0.1 p.p.m.) and BA (2 p.p.m.) for shoot formation. The shoots were transferred onto hormone-free MS medium for root formation. Regenerated plants were potted and cultivated in a greenhouse. Transgenic plants were obtained as described by Ohshima *et al.* (1990).

Extraction of nucleic acids

Total RNA was prepared by classical caesium chloride centrifugation (Sambrook *et al.*, 1989) and poly(A)$^+$ RNA was purified using oligo(dT)-Latex (Daiichi Chemical, Japan). DNAs were prepared as described by Hirochika *et al.* (1992) and their concentration was determined by fluorometry using Hoechst 33258 dye and a Minifluorometer (Hoefer).

Northern and Southern blot hybridization

Total and poly(A)$^+$ RNA (10 and 1 μg, respectively) were fractionated on a 1.0% formaldehyde−agarose gel in MOPS buffer and transferred to nylon membrane as described by Sambrook *et al.* (1989). Preparation of probes and hybridization were as described previously (Hirochika *et al.*, 1992).

Primer extension

The 5′ ends of Tto1 RNA were determined by primer extension analysis as described by Hirochika *et al.* (1987) using the primers shown in Figure 3A and 10 μg of total RNA.

cDNA synthesis and PCR

The first strand cDNA was generated from poly(A)$^+$ RNA isolated from protoplasts of the BY2 cell line using random primers and a cDNA cycle kit (Invitrogen). PCR amplification of cDNA was carried out using primers as described by Hirochika *et al.* (1992). Products amplified by PCR were purified, phosphorylated and cloned into the *Hin*cII site of M13 vectors.

Construction and screening of libraries

The genomic library of the BY2 line was constructed by cloning the partially *Sau*3A digested genomic DNA into the *Bam*HI site of the λ EMBL3 vector. This library was screened using a ^{32}P-labelled Tto1-A sequence (Figure 1A). Positive clones were subcloned into M13 or pBluescript vector.

DNA sequencing

The DNA sequence was determined by the M13 chain termination method (Messing, 1983) using Sequenase (United States Biochemical).

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