The mobility of the tobacco Tnt1 retrotransposon correlates with its transcriptional activation by fungal factors

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Summary
We have analyzed the stress-induced amplification of the tobacco Tnt1 element, one of the rare active plant retrotransposons. Tnt1 mobility was monitored using the retrotransposon-anchored SSAP strategy that allows the screening of multiple insertion sites of high copy number elements. We have screened for Tnt1 insertion polymorphisms in plants regenerated from mesophyll leaf cells, either via explant culture or via protoplast isolation. The second procedure includes an overnight exposure to fungal extracts known to induce high levels of Tnt1 transcription. Newly transposed Tnt1 copies were detected in nearly 25% of the plants regenerated via protoplast isolation, and in less than 3% of the plants derived from explant culture. These results show that Tnt1 transcription is followed by transposition, and that fungal extracts efficiently activate Tnt1 mobility. Transcription appears to be the key step to controlling Tnt1 amplification, as newly transposed Tnt1 copies show high sequence similarities to the subpopulations of transcribed Tnt1 elements. Our results provide direct evidence that factors of microbial origin are able to induce retrotransposon amplification in plants, and strengthen the hypothesis that stress modulation of transposable elements might play a role in generating host genetic plasticity in response to environmental stresses.

Keywords: fungal factor, protoplast, retrotransposon, stress, Tnt1, tobacco.

Introduction
Stress and environmental challenges are known to trigger surprisingly similar defensive mechanisms in different eukaryotic organisms (Taylor, 1998). In particular, recent evidence suggests the possible generation of genetic plasticity in response to stress through the mobilization of retrotransposons, the most widespread eukaryotic mobile elements. Stress and external challenges, including microbial attacks, are known to be major factors activating retrotransposon expression in a wide range of organisms such as yeast (Bradshaw and McEntee, 1989; Rofte et al., 1986), drosophila (Strand and McDonald, 1985), mammals (Liu et al., 1995) and plants (Grandbastien, 1998; Wessler, 1996). So far, the biological significance of the stress modulation of retrotransposons remains unclear. However, it is in agreement with McClintock’s original model that postulates that transposable elements are involved in genome restructuring in response to environmental challenges (McClintock, 1984). A recent study indeed suggests that retrotransposon amplification has been involved in barley adaptative evolution to drought conditions (Kalendar et al., 2000). An alternative, but not exclusive, hypothesis is that activation by microbial challenges might also favor horizontal transmission and allow elements to colonize new hosts. A parallel can be noted between this hypothesis and the recent proposition that stress activation of retroviral replication corresponds to an escape mechanism for the virus from damaged or stressed host cells (Andrews et al., 1998).

Any debate on the putative biological impact of retrotransposon activation implies that stress induces not only expression but also subsequent transposition. However, except for yeast, direct evidence of retrotransposition in
response to stress is scarce and often controversial. This situation partly results from the difficulty in establishing experimental systems to demonstrate mobility in multicellular organisms (Arnault and Dufournel, 1994). Additionally, post-transcriptional inhibition mechanisms are often used by hosts to control potentially deleterious retrotranspositions (Curcio and Garfinkel, 1999; Menees and Sandmeyer, 1996). In particular, there is little evidence for retrotransposon mobilization by stress in plants, where experimental tests for retrotransposition are particularly difficult due to the high copy number of most plant retrotransposons. Examples of transposon insertions were reported in progenies of virus-infected maize plants (Dellaporta et al., 1984; Johns et al., 1985). However, a causal relationship between virus infection and transposition still remains to be established in these cases. So far, a link between transcription and retrotransposition has been demonstrated for a few low copy number elements, in response to in vitro tissue culture (Hirochika et al., 1996; Hirochika, 1993).

The Tnt1 element of tobacco (Nicotiana tabacum) is one of the best characterized plant retrotransposons (Grandbastien et al., 1989b). Tnt1 is not expressed in healthy tobacco tissues, except in roots (Pouteau et al., 1991). Tnt1 expression is strongly induced by stresses such as pathogen attacks and factors of microbial origin, and a tight correlation has been shown between Tnt1 transcription and plant defense responses (Grandbastien et al., 1997; Mhiri et al., 1997; Mhiri et al., 1999; Moreau-Mhiri et al., 1996; Pouteau et al., 1994). In contrast to many other elements, Tnt1 transcription is poorly activated by tissue culture, at least in tobacco (Grandbastien et al., 1997; Hirochika, 1993; Pauls et al., 1994). However, Tnt1 is highly expressed in freshly isolated tobacco protoplasts (Pouteau et al., 1991). A detailed study of the different factors involved in protoplast isolation has shown that wounding, plasmolysis or hormone addition have little effect on Tnt1 transcription, and that Tnt1 expression in protoplasts is a direct response to the Onozuka solution, one of the three components of the cocktail of fungal extracts used to digest cell walls (Pouteau et al., 1991). Onozuka is a crude extract prepared from the fungus Trichoderma viride and contains proteinaceous elicitors that activate defense responses in tobacco, leading to necrotic hypersensitive cell death (Bailey et al., 1990; Lotan and Fluhr, 1990; Yano et al., 1998). Tnt1 expression is detected soon after application (Grandbastien et al., 1997), and Onozuka also activates Tnt1 expression when applied to intact tobacco tissues (Pouteau et al., 1994), indicating that Tnt1 is activated in direct response to elicitors contained in the Onozuka solution. The effect of the fungal extracts is transient, as Tnt1 transcript levels rapidly decrease after their removal, in the early stages of subsequent cell culture (Grandbastien et al., 1997).

Tnt1 is mobile and was originally isolated after transposition into a target gene. However, in spite of a good knowledge of the conditions and mechanisms activating Tnt1 expression, no evidence was yet reported that Tnt1 transcriptional activation is correlated to subsequent Tnt1 mobility. The low level of Tnt1 expression in response to tissue culture stimuli or plasmolysis has allowed us to develop an experimental strategy aimed at testing for the effect of fungal extracts on Tnt1 mobility. We have monitored Tnt1 transposition in tobacco plants regenerated from mesophyll leaf cells, either via protoplast isolation, or as controls, via leaf explant culture. In both procedures, regenerated plants originate from cells that have been submitted to stress stimuli linked to successive steps of wounding, cell dedifferentiation, callus formation and shoot regeneration. The major difference between the two procedures consists of an early step of plasmolysis that does not induce detectable Tnt1 expression, associated with overnight exposure to fungal extracts, including the Onozuka solution known to induce very high levels of Tnt1 expression. The comparison of Tnt1 transposition between each of these two procedures is thus expected to provide an accurate estimate of the effect of the fungal extracts on Tnt1 mobility.

Since Tnt1 copy number has been estimated at several hundred copies (Grandbastien et al., 1989b), we used a high resolution retrotransposon-anchored PCR strategy allowing the simultaneous detection of multiple insertion sites of high copy number elements, described as the Sequence-Specific Amplification Polymorphisms (SSAP) technique (Ellis et al., 1998; Gribbon et al., 1999; Waugh et al., 1997) or as Transposon Display (Casa et al., 2000; Van den Broek et al., 1998). Newly transposed copies have been screened through the appearance of new polymorphic bands in SSAP profiles of regenerated plants. We have also characterized these new SSAP polymorphic bands, in order to analyze the sequences of the population of newly transposed Tnt1 elements and to compare them with the sequences of the population of elements known to be transcribed in the same stress conditions (Casacuberta et al., 1995).

Results

Tnt1 insertion polymorphisms in regenerated plants

The SSAP procedure outlined in Figure 1 was developed to analyze Tnt1 insertion polymorphism in regenerated tobacco plants. Since most Tnt1 elements do not contain EcoRI restriction sites (Grandbastien et al., 1991), EcoRI digestions were performed to avoid internal amplifications from the 3’LTR. A primer pair consisting of an EcoRI primer (E00) and a Tnt1-specific primer (LTR13) was used for SSAP amplifications. The Tnt1-specific primer, LTR13, was

designed in the highly conserved U5 region, 250 pb downstream of the 5' end of the element, and oriented towards the 5' end. The E00/LTR13 primer combination thus allows amplification of variable amounts of 5' flanking genomic DNA, together with a 5' LTR portion that includes the U3 region, previously shown to be highly variable in Tnt1 populations (Casacuberta et al., 1995; Vernhettes et al., 1998). Control SSAP experiments were performed on six tobacco genotypes (Figure 2a). In our experimental conditions, about 80 bands per SSAP profile were observed. These profiles are similar for all genotypes, except for the PBD6 line, which shows a low number of polymorphic bands. These results indicate that the tobacco lines used in this study are closely related. In addition, no SSAP polymorphism was found in different plants of the D8, tl and PBD6 lines and in different leaves of D8 plants, thus indicating no detectable interplant or intraplant variability (data not shown).

Following protoplast isolation or direct explant culture, 20–30 plants were regenerated per genotype, leading to totals of 156 plants and 147 plants, respectively. Tnt1 insertion polymorphism was examined in all the regenerated plants using the SSAP procedure. After protoplast isolation, new SSAP bands were detected in 11.8% to 42.9% of the regenerated plants, depending on the genotype (Table 1). SSAP profiles obtained for a few representative plants are illustrated in Figure 2b. χ² tests of homogeneity show that intergenotype differences are not statistically significant ($\chi^2_{\text{diff}} = 6.37$, $P = 0.27$). The presence of the tl somatic instability, known to increase the level of spontaneous mutations (Grandbastien et al., 1989a), does not lead to a significant increase in new SSAP bands. Altogether, an average of 24.4% of all plants regenerated via protoplast isolation contain new SSAP bands. In contrast, after direct explant culture, new SSAP bands were detected in a much lower percentage of the regenerated plants, ranging from 0% to 6.6% (Table 1). The Fisher’s exact test demonstrates that intergenotype differences are not statistically significant either ($P = 0.41$). An average of 2.7% of all plants regenerated via explant culture contain new SSAP bands. Altogether, a significant difference ($\chi^2_{\text{diff}} = 29.68$, $P < 0.001$) is found between plants regenerated via protoplast isolation or via explant culture, indicating a 9-fold increase in the appearance of new SSAP bands in response to the protoplast isolation.
procedure. The number of new SSAP bands revealed on E00/LTR13 profiles varied from one to seven per plant (Table 1), with a mean number of bands of 2.45 and 1.5 for plants regenerated via protoplast isolation or explant culture, respectively. Due to the low number of explant-derived plants containing new SSAP bands, it is not possible to determine if the numbers of new SSAP bands per plant statistically differ between the two populations of regenerated plants.

Thirty-eight new SSAP bands isolated from 22 plants regenerated via protoplast culture, and three new SSAP bands isolated from three plants regenerated via explant culture, were sequenced after re-amplification from the gel. The transpositional nature of each new SSAP band was tested by direct PCR performed using the LTR13 primer and primers designed in each flanking genomic sequence. Thirty-eight out of 41 new SSAP bands were confirmed to correspond to transposition events (Table 1). The remaining three bands, obtained from three different protoplast-derived regenerants, could not be identified as transposed copies. In these three cases, however, no consensus sequence was obtained from the few clones recovered from the cloning step (see Experimental procedures), suggesting that the failure to identify the transposed copy represents the background of technical problems inherent in our cloning strategy. Taken together, our results demonstrate that the vast majority (93%), and possibly all, of the new SSAP bands detected in regenerated plants correspond to newly inserted Tnt1 copies.

Table 1. Tnt1 insertion polymorphisms in regenerated tobacco plants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Plants regenerated from leaf protoplasts</th>
<th>Plants regenerated from leaf explants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of tested plants</td>
<td>Plants with new bands</td>
<td>New bands per profile</td>
</tr>
<tr>
<td>Gat</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>D8</td>
<td>33</td>
<td>7</td>
</tr>
<tr>
<td>tl</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td>D8 × tl</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td>Sam</td>
<td>27</td>
<td>8</td>
</tr>
<tr>
<td>PBD6</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>156</td>
<td>38</td>
</tr>
</tbody>
</table>

1 confirmed transpositions/number of tested bands; nt = non-tested

subfamily, characterized by the BI and BII regulatory sequences involved in Tnt1 stress-activation (Casacuberta and Grandbastien, 1993; Vernhettes et al., 1997), is expressed in protoplasts (Casacuberta et al., 1995). Furthermore, protoplast-specific transcripts are not a unique sequence, but a population of different, albeit very closely related, RNAs (Casacuberta et al., 1995). Since the first half part of the 5′LTR is amplified by the E00/LTR13 primer pair (Figure 1), it is possible to compare the U3 regions of the Tnt1 copies that have transposed in protoplast-derived plants with the U3 regions of previously characterized Tnt1 protoplast-specific transcripts. The unexpected characterization of preexisting insertions (see Experimental procedures) also allowed the comparison of newly transposed Tnt1 copies with ancient copies that inserted earlier during tobacco evolution.

All the newly transposed copies analyzed belong to the Tnt1A1 group (Figure 3a). Furthermore, a striking correlation was found between the U3 sequences of transcribed and transposed copies. We have previously shown that two major U3 subpopulations could be defined in the protoplast-specific transcript population, the P23 and the P1 RNAs, each representing 24% of the population, and both containing four BII regulatory repeats (Casacuberta et al., 1995). The P1 and P23 RNAs differ in the U3 region by four signature nucleotide changes only (G, A, A, T) (Figure 3a) and the U3 regions of the remaining RNAs differ from P1 and P23 by one or two single nucleotide changes only, as well as by the sporadic deletion of a complete BII repeat, that may be considered as a single mutational event generated during the reverse transcription process (Casacuberta et al., 1995). Our results show that most of the newly transposed copies can also be grouped in P1-type (four copies) and P23-type (24 copies) sequences. Furthermore, 10 (30%) of the newly transposed Tnt1 copies are identical to the P23 sequence. A third subpopulation of newly transposed copies is closely

related to the Tnt1–94 element originally isolated after insertion into the nitrate reductase encoding gene and characterized by an atypical third BII repeat (Grandbastien et al., 1989b) (Figure 3a, six lower lines). Interestingly, transcripts of this type were never characterized in protoplasts. Finally, it is noticeable that Tnt1 copies

Figure 3. Sequence variability of the partial LTR sequences of newly transposed Tnt1 copies characterized in protoplast-derived plants (a) and of preexisting Tnt1 insertions (b).

Sequences have been aligned on the d161tr transposed copy, chosen as a reference because of its identity to the major P23 Tnt1 RNA species. Sequences of the P23 and P1 Tnt1 RNA species as well as of the Tnt1–94 mobile copy are included (the P23 and P1 RNA sequences terminate at the end of the R region). Dashes and blanks indicate sequence identity and deletions, respectively. Nucleotides that differ from the reference sequence are shown in capital letters. Lower case letters and stars indicate that the 5’ end of the LTR could not be determined, since no homology to previously characterized Tnt1 LTRs could be detected upstream from the sequences shown by dashes. Blanks have been introduced in the reference sequence to allow for nucleotide insertions. Black boxes indicate important features of the Tnt1A LTR (BI and BII boxes, TATA box, R region). The subfamily and group of each sequence, as defined in Vernhettes et al. (1998) are indicated in brackets.

newly transposed in the same regenerant belong usually, but not exclusively (e.g. s81tr and s82tr), to the same subpopulation type.

These data indicate that there is a good correlation, at the level of the regulatory U3 region, between the Tnt1 transcripts generated by protoplast isolation and the Tnt1 copies that have successfully completed the retrotransposition cycle. However, a significant difference between transcribed and transposed copies is found with respect to the number of BII repeats. We have previously shown that 90 percent of transcribed elements contain 4 BII repeats, indicating that BII sequences were essential for Tnt1 activation in protoplasts (Casacuberta et al., 1995). Surprisingly, a significantly lower proportion of newly transposed copies containing four BII repeats (64%) has been observed in this work (χ²cal = 8.48, P < 0.001). In addition, one of the newly transposed copies, t42tr, contains only two BII repeats, although transcripts containing fewer than three BII repeats have never been characterized (Casacuberta et al., 1995).

In contrast to newly transposed Tnt1 copies, the regulatory regions of pre-existing Tnt1 insertions are much more heterogeneous (Figure 3b). While most of them (75%) belong to the Tnt1A1 group, only four of them belong to the P23-type and one of them to the P1-type. All the remaining pre-existing Tnt1A1 copies contain more than four bp changes. Only six (33%) of them contain four BII repeats, and several others display larger-scale modifications such as insertions or apparent 5’ truncations (dp162a, g72c, s82c and gp94b). The remaining ancient Tnt1 insertions belong either to the Tnt1B (17%) or to the Tnt1C (8%) subfamilies.

Discussion

Microbial factors activate retrotransposon amplification

Using the SSAP strategy, we have monitored the amplification of one of the few plant retrotransposons known to be active, the tobacco Tnt1 element. Here we report that the protoplast isolation procedure activates Tnt1 transposition efficiently in tobacco, since a nine-fold increase in Tnt1 mobility is observed in plants regenerated via protoplast isolation, compared with the Tnt1 mobility observed in control plants regenerated via explant culture. Since transcription is a prerequisite for the transposition of retroelements, this sharp increase in Tnt1 mobility results necessarily from one or several protoplast-specific factors able to strongly activate Tnt1 expression. Previous studies have shown that the major factor activating Tnt1 expression during the protoplast isolation procedure is the initial overnight application of Onozuka fungal extracts (Pouteau et al., 1991). The subsequent early steps of protoplast-derived cell culture are expected to have very little impact on Tnt1 transposition, since Tnt1 transcript levels decrease strongly in the few hours following removal of the fungal extracts (Grandbastien et al., 1997), and no Tnt1 transcript could be detected by Northern analysis in tobacco cell cultures (Hirochika, 1993). Furthermore, Tnt1 is not expressed in tobacco calli (Pauls et al., 1994). As other factors involved in the production of the regenerated plants were also involved in the generation of the control plants from leaf explants, it can therefore be assumed that the nine-fold increase in Tnt1 mobility observed in plants regenerated from leaf protoplasts is specifically attributable to the effect of the cell wall hydrolyzing fungal extracts, most essentially the Onozuka solution. Our results therefore show that Tnt1 mobility correlates to its transcriptional activation by factors of microbial origin.

New insertions were detected in nearly 3% of the control plants regenerated from leaf explants. This demonstrates that stimuli other that microbial factors are also able to activate Tnt1 mobility. This background mobility could result from the original wounding step, since previous studies showed that, although no transcript could be detected by Northern analysis in shredded tobacco leaf tissues (Pouteau et al., 1991), a low level of expression was detected in mechanically wounded tobacco leaves through the use of a reporter gene placed under control of the Tnt1 LTR (Mhiri et al., 1997). Alternatively, background transpositions could result from a low level of activation during tissue culture. Although no Tnt1 transcript could be detected by Northern analysis, a small increase in Tnt1 copy number was indeed observed in established cell cultures (Hirochika, 1993). It is noticeable that new SSAP bands were usually detected in a reproducible manner in an SSAP experiment performed with DNA isolated from duplicate cuttings of the regenerated plantlets (data not shown). This suggests that the regenerated plants are probably not chimaeric for the new insertions, and that the transpositions that we detected have occurred very early in the regeneration process. They might therefore originate from the original wounding step rather than from the successive rounds of transposition expected from a response to tissue-culture stimuli. Finally, we cannot exclude the possibility that background transpositions could have a somatic origin in the leaves used for explant and protoplast isolation, due to a possible low background of Tnt1 expression in mature leaves. No interplant or intraplant polymorphisms were detected in our control analyzes, but somatic transpositions occurring in a few leaf cells would not be detectable by the SSAP procedure.

Interestingly, microbial-induced transposition levels measured in this work are strikingly high, since transpositions specifically induced in protoplasts have occurred in over one protoplast out of five. Due to competitive PCR amplification, the E00 primer, which contains no additional selective nucleotide in 3’, reveals only a fraction (80) of all
tobacco Tnt1 insertions. The total number of Tnt1 insertions in tobacco was indeed estimated to be more than 500 through SSAP analyzes using EcoRI primers containing two additional selective nucleotides (unpublished data). It is therefore likely that the total number of newly transposed Tnt1 copies that we observed in each plant on E00/ LTR13 profiles is also underestimated. The high level of Tnt1 transposition observed in our in vitro experimental strategy opens the perspective to assess efficiently the genetic impact of stresses such as microbial attacks or generalized abiotic stresses applied on whole plants, since the plant germline is not sequestered early and somatic transpositions can be transmitted to the progeny in plants. Although the functional consequences of the new Tnt1 insertions remain to be established, insertions of transposable elements are thought to be a major source of genetic diversity, by generating direct gene mutation or genome structural rearrangements (Kidwell and Lisch, 2000). Our work represents the first direct demonstration that factors of microbial origin, known to activate plant defense responses, are also able to generate plant genomic diversity through retrotransposition amplification. Our results therefore strengthen the hypothesis that stress modulation of transposable elements might play a role in generating host genetic plasticity in response to environmental stresses (McClinstock, 1984). Interestingly, T. viride does not invade tobacco tissues, but merely activates tobacco defense responses and hypersensitive cell death. A successful pathogen infection is thus not a prerequisite for Tnt1 amplification, and mobilization might also result from incompatible or non-host plant–microbe interactions, provided that tobacco defense responses are activated. Since such ‘unsuccessful’ microbial challenges are quite frequent in natural conditions, and since resistant or non-host plants are much more likely to produce progenies than heavily infected plants, the evolutionary impact of retrotransposon activation by microbial challenges might be quite significant.

Transcription is a key step controlling Tnt1 amplification in response to pathogen factors

Previous studies have shown that the population of tobacco Tnt1 transcripts displays a structure similar to the populations of closely related but different viral genomes referred to as ‘quasi-species’, and that the LTR U3 region is important for expression in protoplasts (Casacuberta et al., 1995). We show here a striking similarity between the U3 sequences of Tnt1 elements transcribed in protoplasts and the U3 sequences of Tnt1 copies that have subsequently completed their retrotransposition cycle and inserted into the tobacco genome. Interestingly, the sharp contrast between the populations of newly transposed copies and the more variable pre-existing insertions allows us to determine with little error, by visual analysis of U3 regions, whether a given Tnt1 element has been recently active or is an ancient insertion. The effect of fungal extracts on Tnt1 expression is transient, since their removal after the overnight treatment is rapidly followed by a sharp decrease in Tnt1 transcript levels (Grandbastien et al., 1997). This indicates that induced transpositions have mostly occured during, or shortly after, the application of the fungal extracts, at the single cell level. It can thus be assumed that transpositions in each cell have occurred in a single transposition burst. Interestingly, new Tnt1 copies detected in the same regenerant can belong to different subpopulations, indicating that different master copies can be simultaneously active within the same cell.

Since the population of transposed copies is expected to reflect template RNAs that succeed in completing the downstream steps of the retrotransposition process, the tight correlation between transcribed and transposed pools suggests that there is very little or no template selection for Tnt1 reverse transcription. Transcription appears therefore to be a key step controlling Tnt1 retrotransposition, and no specific post-transcriptional control, for instance at the reverse transcription or the integration steps, seems to further select for particular Tnt1 types, at least in pathogen-related activating conditions. It is, however, interesting to note that newly transposed copies contain a significantly lower number of BI repeats, compared with RNA templates. Deletion of repeated sequences frequently occur during retroviral replication and retrotransposition (Pathak and Temin, 1990; Xu and Boeke, 1987). We have previously suggested that BI deletions during reverse-transcription could be involved in the control of active Tnt1 populations (Casacuberta et al., 1995). The significant decrease in the proportion of 4-BII elements that we observe after completion of the reverse transcription cycle strengthens this hypothesis. Interestingly, an unexpected 2-BII transposed copy appears as a possible single direct deletion derivative of two adjacent BI. However, since we also observed in this work the frequent transposition of Tnt1–94-type copies, poorly represented at the transcriptional level (Casacuberta et al., 1995), we cannot exclude the possibility that 3-BII elements are in fact more efficiently reverse transcribed than 4-BII elements.

SSAP is a very efficient tool for monitoring retrotransposon behavior and genomic impact

Most plant retrotransposons are present in high copy number in the genome, and information on their activity is mostly available through transcript analyzes (Grandbastien, 1998). To date, direct retrotransposition has only been monitored by characterization of insertion

mutants (Grandbastien et al., 1989b; Varagona et al., 1992; White et al., 1994), or for low copy number elements (Hirochika et al., 1996; Hirochika, 1993). We show here that the SSAP strategy is an efficient tool to detect and quantify the mobility of high copy number elements. SSAP provides an interesting alternative to reverse transcription tests based on intron splicing from indicator genes (Heidmann and Heidmann, 1991), since complete retrotransposition events, up to the insertion step, are directly detected. Consequently, results can be directly interpreted in terms of genomic impact. In addition, SSAP provides a tool for isolation of new mobile retrotransposon copies when only partial LTR data are available, since it allows the detection of transposed copies without any need for insertional mutants for a known target gene. Finally, SSAP performed with carefully designed primers allows a direct and easy access to LTR sequences, the most variable region of retroviruses and retrotransposons. In addition to polymorphism studies and retrotransposition assays, SSAP can therefore be used to correlate the activity and the population evolution of retrotransposons and endogenous retroviruses, and to study more efficiently their behavior and impact on host genomes.

Experimental procedures

Plant material

Six tobacco genotypes were used: (1) N. tabacum cv Xanthi: the XHFD8 line (‘D8’), a homozygous diploid line obtained from an another culture-derived haploid line (Bourgin and Missonier, 1973); the t-1086 line (‘t’), carrying the somatically instable t mutation (Deshayes, 1979). The t mutation, originally obtained on N. tabacum cv Samsun (Dulieu, unpublished), was transferred to N. tabacum cv Xanthi by successive backcrosses (Deshayes, 1976). The F1 hybrid between the XHFD8 and t-1086 lines was also used (‘D8xtl’). (2) N. tabacum cv Gatersleben (‘Gat’). (3) N. tabacum cv Samsun (‘Sam’) and (4) N. tabacum cv PBD6 (‘PDB6’), both provided by the Institut du Tabac, ALTADIS, Bergerac, France.

Plants were cultured in a shaded greenhouse and fully expanded young leaves of the six genotypes were sampled to obtain leaf explants and mesophyll protoplasts. Leaf tissues were simultaneously sampled from similar plants for both procedures.

Protoplast-derived regenerants

Leaves were sterilized, wounded and placed in medium T0 (Bourgin et al., 1979) containing cell wall hydrolases (Onozuka R10, Yakult Biochemicals Co. Ltd, Tokyo, Japan, 0.1%; Macerozyme R10, same origin, 0.02%; Driselase, Sigma, St Quentin Fallavier, France, 0.05%) for a standard overnight (16 h) enzymatic digestion. Protoplasts were rinsed from the digestion medium, cultured in medium T0 as described (Bourgin et al., 1979) and subcultured in medium C as previously described (Muller et al., 1983). Derived colonies were plated onto solidified medium R4M0 medium (Bourgin et al., 1979), allowing callus formation and callus-derived shoot regeneration. Regenerated shoots were rooted on B medium (Bourgin et al., 1979). Each regenerated plant was produced from a different callus, that is from a different protoplast.

Explant-derived regenerants

Leaves were sterilized, and wounded leaf pieces were placed onto solidified medium R4M0 medium (Bourgin et al., 1979), allowing callus formation and callus-derived shoot regeneration at wound sites. Regenerated shoots were rooted on B medium (Bourgin et al., 1979). Calli produced at different positions of the same leaf explant were separated at early stages. Each regenerated plant was obtained from a different callus.

SSAP procedure

Plant DNA was extracted from leaf material of 5±6 leaves in vitro plants, according to the Doyle and Doyle CTAB method (Doyle and Doyle, 1990). After an RNAse treatment, RNA-free genomic DNA was extracted with phenol:chloroform, salt-precipitated and re-suspended in TE (10 mM Tris, 0.1 mM EDTA). Genomic DNA (0.5 µg) was digested with 5 U EcoRI (Gibco BRL, Cergy-Pontoise, France) in a 25-µ1 volume of RL buffer (10 mM Tris-acetate pH 7.5, 10 mM magnesium acetate, 50 mM potassium acetate, 5 mM DTT) at 37°C overnight. The restricted DNA was diluted with one volume of ligation mix comprising 50 pmols of EcoRI adaptors (Figure 1), 0.4 mM ATP, 1 U of EcoRI and 1 U of T4 DNA ligase (Pharmacia, Orsay, France) in RL buffer. Ligation was performed at 37°C for 3 h. Ligated DNA was finally diluted with 10 mM Tris-HCl, 0.1 mM EDTA pH 7.5 to the final concentration of 2.5 ng µl−1. A 5-µl aliquot of the diluted ligation mix was amplified in a 25-µl reaction containing 35 ng of 32P-labeled LTR13 primer (5′-CTTATACCTTGTCGTGAAACC-3′, +296 to +265 of Tnt1-94 (Grandbastien et al., 1989b), 50 ng of EcoRI primers (Figure 1), 0.24 mM of each dNTP, 1.5 mM MgCl2 and 1.5 U of Taq DNA polymerase (Perkin Elmer, Courtaboeuf, France) in its reaction buffer. PCR was carried out using the following conditions: 5 min at 94°C, 13 cycles of 30 sec at 94°C, 30 sec at 65°C (~0.7°C per cycle), 2 min at 72°C, 25 cycles of 30 sec at 94°C, 30 sec at 56°C and 2 min at 72°C, followed by a final extension step at 72°C for 10 min.

After PCR, samples were diluted with one volume of loading dye (95% formamide, 0.05% xylene cyanol FF and 0.05% bromophenol blue), heat denatured at 94°C for 5 min and directly cooled on ice. For each sample, a 6-8 µl aliquot was loaded on a 6% denaturing polyacrylamide gel (20 × 40 × 0.04 cm) and pre-run at 35 W for 45 min. Samples were then run at 35 W for 4 h in 1xTBE. Gels were transferred to Whatman 3 MM paper and vacuum dried at 65°C for 1 h. Dried gels were exposed to X-ray films overnight or for 48 h, depending on the signal intensity.

Isolation and characterization of new SSAP bands

New SSAP bands that were clearly separated from non-polyorphic bands were selected, excised from the dried gels using a razor blade and re-suspended in 100 µl of water. DNA was eluted from the bands by boiling for 15 min, salt-precipitated and re-suspended in 10 µl of sterile water. A 5-µl sample was then used as DNA template in PCR amplification using the SSAP condition and primers described above. Amplification products were separated on 2% agarose gels. Bands with the expected molecular weight were excised from the agarose gels and cloned in pGEMT vector according to the manufacturer’s recommendations.
(Promega, Charbonnières, France). Sequencing was performed using the Abi prism automated sequencer. The nucleotide sequence data of these clones have been submitted to the DDBJ/EMBL/Genbank databases under the accession numbers: AF401683 to AF401739.

Five to six clones per SSAP band were usually analyzed. In some cases, minor contaminating clones were obtained in addition to a major consensus clone. Primers (sequence available upon request) were designed from the flanking genomic regions of consensus clones. Direct PCRs were performed using these primers and the LTR13 primer. In each case DNA from the regenerant, from which the new SSAP band was characterized, was tested against control DNA samples from other regenerants and/or from original genotypes. A new insertion was indicated when a PCR product of the expected molecular weight was obtained with DNA from the corresponding regenerant, but not with other control DNA samples. One regenerant was lost and the transpositional origin of two new SSAP bands could only be indirectly tested by negative PCR results on control DNA samples. For two bands isolated from protoplast-derived plants, only 2 bp of adjacent flanking sequences were found. However, these bands were assumed to be new insertions, since they each generated 5-6 identical clones. Most minor contaminating clones were also tested, and shown to correspond, as expected, to pre-existing Tnt1 insertions.

All sequenced copies were named as follow: the first letter(s) indicate(s) the genotype (g and gp = Gay; d and dp = D8; t = tl; x = D8xt; s = sam; pb = PB6); the following numbers (ranging from 1 to 23) indicate(s) the regenerant and the final number identifies the SSAP band. In the case of transposed copies, ‘tr’ was added to the name. In the case of pre-existing insertions (minor clones), a lower-case letter corresponding to each clone (ranging from ‘a’ to ‘i’) was added. For instance, the s231tr, s322tr and s23tr sequences correspond to three newly transposed copies, characterized from the same regenerant (numbered 23) obtained from leaf protoplasts of the cv. Samsun line, while the s231a sequence correspond to a pre-existing insertion isolated together with the s23trt new band.

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References


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