Autonomous Transposition of the Tobacco Retrotransposon Tto1 in Rice

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The complete nucleotide sequence of the tobacco retrotransposon Tto1, one of the few active retrotransposons of plants, was determined. The sequence analysis suggests that Tto1 carries all functions required for autonomous transposition through reverse transcription. Gene organization and the nature of the transcription product suggest that Tto1 uses a gene expression mechanism different from those employed by retroviruses and most retrotransposons to regulate Gag and Pol stoichiometry.

Tto1 was introduced into rice to study its autonomous transposition in heterologous hosts. Transcription and transposition of Tto1 were observed in rice cells. To probe the autonomous transposition through reverse transcription, a modified Tto1 retrotransposon in which part of a reverse transcriptase gene was replaced with an intron-containing hygromycin resistance gene was constructed and introduced into rice cells. Loss of the intron was observed only when intact Tto1 was cotransfected. These results indicate that Tto1 can transpose autonomously through reverse transcription and that the host factors required for transposition are conserved among monocots (class Magnolilopaedia; rice) and dicots (class Liliopsida; tobacco), which diverged ~200 million years ago. These findings are discussed in relation to the regulation and evolution of retrotransposons and the possible use of Tto1 as a molecular genetic tool.

INTRODUCTION

Retrotransposons are a major class of eukaryotic transposable elements whose structure resembles that of the integrated copies of retroviruses. Retrotransposons have been found in a wide range of species, including Drosophila (Bingham and Zachar, 1989), yeasts (Boeke, 1989), filamentous fungi (McHale et al., 1992), slime molds (Rothnie et al., 1991), nematodes (Aebly et al., 1988), and plants (Grandbastien, 1992). These data strongly suggest that retrotransposons are ubiquitous components of eukaryotic genomes. With the help of the polymerase chain reaction (PCR), it has become possible to assess the ubiquity of retrotransposons. For example, retrotransposons have been found in >100 plant species, indicating that retrotransposons are ubiquitous in plants (Flavell et al., 1992; Voytas et al., 1992; Hirochika and Hirochika, 1993). By using the same strategy, the presence of retrotransposons has also been determined in fish (Flavell and Smith, 1992), amphibians, and reptiles (Flavell et al., 1995).

The presence of long terminal repeats (LTRs) and an internal domain encoding proteins analogous with the Gag and Pol proteins of retroviruses characterizes retrotransposons. The Pol protein has conserved domains characteristic of integrase, reverse transcriptase, and RNase H (Mount and Rubin, 1985; Toh et al., 1985). By sequence homology and the order of these domains, retrotransposons can be divided into two groups, the Ty1-copia and Ty3-gypsy groups (Doolittle et al., 1989; Xiong and Eickbush, 1990). Retrotransposons transpose via an RNA intermediate, as is clearly shown in yeast (Boeke et al., 1985). In contrast with retroviruses that have the envelope (env) gene, retrotransposons lack an infection step in their life cycle. Therefore, retrotransposons are thought to be inherited in the same way as other genes. The wide distribution of retrotransposons is consistent with this view. However, the distribution of some retrotransposons does not follow the accepted phylogeny of species groups, suggesting that horizontal transmission of these retrotransposons occurred across different species (Doolittle et al., 1989; Xiong and Eickbush, 1990; Konieczny et al., 1991; Flavell et al., 1992; Voytas et al., 1992; Hirochika and Hirochika, 1993). Clear examples of horizontal transmission have been shown with the P (Engels, 1992) and mariner (Robertson, 1993) elements of Drosophila. These are DNA-type elements distinct from retrotransposons. However, the frequency and mechanism of horizontal transmission are largely unknown.

To examine the probability of horizontal transmission, the vectors for transmission and the ability of the elements to transpose in heterologous hosts must be considered. Although the involvement of baculoviruses (Miller and Miller, 1982), mites (Houck et al., 1991), and plant RNA viruses (Hirochika and
Hirochika, 1993) as vectors has been proposed, no direct evidence has been reported. Furthermore, only a few transposable elements have been examined for their ability to transpose in heterologous hosts. Tff of the budding yeast Schizosaccharomyces cerevisiae cannot transpose in the fission yeast Schizosaccharomyces pombe due to its inability to use a heterologous tRNA as a primer for reverse transcription (Keeney et al., 1995). The transposition of the DNA-type transposable elements Activator and Suppressor-mutator/Enhancer of the monocot plant maize has been demonstrated in heterologous plants, including dicot plants (Haring et al., 1991).

Transposable elements are useful molecular genetic tools for analyzing genes. However, such tools are available only in a limited number of species in which active endogenous transposable elements are found. The ability to transpose in heterologous hosts makes it possible to use transposable elements in a wide range of species. For example, Activator has been used successfully for transposon tagging and enhancer trapping in diverse plant species (Jones et al., 1994; Whitham et al., 1994; Springer et al., 1995).

Although many retrotransposons of plants have been reported, only a few of them have been demonstrated to be mobile. Bst of maize was isolated as an insertion into an alcohol dehydrogenase (Adh) gene (Johns et al., 1985). However, only nonautonomous Bst elements, in which part of the pol gene was replaced with a plasma membrane proton ATPase gene fragment, were identified (Bureau et al., 1994; Jin and Bennetzen, 1994). Tnt7 of tobacco was isolated as an insertion in a nitrate reductase gene (Grandbastien et al., 1989). Its sequence suggests that Tnt1 is an autonomous element. Stoner, B5, G, and Hopscotch elements have been found in spontaneous mutant alleles of the maize waxy gene (Varagona et al., 1992; White et al., 1994). The tobacco retrotransposon Tto7 (Hirochika, 1993) and the recently isolated retrotransposon of rice, Tos77 (Hirochika et al., 1996), are mobile in cultured cells. Only Tnt1 (Pouteau et al., 1991), Tto7 (Hirochika, 1993), and Tos77 (Hirochika et al., 1996) are known to be transcriptionally active.

Recently, the mobility of the tobacco retrotransposon Tnt1 in the heterologous plant Arabidopsis has been reported (Lucas et al., 1995). This result shows that host factors required for transposition are conserved between tobacco and Arabidopsis. In this study, Tto7 was introduced into rice to test the conservation of host factors between dicot and monocot plants. We show that Tto7 can transpose autonomously in rice and could also be used as a gene transfer vector.

RESULTS

Structure and Expression of the Tobacco Retrotransposon Tto7

The Tto7-1 element is a transposed copy generated during culture of tobacco cells (Hirochika, 1993). This and the partial sequencing of terminal regions, including the LTRs, indicate that Tto7-1 carries all cis elements required for transposition (Hirochika, 1993). Here, we determined the nucleotide sequence of the entire element. The total size of Tto7-1 is 5300 bp, and one open reading frame (ORF) of 1338 amino acids was found between LTRs of 574 bp (Figure 1A). The overall organization of the Tto7-1 ORF is very similar to the copia element of Drosophila (Mount and Rubin, 1985) and Tnt1 (Grandbastien et al., 1989). Both copia and Tnt1 have one ORF of similar size (1328 and 1409 amino acids in Tnt1 and copia, respectively). A zinc finger motif characteristic of the Gag protein and sequences homologous to reverse transcriptase, and RNase H, characteristic of the Pol protein, were found in that order. Between the Gag and Pol proteins, a

Figure 1. Structure and Expression of Tto7.

(A) Structural features deduced from the complete nucleotide sequence. The sequence of the LTR and the transcription start point have been described previously (Hirochika, 1993). The thick arrow indicates the transcript of Tto7, and the open arrow indicates the longest ORF starting with the ATG codon. The Gag, INT (integrase), RT (reverse transcriptase), and RH (RNase H) domains are indicated. aa, amino acid. The complete nucleotide sequence of Tto7-1 has been submitted to DDBJ as accession number D83003.

(B) RNA gel blot analysis using different portions of Tto7-1 as probes. Total RNA was prepared from BY2 cells (Nagata et al., 1981) and analyzed by using 32P-labeled probes A to D. Restriction sites used to prepare the probes are indicated. The positions of the RNA length markers are shown.
sequence homologous to the protease was found. The homologies of the Ttol integrase domain (amino acid residues 420 to 720) with copia and Tntl were 36 and 56%, respectively. The Ttol reverse transcriptase domain (amino acid residues 810 to 1338) shows a similar degree of homology: 37% with copia and 66% with Tntl. These data indicate that Ttol and Tntl are more closely related to each other than to copia. The sequence homology and the order of the domains indicate that Ttol belongs to the Tyl-copia group (Doolittle et al., 1989; Xiong and Eickbush, 1990). The presence of all the sequence motifs involved in the enzyme activities necessary for transposition strongly suggests that Ttol-1 is an autonomous element.

Gag and Pol proteins are encoded by two different ORFs in retroviruses and most retrotransposons (Varmus and Brown, 1989; Voytas and Boeke, 1993). Translational frame shifting resulting in the Gag–Pol fusion regulates the production of the Pol protein (Varmus and Brown, 1989; Voytas and Boeke, 1993). This regulation, leading to overproduction of the Gag protein relative to the Pol protein, is necessary for the formation of functional virus and virus-like particles. In contrast, copia uses a splicing mechanism to regulate the overproduction of Gag (Brierley and Flavell, 1990; Yoshioka et al., 1990). Therefore, we examined the possibility that a splicing mechanism is also involved in the regulation of Ttol.

Total RNA was prepared from cultured cells of tobacco in which transcription and transposition of Ttol are activated (Hirochika, 1993; Hirochika and Otsuki, 1995). Analysis by RNA gel blotting using different portions of Ttol-1 as probes showed that only the full-length 5.1-kb RNA was detected (Figure 16). No spliced RNA was detected even by a more sensitive reverse transcriptase–PCR assay (data not shown). These results indicate that Ttol uses a regulatory mechanism other than splicing.

Promoter Activity of a Ttol LTR in Rice Protoplasts

The first step in the transposition of retrotransposons is transcription, which is the major regulatory step of the tobacco retrotransposons Ttol (Hirochika, 1993) and Tntl (Pouteau et al., 1991) and the rice retrotransposon Tosl7 (Hirochika et al., 1996). In tobacco, the transcription of Ttol is activated in cultured cells and further enhanced in protoplasts derived from them (Hirochika, 1993). Therefore, the promoter activity of Ttol was examined in protoplasts of cultured cells of rice. The promoter activity was monitored by assaying the expression of the chloramphenicol acetyltransferase (CAT) gene fused to the LTR (Figure 2). Relatively strong activity was observed (Figure 2B; compare pLTRCAT-1 and p35SCAT): the Ttol LTR was stronger than the cauliflower mosaic virus 35S promoter, which is one of the most active promoters (Odell et al., 1985; Kay et al., 1987). In tobacco protoplasts (Figure 2A), the same construct showed slightly weaker activity than did the 35S promoter. To examine whether the same sequences in the LTR contribute to the promoter activity in both rice and tobacco, deletion derivatives were constructed and their activity compared. The deletion of −164 to −151 had a strong effect in both species, though the reduction was less in tobacco. Based on these results, we conclude that although the contribution of the cis regulatory region to promoter activity is different in rice and tobacco, the promoter activity in rice is strong enough to support the transposition of Ttol-1.

Transposition of Ttol-1 in Rice Cells

The plasmids used for the transposition assay in rice are shown in Figure 3. pSKTtol carries the complete Ttol-1 sequence cloned in the pBluescript SK + vector. A 36-bp deletion, which has almost no effect on the promoter activity in rice (Figure
pSKTtol

pSKTtol(-36)

pSKTtol(-1.5)

pSKTtol-Hyg

Figure 3. Structure of the Plasmids Used for the Transposition Assay. Tto1-1 and its derivatives were cloned into the pBluescript SK+ vector. Only Tto1 sequences are shown.

which does not cut Tto1-1, and ligated. The fragments carrying both flanking sequences were cloned after amplification by inverse PCR. The sequence of one clone is shown in Figure 5. As expected, the complete 36-bp sequence was recovered at the 5' end. This Tto1 copy was flanked by direct repeats of 5 bp. This was shown, by analyzing an empty target site, to be the consequence of a duplication of the target sequence during the insertion into the genome (Figure 5). The 5-bp target sequence duplication is typical of Tto1 transposition in tobacco (Hirochika, 1993). These results clearly show that the transposition of Tto1 occurred in rice. DNA gel blot analysis using the cloned flanking sequence detected the band shift only in the subclone 5 from which the flanking sequence was amplified (data not shown), indicating that transposition of Tto1 occurred during subculture.

As a control, pSKTtol(-1.5) (Figure 3), a derivative of pSKTtol(-36) carrying a deletion in the pol region, was introduced into rice. From the resulting 30 hygromycin-resistant calli examined, no fragment was amplified by PCR with the primers used for the analysis of pSKTtol(-36). These results indicate that the pol region is necessary for the transposition of Tto1-1 in rice. The results also indicate that endogenous rice retrotransposons (Hirochika et al., 1992, 1996) cannot help to transpose Tto1-1. This was also confirmed by the experiment described in the next section. Thus, the mobility of Tto1-1 in rice indicates that Tto1-1 is transposed autonomously.

Figure 4. DNA Gel Blot Analysis of Transposition of Tto1 in Rice. pSKTtol(-36) was mixed with pUCHyg and electroporated into rice protoplasts. DNAs from one positive callus (original) and clones 1 to 12 derived from this callus were digested with HindIII and analyzed by DNA gel blotting, using a 32P-labeled XbaI (nucleotide 4284)-PstI (nucleotide 4688) fragment. Arrowheads indicate bands that are absent in the original callus. Molecular length markers are given at left in kilobases.
Transposition of \( Tto1 \) in Rice 729

M-bp deletion

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**Figure 5.** Analysis of Sequences Flanking the Transposed \( Tto1 \) and the Empty Target Site.

The flanking sequences were amplified from the subclone 5 genomic DNA (Figure 4) by inverse PCR, and the empty target site was amplified from the control genomic DNA by PCR. Amplified fragments were sequenced after cloning into M13mp18. The site of the insertion target sequence (TS) and the resulting sequence duplication are indicated by underlining.

**Transposition of \( Tto1 \) through Reverse Transcription**

To probe transposition through reverse transcription, a modified \( Tto1 \) retrotransposon was constructed in which a part of the pol gene was replaced with an intron-containing HPT gene (pSKTto1-Hyg; Figure 3). If \( Tto1 \) transposes through reverse transcription, loss of the intron would be expected (Figure 6A), as has been demonstrated for \( Ty1 \) (Boeke et al., 1985). Because pSKTto1-Hyg has a defect in the pol gene, we expected it to transpose only when the Pol protein encoded by \( Tto1 \) was supplied. Thus, pSKTto1-Hyg was cotransfected with pSKTto1(-36) carrying the intact pol gene, and the resulting hygromycin-resistant calli were analyzed. Loss of the intron was assayed by PCR, using primers 1 and 3 shown in Figure 6A. The PCR products were analyzed after cutting with PstI to distinguish clearly the intron-containing and intron-less fragments. Expected lengths of the intron-containing and intron-less fragments were 495 and 423 bp, respectively. A fragment of \( \sim 420 \) bp was found in three of 10 hygromycin-resistant calli examined (Figure 6B). In these three calli, another fragment of 280 bp was also amplified. The structure of the 420- and 280-bp fragments was examined by sequencing. As shown in Figure 7A, the intron sequence of 72 bp was lost in the 420-bp fragment. In the 280-bp fragment, a sequence of 141 bp in the HPT gene (Figure 7B), as well as the intron sequence, was deleted. The 141-bp sequence is intron-like, because both junction sequences match the corresponding region of the RNA splicing consensus sequence (Mount, 1982). The deletion of the intron and the intron-like sequence can be readily explained by splicing followed by reverse transcription.

In the calli in which the loss of the intron was observed, no intron-containing fragment was detected (Figure 6B). This was further confirmed by PCR, using primers 2 and 3. The reaction was designed to amplify only the intron-containing fragment (Figure 6C), which indicates that the \( Tto1 \) carrying the HPT gene transposed directly from the plasmid vector to the rice genome via an RNA intermediate. If it had transposed

**Figure 6.** PCR Analysis of the Loss of an Intron during Transposition.

(A) Structure of the 35S-intron-HPT region of pSKTto1-Hyg introduced into rice and its intron-less derivative expected from the transposition through RNA. Two sets of primers indicated by small arrows were used to detect the loss of an intron. The PstI site in the HPT gene was used to distinguish clearly intron-containing and intron-less fragments. Sizes of expected fragments are indicated.

(B) PCR analysis using the primers 1 and 3. pSKTto1-Hyg was mixed with pSKTto1(-1.5) or pSKTto1(-36) and electroporated into rice protoplasts. Ten individual hygromycin-resistant calli obtained from each transfection were subjected to PCR analysis. Lanes 1 contain HindIII-digested pX714 as a size marker; lanes 2 to 11 contain PCR products digested with PstI. Sizes of amplified fragments are indicated on the right of the panel.

(C) PCR analysis using primers 2 and 3. The hygromycin-resistant calli used in (B) obtained from the transfection with pSKTto1-Hyg and pSKTto1(-36) were subjected to the PCR analysis. Lane 1 contains HindIII-digested pX714 as a size marker; lanes 2 to 11 contain PCR products digested with PstI. Sizes of amplified fragments are indicated on the right of the panel.
Ttol-1.

The 280-bp fragment should have been detected.

As a control, pSKTto1-Hyg was cotransfected with pSKTto1(-1.5) lacking the intact pol gene. The resulting hygromycin-resistant calli were examined by PCR (Figure 6B). In the 10 calli examined, only the intron-containing fragment was obtained. The analysis of another 10 calli gave the same result. These data indicate that the loss of the intron depends on cotransfection with the intact Tto1-1 sequence, confirming our previous conclusion that endogenous rice retrotransposons cannot help to transpose Tto1-1.

**DISCUSSION**

The complete nucleotide sequence of Tto1-1 was determined. Sequence analysis showed that Tto1-1 carries all the structural features characteristic of retrotransposons. Transposition assays in the heterologous host plant species rice demonstrated that Tto1-1 is an autonomous element. In rice, we have recently found retrotransposons activated by tissue culture (Hirochika et al., 1996). To exclude the possibility that these endogenous retrotransposons supply trans-acting factors to help in the transposition of Tto1-1, we assessed the ability to transpose a derivative of Tto1-1 carrying a deletion in the coding region. The transposition of the deletion derivative was observed only when intact Tto1-1 was cotransfected (Figures 4 and 7), indicating that endogenous retrotransposons cannot help to transpose Tto1-1. Thus, the mobility of the introduced Tto1-1 in rice indicates that it can transpose autonomously. Although the mobility of the tobacco retrotransposon Tnt1 in the heterologous plant Arabidopsis has been shown by Lucas et al. (1995), the possibility that endogenous elements assisted transposition was not ruled out. Therefore, Tto1 is the first plant retrotransposon that has been shown to be an autonomous element. The copy number of Tto1 in tobacco is ~30 (Hirochika et al., 1993). These endogenous copies complicate analysis of Tto1. Studies of this element in rice, in which endogenous retrotransposons do not supply trans-acting factors for the transposition of Tto1, will facilitate structure-function analysis.

In retroviruses and most retrotransposons, Gag and Pol proteins are encoded by two separate ORFs (Varmus and Brown, 1989; Voytas and Boeke, 1993). These ORFs are often expressed as a fusion protein due to suppression of the stop codon or ribosome frame shift between the ORFs. As a consequence of this regulation, the level of the Pol protein is 4 to 20% of that of the Gag protein. In contrast, the Gag and Pol proteins are encoded by a single ORF in Tto1. Similarly, Tnt1 (Grandbastien et al., 1989) and Hopscotch of maize (White et al., 1994) carry only one ORF. The copia element of Drosophila also carries only one ORF; in which the Pol production is regulated by a splicing mechanism (Brierley and Flavell, 1990; Yoshioka et al., 1990). Major RNAs of copia are 5 and 2 kb in cultured cells, and the 2-kb RNA encoding the Gag protein is generated by splicing the 5-kb RNA. No spliced Tto1 RNA was detected in tobacco (Figure 1B) or transgenic rice (data not shown), suggesting that mechanisms other than splicing regulate the overproduction of Gag relative to Pol. Similarly, no spliced RNA of Tnt1 was detected (Pouteau et al., 1991), and the possible regulatory mechanism has been discussed (Lucas et al., 1995). In the retrotransposon Ttf of S. pombe, a third mechanism has been reported: the translation of a fusion protein followed by selective degradation of Pol protein (Levin et al., 1993). Although Ttf is a Ty3-gypsy-type element, the Ty1-copia-type retrotransposons of plants might also use this mechanism. However, we cannot exclude the possibility that such regulation is unnecessary for the transposition of plant retrotransposons.

Transposable elements are useful molecular genetic tools to analyze genes. For example, the P element has been used for diverse purposes, such as gene transfer, insertion mutagenesis, enhancer trapping, and gene cloning (Engels, 1989). Although the first transposon tagging in eukaryotes was accomplished using copia (Bingham et al., 1981), retrotransposons have been used in only a limited number of cases. This seems due mainly to the low efficiency of transposition.
Ty1 has been modified to increase transposition efficiency and has been used for gene tagging and as a gene transfer vector (Boeke, 1989). Retrotransposon vectors have a unique character: it is possible to increase the copy number of the introduced genes because retrotransposons undergo replicative transposition. As shown in this study (Figure 6), it eventually will be possible to use TtOl as a gene transfer vector, though currently the efficiency of transfer is not high enough for routine use. Because the transposition of plant retrotransposons is regulated primarily at the transcriptional level (Pouteau et al., 1991; Hirochika, 1993; Hirochika et al., 1996), it may be possible to increase the frequency by modifying the promoter elements. Another possible use of TtOl is to develop a site-selected mutagenesis system (Balling and Benzer, 1989; Kaiser and Goodwin, 1990; Koes et al., 1995).

The presence of various barriers to transposition in heterologous hosts has been reported. For example, the P element can transpose in all drosophilid species tested but not in the related tephritid species (O'Brocha and Handler, 1988). Drosophilids and tephritids diverged ~120 million years ago. Because the transcript of the transposase gene was observed in the tephritid species, involvement of nonconserved host-encoded factors in the transposition of P elements has been suggested. The barrier for the transposition of Ty1 in the fission yeast system has been shown to be a structural difference in the primer tRNA used for reverse transcription (Keeney et al., 1995). Although the LTR of copia is as active as the 35S promoter in rice protoplasts (Ou-Lee et al., 1986), copia may not be able to transpose in plants because of the same problem. All of the known plant retrotransposons use the initiator tRNA as a primer for reverse transcription (Grandbastien, 1992). Recently, one exception has been reported (Hu et al., 1995). As we discussed previously (Hirochika et al., 1992), the use of the initiator tRNA as the primer may help horizontal transfer because the initiator tRNA is highly conserved (Sprinzi et al., 1987).

Tnt1 of tobacco can transpose in Arabidopsis (Lucas et al., 1995). Both tobacco and Arabidopsis are dicot species and belong to the class Magnoliopsida, whereas the monocot rice belongs to the class Liliopsida. Here, we have shown that there is no barrier for the transposition of TtOl even between monocots and dicots that diverged ~200 million years ago (Wolfe et al., 1989). Many examples showing differences in the gene expression machinery between monocots and dicots have been reported. For example, the gene for the small subunit of ribulose bisphosphate carboxylase of wheat is not transcribed in tobacco (Keith and Chua, 1986). Differences in splicing and termination have also been reported (Keith and Chua, 1986; Goodall and Filipowicz, 1991). Our current data indicate that these differences do not interfere with the transposition of TtOl and that host factors required for transposition of TtOl are conserved. The data also indicate that the horizontal transfer of retrotransposons, inferred from the phylogenetic analysis based on the sequence comparison, is possible if a vector is available.

The known plant retrotransposons are highly regulated mainly at the transcriptional level. In contrast to yeast and Drosophilila retrotransposons, plant retrotransposons have been found to be inactive under normal growth conditions but can become active under stress conditions, such as tissue culture (Hirochika, 1993; Hirochika et al., 1996) infection by bacteria, fungi (Pouteau et al., 1994), and viruses (Hirochika, 1995). The promoter activity of TtOl was examined by fusing the LTR to the CAT gene (Figure 2). The activity is stronger in rice than that of the 35S promoter, which is one of the most active promoters (Odell et al., 1985; Kay et al., 1987). Our current data involving deletion analysis suggest that the cis elements used are different between tobacco and rice. The use of a cis regulatory element not used in the original host in the heterologous plants will lead to a change in the regulation of retrotransposons. The TtOl LTR is transcriptionally active in the leaves of regenerated rice plants (H. Hirochika, Y. Ostuki, and K. Sugimoto, unpublished data), although it was completely inactive in tobacco leaves (Hirochika, 1993). Altered transcription of Tnt1 in Arabidopsis has also been reported (Lucas et al., 1995). These results suggest that retrotransposons behave differently in the new hosts. As a consequence of the deregulation, a burst of transposition may be induced. The very high copy number of many plant retrotransposons (Wessler et al., 1995), for example, 100,000 copies for BIS-1 of barley, may have been attained by this mechanism. Further studies on the behavior of the introduced retrotransposons should contribute to our understanding of the interaction between retrotransposons and hosts and their evolution.

METHODS

Plant Materials

Calli of rice were induced and cultured as described by Hirochika et al. (1996). The Oc cell line of rice (Baba et al., 1986) was cultured in AA medium (Müller and Grafe, 1978). Rice protoplasts were prepared as described by Sugimoto et al. (1994). BY2 cells of tobacco were cultured and treated to prepare protoplasts as described by Nagata et al. (1981).

Extraction of Nucleic Acids and RNA and DNA Gel Blot Hybridization

Cells were frozen and homogenized in liquid nitrogen, and total RNA was extracted using Isogen (Nippongene, Toyama, Japan). Poly(A)+ RNA was purified using oligo(dT)-latex (Daichichi Chemical, Tokyo, Japan). Total DNAs were prepared, and their concentration was determined as described by Hirochika (1993). Blotting, preparation of probes, and hybridization were as described previously (Hirochika et al., 1992).
DNA Sequencing

DNA fragments derived from the clone Tto-1 (Hirochika, 1993) were subcloned into M13mp18 or M13mp19 vectors after digestion with restriction enzymes or exonuclease III, followed by mung bean nuclease, using a deletion kit (Takara, Kyoto, Japan). DNA sequences were determined by using a Taq DyeDeoxy Terminator Cycle Sequencing Kit (ABI, Foster City, CA) and an autosequencer (model 370; ABI) or manually determined by the chain termination method, using Sequenase (Amersham, Buckinghamshire, UK).

Sequence Analysis

Handling of primary sequences and multiple sequence alignment were performed using the GeneWorks 2 software (IntelliGenetics, Mountain View, CA).

Construction of CAT Plasmids

p35SCAT was constructed as follows. The Xbal-Sacl fragment carrying 9-glucuronidase gene of pBII221 (CLONTECH, Palo Alto, CA) was replaced with the Xbal-Sacl polylinker fragment derived from pUC19 to produce p35SSt. A Sau3A fragment carrying the chromosomal acetyltransferase (CAT) gene derived from pCAT-A (Hirochika et al., 1987) was flanked with the Klenow fragment of DNA polymerase I and cloned into the SmaI site of p35SSt, resulting in p35SCAT. The promoter region of Tto-1 was prepared by polymerase chain reaction (PCR) amplification, using primers Tnt2-L (5'-CTCTAGAATTTCATCTGTATT-3'), corresponding to nucleotides -22 to -3 from the 5' end of Tto-1) and LTR-2 (5'-GCCAAAATATCCGACTAGT-3', corresponding to nucleotides 329 to 310 of Tto-1). The amplified fragment was cloned between SmaI and SphI sites of p35SCAT blunt ended with T4 DNA polymerase, resulting in pLTRCAT-1. Deletion plasmids (pLTRCAT-2 to pLTRCAT-6) were constructed as follows. The promoter fragments with different deletions were amplified using forward primers with an Xbal site at the 5' end and the reverse primer LTR-2. After digestion with Xbal and Spel, the amplified fragments were cloned between the Xbal and Spel sites of pLTRCAT-1. The deletion end points of the resulting plasmids pLTRCAT-2, pLTRCAT-6, pLTRCAT-5, pLTRCAT-3, and pLTRCAT-4 were -161, -151, -132, -96, and -37, respectively, from the transcription start site (Hirochika, 1993).

PCR Analysis of Transposition

The oligonucleotides LTR-11 (5'-TCTAGGTTTTCCTAAAATATAGG-3', nucleotides 1 to 25) and Tnt2-gag (5'-GGATGAATAGTACTCGTACGT-ATG-3'; nucleotides 630 to 606) were used to amplify the 5' end recovered after transposition of Tto-1 del carried on the plasmid pSKTtol-Hyg. Sequences flanking transposed Tto-1 (target site sequences) were amplified by inverse PCR. Approximately 1 μg of a total DNA from the transgenic calli was digested with HindIII, which does not cut Tto-1. Digested DNA was ligated and purified as described by Sugimoto et al. (1994). Two sets of primers were used for two-step PCR. The primers LTR-17 (5'-TGGTGTCGGATTTGACG-3', nucleotides 440 to 462) and LTR-10 (5'-ATTGCATCATGTCATCGACT-3', nucleotides 86 to 65) were used for the first PCR, and the primers LTR-14 (5'-TCCATCTGTCG-CAGTAGTTTAGTG-3', nucleotides 535 to 558) and LTR-16 (5'-CTTCATCCACATCAACCA-3', nucleotides 44 to 22) were used for the second reaction.

To analyze the loss of an intron during transposition of Tto-1-Hyg carried on the plasmid pSKTtol-Hyg, two sets of primers were used (see Figure 6A). Primers 1, 2, and 3 are 5'-CGCTCTCTTACTAAAGAAG-3', corresponding to -40 to -20 of the 35S promoter; 5'-AGTAGTAAATGTCAGT-3', corresponding to the junction between exon 1 and intron 1 of the phaseolin gene (Slightom et al., 1983); and 5'-CCATCTGTCACGGTTAC-3', corresponding to the 3' end of the HPT gene (Griffith and Davies, 1983), respectively. Amplification reactions consisted of 30 cycles of 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C. PCR products were cloned into the HindII site of M13mp18 or M13mp19.

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