

Identification of Active Transposon *dTok*, a Member of the *hAT* Family, in Rice

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Recent completion of the sequencing of the rice genome has revealed that it contains >40% repetitive sequences, most of which are related to inactive transposable elements. During the molecular analysis of the *floral organ number1/multiple pistil 2* (*fon1/mp2*) mutant, we identified an active transposable element *dTok0* that was inserted at the kinase domain of *FON1*, a homolog of *CLAVATA1*. Insertion of the element into *FON1* generated an 8 bp duplication of its target sites, which is one of the major characteristics of the *hAT* family of transposons. The *dTok0* element was actively transposed out of the *FON1* gene, leaving 5–8 bp footprints. Reinsertion into a new location was observed at a low frequency. Analysis of the genome sequence showed that the rice cultivar ‘Nipponbare’ contains 25 copies of *dTok* elements; similar numbers were present in all the *Oryza* species examined. Because *dTok0* does not encode a transposase, enzyme activity should be provided in trans. We identified a putative autonomous transposon, *Tok1* that contains an intact open reading frame of the *Ac*-like transposase.

Keywords: *hAT* superfamily — *floral organ number 1/multiple pistil 2* — *Oryza* — Rice — Transposon.

Abbreviations: *Ac*, Activator; AFLP, amplified fragment length polymorphism; *Ds*, Dissociation; *FON1*, floral organ number1; MITE, miniature inverted repeat transposable element; *fon1/mp2*, floral organ number1/multiple pistil 2 mutant; MULE, mutator-like transposable element; ORF, open reading frame; TE, transposable element; TIR, terminal inverted repeat.

Introduction

Both eukaryotic and prokaryotic genomes possess numerous transposable element (TE) families (Nekrutenko and Li 2001, Feschotte et al. 2002, Tu and Coates 2004). TEs, the single largest component in the genetic materials of most eukaryotes, account for 45% of the human genome (Lander et al. 2001) and >80% in some plant species

(Vicent et al. 1999, Meyers et al. 2001). The rice genome sequence contains >40% repetitive sequences, with most being related to TEs (Goff et al. 2002, Yu et al. 2002). The most frequent TEs are retrotransposons, which comprise approximately 15% of the rice genomic DNA (Jiang et al. 2004a). Miniature inverted repeat transposable elements (MITEs) are also abundant, making up 6% of that genome.

Most of these sequences in higher eukaryotes are apparently inactive TEs; only a tiny fraction is active under normal conditions. In rice, the usually silent *copia*-like retroposon, *Tos17*, is activated by tissue culture (Hirochika et al. 1996). In contrast to other plant retrotransposons, its copy number is low: 1–5 copies depending on the cultivar. During tissue culture, however, this number increases to at least 30. These features make the element suitable as an insertional mutagen of the rice genome (Hirochika 2001). Another retroposon, *Karma*, is also active in cultured rice cells in response to DNA hypomethylation (Komatsu et al. 2003).

Evolutionary studies suggest that some MITEs in plant and animal genomes have spread recently (Zhang and Hong 2000, Feschotte et al. 2002). For example, a sequence classified as a *Tourist*-like MITE, called *miniature Ping* (*mPing*), is present in about 70 copies in japonica rice (cv. Nipponbare) and at least 14 copies in indica rice (cv. 93-11) (Goff et al. 2002, Yu et al. 2002). These elements transpose actively by cell culture, anther culture and γ -ray irradiation (Jiang et al. 2003, Kikuchi et al. 2003, Nakazaki et al. 2003).

Since the discovery of the TEs *Activator* (*Ac*) and *Dissociation* (*Ds*) from maize (McClintock 1947, McClintock 1948), *Ac/Ds*-like elements have been identified from other plants, animals and fungi. The first two *Ac*-like elements identified were *Tam3* from snapdragon (Hehl et al. 1991) and *hobo* from *Drosophila melanogaster* (Calvi et al. 1991). In many plant species, including *Arabidopsis*, tobacco, petunia, potato and rice, *hAT* elements have been identified and characterized (Koster-Topfer et al.

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1990, Tsay et al. 1993, Grappin et al. 1996, Fujino et al. 2005). In maize, *Ac* is stable during the vegetative growth stages, but is transposed shortly after replication (Ros and Kunze 2001). *Mx*, another member of the *hAT* superfamily in maize, is induced by X-rays (Xu and Dooner 2005). The activity of the *Arabidopsis* transposon *Tag1* is restricted to germinal events, with no detectable vegetative excision (Galli et al. 2003). The *nDart* element, a member of the *hAT* transposons recently identified from rice, is excised at high frequency in vegetative tissues (Fujino et al. 2005). In other species, the snapdragon *Tam3* is inactive at high temperatures (around 25°C), but activated by low-temperature treatments (around 15°C) (Martin et al. 1989, Hashida et al. 2003). All of these results have made it possible for researchers to use *Ac*, *Tam3* and *Tag1* as insertional mutagens in their host and heterologous plants (Martin et al. 1989, Liu et al. 1999, Ki et al. 2002).

Here, we report identification of *dTok*, a *Ds*-like element, from the *fon1/mp2* mutant. Unlike the *nDart* element, *dTok* is inactive in most vegetative organs but is occasionally active in reproductive organs.

Results

The naturally occurring mp2 mutant is allelic to fon1-3

We have previously identified two alleles, *fon1-3* and *fon1-4*, which were generated by insertion of T-DNA and *Tos17*, respectively, into the *FON1* gene, an ortholog of *clv1* (Moon et al. 2006). The point mutations, *fon1-1* and *fon1-2*, in the gene disrupted meristem balance, resulting in alteration of floral organ numbers and the architecture of primary rachis branches (Suzaki et al. 2004). Our knockout alleles, *fon1-3* and *fon1-4*, displayed a strong phenotype of multiple pistils and stamens. These phenotypes are similar to those of multiple pistil mutants, *mp1* and *mp2*, which were identified as naturally occurring mutants (Heu and Suh, 1976). To examine whether they are in an allelic relationships, we cross-fertilized the T-DNA-tagged *fon1-3* line with the naturally occurring mutant line carrying *mp1* or *mp2*. When *fon1-3* carpels were fertilized with *mp1* pollen grains, all the progeny exhibit only a single seed, indicating that the T-DNA mutant and *mp1* are not allelic. However, when the T-DNA-tagged line was pollinated with the *mp2* line, their progeny produced the phenotypes of multiple floral organ numbers and numerous seeds (Fig. 1A). This complementation experiment demonstrated that the T-DNA-driven *fon1-3* mutant is allelic to the previously reported *mp2* mutant. Sequencing of the region containing the *FON1* gene of this *fon1/mp2* mutant revealed a 670 bp insertion in the first exon, which disrupted the kinase domain of the protein (Fig. 1B).

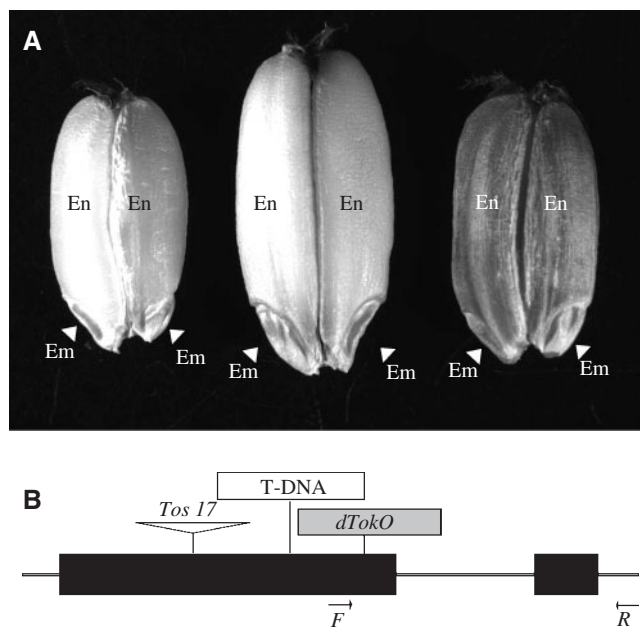


Fig. 1 Phenotypes and positions of the insertional alleles in *FON1*. (A) Seed morphology of *fon1-3* (left), *mp2* (middle) and *fon1-3/mp2* (right). Em, embryo; En, endosperm. (B) Schematic representation of the *FON1* gene and insertional alleles. Relative insertion positions of T-DNA (*fon1-3*), *Tos17* (*fon1-4*) and *dTok0* (*mp2*) are represented. Dark-filled boxes are exons; the inter-line is an intron. F1 and R1 are primers used to test the status of *dTok0* in *FON1*. The scale bar represent 200 bp.

The insertion element in fon1/mp2 is a member of the hAT family of transposons

The 670 bp sequence present in the *fon1/mp2* mutant contained an 8 bp target site duplication, 18 bp terminal inverted repeat (TIR) sequences and an approximately 200 bp subterminal domain (Fig. 2A). The subterminal sequences at both ends contained 12 and 16 copies of repetitive ACGG and TCGG tetranucleotides, which are known as the binding sequences of *Ac* transposase (Becker and Kunze 1997). These characteristics indicated that the 662 bp sequence is a TE, which we named *dTok0* (Tok is 'jump' in Korean).

Our database search of the 'Nipponbare' genomic sequences identified 25 regions highly homologous to the *dTok* sequence present in the *FON1* gene. These we called *dTok1-dTok25*, starting from the short arm of chromosome 1 (Fig. 2B). Among those 25 regions, 16 were located near the centromeres or telomeres. The insertion element present in the *FON1* gene was identical to *dTok15* on chromosome 9. The *dTok* elements had similar lengths, ranging between 631 and 668 bp (Supplementary material 1). Compared with *dTok0*, there were 1–16 bp of insertions, 1–26 deletions and base substitutions. However, overall homology was quite high (at least 90%). The 18 bp

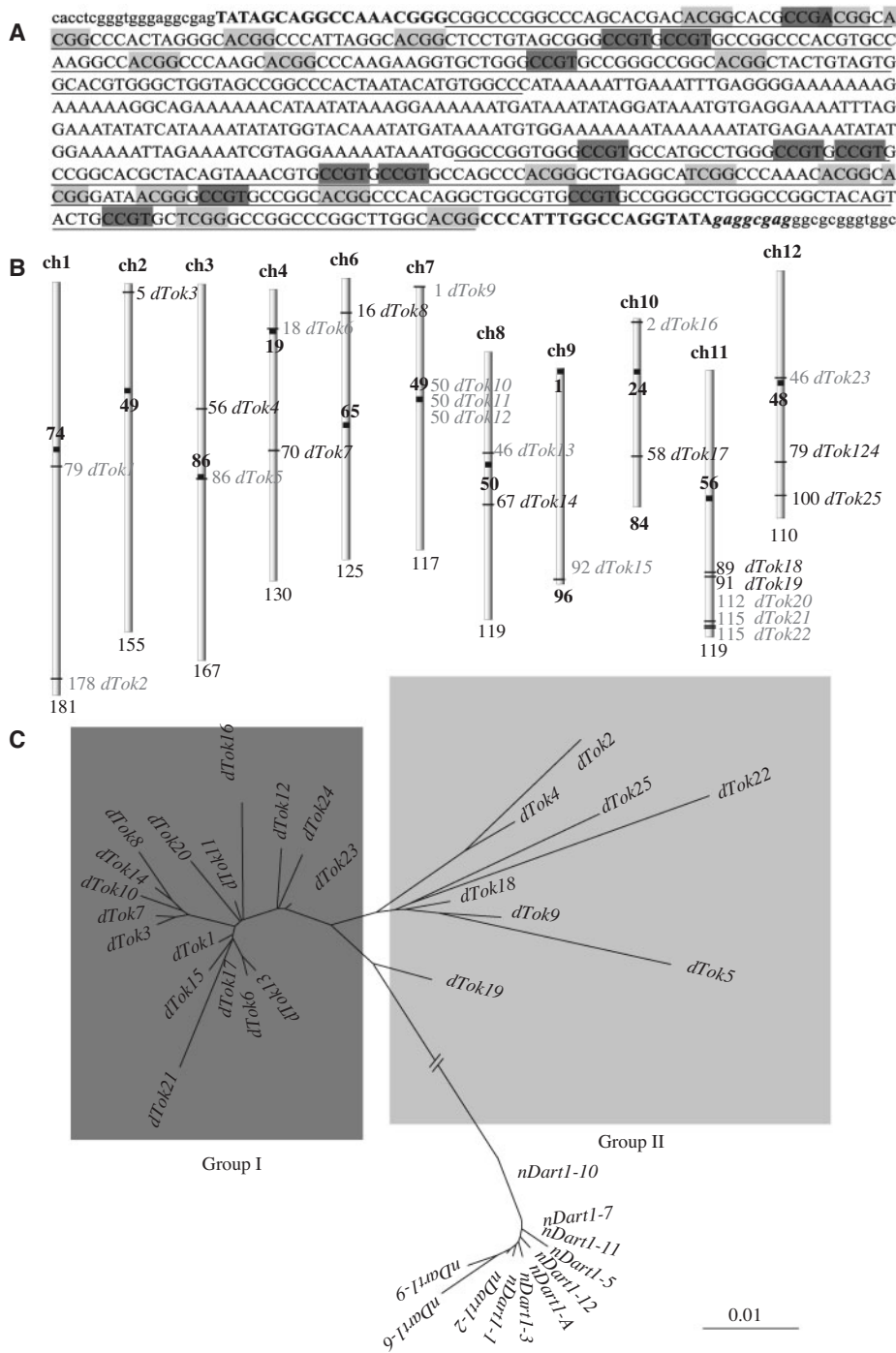


Fig. 2 Sequence, chromosomal location and phylogenetic relationship of *dTok* elements in cv. Nipponbare. (A) The sequence of *dTok0* inserted in *FON1*. *FON1* sequence and target site-duplicated sequence are represented by lower case letters and italics, respectively. The *dTok0* sequence is marked by upper case letters. Terminal invert repeats and the subterminal domain are represented by bold face and underlining, respectively. ACGG and TCGG tetranucleotide sequences, which are putative Ac-binding sites, are shown on both strands marked by light gray boxes or dark-gray boxes. (B) The location of 25 *dTok* elements on ‘Nipponbare’ chromosomes. Longitudinal bars indicate chromosomes. Centromeres are represented by black rectangles. Numbers indicate chromosomal locations of *dTok* elements. (C) Phylogenetic analysis of *dTok* elements and *nDart*. Sequences were aligned using ClustalX, and the tree was constructed using the neighbor-joining program in MEGA version 2.1. *dTok* in different locations between japonica and indica belong to group I. Except for *dTok1*, elements present in the same location between japonica and indica belong to group II. The scale bar corresponds to 0.01 nucleic acid substitutions per base. The length of the line between *dTok* and *nDart* was reduced to 1/20 of the actual distance.

TIR sequences were well conserved, except that the third base A from one end was substituted by T or C in eight *dTok* sequences. All the elements generated 8 bp duplications at the target sites. In three of them, *dTok4*, *dTok22* and *dTok25*, a one nucleotide mismatch was present in the duplicated sequences.

Comparison of the genomic sequences for ‘Nipponbare’ and ‘93-11’ revealed that seven elements (*dTok1*, 2, 4, 5, 9, 19 and 25) were present in the same chromosome loci between two cultivars, while eight others (*dTok3*, 6, 8, 10, 11, 14, 20 and 24) occurred in different loci. The remaining 10 elements present

in 'Nipponbare' were not found in the '93-11' genome sequence database.

Phylogenetic analyses of the *dTok* elements enabled us to divide them into two groups (Fig. 2C). Those in the first group were more closely related to each other than those in the second, which was more divergent. Except for *dTok1*, the *dTok* elements present in the same location between the japonica and indica cultivars belonged to group II. In contrast, all the *dTok* elements found in different locations between the two cultivars belonged to group I.

dTok0 is an active transposable element

Since the *fon1/mp2* mutant was isolated in 1976, its progeny have been independently maintained in several locations, and have been propagated through at least 10 generations. Here, we examined the stability of *dTok0* in 14 independently maintained *fon1/mp2* lines (Fig. 3A). For preparation of DNA, >5 seeds from each line were germinated and pooled at the seedling stage. PCR amplification of the region carrying *dTok0* showed that the TE remained intact in line 7. However, in lines 1, 9, 12 and 13, only the small fragment was amplified, indicating that *dTok0* had jumped out from the region. In the remaining nine lines, both small and large fragments were amplified. The relative intensity of the bands differed among the lines, with the smaller band being stronger in

line 2, but weaker in lines 4 and 5. This reflected the difference in populations of *dTok0* deletions among those lines.

Sequencing the small fragments amplified from individuals of various stocks revealed that the *dTok0* element was deleted, and that an eight nucleotide footprint was present at the deletion sites for the majority of the lines (Fig. 3D). In addition, we found five or seven nucleotide footprints. Because these extra sequences caused frameshift mutations in *FON1*, deletion of the transposon did not result in recovery of gene function. Therefore, these results indicate that *dTok0* has been actively transposed out from the *FON1* gene in most of the lines during maintenance.

We observed that the *fon1/mp2* mutant line occasionally reverts to the wild-type phenotype, generating single seeds. PCR amplification of the revertants showed that the length of the *dTok0*-containing fragment was reduced to that of the wild type in that case (Fig. 3B). Sequence determination of the revertants also indicated that the *dTok0* element jumped out from the *FON1* gene, leaving 6 bp footprints. Therefore, the *FON1* protein in the revertants gained two amino acids that apparently did not affect the protein's functional role.

The observations described above demonstrate that *dTok0* is an active element. To determine the conditions for TE transposition, we used *fon1/mp2* plants that carried the intact *dTok0* sequence on both chromosomes. DNA was prepared from each leaf according to the position and examined for reduction in length. Nevertheless, we found no indication that the element had moved out from *FON1* (data not shown). It was also found that various stress treatments, e.g. tissue culture, cold, heat or γ -rays, did not induce this transposition.

Since *dTok0* was not active during the vegetative growth, we examined the mobility of *dTok0* during the reproductive phase. We grew the *dTok0* homozygous plants to maturity and collected their seeds. The resultant seedlings from individual seeds were then examined via PCR for TE mobility (Fig. 3C). Among the 52 progeny examined, band deletion was detected in 32. Interestingly, none of the progeny lost *dTok0* on both chromosomes, suggesting that the transposition occurred in either the male or the female gametophyte, but not in both. Sequence analysis of those deletion sites showed that the progeny carried different footprints, thereby demonstrating that transposition had occurred in reproductive organs.

Because *dTok0* frequently jumps out from the *FON1* gene, we examined whether this element then inserts into a new locus. Amplified fragment length polymorphism (AFLP) analysis of 13 progeny from the *fon1/mp2* line indicated that the element did indeed re-insert in a new locus for one of the progeny (Fig. 4, sample 11).

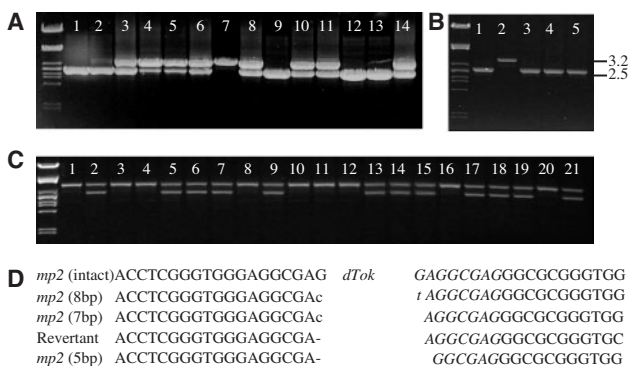


Fig. 3 Stability of *dTok0* in *FON1/MP2*. (A) Status of *dTok0* in 14 independently maintained lines. The upper 3.2 kb bands represent the *FON1/MP2* gene with *dTok0*, whereas the lower 2.5 kb bands indicate loss of TE. PCR was performed using the F1 and R1 primers shown in Fig. 1B. The numbers represent individually maintained *fon1/mp2* lines. (B) The status of *dTok0* in wild-type revertants. Upper and lower bands are with and without *dTok0*, respectively. 1, Wild type; 2, *fon1/mp2* with homozygous *dTok0*; 3–5, three independent isolates of wild-type revertants from *fon1/mp2*. (C) Status of *dTok0* in progeny of the *fon1/mp2* plant with homozygous *dTok0*. 1, A parental *fon1/mp2* plant; 2–21, independent progeny from the parental plant. (D) Footprints of *dTok0*. Italicized characters indicate duplicated sequence at the *dTok0* target site. Dashes and lower case characters indicate deleted sequences and base changes due to *dTok0* jumping.

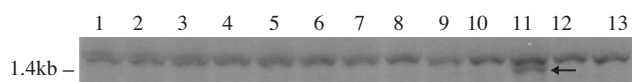


Fig. 4 Insertion of *dTok0* into a new locus. Thirteen *fon1/mp2* progeny that lost *dTok0* were analyzed by the transposon display method. Insertion into a new chromosomal location is indicated by an arrow.

Tok might mobilize *dTok* elements

Because *dTok* elements are small and have no obvious open reading frames (ORFs), they are probably non-autonomous TEs. Searching the putative autonomous elements from the ‘Nipponbare’ genome database identified nine sequences that share TIRs and subterminal domains with *dTok* (Supplementary material 2). These sequences also carried the region that potentially encodes *Ac*-type transposase, with six blocks conserved in the *hAT* superfamily (Rubin et al. 2001) (Fig. 6B). They were also flanked by 8 bp target site duplications. We named these elements *Tok1–Tok9*, starting from the short arm of chromosome 1 (Fig. 6C). Among the nine elements found in ‘Nipponbare’, only one, *Tok6*, was located on the same loci in the indica cultivar of ‘93-11’.

Full-length cDNAs for *Tok1* and *Tok4* are present in the KOME database. Interestingly, the cDNA sequences start at the upstream region of the *Tok1* (3.9 kb apart from the TIR) and *Tok4* element (7.4 kb apart from the TIR), indicating that the transcripts are expressed by the promoters located outside of the TIR (Fig. 6A). However, the *Tok4* cDNA in the KOME database may encode inactive transposase, since three out of six conserved boxes in the *hAT* superfamily transposases are not present. However, we could not exclude the possibility that the KOME cDNA is a product of alternative splicing and that there are other transcripts that encode a functional transposase. On the other hand, *Tok1* appears to encode an intact transposase (Fig. 6B). There are five exons and only one is located between TIRs of *Tok1*. The rest (four exons) are transcribed from the region outside the *Tok* element. Hashida et al. (2003) has reported similar results with snapdragon, in which some of the *Tam3* transcripts were larger than expected.

dTok elements are universally present in *Oryza* genomes

To examine the presence and copy number of the *dTok* element in several *Oryza* genomes (Table 1), we performed Southern blotting. However, due to *dTok0*-related sequences, the background was high. Therefore, we performed transposon display analysis with 12 *O. sativa* varieties (Fig. 5A, samples 1–12), and one each from the species of *O. glaberrima*, *O. rufipogon*, *O. nivara*, *O. meridionalis*, *O. longistaminata*, *O. glumaeatula* and

Table 1 *Oryza* species used in transposon display AFLP analysis

Subgenus	Species	Variety	Genome
<i>sativa</i>	<i>sativa</i>	Nipponbare	AA
<i>sativa</i>	<i>sativa</i>	Ilpum	AA
<i>sativa</i>	<i>sativa</i>	Hwaseong	AA
<i>sativa</i>	<i>sativa</i>	Wonju	AA
<i>sativa</i>	<i>sativa</i>	Hongseong	AA
<i>sativa</i>	<i>sativa</i>	Hampyeong	AA
<i>sativa</i>	<i>sativa</i>	Yooshin	AA
<i>sativa</i>	<i>sativa</i>	Baekyang	AA
<i>sativa</i>	<i>sativa</i>	Tongil	AA
<i>sativa</i>	<i>sativa</i>	IR6	AA
<i>sativa</i>	<i>sativa</i>	IR36	AA
<i>sativa</i>	<i>sativa</i>	IR72	AA
<i>sativa</i>	<i>glaberrima</i>	Gbessama 2	AA
<i>sativa</i>	<i>rufipogon</i>		AA
<i>sativa</i>	<i>nivara</i>	MY-90-W75	AA
<i>sativa</i>	<i>meridionalis</i>	OR50	AA
<i>sativa</i>	<i>longistaminata</i>		AA
<i>sativa</i>	<i>glumaeatula</i>	Arroz dopato	AA
<i>sativa</i>	<i>barthii</i>		AA
<i>australienses</i>	<i>australiensis</i>		EE
<i>latifoliae</i>	<i>rhizomatis</i>	Uru wee	CC
<i>latifoliae</i>	<i>officinalis</i>		CC
<i>latifoliae</i>	<i>eichingeri</i>		CC
<i>latifoliae</i>	<i>alta</i>		CCDD
<i>latifoliae</i>	<i>grandiglumis</i>		CCDD
<i>latifoliae</i>	<i>latifolia</i>		CCDD
<i>latifoliae</i>	<i>minuta</i>		BBCC
<i>latifoliae</i>	<i>punctata</i>		BBCC
<i>ridleyanae</i>	<i>ridleyi</i>		HHJJ
<i>ridleyanae</i>	<i>longigulumis</i>		HHJJ
<i>meyerianae</i>	<i>meyeriana</i>	Kurocawayan	GG
<i>meyerianae</i>	<i>granulate</i>	Kadaka	GG

O. barthii (samples 13–19), all of which are AA genomes. In addition, we analyzed three CC genomes (*O. rhizomatis*, *O. officinalis* and *O. eichingeri*; samples 21–23), one EE genome (*O. australienses*; sample 20) and two GG genomes (*O. meyeriana* and *O. granulata*; samples 31 and 32). Because the *Oryza* genus also contains tetraploid genomes, we included the following: two BBCC (*O. minuta* and *O. punctata*), three CCDD (*O. alta*, *O. grandiglumis*, and *O. latifolia*) and two HHJJ (*O. ridleyi* and *O. longiglumis*) tetraploids (samples 24–30).

This analysis demonstrated that the complexity of the element is well conserved in most of the AA genomes (samples 1–17). Several bands were present at the same position, indicating that they are stabilized early during AA genome evolution. Unlike the majority of AA-type species,

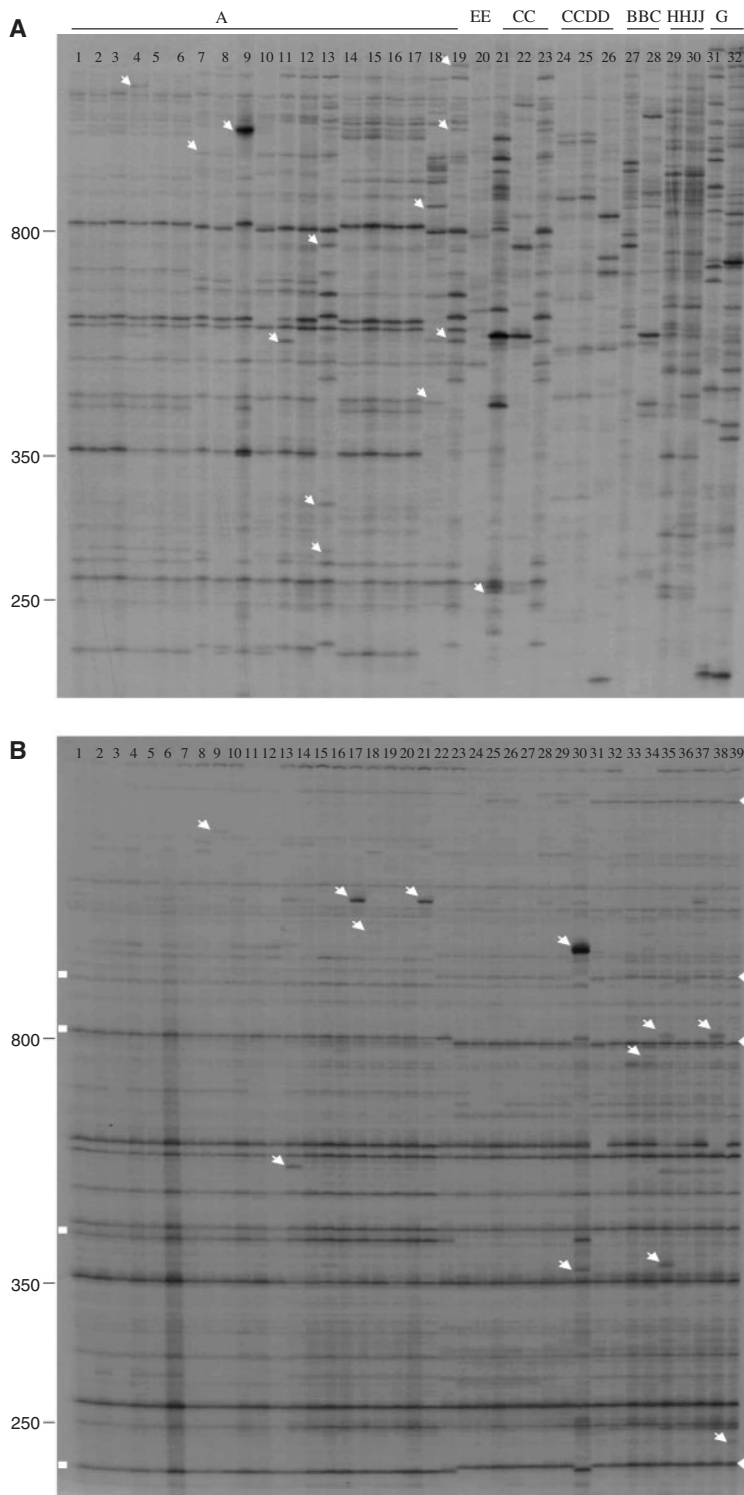


Fig. 5 Analyses of *dTok* in the *Oryza* genus. (A) Transposon display of *dTok* in the *Oryza* genus. White rectangles mark conserved bands in the AA genome. Arrowheads indicate bands specific to one or a few cultivars in the AA genome. Lines 1–32 are the *Oryza* species listed in Table 1. Lines 1–19, AA genome; 20, EE genome; 21–23, CC genome; 24–26, CCDD genome; 27–28, BBCC genome; 29–30, HHJJ genome; 31 and 32, GG genome. Size markers are shown on the left. (B) Transposon display analysis of *dTok* in *O. sativa*. Japonica-specific bands are marked by rectangles; indica-specific bands, by triangles. Bands specific to one or a few cultivars are indicated by arrowheads. Samples from 1 to 39 are Nipponbare, Ilpum, Dongjin, Suwon 330, Suwon 387, Suwon 383, Suwon 396, Nakdong, Suwon 303, Suwon 433, Suwon 427, Dongan, Wonju, Hongseong, Hampyeong, Bonghwa, Namwon, Songtan, Muju, Cheongsong, Hengseong, Nong-an, Sujeong, Nopung, Joongwon, Yooshin, Youngpoong, Pungsan, Baekyang, Tongil, IR 6, IR 26, IR 28, IR 29, IR 32, IR 36, IR 56, IR 60 and IR 72.

the band patterns for *O. glaberrima* (sample 13), *O. glumaeatula* (sample 18) and *O. barthii* (sample 19) genomes were quite different from the rest of the AA genome. Although most were widely present throughout the

AA genomes, we also noted species- or variety-specific bands, with the latter probably having resulted from more recent movement of *dTok* elements after species diversification.

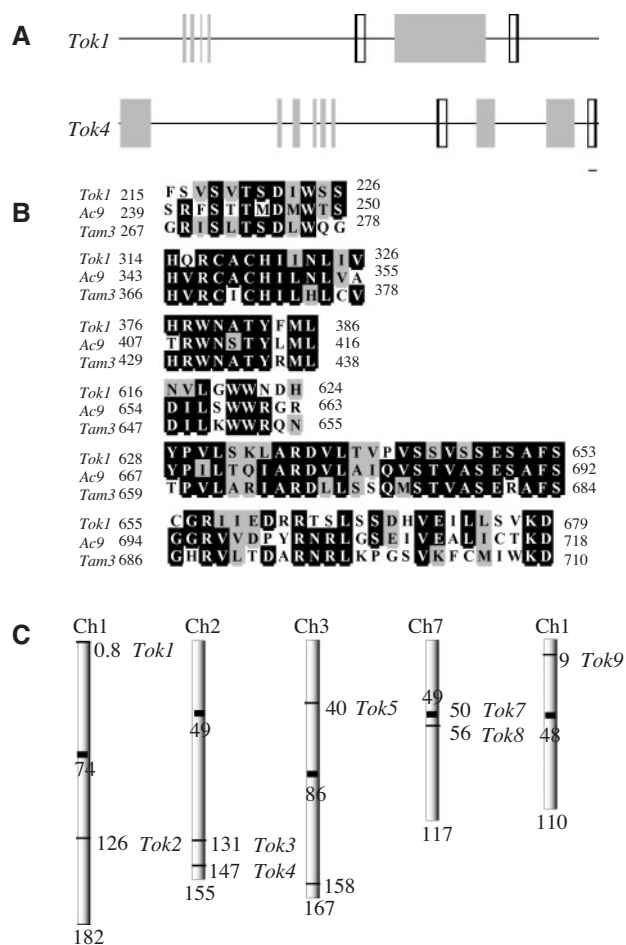


Fig. 6 *Tok* in cv. Nipponbare. (A) Schematic representation of *Tok1* and *Tok4*. Gray filled boxes indicate exons and open boxes indicate subterminal domains. TIRs are represented by black bars. (B) Amino acid alignment of six conserved boxes in *hAT* superfamily transposases. *Ac9*, *Tam3* and *Tok1* are aligned. (C) Location of nine *Tok* elements on 'Nipponbare' chromosomes. Longitudinal bars indicate chromosomes. Centromeres are represented by a black rectangle. Numbers indicate the chromosomal locations of *Tok* elements. The scale bar represents 200 nucleotides.

In the remaining *Oryza* species, as many or even more *dTok* elements were found than those observed in the AA-type genomes; their band patterns were also quite divergent (Fig. 5A). Among the CC genomes (samples 21–23), the number of conserved bands was small and their patterns differed significantly. Similar diversification was observed in the GG genomes, which also contained more bands (samples 31 and 32). The *dTok* elements were also significantly differentiated in the tetraploid genomes (samples 21–30).

Most *dTok* elements are stable in *O. sativa*

We further investigated the polymorphism of *dTok* among 39 *O. sativa* cultivars—21 japonica (Fig. 5B,

samples 1–21), nine indica (samples 31–39) and nine Tongil (samples 22–30)—and found several *dTok* elements conserved in all those species. These are probably ancient elements that were stabilized early on. We also identified at least four japonica-specific and four indica-specific *dTok* elements. Samples 21–30 were from Tongil varieties that were bred by crossing indica and japonica species; as expected, they generally showed intermediate band patterns between the two. However, 'Nong-an' (sample 22) and 'Tongil' (sample 30) were related more to japonica, while the rest were closer to indica. Cultivar-specific elements existed, but were rare. These results again indicate that only a small number of *dTok* elements are probably active transposons.

Tok-related sequences are present in AA-type *Oryza* genomes

To assess the presence and complexity of *Tok* in other *Oryza* species, we performed AFLP analysis, using conserved sequences (Fig. 7A), and found that *Tok* elements were present in most of the AA genomes. The numbers and positioning of their bands were identical among six japonica varieties (Fig. 7A, samples 1–6). Therefore, it appears that the *Tok* sequences stabilized after species diversification. Examination of three indica varieties (samples 10–12) showed that the band pattern was also conserved among them, though it differed from that of the japonica rice. For example, two bands appeared in 'IR36' (sample 11), in contrast to 'IR6' (sample 10) and 'IR72' (sample 12). Patterns for Tongil rice were intermediate between the japonica and indica types, with the 'Tongil' variety (sample 9) being more closely related to the japonica, and 'Yousin' (sample 7) and 'Baekyang' (sample 8) more similar to indica. For the other AA-type *Oryza* species (samples 13–19), the number of bands visualized here ranged from one to three, except for *O. glumaeatula* (sample 18), where no AFLP fragment was detected.

In contrast to the frequent appearance of *Tok* elements in the AA-type genomes, they were not detectable in other *Oryza* species. To confirm the transposon display data, we amplified the region containing the TIRs and subterminal domain by PCR, using specific primers. Amplified bands were observed only from the AA genomes, including *O. glumaeatula*, further demonstrating that *Tok* is commonly present in those genomes but not in other types (data not shown).

Finally, using primer sets in the coding sequence, we were able to locate the *Tok*-like transposase in all *Oryza* species examined (Fig. 7B).

Discussion

dTok0 is a member of *Ac*-type TEs

TEs are the single largest component in the genetic materials of most eukaryotes, and have played an

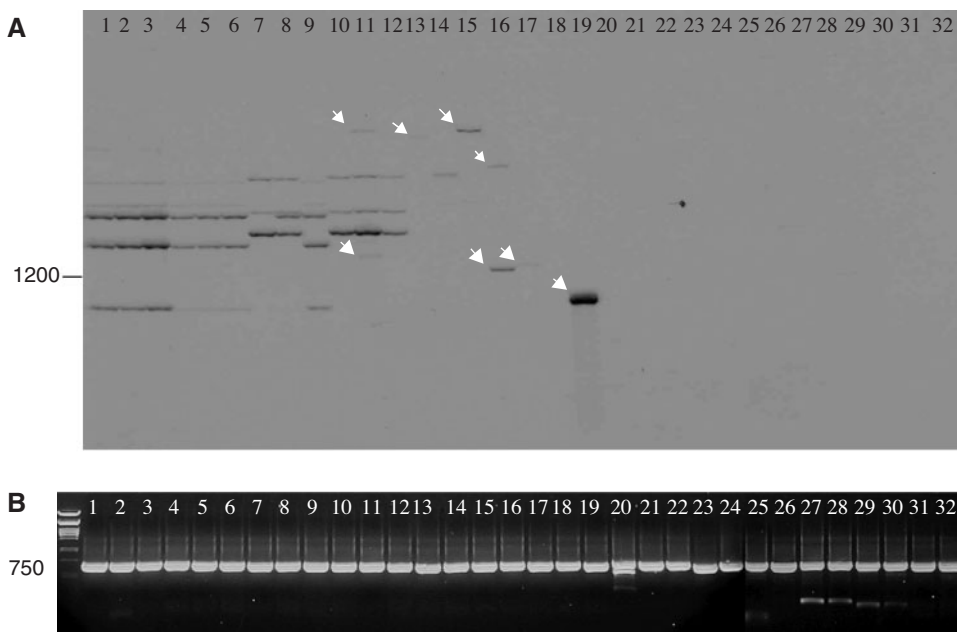


Fig. 7 Analyses of *Tok* in the *Oryza* genus. (A) Transposon display of *Tok* in the *Oryza* genus. Arrowheads indicate bands specific to one or a few cultivars in the AA genome. Lines 1–32 are the *Oryza* species listed in Table 1. Lines 1–19, AA genome; 20, EE genome; 21–23, CC genome; 24–26, CCDD genome; 27–28, BBCC genome; 29–30, HHJJ genome; 31 and 32, GG genome. Size markers are shown on the left. (B) PCR analysis of *Tok* in the *Oryza* genus.

important role during their evolution. Rice genome contains 90,000 MITEs and they are mainly distributed in the gene-rich region (Jiang et al. 2004a). The rice genome also contains 3,000 Pack-MULEs, mutator-like transposable elements (MULEs) carrying fragments derived from >1,000 cellular genes. Pack-MULEs form new ORFs, some of which are expressed as chimeric transcripts. This suggests that TEs play an important role in the evolution of genes (Jiang et al. 2004b).

TE amplification is responsible for a large proportion of the variation in genome sizes among plant species (SanMiguel et al. 1998, Kalendar et al. 2000). However, only a few TEs are currently active (Feschotte et al. 2002). The *dTok0* element found in this study shares several characteristics with *hAT* family TEs. It generates an 8 bp target site duplication, and contains TIRs and subterminal domains. The latter comprises a number of tetranucleotide sequences, ACGG and TCGG, which are *Ac* transposase-binding site (Becker and Kunze 1997). Although *Ac* was the first active TE identified in eukaryotic organisms, active *Ac*-like elements have been found in dicotyledonous species such as snapdragon and *Arabidopsis*, as well as in some animals (Kempken and Windhofer 2001). Recently, an active *Ac*-like element, *nDart*, has been isolated from rice. The element was actively excised from the Mg-protoporphyrin IX methyltransferase gene at a high frequency in leaf tissues (Fujino et al. 2005). Our *dTok0* element is different from *nDart* since the former is apparently not active during the vegetative stages but becomes active in the reproductive phase. Comparison between *nDart1-1* and *dTok0* reveals that they have 59%

identity in DNA sequence. The sequence identity is higher in the TIR and subterminal domain, i.e. 70%. Phylogenetic tree analysis suggests that *dTok* and *nDart* elements are distinctive members of the *hAT* superfamily.

Most dTok elements are stable and ubiquitously present in Oryza

At least 25 copies of *dTok* elements exist in 'Nipponbare' rice. AFLP analyses showed that most of the elements are highly conserved in the japonica genomes, indicating that they were stabilized during the early stages of genome evolution, but most probably are now inactive. However, some of the bands identified here are possibly still active, and are specific to one or only a few cultivars (Fig. 5B, arrows), probably the result of recent transposition of the *dTok* elements.

Our AFLP analysis also demonstrated that the numbers of *dTok* elements are similar between indica and japonica species. The chromosomal positioning of several of these is also well conserved among species. Genome sequence analysis indicated that seven *dTok* elements present in the 'Nipponbare' genome are found in the same chromosomal locations for indica '93-11'. As with the japonica types, elements may be specific only to one or a few varieties. Interestingly, the diversity is greater among indica varieties, suggesting that genetic variation may be generally higher in indica than in the japonica species. Alternatively, japonica varieties that we have used are more closely related to each other.

Because the Tongil lines were generated by crossing indica to japonica, both parents should have contributed to

the Tongil genome. Our analyses revealed that the *dTok* distribution patterns are more similar to that of *indica* in most lines. However, two of them show a pattern similar to *japonica*. Nonetheless, the total number of *dTok* elements remains unchanged.

dTok elements occur in all the *Oryza* species examined here. Although distribution patterns are similar among the *sativa* varieties, they are quite divergent in other species. For example, only a few common bands are observed among the three CC genomes (Fig. 5A, samples 21–23) or two GG genomes (samples 31 and 32). Therefore, conservation of the *dTok* element in *O. sativa* is probably due to the cross-relationship among cultivated varieties. It is interesting to note that the primers used for the transposon display AFLP analysis were conserved in all species. This probably did not arise from the random amplification of genomic DNA because the analysis used two adjacent primers—19mer and 24mer. Because the chance of having those sequences within such short distances is extremely low, most of the visualized bands were probably TE specific.

dTok0 is an active TE

The *dTok0* element is identified by an insertional mutation of the active gene *FON1*. During line maintenance, this element is frequently transposed out. However, most of the progeny still retain the *fon1/mp2* mutant phenotypes because the transposition generates footprints, which cause frameshift mutations. Occasionally, that mutant generates wild-type revertants that contain six nucleotide footprints. Our results are consistent with previous observations that excision of *Ds* from different sites gives rise to various predominant footprint types (Scott et al. 1996). The instability of the *dTok0* element indicates that this TE has been active during line maintenance for the last 30 years. The active status of this element is evident because propagation of the line carrying intact *dTok0* in the *FON1* gene results in frequent loss of the transposon.

Our attempts to identify *dTok0* transposition during vegetative growth under various stress conditions were unsuccessful. Therefore, the element is apparently stable in those growth stages. However, this element is apparently quite active during the reproductive phase, as evidenced by its frequent transposition out seen in the progeny. Differences in footprints among the progeny suggest that transposition occurs after either reproductive organ formation or meiosis. The fact that none of the progeny lost both copies of *dTok0* also implies that transposition occurs in either the male or the female floral organs. For *Mu* and *P* elements, excision of transposons from the homozygous copy initiates gap repair, resulting in duplication of intact or partially deleted transposons at the excised locus (Lisch et al. 1995). Therefore, we cannot eliminate the possibility that *dTok* excision and gap repair occurred in

somatic tissues. However, Yamashita et al. (1999) reported that *Tam3* probably failed to perform gap repair because of its hairpin structure at both ends. This led us to suppose that the excision of *dTok0* was not due to gap repairs, since *dTok* possesses the secondary structure at its ends. In addition, we did not find partially deleted *dTok0* sequences at the *FON1* locus.

Transpositions for most other *hAT* elements, such as maize *Ac* and *Arabidopsis Tag1*, are also germinal events, with no detectable vegetative excision (Ros and Kunze 2001). However, snapdragon *Tam3* is activated in vegetative tissues by low temperature treatments (Martin et al. 1989, Hashida et al. 2003), while maize *Mx* is influenced by X-rays (Xu and Dooner 2005). *Ac/Ds* transposes in the somatic cells when the transposase is ectopically expressed in the vegetative cells (Hehl and Baker 1989). Although we do not rule out the possibility that the *dTok* elements become active in vegetative cells under specific stress treatments, our data indicate that the element is likely to be active in reproductive tissues.

Even though *dTok0* is active, our AFLP analysis indicates that most *dTok* elements including *dTok15* on chromosome 9 are not active. Therefore, the *dTok0* element must somehow become activated in mutant lines, possibly by alteration in methylation status. The *fon1/mp2* mutant was originally isolated from hybrid lines between a *japonica* rice cultivar, ‘Suwon 224’, and a *javanica* rice cultivar, ‘Malagkit Sinaguig’ (Heu and Suh 1976). Therefore, it is most likely that *dTok0* activation is due to this cross between distantly related varieties. The maize inbred lines ‘B73’ and ‘Mo17’ both lack active TEs, whereas active transposons are certainly present in wild populations (van der Walt and Brink 1969, Cormack et al. 1988). Therefore, one possibility is that the *dTok0* element was transferred from the *javanica* rice cultivar, one that is more closely related to wild varieties. Alternatively, *dTok0* is of *japonica* origin and is activated during the crossing. In either case, once the activation state is achieved, it must be maintained in the progeny because *dTok0* is still transposed out from the *FON1* locus.

dTok0 is a potential insertional mutagen

Transposons are utilized for generating insertional mutants in a wide variety of organisms, including plants, animals and microorganisms (Martin et al. 1989, Llamas et al. 2000, Ki et al. 2002, Kawakami et al. 2004, Tsugane et al., 2006). *Ac/Ds* has been successfully used in the construction of a large number of mutants in *Arabidopsis* and rice (Ki et al. 2002, Kuromori et al. 2004). However, its introduction into most plant species requires tissue culture procedures that often create mutations not associated with the transposon insertions (Kaeppler and Phillips 1993, Phillips et al. 1994, Hirochika et al. 1996). Because this

background mutation frequency is usually quite high, forward screening of the insertional mutant populations often results in non-*Ac/Ds* mutations. Such an obstacle hampers reliance on transposon tagging in most plant species. To avoid these tissue culture-driven mutations, one could use an endogenous transposon, such as *dTok0*, for rice. To this end, conditions for the activation of *dTok0* should be further investigated. For example, constitutive expression of *Tok1* transposase may activate *dTok0* in vegetative tissues. It will be important to evaluate whether other *dTok* elements are also activated by ectopic expression of *Tok* transposases. Other sequences similar to *dTok* and *Tok* elements exist in the rice genome (Goff et al. 2002, Yu et al. 2002). They are potentially active transposons that can also be used for background-free insertional mutagenesis.

Materials and Methods

Maintenance of *fon1/mp2* lines

Since the isolation of the *fon1/mp2* mutant in 1976, the mutant was crossed with KhaoLo, a Thai rice variety. Several *fon1/mp2* plants selected in the F₂ population were generation-advanced to F₁₀ lines by phenotypic selection of multiple pistils, and 14 homozygous lines have been independently maintained.

Genotyping the *fon1/mp2* plants

All PCRs were carried out in 50 µl of a mixture containing 20 ng of plant DNA or 2 ng of cDNA, 10× ExTaq buffer, 0.2 mM dNTP, 0.5 U of ExTaq polymerase and 1 µM of the primers (Jung et al. 2005). The protocol included 35 cycles of 94°C for 60 s, 60°C for 60 s and 72°C for 90 s. Primers for genotyping were F1 (5'-CGTTCCAGAAGCTGGAGTTC-3') and R1 (5'-TTTGGATTTCGATTCTGCACTG).

Primers, Tok F (5'-TGATATGGCATGCACAACGCT-3') and Tok R (5'-ATGGAAGGCAGGTAACATTCG-3') were used for amplification of *Tok*.

Transposon display AFLP analysis

DNA was extracted from leaf tissues of a single plant and 50 ng of the DNA was digested to completion for 8 h at 37°C in 40 µl containing 5 U of *MseI* and 20 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol and 100 µg ml⁻¹ bovine serum albumin (BSA). After digestion, 50 pmol of adaptors (5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3') were added and ligated. PCR was performed by using the *MseI* pre-selective primer (5'-GACGATGAGTCCTGAGTAA-3') complementary to the adaptors and another primer complementary to an internal *dTok* (5'-ACTAGTACATGTGGCCCAT-3') or *Tok* (5'-ACAGCGGTCGTTGGATTAGC-3'). Reactions were in 50 µl containing 0.2 mM dNTPs, 1 U of EF Taq DNA polymerase and reaction buffer. The temperature cycling parameters were as follows: an initial cycle at 72°C for 2 min and 94°C for 3 min followed by 24 cycles at 94°C for 30 s, 59°C for 30 s and 72°C for 1.5 min, and a final cycle at 72°C for 5 min. Selective amplification was performed in 20 µl of reaction solution containing 5 µl of the 20-fold diluted pre-selective amplification products, 8 pmol of pre-selective primer *MseI*, 1.25 pmol ³³P-labeled selective primer of *dTok*

(5'-TAAATGGGCCCGTGGGCCGTGCCA-3') or *Tok* (5'-GTCGTTTGACTAGCTGTTCCGGCCG-3'), 0.2 mM dNTPs, 1 U of EF Taq DNA polymerase and reaction buffer. PCR conditions used for the 'touchdown' reaction were 94°C for 5 min followed by 94°C for 30 s, 73°C for 30 s and 72°C for 1.5 min. In subsequent cycles, the annealing temperature was reduced from 72 to 64°C in 1°C increments each cycle. Twenty-seven cycles were then performed at the 64°C annealing temperature, followed by a final cycle at 72°C for 5 min. The reaction was stopped by adding 20 µl of denaturing buffer (98% deionized formamide, 10 mM EDTA, pH 8.0, 0.025% xylene cyanol, 0.025% bromophenol blue). After denaturation, 3 µl of the mixture was loaded on a 5% denaturing acrylamide-bisacrylamide gel. After samples were electrophoresed (30 mA constant) in a 45 cm gel for 6 h, the gel was transferred to filter paper, dried and exposed to X-ray film for 24 h.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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