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Two-Step Regulation and Continuous Retrotransposition of the Rice LINE-Type Retrotransposon *Karma*

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Here, we report the identification of *Karma*, a LINE-type retrotransposon of plants for which continuous retrotransposition was observed in consecutive generations. The transcription of *Karma* is activated in cultured cells of rice upon DNA hypomethylation. However, transcription is insufficient for retrotransposition, because no increase in the copy number was observed in cultured cells or in the first generation of plants regenerated from them. Despite that finding, copy number increase was detected in the next generation of regenerated plants as well as in later generations, suggesting that the post-transcriptional regulation of *Karma* retrotransposition is development dependent. Our results indicate that two different mechanisms, one transcriptional and the other developmental, control the mobilization of *Karma*. In addition, unlike other known active plant retrotransposons, *Karma* is not subject to de novo methylation, and retrotransposition persists through several generations.

INTRODUCTION

The completion of the rice genome draft sequences revealed that retrotransposons account for >15% of the genome of rice (Goff et al., 2002; Yu et al., 2002). In contrast to DNA-type transposable elements, retrotransposons encode a reverse transcriptase (RT) activity and move by a replicative mechanism that involves an RNA intermediate. Thus, retrotransposons contributed greatly to the expansion and the evolution of the genome (reviewed by Kumar and Bennetzen, 1999; Prak and Kazazian, 2000; Feschotte et al., 2002).

Despite the fact that retrotransposons exist in high copy numbers in the genomes of most eukaryotes, the great majority of them are inactive or defective, and only a small portion of them retain the ability to retrotranspose (reviewed by Grandbastien, 1998; Kumar and Bennetzen, 1999). In plants, direct evidence for retrotransposition was demonstrated for only a few elements, such as *Tos17* of rice (Hirochika et al., 1996) and *Tto1*, *Tto2*, and *Tnt1* of tobacco (Hirochika, 1993; Lucas et al., 1995). This finding may reflect technical problems, because special techniques, such as transposon display, are required to monitor the mobility of highly repetitive elements (Melayah et al., 2001). In some cases, retrotransposition was detected only with the finding of clear-cut evidence, such as gene disruption associated with mutant phenotypes (Johns et al., 1985; Grandbastien et al., 1989; Varagona et al., 1992; Schwarz-Sommer et al., 1997).

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Recently, a survey of >400,000 EST sequences of maize identified only 56 retrotransposon cDNAs, supporting the notion that most retrotransposons are inactive (Meyers et al., 2001). This search also showed that most retrotransposons found in EST libraries are low-copy elements in genomic sequences, whereas only a few of the high-copy elements are found in EST libraries. Even active retrotransposons are subject to tight regulation, so that retrotransposition occurs only rarely. A well-studied aspect of the regulation of plant retrotransposon activities is the induction of their transcription by biotic or abiotic stresses (Johns et al., 1985; Grandbastien et al., 1989; Hirochika, 1993). Epigenetic gene silencing through methylation also is an important regulatory mechanism that suppresses the activity of retrotransposons (Hirochika et al., 2000; Lindroth et al., 2001; Tompa et al., 2002). Recently, RNA silencing and RNA-directed DNA methylation of retrotransposons were demonstrated (Hamilton et al., 2002; Llave et al., 2002; Zilberman et al., 2003).

Retrotransposons can be classified as either long terminal repeat (LTR) retrotransposons or non-LTR retrotransposons, depending on the presence or absence of terminal repeats. Retrotranspositionally active elements identified from plants to date all belong to the LTR subclass, and very limited information is available concerning non-LTR retrotransposons. Plant genomes contain both the long interspersed elements (LINEs) and short interspersed elements (SINEs) types of non-LTR retrotransposons; however, these are less abundant than LTRs (Noma et al., 1999; Le et al., 2000; Turcotte et al., 2001). This is in sharp contrast to the situation in mammals, in which non-LTR retrotransposons are predominant. For example, the human L1, a LINE element, comprises 17% of the genome (Prak and Kazazian, 2000). The mechanism of mammalian L1 retrotransposition has been studied extensively (reviewed by Ostertag and Kazazian, 2001), and recently, the mobilization of

SINEs by LINEs was demonstrated in eels (Kajikawa and Okada, 2002).

To fill the void of knowledge about plant non-LTR retrotransposons, isolation of active copies has been long desired. Here, the identification of *Karma*, a mobile LINE element in plants, is described. *Karma* activation in rice plants is controlled in two steps: the first occurs at transcription, and the second is post-transcriptional and development dependent. Interestingly, once the retrotransposition of *Karma* starts in the progeny of tissue culture–derived plants, *Karma* remains active for generations.

RESULTS

Karma Is a Novel LINE-Type Retrotransposon

Karma was identified as an insertion in a mutant allele of the *FRIZZY PANICLE2* locus (Komatsu et al., 2001, 2003). DNA sequence analysis revealed that this insertion represents a novel rice non-LTR retrotransposon of the LINE group, which was designated *Karma*. *Karma* is 7080 bp long and has a 5′ untranslated region of 39 bp, two nonoverlapping open reading frames of 3156 and 3594 bp, which are separated by an intergenic spacer of 220 bp and translated in different frames, and a 3′ untranslated region of 71 bp; it is terminated by a poly(A) tail of 11 bases (Figure 1A). Domains conserved in LINE elements are observed in *Karma*. Short Cys-rich motifs are present at each open reading frame, and an endonuclease and a reverse transcriptase domain are found at the second open reading frame. *Karma* is related more closely to other plant LINEs, in particular to the maize *Cin4* element (Schwarz-Sommer et al., 1997), with which it shares 37% amino acid identity in the reverse transcriptase domain (Figure 1B). Interestingly, all known plant LINEs belong to the L1 clade (Figure 2), which includes the mouse and human *LINE-1* elements (Loeb et al., 1986; Dombroski et al., 1991).

Karma Distribution Varies between Rice Subspecies

The number of *Karma* copies in the genome of *japonica*, *javanica*, and *indica* subspecies of rice was examined first by DNA gel blot analysis (Figures 3A and 3B). As indicated in Figure 1A, DNA was digested with EcoRV, and blots were hybridized with probes A and B from the 5′ and 3′ ends of *Karma*, respectively. In the eight *japonica* cultivars analyzed, only one fragment was hybridized by probe A (Figure 3A), whereas three fragments were detected by probe B (Figure 3B). All fragments were conserved in the eight cultivars, suggesting that *japonica* rice has one full-length and two 5′ truncated copies of *Karma*. In the five *indica* cultivars analyzed, no fragments hybridized to probe A, whereas one or two fragments were detected by probe B, suggesting that *indica* rice has only 5′ truncated *Karma* copies. Three cultivars of *javanica*, which also is a cultivated rice subspecies, were analyzed. One fragment detected by probe A and three fragments detected by probe B were conserved in the three *javanica* cultivars, suggesting that *javanica* rice also has one full-length and two 5′ truncated copies of *Karma*. An additional fragment was detected by both probes in one of the *javanica* cultivars.

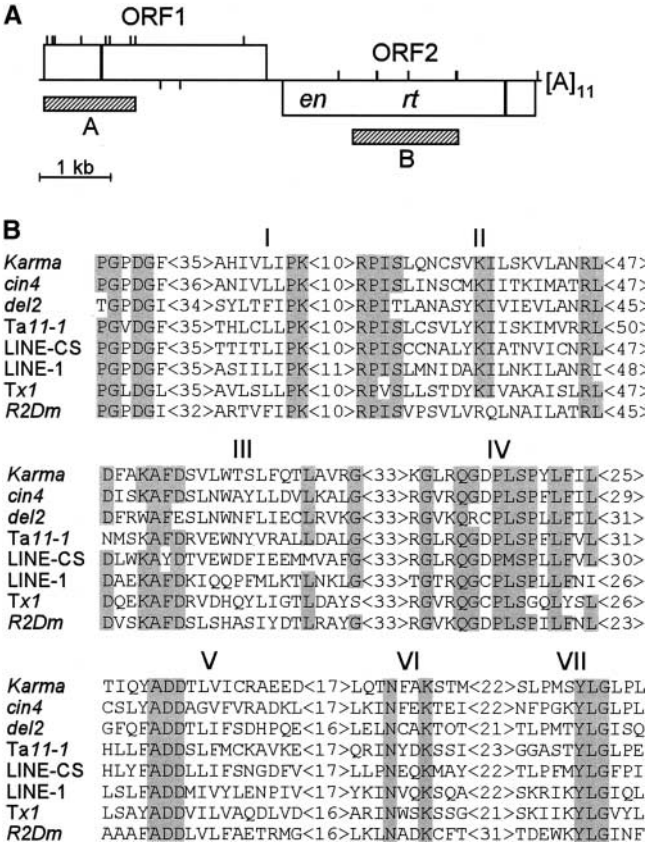

Figure 1. Conserved Domains in *Karma*.

(A) *Karma* structure. Open boxes depict the two open reading frames of *Karma* (ORF1 and ORF2). ORF2 contains the endonuclease (*en*) and reverse transcriptase (*rt*) domains. Black boxes depict Cys-rich motifs. Vertical lines above the boxes depict the sites of HpaII and MspI cleavage, and vertical lines below the boxes depict the sites of EcoRV cleavage. Hatched boxes depict probes A (1317 bp) and B (1498 bp) used in DNA gel blot analysis. Bar = 1 kb.

(B) Alignment of the conserved reverse transcriptase domain. Gray boxes indicate residues conserved in at least seven of the eight sequences depicted. I to VII denote the seven domains characteristic of reverse transcriptases. Retrotransposon sequences are from rice *Karma*, maize *cin4*, lily *del2*, Arabidopsis *Ta11-1*, *Cannabis* LINE-CS, human LINE-1, frog *Tx1*, and fruit fly *R2Dm*.

The full-length *Karma* copies conserved in *japonica* and *javanica* cultivars separated into bands of different sizes in DNA gel blot analyses. Sequencing of their flanking regions and rough mapping using recombinant inbred lines confirmed that they are localized at different positions in the genome. The *japonica* full-length copy is 7080 bp long and is localized on chromosome 11, whereas the *javanica* full-length copy is 7068 bp long and is localized on chromosome 1. PCR amplifications demonstrating the divergence in *japonica* and *javanica* full-length copy locations are shown in Figure 3C. Nondisrupted PCR products

also were sequenced and showed no deletions or truncations that could indicate genome rearrangements (data not shown). Both copies were flanked by direct repeats and were terminated by poly(A) tails of different lengths (Figure 3D).

With the publication of draft sequences for the *japonica* and *indica* rice genomes (Goff et al., 2002; Yu et al., 2002) and constant updates toward their completion, it was possible to assess the structures and locations of *Karma* copies present in both subspecies. BLAST (Basic Local Alignment Search Tool) searches in public databases revealed that the *japonica* cv Nipponbare indeed has only one full-length *Karma* copy (Table 1). Nine 5′ truncated copies with identities ranging from 66 to >99% were found. Although not all were flanked by target site duplications, the nine elements were terminated by poly(A) repeats. 5′ truncated copies with lengths within the hybridization range of probe B also were detected by DNA gel blot analysis in lower stringency conditions (data not shown). Meanwhile, BLAST searches of the *indica* cv 93-11 database revealed the presence of 5′ truncated copies only, some of which were highly identical to 5′ truncated copies found in the *japonica* database (Table 2).

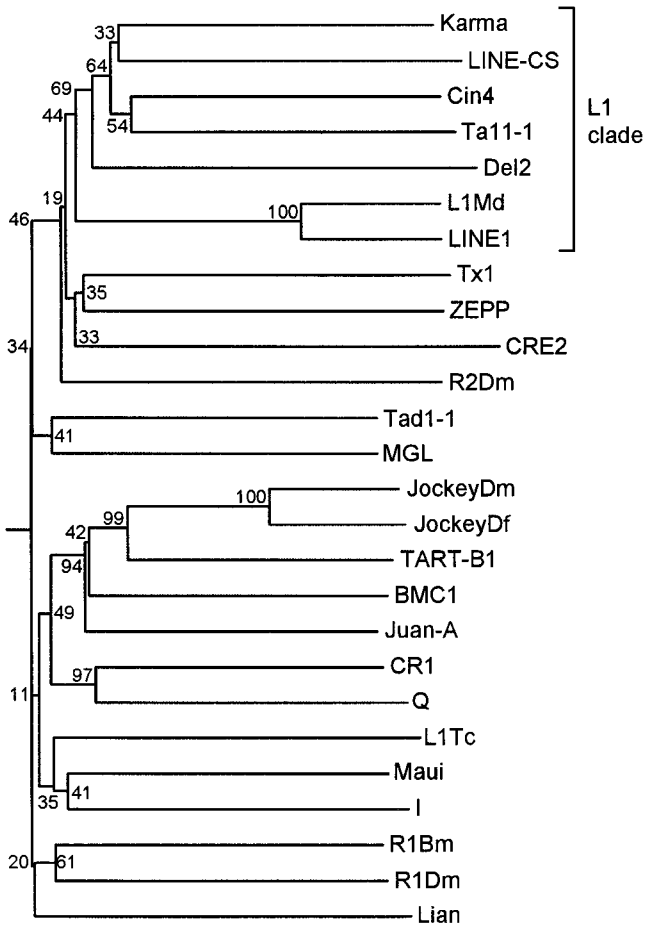

Figure 2. Phylogenetic Analysis of 26 LINE Reverse Transcriptase Sequences.

Sequences analyzed were from fruit fly I element, frog *Tx1*, fruit fly *R2Dm*, mouse L1Md, human LINE-1, rice *Karma*, lily *del2*, maize *cin4*, Arabidopsis *Ta11-1*, *Cannabis* LINE-CS, green alga ZEPP, trypanosome CRE2, mosquito Juan-A, silkworm BMC1, fruit fly TART-1, fruit fly JockeyDm, fruit fly JockeyDf, chicken CR1, mosquito Q, trypanosome L1Tc, pufferfish Maui, rice blast fungus MGL, fungi Tad1-1, mosquito Lian, silkworm R1Bm, and fruit fly R1Dm. The L1 clade is indicated. Phylogeny was constructed using the neighbor-joining algorithm from distance matrices according to Kimura's two-parameter method (Kimura, 1983). Branch lengths are proportional to genetic distance. Bootstrap values are indicated as a percentage of 1000 replicates.

Karma Distribution Varies in *Oryza* Species

The number and distribution of *Karma* copies also diverged in accessions of *Oryza rufipogon* (Figure 4), the proposed wild ancestor of rice (Wang et al., 1992; Khush, 1997; Bautista et al., 2001), and other *Oryza* species. This divergence suggests that *Karma* transposition may have been activated occasionally during the course of rice domestication. However, the possibility that the difference in band sizes results from genome rearrangements cannot be excluded. A conserved pattern of distribution was not observed among *O. rufipogon* accessions from China or tropical Asia, indicating that the divergence in *Karma* copy distribution occurred before the domestication of rice. Accordingly, hybridization also was observed in *O. longistaminata* but not in other *Oryza* species besides *O. rufipogon* (Figure 4).

Karma Retrotransposes in Regenerated Plants but Not in Cultured Cells

Because retrotransposons move in a "copy-and-paste" manner, an increase in copy number indicates the occurrence of retrotransposition. No increase in copy number was detected in 54 independent lineages of cells cultured for 5 months to 4 years (Table 3, Figure 5A), suggesting that *Karma* did not retrotranspose in cultured cells. *Karma* retrotransposition also was not detected in leaves of 13 independent R0 seedlings, which were regenerated from cultured cells. However, an increase in *Karma* copy numbers was observed in plants of R1 lineages (Table 3, Figure 5B). Most interestingly, we detected additional copy number increases in regenerated plants of consecutive generations up to R6, which was the most advanced generation analyzed (Table 3). Although we did not analyze the same lineage for more than two subsequent generations, the transmission of new insertions of the parental plant to the progeny was observed, as shown in Figures 5C and 5D. Lineages in which the copy number did not increase in the R1 generation but increased in R2 also were observed (data not shown).

To determine if the new copies actually resulted from retrotransposition and not from DNA rearrangements, the sequences flanking five new copies were recovered using inverse PCR. As shown in Table 4, the five new copies had short or long 5′ deletions, were terminated by poly(A) tails of variable lengths, and were flanked by direct repeats of different lengths, indicating the formation of target-site duplications. These three features have been reported extensively for mammalian L1 element retrotransposition (Luan et al., 1993; Ostertag and Kazazian,

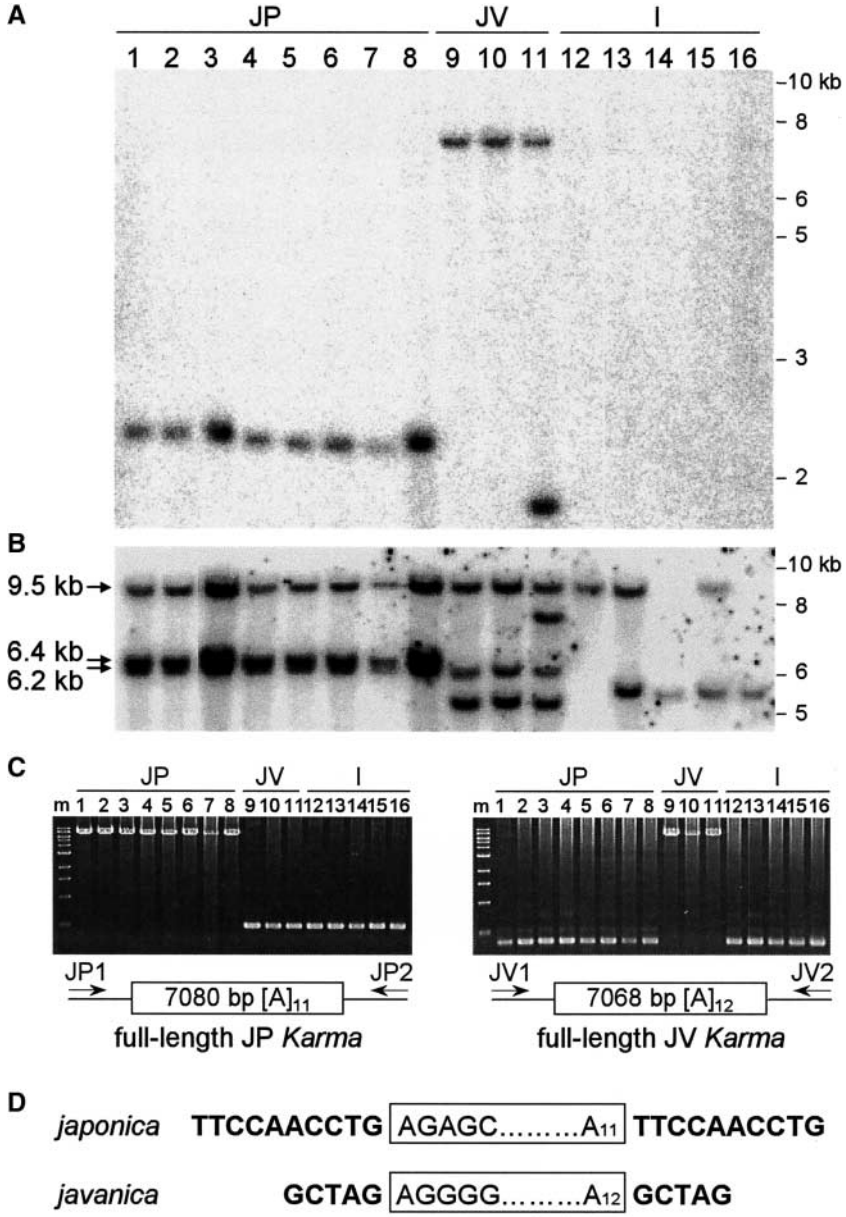

Figure 3. *Karma* Copies in *japonica*, *javanica*, and *indica* Cultivars.

Lanes 1 to 8, *japonica* (JP) cultivars Asominori, Kinmaze, Nipponbare, Notohikari, Shiokari, Taichung65, Toride, and Kinoshitamochi; lanes 9 to 11, *javanica* (JV) cultivars 242, 532, and 647; lanes 12 to 16, *indica* (I) cultivars IR24, 108, 419, C5444, and C8005. The number of *Karma* copies was examined by DNA gel blot analysis of leaf DNA digested with EcoRV.

(A) Fragments hybridized by probe A as indicated in Figure 1A.

(B) Fragments hybridized by probe B as indicated in Figure 1A. Arrows indicate the fragments conserved in the eight *japonica* cultivars and their respective sizes.

(C) PCR using primers flanking the full-length copies of *Karma* in *japonica* and *javanica* rice confirms that they are localized at different positions in the genome. Left gel, PCR using primers flanking the *japonica* full-length *Karma* copy (JP1 and JP2) results in products of 7562 bp in *japonica* cultivars and 471 bp in *javanica* and *indica* cultivars. Right gel, PCR using primers flanking the *javanica* full-length *Karma* copy (JV1 and JV2) results in products of 7454 bp in *javanica* cultivars and 374 bp in *japonica* and *indica* cultivars. The molecular mass markers (m) indicate, from top to bottom, 10, 8, 6, 5, 4, 3, 2, 1.5, 1, and 0.5 kb.

(D) Direct repeats flanking the full-length *Karma* copies of *japonica* and *javanica*.

Table 1. Results of BLAST Searches of the *japonica* (cv Nipponbare) Genomic Database

Hit (PAC)	Chromosome	cM	Length	Identity (%)[a]	Position[b]		Target Site Duplication[c]
AC134924	11	8.6	7080 [A]11	100	17,912	+	TTCCAACCTG/TTCCAACCTG
AC135926	5	54.6	5764 [A]11	99.6	83,058	−	None
AC138454	11	7.5	2126 [A]7	99.5	153,303	+	CTTGTAAAG/CTTGTTAAG
AP004309	7	94.7	2536 [A]14	81	82,517	+	None
BX000506	12	9.7	2822 [A]9	97	57,254	+	None
AP005288	2	11.6	66 [A]6	92	31,725	+	TTTATATGGGTTCT/TTTATATGGGTTCT
AC120539	11	19.8	577 [A]7	66	163,784	+	None
AC135429	5	80.7	691 [A]10	69	106,341	+	TTACTC/TTACTC
AL607005	4	52.6	106 [A]11	76	74,011	+	CAAAT/CAAAT
AP005776	2	10.8	187 [A]8	70	106,179	+	TAGG/TAGG

cM, centimorgan; PAC, P1 artificial chromosome.

[a] Percentage of identity to the full-length *Karma* copy isolated from *japonica*.

[b] Position of the first nucleotide and direction within the PAC clone; +, upper strand; −, lower strand.

[c] 5′ flanking/3′ flanking duplications. Different bases within the duplications are underlined.

2001) and indicate that the new copies of *Karma* indeed resulted from retrotranspositional events.

Karma Transcription Is Insufficient for Transposition

The transcription of *Karma* in regenerated plants and cultured cells was analyzed by RT-PCR (Figure 6A). Whereas *Karma* was not transcribed in leaves of normal plants, transcription was detected in leaves of regenerated plants. Transcription also was observed in cells cultured for 1 month to 4 years. The fact that *Karma* retrotransposition was not observed even though it was transcribed suggests that transcription is insufficient for the retrotransposition of *Karma*. This also was the case for R0 plants, in which transcription, but not retrotransposition, was detected (data not shown).

Karma Is Hypomethylated in Cultured Cells and Regenerated Plants

Karma methylation levels were examined by DNA gel blot analysis using the methylation-sensitive HpaII and the methylation-insensitive MspI restriction enzymes. HpaII does not cleave methylated CCGG sequences, whereas its isoschizomer, MspI, cleaves C5mCGG but not 5mCCGG sequences. Correlating with transcription, *Karma* methylation levels in cultured cells and regenerated plants were lower than those in wild-type plants (Figure 6B). In wild-type plants, the fragment of *Karma* hybridized by probe A was not digested with HpaII, indicating that *Karma* is methylated under normal conditions. A slight decrease in methylation levels was observed in cells cultured for 1 month. In cells cultured for 5 months, 2 years, and 4 years, methylation levels decreased considerably. Hypomethylation of *Karma* also was observed in lineages of regenerated plants from different generations, indicating that *Karma* was not subject to de novo methylation in them.

DISCUSSION

Karma Retrotransposition Continues through Several Generations

Although LINE elements are found in the genomes of several plant species, evidence of recent retrotransposition was not observed previously. We demonstrated that *Karma* transcrip-

Table 2. Results of BLAST Searches of the *indica* (cv 93-11) Genomic Database

Hit (Contig)	Length	Identity (%)[a]	Position[b]		Target Site Duplication	Similar *japonica* Copy	Identity (%)[c]
1,041	5063 [A]12	99	6,488	+	None		
144	2536 [A]13	81	13,682	+	None	AP004309	99.6
23,198	4121	62	3,959	−	None		
15,747	2336	68	2,560	−	None		
5,471	2825 [A]9	67	10,621	−	None	BX000506	98.8
98,735	52 [A]6	94	342	−	TTTATATGGGTTCT/TTTATATGGGTTCT	AP005288	100
19,342	264 [A]7	70	4,506	+	None	AC120539	99.6
9,831	123 [A]11	76	5,668	−	CAAAT/CAAAT	AL607005	99.0

[a] Percentage of identity to the full-length *Karma* copy isolated from *japonica*.

[b] Position of the first nucleotide and direction within the contig; +, upper strand; −, lower strand.

[c] Percentage of identity between the *indica* and *japonica* elements.

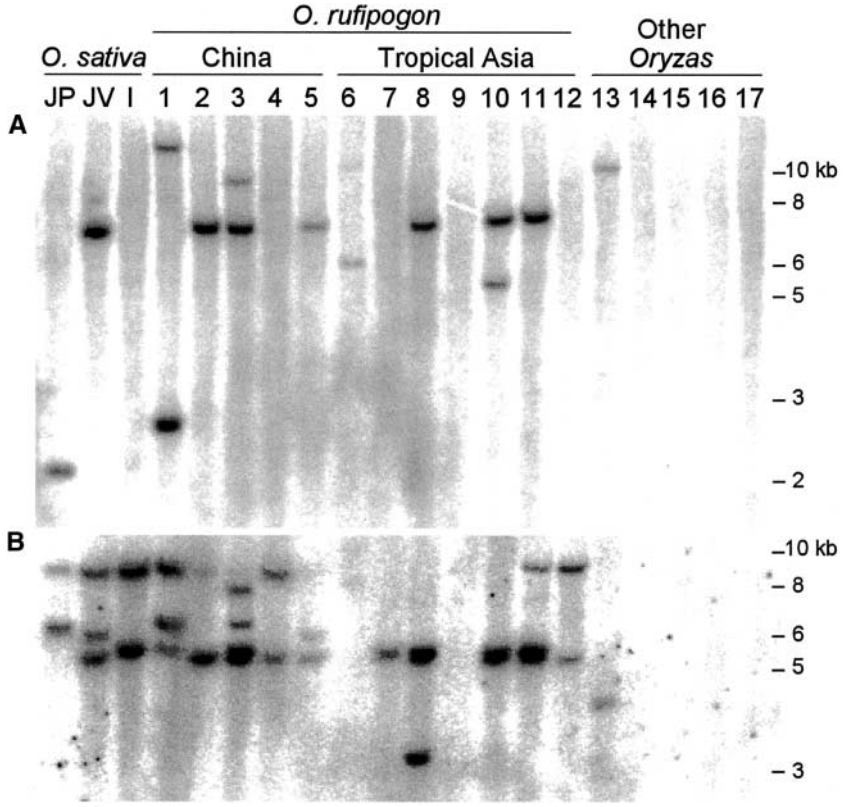

Figure 4. Distribution of *Karma* Copies in *Oryza* Accessions.

The number of *Karma* copies was examined by DNA gel blot analysis using EcoRV. Lane JP, *japonica* cv Notohikari; lane JV, *javanica* cv 242; lane I, *indica* cv IR24; lanes 1 to 12, *O. rufipogon* accessions W1956, W1962, W1964, W1965, W1967, W108, W120, W149, W593, W630, W1972, and W1976, respectively; lanes 13 to 17, *O. longistaminata*, *O. glumaepatula*, *O. meridionalis*, *O. glaberrima*, and *O. barthii*, respectively. *O. rufipogon* accessions from China or tropical Asia are indicated.

(A) Fragments hybridized by probe A as indicated in Figure 1A.

(B) Fragments hybridized by probe B as indicated in Figure 1A.

tion is activated by tissue culture and that *Karma* retrotransposes continuously in regenerated plants. Unlike plant retrotransposons reported to date, the transcription of *Karma* is not silenced after activation, and hypomethylated states persist through several generations. Although the precise mechanisms of the epigenetic regulation of transposons in general remain unclear, mobilization often is associated with hypomethylation and transcriptional activation (Hirochika et al., 2000; Lindroth et al., 2001; Tompa et al., 2002). Recently, it was demonstrated that short interfering RNAs derived from retroelements are found in wild-type Arabidopsis and tobacco plants and that long short interfering RNAs are correlated specifically with retroelement DNA methylation but not with mRNA degradation (Hamilton et al., 2002; Llave et al., 2002; Zilberman et al., 2003). We observed an inverse correlation between *Karma* methylation and mRNA levels. However, whether *Karma* methylation prevents transcription or RNA degradation sets *Karma* methylation in wild-type plants, and the specific effect of tissue culture in these processes, remain to be determined.

The persistence of *Karma* hypomethylation through generations may result from the low activity of plant de novo methyltransferases (Vongs et al., 1993; Kakutani et al., 1999). Alternatively, it may be a consequence of the small number of additional *Karma* copies present in early generations of regenerated plants. The increase in copy number correlates positively with both the restoration of DNA methylation levels and

Table 3. Insertion Frequency of Active *Karma* Elements

Generation	Number of Plants Tested[a]	Number of New Insertions	New Insertions per Plant
R0 (callus)	54	0	0
R0 (plants)	13	0	0
R1	15	4	0.27
R2	16	5	0.31
R3	15	10	0.67
R4	23	18	0.78
R5	5	2	0.40
R6	9	2	0.22

[a] Sum of plants from different lineages.

the inactivation of the *Tto1* LTR retrotransposon already in the R0 generation (Hirochika et al., 2000). Also, a decrease in transcription of the *Drosophila I* LINE element was associated with increasing copy numbers, indicating the occurrence of repeat-induced gene silencing (Chaboissier et al., 1998). Therefore, the possibility that the greater accumulation of new insertions through the generations will eventually lead to the inactivation of *Karma* cannot be excluded.

Regulation of *Karma* Retrotransposition

The general steps of retrotransposition include transcription, RNA processing, mRNA export, translation, post-translational modifications, entry into the nucleus, reverse transcription, and integration (reviewed by Grandbastien, 1998; Ostertag and Kazazian, 2001). Any of these steps can limit the activity of retrotransposons. The developmental and environmental regulation of the transcription of plant LTR retrotransposons has been studied extensively and involves the alternating use of different *cis*-regulatory sequences present in the LTRs (Suoniemi et al., 1996; Casacuberta et al., 1997; Grandbastien et al., 1997; Takeda et al., 1999). Although the mechanisms of LINE retrotransposition in plants are unknown, studies in mammals demonstrated that an internal promoter localized in the 5′ untranslated region is required for the in vitro transcription of human L1 elements (Swergold, 1990) and suggested that the

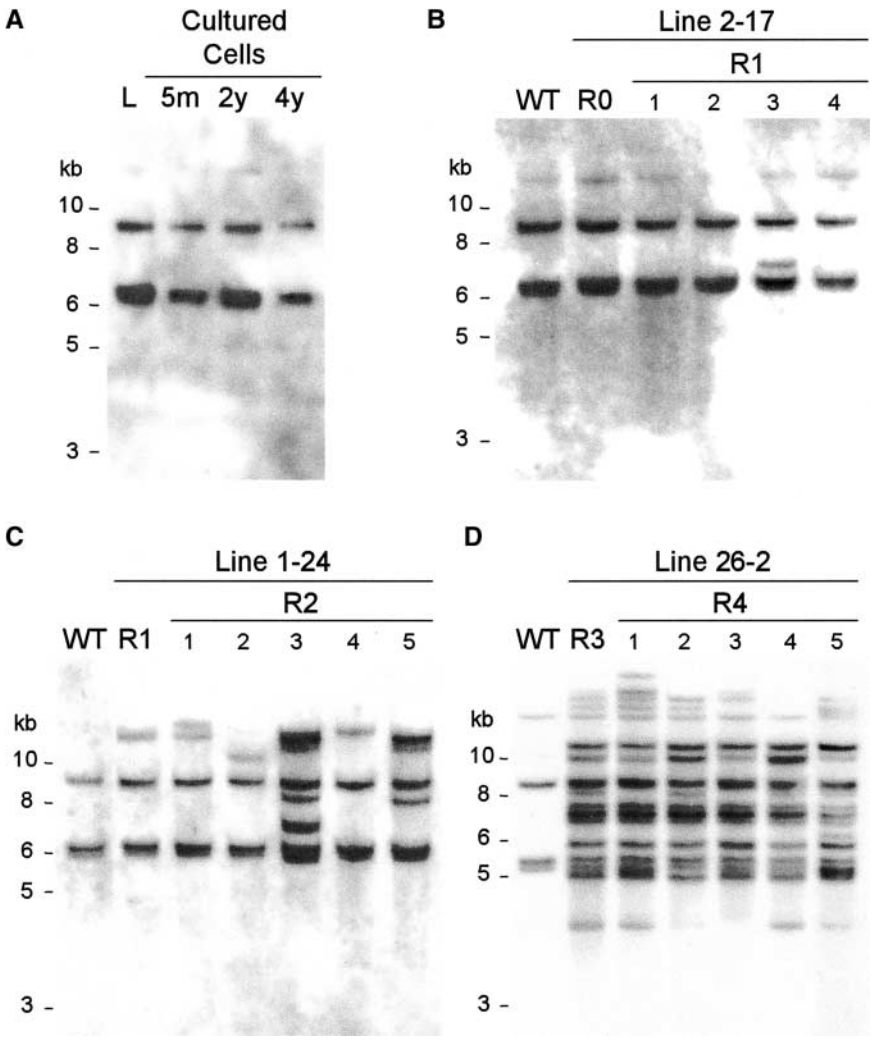

Figure 5. *Karma* Transposition in Cultured Cells and Regenerated Plants.

The number of *Karma* copies was examined by DNA gel blot analysis using EcoRV and probe B as indicated in Figure 1A.
(A) DNA extracted from wild-type plant leaves (L) and cells cultured for 5 months (5m), 2 years (2y), and 4 years (4y).
(B) DNA extracted from leaves of a wild-type (WT) plant and line 2-17 plants, which were regenerated from cultured cells (R0) and four plants of the subsequent generation (R1).
(C) DNA extracted from leaves of a wild-type plant, an R1 regenerated plant of line 1-24, and five plants of its progeny (R2).
(D) DNA extracted from leaves of a wild-type plant, an R3 regenerated plant of line 26-2, and five plants of its progeny (R4).

Table 4. Analysis of New *Karma* Insertions

Designation	Insertion Site[a]	Length (bp)	Target Site Duplication
Karma-1	Chromosome 7, 105.7 to 115.5 cM (AP004570)	7074 $[A]_8$	AGATGTCTAGCT/AGATGTCTAGCT
Karma-2	Chromosome 2, 114 to 118.1 cM (AP004255)	7072 $[A]_{11}$	CATCTATGGAAA/CATCTATGGAAA
Karma-3	Chromosome 11, 8.6 cM (AC123526)	4347 $[A]_{10}$	CAAACTGGACAG/CAAACTGGACAG
Karma-4	Chromosome 3, 31 cM (AC104473)	7063 $[A]_3$	AAAAATAATTTTT/AAAAATAATTTTT
Karma-5	Chromosome 1, 143.7 cM (AC123526)	4051 $[A]_{15}$	CAAT/CAAT

[a] Inferred by the position of the P1 artificial chromosome clone (in parentheses) corresponding to the region of insertion. cM, centimorgan.

expression of mouse L1 elements is germ line specific (Branciforte and Martin, 1994; Trelogan and Martin, 1995).

In the case of *Karma*, we showed that transcripts were not detected in leaves of wild-type plants but were observed in cultured cells and leaves of regenerated plants. Therefore, although we did not analyze the transcription of *Karma* in other tissues of wild-type plants, the suppression of transcription or the degradation of transcripts might be the first stage in the regulation of *Karma* retrotransposition. However, transcription is required but is insufficient for the retrotransposition of *Karma*, because new copies were not detected in cultured cells or in the R0 generation of regenerated plants despite the fact

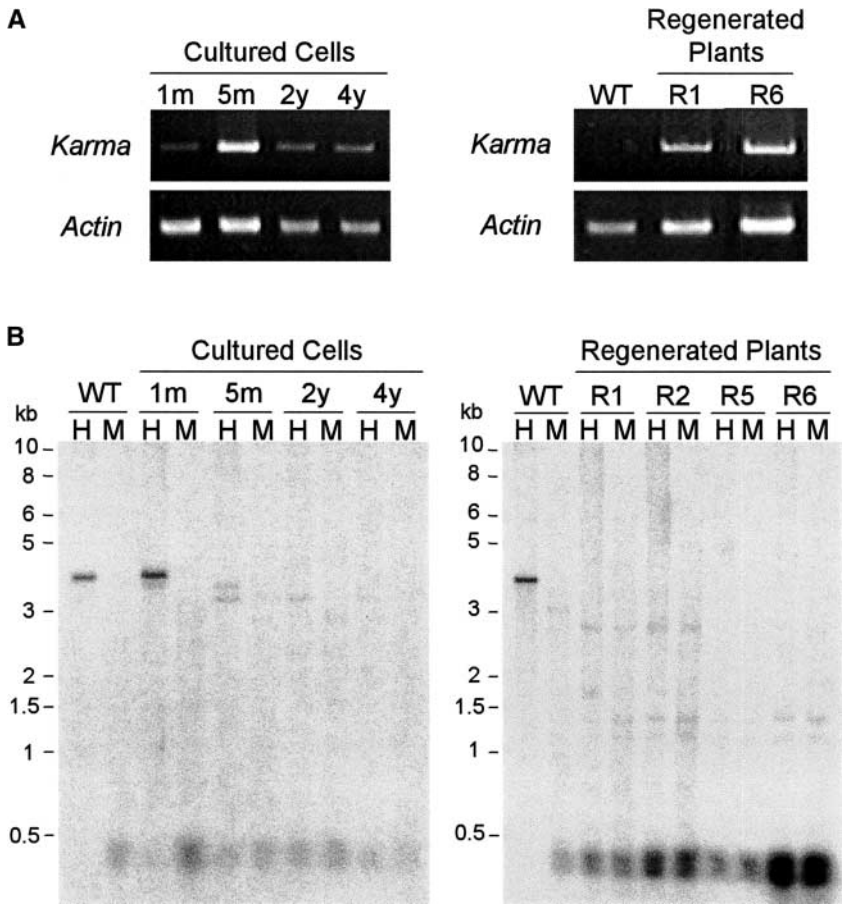

Figure 6. *Karma* Transcription and Hypomethylation in Cultured Cells and Regenerated Plants.

(A) RT-PCR of poly(A) mRNA extracted from cells cultured for 1 month (1m), 5 months (5m), 2 years (2y), and 4 years (4y), wild-type leaves (WT), and leaves of line 2-17 R1 regenerated plants and line 26-6 R6 regenerated plants. Top gels depict *Karma* expression, and bottom gels depict actin expression as a loading control.

(B) DNA gel blots of cultured cells and leaf DNA treated with HpaII (H) or MspI (M) and hybridized with probe A (Figure 1A) were used to depict the methylation status of the 5′ region of the full-length *Karma* copy in wild-type plants, cultured cells, and regenerated plants. Cultured cells of 1 month (1m), 5 months (5m), 2 years (y), and 4 years (4y) and leaf DNA of lines 2-17 (R1 and R2) and 26-6 (R5 and R6) were used.

that transcripts were observed. Therefore, post-transcriptional regulation may prevent additional steps of *Karma* retrotransposition. Evidence of transcription but no recent integration has been reported for some LTR and SINE retrotransposons (Bi and Laten, 1996; Deragon et al., 1996; Turcich et al., 1996; Meyers et al., 2001), suggesting that the regulation of post-transcriptional steps prevents the complete retrotransposition of these elements. The fact that an increase in *Karma* copy number was observed in regenerated plants of the R1 and later generations indicates that *Karma* retrotransposition also is regulated developmentally. Tight control of retrotransposition is necessary to prevent an indiscriminate increase in copy number that could be deleterious to the host. The restriction of post-transcriptional steps to certain stages of the host development could be such a control. Although the precise mechanisms for the development-dependent regulation of *Karma* remain elusive, the two-step regulation may have evolved to minimize defects to hosts and to ensure the effective transmission of retrotransposed copies to offspring.

METHODS

Plant Materials and Cell Culture

Regenerated rice (*Oryza sativa*) plants were obtained from 18 independent transgenic callus lineages that were transformed and cultivated as described previously (Izawa et al., 1991; Hiei et al., 1994; Nakagawa et al., 2002).

DNA Extraction and DNA Gel Blot Analysis

Extraction of total DNA from calli and mature leaves, blotting, preparation of probes, and hybridization were performed as described previously (Enoki et al., 1999). Probes were amplified by PCR using the following primers: for probe A, 5′-TTGGTGAATAGGGAAACGTGG-3′ and 5′-CGCATTCTCACTAACCTCCATG-3′; for probe B, 5′-CAGGCCATCCAAAGCAAGG-3′ and 5′-CGCATTCTCACTAACCTCCATG-3′.

Characterization of *Karma* Copies by Inverse PCR

Two micrograms of genomic DNA extracted from leaves was digested with EcoRV and self-ligated using the DNA Ligation Kit Version 2 (Takara Bio, Otsu, Japan).

Ligated DNA was subjected to PCR using the following primer pairs: 5′-CGCATTCTCACTAACCTCCATG-3′ and 5′-GATGTGATTGCCATGTTGGAG-3′ (full-length 5′ flanks); 5′-AGGAGATTGTCAGCGAGAAGTG-3′ and 5′-GATGTGATTGCCATGTTGGAG-3′ (5′ truncated 5′ flanks); and 5′-CGGACAGCATAGTGTTGTGTTG-3′ and 5′-GAATGTTGTTGTGGTTTGCAATG-3′ (3′ flanks). The full-length *Karma* copies of *japonica* and *javanica* cultivars were amplified using primers JP1 and JP2 (5′-TAGCTCCGAAAGCAACTACAGAG-3′ and 5′-CAGGCAGGAACTGAGGAAAG-3′, respectively) and JV1 and JV2 (5′-TGAGAAGGCCTTCTTCCTTTG-3′ and 5′-TACGAGTATGCAGATGGCCC-3′, respectively). PCR samples of 20 μL contained 0.5 units of ExTaq DNA polymerase (Takara Bio), 1× PCR reaction buffer (Takara Bio), 0.2 mM of each deoxynucleotide triphosphate, 10 μM of each primer, 4% DMSO, and ~30 ng of genomic DNA. PCR conditions included an initial step at 94°C for 2 min, 30 cycles of amplification (30 s at 94°C, 30 s at 60°C, and 2 min at 72°C), and a final step at 72°C for 5 min.

DNA Sequence Analysis

PCR products were sequenced directly with the Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Foster City, CA) and analyzed with an Applied Biosystems Prism 3100 Genetic Analyzer.

Mapping and Basic Local Alignment Search Tool Analysis

Rough mapping of the *japonica* and *javanica* full-length *Karma* loci was performed using cleaved amplified polymorphic sequence markers, which were constructed based on sequence information provided by the National Institute of Agrobiological Sciences (Tsukuba, Japan). BLAST (Basic Local Alignment Search Tool) searches (Altschul et al., 1990) were performed on National Institute of Agrobiological Sciences and University of Tokyo servers using the National Center for Biotechnology Information, Rice Genome Research Program, and Beijing Genomics Institute BLAST services. Chromosomal locations of the P1 artificial chromosome clones were obtained from the servers of International Rice Genome Sequencing Project members. Phylogenetic analyses were performed using CLUSTAL W (Thompson et al., 1994) from the Pasteur Institute suite of online programs. Pairwise alignment was performed using the LFasta local alignment tool from the Pole Bio-Informatique Lyonnais World Wide Web server.

RNA Isolation and Reverse Transcription PCR Analysis

Total RNA was isolated using the guanidinium method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ mRNA was obtained using the Oligotex-Mag mRNA Purification Kit (Takara Bio) according to the manufacturer's instructions. For reverse transcription PCR analysis, 1 μg of poly(A)$^+$ mRNA was reverse transcribed using RAV2 reverse transcriptase (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions. The product of the first-strand cDNA synthesis reaction was amplified by PCR using the same primers that were used for probe B of the DNA blots. Primers 5′-CAATCGTGAGAAGATGACCC-3′ and 5′-GTCCATCAGGAAGCTCGTAGC-3′ were used to amplify actin cDNA as a loading control.

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact J. Kyozuka, akyozuka@mail.ecc.u-tokyo.ac.jp.

Accession Numbers

The accession numbers for the sequences mentioned in this article are as follows: rice *Karma*, AB81316; maize *cin4*, Y00086; lily *del2*, Z17425; Arabidopsis *Ta11-1*, L47193; *Cannabis* LINE-CS, AB013908; human LINE-1, M80340; frog *Tx1*, M26915; fruit fly *R2Dm*, X519667; fruit fly I, M14954; mouse L1Md, AAA66024; green alga ZEPP, D899938; trypanosome CRE2, U19151; mosquito Juan-A, U87543; silkworm BMC1, AB018558; fruit fly TART-1, U14101; fruit fly JockeyDm, M22874; fruit fly JockeyDf, M38437; chicken CR1, U88211; mosquito Q, U03849; trypanosome L1Tc, X83098; pufferfish Maui, AF086712; rice blast fungus MGL, AF018033; fungi Tad1-1, L25662; mosquito Lan, U87543; silkworm R1Bm, M19755; and fruit fly R1Dm, X51968.

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