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Early Embryogenesis-Specific Expression of the Rice Transposon Ping Enhances Amplification of the MITE mPing

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Abstract

Miniature inverted-repeat transposable elements (MITEs) are numerically predominant transposable elements in the rice genome, and their activities have influenced the evolution of genes. Very little is known about how MITEs can rapidly amplify to thousands in the genome. The rice MITE mPing is quiescent in most cultivars under natural growth conditions, although it is activated by various stresses, such as tissue culture, gamma-ray irradiation, and high hydrostatic pressure. Exceptionally in the temperate japonica rice strain EG4 (cultivar Gimbozu), mPing has reached over 1000 copies in the genome, and is amplifying owing to its active transposition even under natural growth conditions. Being the only active MITE, mPing in EG4 is an appropriate material to study how MITEs amplify in the genome. Here, we provide important findings regarding the transposition and amplification of mPing in EG4. Transposon display of mPing using various tissues of a single EG4 plant revealed that most de novo mPing insertions arise in embryogenesis during the period from 3 to 5 days after pollination (DAP), and a large majority of these insertions are transmissible to the next generation. Locus-specific PCR showed that mPing excisions and insertions arose at the same time (3 to 5 DAP). Moreover, expression analysis and in situ hybridization analysis revealed that Ping, an autonomous partner for mPing, was markedly up-regulated in the 3 DAP embryo of EG4, whereas such up-regulation of Ping was not observed in the mPing-inactive cultivar Nipponbare. These results demonstrate that the early embryogenesis-specific expression of Ping is responsible for the successful amplification of mPing in EG4. This study helps not only to elucidate the whole mechanism of mPing amplification but also to further understand the contribution of MITEs to genome evolution.

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Introduction

Transposable elements (TEs) are DNA sequences that are capable of jumping from one genomic locus to another and make up a large fraction of eukaryotic genomes. More than 80% of the maize (Zea mays) and barley (Hordeum vulgare) genomes are composed of TEs [1], [2], and they constitute 33% and 14% of the genomes of rice (Oryza sativa) and Arabidopsis (Arabidopsis thaliana), respectively [3], [4]. TEs are harmful to the host because their mobilities perturb genome stability, whereas they play greatly generative roles in genome evolution such as alternation of gene structure, change of expression pattern, and rearrangement of chromosome structure [5], [6].

TEs are classified into two groups according to their transposition mechanisms: class I elements (retrotransposons) that transpose through a copy-and-paste mechanism via an RNA intermediate, and class II elements (transposons) that transpose through a cut-and-paste mechanism without undergoing an RNA intermediate. Class I elements easily attain tens of thousands of copies, whereas the majority of class II elements cannot amplify themselves to 50 copies at most. Unlike other class II elements, miniature inverted-repeat transposable elements (MITEs) have the capacity to amplify themselves to high copy numbers (hundreds or thousands) [7]–[9]. In the rice genome, MITEs are numerically predominant TEs [10], constituting 8.6% of the genome [11]. Because MITEs are too short (<600 bp) to encode any protein, their transpositions must depend on the proteins encoded by the autonomous elements. Well-studied MITEs are classified into the Stowaway and Tourist families, which belong to the Tc1/mariner and PI/Puf/Harbinger superfamilies, respectively. Because MITEs are mainly deployed in gene-rich regions [10], [12] and affect gene expression [13], they are considered to play an important role in genome evolution. However, little is known about how MITEs attain high copy numbers.

Miniature Ping (mPing) is the first active MITE identified in the rice genome [14]–[16]. Although MITEs are deployed in the genome at a high copy number, the copy number of mPing exceptionally remains at a low level in most rice cultivars: indica and tropical japonica cultivars have fewer than 10 copies, and temperate japonica cultivars including Nipponbare have approximately 50 copies [14]. The transposition of mPing is suppressed in most rice cultivars, but, like other TEs, mPing is activated by...
**Author Summary**

Transposable elements are major components of eukaryotic genomes, comprising a large portion of the genome in some species. Miniature inverted-repeat transposable elements (MITEs), which belong to the class II DNA transposable elements, are abundant in gene-rich regions, and their copy numbers are very high; therefore, they have been considered to contribute to genome evolution. Because MITEs are short and have no coding capacity, they cannot transpose their positions without the aid of transposase, provided in trans by their autonomous element(s). It has been unknown how MITEs amplify themselves to high copy numbers in the genome. Our results demonstrate that the rice active MITE mPing is mobilized in the embryo by the developmental stage-specific up-regulation of an autonomous element, Ping, and thereby successfully amplifies itself to a high copy number in the genome. The short-term expression of Ping is thought to be a strategy of the mPing family for amplifying mPing by escaping the silencing mechanism of the host genome.

Plants have acquired the silencing mechanism of TEs in germ cells. In Arabidopsis, for example, TEs are activated specifically in the vegetative nucleus of the pollen, and siRNAs from the activated TEs accumulate in the sperm cells [26]. On the basis of these results, Slukhin and colleagues proposed that siRNAs derived from TEs activated in the vegetative nucleus silence TEs in the sperm cells [26]. We conceived that mPing might overcome such a silencing mechanism in EG4. To confirm this hypothesis, we developed two F₁ populations from reciprocal crosses between the mPing-active strain EG4 and the mPing-inactive cultivar Nipponbare, and investigated the transposition activity of mPing by transposon display (TD) analysis. Success of reciprocal crosses was confirmed by PCR analysis using locus-specific primers (Figure S1A). One of the results of TD analysis using two selective bases is shown in Figure 1A; all 16 possible primer combinations were analyzed. The banding patterns of F₁ plants were almost the same as those of EG4. The bands that appeared in all F₁ plants but not in the parental EG4 plant were derived from another parental Nipponbare plant (Figure S1B). Furthermore, the bands that appeared in only one of eight F₁ plants but not in the parental EG4 plant are herein referred as de novo insertions. These bands were confirmed not to be PCR artifacts by sequence and locus-specific PCR analysis (Table S1 and Figure S2). We detected 15.3 de novo insertions per plant in the selfed progenies of EG4, whereas Nipponbare yielded no de novo insertions in the selfed progenies (Figure 1B). This confirmed that mPing is active in EG4 under natural growth conditions but inactive in Nipponbare. If mPing was specifically activated in the pollen of EG4, we could obtain de novo insertions only in the F₁ plants from the Nipponbare/EG4 cross. However, we obtained de novo insertions in both Nipponbare/EG4 and EG4/Nipponbare populations (Figure 1B). Moreover, there was no significant difference in the number of de novo insertions per plant between the two F₁ populations. This indicates that the activating factor(s) for the mPing transposition is present in both male and female gametes of EG4.

**Transpositions of mPing during ontogeny of EG4 plants**

We performed TD analysis of mPing using genomic DNA samples extracted from endosperm, radicle, and leaf blades of eight progenies (S₁) derived from a single parental EG4 plant (S₀), and investigated the mPing transposition during ontogeny of rice plants (Figure 2A). One of the results of TD analysis using two selective bases is shown in Figure S3; all 16 possible primer combinations were analyzed. We examined de novo insertions in the same way as described above. Consequently, a total of 228 de novo insertions were detected. These insertions were divided into five groups (Figure 2B); (1) endosperm-specific insertions that appeared only in the endosperm sample, (2) radicle-specific insertions that appeared only in the radicle sample, (3) leaf-specific insertions that appeared only in one sample from the 1st to 3rd leaf blades, but not in the 4th and 5th leaf blades, (4) shoot-specific insertions that appeared in at least one sample of 1st, 2nd, and 3rd leaf blades, and in at least one sample of 4th and 5th leaf blades, and (5) radicle/shoot-specific insertions that appeared in both radicle and leaf blade samples. These de novo insertions were confirmed by sequence and locus-specific PCR analysis (Table S2 and Figure S4). Numbers of each insertion obtained in this study are summarized in Figure 2C. Plant development is divided roughly into three successive phases: embryogenesis, vegetative phase, and reproductive phase. If mPing transposed in the SAM of the S₀ plant during vegetative and/or reproductive phases, the de novo insertions would segregate according to Mendel’s law among the S₁ progenies. We obtained no band that appeared in at least two S₁ progenies and was not seen in the S₀ plant. This indicates that the transmissible insertion of mPing to the next generation seldom (or never) arises during the vegetative and reproductive phases.
Flowering plants have evolved a unique reproductive process called double fertilization. In this process, either of two sperm cells in pollen fuses with either an egg cell or a central cell in the ovule, and then the egg cell fertilized with the sperm cell initiates embryogenesis [27]. In rice, the SAM and radicle are regionalized in the embryo 3 DAP, and three leaves and the radicle are already present in the mature embryo [28]. We detected only three radicle/shoot-specific insertions (Figure 2C), indicating that mPing scarcely transposes during the period from the onset of gametogenesis to the early stage (until 3 DAP) of embryogenesis. Among the 228 de novo insertions, 116 and 17 were shoot-specific and leaf-specific insertions, respectively (Figure 2C). This indicates that mPing actively transposes in the embryo during the period from the regionalization of SAM and radicle (at 3 DAP) to the formation of the 3rd leaf primordia (at 8 DAP). Of the 133 shoot- and leaf-specific insertions, 108 were of the 1st leaf blade (Figure 2D). Since the 1st leaf primordium is formed at 5 DAP, the most active phase of the mPing transposition was considered to be from 3 to 5 DAP. We detected a large number of radicle-specific insertions as well as shoot-specific insertions, and the sum of these insertions accounted for 90% of all insertions detected in this study (Figure 2C). Taken together, we concluded that mPing in EG4 most actively transposes in the 3 to 5 DAP embryo.

Endosperm is a triploid tissue that is produced by fusing a central cell containing two polar nuclei with one of two sperm cells in no particular order. The endosperm formation occurs in parallel with embryogenesis. The endosperm-specific insertions result from the mPing transposition occurred in either gametogenesis or endosperm formation. We observed only two endosperm-specific insertions (Figure 2C), supporting that mPing scarcely transposes during the period from the onset of gametogenesis to the early stage of embryogenesis. The relationship between the banding patterns obtained in TD analysis and the timing of mPing transposition is summarized in Figure S5.

Inheritance of de novo mPing insertions to the next generation

In order for mPing to amplify, the de novo insertions must be transmitted to the next generation. We performed TD analysis using 12 progenies ($S_2$) derived from the main culm and the primary tiller of a single selfed parent ($S_1$) to investigate whether the de novo insertions detected in ontogenical analysis are inheritable (Figure S6). Both radicle-specific and leaf-specific insertions in the $S_1$ plants were not detected in the $S_2$ progenies ($0$ of $13$, $0$ of $2$, respectively). In contrast, $85\%$ ($11$ of $13$) of the shoot-specific insertions that were detected in the $S_1$ plants also appeared in the $S_2$ progenies. This value ($85\%$) is consistent with the estimated number of inheritable de novo insertions in our previous report [21]. Thus most of the de novo insertions that arose in the 3 to 5 DAP embryo were successfully inherited to the next generation.

Excisions of mPing during ontogeny of EG4 plants

We have already determined the sites of all mPing insertions (1163 in total) in the EG4 genome [13], and have investigated mPing excisions in a small EG4 population using locus-specific
Figure 2. De novo mPing insertions during rice ontogeny. (A) Experimental setup for the ontogenical analysis to determine the timing of mPing transposition in EG4. Eight progenies (S1) derived from a single parental EG4 plant (S0) were grown in a greenhouse. Endosperm, radicle, and leaf blades (1st to 5th) of each S1 plant were sampled for DNA extraction. S2 seeds were harvested from the main culm and the primary tiller of each S1 plant to investigate the inheritance of de novo mPing insertions. The 2nd leaf blade of S0 and S2 plants was also sampled. Detailed information is provided in the ‘Materials and Methods’. (B) mPing insertions were detected by transposon display. Representative images of shoot-, leaf-, endosperm-, radicle-, and radicle/shoot-specific insertions are shown. White arrowheads indicate the bands representing the de novo mPing insertion. E: endosperm, R: radicle, L1–L5: 1st to 5th leaf blades. (C) The number of organ-specific de novo insertions in EG4. All 16 possible primer combinations were analyzed. En: endosperm-specific insertion, RS: radicle/shoot-specific insertion, R: radicle-specific insertion, Shoot: shoot-specific insertion, Leaf: leaf-specific insertion. (D) Percentage of leaf positions where the first de novo mPing insertion was found. Shoot: shoot-specific insertion, Leaf: leaf-specific insertion.

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primer pairs [29], [30]. Here we examined the timing of the \( \text{mPing} \) excision with locus-specific PCR using the genomic DNA samples that were used for the ontogenetical analysis of the \( \text{de novo} \) insertion. We randomly chose 48 markers for this study (Table S3). We divided the \( \text{mPing} \) excisions into five types with the same criteria as those used for the \( \text{de novo} \) insertions: endosperm-, radicle-, leaf-, shoot-, and radicle/shoot-specific excisions (Figure S7). There were no endosperm-specific and radicle/shoot-specific excisions, indicating that no \( \text{mPing} \) transposition occurs during the period from the onset of gametogenesis to the early stage of embryogenesis. We detected seven radicle-specific, six leaf-specific, and three shoot-specific excisions. All shoot-specific excisions were detected from the 1st leaf blade sample. These results indicate that, like the \( \text{de novo} \) insertion, the \( \text{mPing} \) excision also occurs during the period from the regionalization of the SAM and radicle to the formation of the first leaf primordium, although we cannot completely rule out the possibility that these excisions occur also in somatic cells of mature tissues. Thus, in addition to the experimental results of the \( \text{de novo} \) insertion, we concluded that \( \text{mPing} \) of EG4 was most actively transposing in the 3 to 5 DAP embryo.

Expression pattern of \( \text{Ping} \) in EG4

Both \( \text{Ping} \) and \( \text{Pong} \) provide a Myb-like protein and a transposase, which are encoded by their ORF1 and ORF2, respectively (Figure 3A), and have been considered as autonomous elements responsible for the \( \text{mPing} \) transposition. We investigated the expression of \( \text{Ping-ORF1} \), \( \text{Ping-ORF2} \), \( \text{Pong-ORF1} \), and \( \text{Pong-ORF2} \) during embryogenesis to evaluate which autonomous element plays a predominant role in driving the \( \text{mPing} \) transposition in EG4. Reverse transcription-PCR analysis revealed that \( \text{Ping-ORF1} \) and \( \text{Ping-ORF2} \) constitutively expressed in the ovary during embryogenesis (Figure 3B). On the other hand, no transcriptions of \( \text{Ping-ORF1} \) and \( \text{Ping-ORF2} \) (Figure 3B) were observed. This strongly suggests that \( \text{Pong} \) predominantly controls the \( \text{mPing} \) transposition in EG4.

We performed real-time quantitative PCR (qPCR) analysis to compare the expression level of \( \text{Ping-ORF1} \) and \( \text{ORF2} \) between EG4 and Nipponbare during embryogenesis. In all developmental stages from 1 to 6 DAP, the expression levels of both \( \text{Ping-ORF1} \) and \( \text{ORF2} \) were higher in EG4 than in Nipponbare (Figure 3C, D). Since EG4 harbors seven copies of \( \text{Ping} \), whereas Nipponbare has only one copy (Table S4), the difference in the expression levels between EG4 and Nipponbare is considered to be attributable to the different copy number of \( \text{Ping} \). However, we found that \( \text{Ping} \) of EG4 showed different expression patterns from that of Nipponbare. In Nipponbare, the expression level of \( \text{Ping-ORF1} \) and \( \text{ORF2} \) gradually declined until 3 DAP, and restored to the basal level at 6 DAP. In contrast, in EG4, the expression levels of both \( \text{Ping-ORF1} \) and \( \text{ORF2} \) rapidly increased, with a peak at 3 DAP (Figure 3C, D). The ratio of relative expression level (EG4/ Nipponbare) clearly demonstrated that \( \text{Ping} \) might be up-regulated in a developmental stage-specific manner in the ovary of EG4 (Figure 3E). Since \( \text{mPing} \) transposed during the period from 3 to 5 DAP, the rapid increase in \( \text{Ping} \) expression most likely drive the \( \text{mPing} \) transposition.

Accumulation of \( \text{Ping} \) transcripts in the embryo triggers \( \text{mPing} \) transposition

We investigated the spatial expression pattern of \( \text{Ping} \) by \textit{in situ} hybridization using \( \text{Ping} \)-specific probes. The probe positions were indicated in Figure 3A. The \( \text{Ping} \) transcripts were detected in all tissues, viz. embryo, endosperm, and ovary wall, in both EG4 and Nipponbare (Figure 4A–C, S8). Among the tissues, the 3 DAP embryo of EG4 yielded an exceptionally strong signal, indicating a high accumulation of \( \text{Ping} \) transcripts (Figure 4A), whereas the 5 DAP embryo showed a much lower accumulation of \( \text{Ping} \) transcripts in EG4 (Figure 4D–F). Such a drastic change in accumulation quantity of \( \text{Ping} \) transcripts with the advance of embryogenesis was consistent with the change in the expression quantity of \( \text{Ping} \) with the advance of embryogenesis, which was investigated by real-time qPCR (Figure 3C–E). These results suggest that the tissue- and developmental stage-specific accumulation of the \( \text{Ping} \) transcripts triggers \( \text{mPing} \) transposition at this stage in EG4. To confirm this hypothesis, we evaluated the spatial expression pattern of \( \text{Ping} \) in the SAM during the vegetative phase. As described above, \( \text{mPing} \) hardly transposes in the SAM during this phase. The \( \text{Ping} \) transcripts were detected in all tissues including the SAM, and, as expected, there was no obvious difference in the signal intensity between EG4 and Nipponbare (Figure 4G–I). Thus the \( \text{Ping} \) transcripts proved to accumulate developmental stage-specifically only in the tissue where \( \text{mPing} \) actively transposes. We therefore concluded that the high accumulation of \( \text{Ping} \) transcripts triggers the transposition of \( \text{mPing} \) in the 3 DAP embryo of EG4.

SNP in an intronic region of \( \text{Ping-ORF1} \)

EG4 has seven \( \text{Ping} \) elements (\( \text{Ping-1} \) to -7), whereas Nipponbare has only one (\( \text{Ping-N} \)) (Table S4). When we sequenced and compared all \( \text{Ping} \) elements, a single nucleotide polymorphism (SNP) in the first intronic region of \( \text{Ping-ORF1} \) was detected between EG4 and Nipponbare (Figure 5A). \( \text{Ping-N} \) has a ‘T’ nucleotide on the SNP region, whereas all \( \text{Ping} \) elements in EG4 have a ‘C’ nucleotide. We named the former ‘T-type \( \text{Ping} \)’ and the latter ‘C-type \( \text{Ping} \)’.

In addition to EG4, several Aikoku and Gimbozu landraces (hereafter AG strains) are known to exhibit high \( \text{mPing} \) activity [21]. We investigated the SNP-type of \( \text{Ping} \) and the copy number of \( \text{Ping} \) and \( \text{mPing} \) in 93 AG strains, and evaluated the effect of C-type \( \text{Ping} \) on the \( \text{mPing} \) activity. These 93 AG strains were divided into three groups according to the SNP-type of the \( \text{Ping} \) allele (Table S4); strains harboring C-type \( \text{Ping} \) strains harboring T-type \( \text{Ping} \) and strains harboring no \( \text{Ping} \). The strains with C-type \( \text{Ping} \) had more \( \text{mPing} \) copies than those with T-type \( \text{Ping} \) or no \( \text{Ping} \) (Figure 5B, Steel–Dwass test, \( p<0.01 \)). This implies that the C-type \( \text{Ping} \) could drive the \( \text{mPing} \) transposition. We further investigated the expression patterns of \( \text{Ping-ORF1} \) and \( \text{ORF2} \) in two \( \text{mPing} \)-active strains (A119 and A123) and two \( \text{mPing} \)-inactive strains (A105 and G190) during embryogenesis (from 1 to 6 DAP). A119 and A123 have six and ten copies of C-type \( \text{Ping} \), whereas both A105 and G190 have one copy of T-type \( \text{Ping} \) (Table S4). Expression analysis revealed that A105 and G190 kept low expression levels of \( \text{Ping} \), respectively, and both A105 and G190 have one copy of T-type \( \text{Ping} \) (Figure 5B). This indicates that, in EG4, A119, and A123, the developmental stage-specific expression of \( \text{Ping} \) is controlled by the same factor(s) described in the Discussion.

Discussion

Our final goal was to elucidate how MITEs attain their high copy numbers in the genome. To this end, we chose \( \text{mPing} \) which is the only active MITE identified in rice, as a material and analyzed the timing of \( \text{mPing} \) transposition in the \( \text{mPing} \)-active strain EG4. Consequently, we successfully found one mechanism of the \( \text{mPing} \) amplification; \( \text{mPing} \) most actively transposes during the period from the regionalization of the SAM and radicle to the formation of the first leaf primordium (3 to 5 DAP) by the
developmental stage-specific up-regulation of the autonomous element Ping. The transpositions of TEs are categorized into germinal and somatic types according to the type of cells where the transposition takes place. LORE1a in Lotus japonicus is activated in plants regenerated from de-differentiated cells and transposes in male germ cells by the pollen grain-specific LORE1a transcription, resulting in the asymmetric transposition of LORE1a in the reciprocal crosses between the active and non-active lines [31]. Tag1 in Arabidopsis shows germinal transposition activity in both male and female germ cells. Consequently, the reciprocal crosses show symmetric transposition of Tag1 [32]. These results demonstrate that the transposition activity in reciprocal crosses reflects the tissue specificity of germinal transposition. In this study, reciprocal crosses between EG4 and Nipponbare showed the same mPing transposition activity, which may suggest that mPing in EG4 transposes in both male and female germ cells. However, we obtained only a few de novo endosperm-specific and radicle/shoot-specific insertions in the ontogenical analysis, although we detected a number of de novo shoot-specific and radicle-specific insertions. We therefore concluded that most mPing transposes not in germ cells but in somatic cells after pollination. Somatic transposition

Figure 3. Ping expression during seed development. (A) Structure of the Ping and Pong elements. Terminal inverted repeats are indicated by black triangles. Boxes represent ORF1 and ORF2, respectively. Gray horizontal arrows indicate the direction of transcription. RNA probes used are indicated below the ORFs. (B) Reverse-transcription PCR analysis of Ping-ORF1, Ping-ORF2, Pong-ORF1, and Pong-ORF2. Numbers in parentheses are PCR cycle numbers. PC: positive control (0.1 ng genomic DNA), NC: negative control (non-reverse-transcribed RNA). (C) Real-time quantitative PCR of Ping-ORF1 and (D) Ping-ORF2. The expression level in the Nipponbare ovary just after pollination was set as 1. The results are presented as means of three biological replicates. Bars indicate SE. (E) The ratio of Ping expression level of EG4 to that of Nipponbare. The means in (C) and (D) were used for calculation. doi:10.1371/journal.pgen.1004396.g003
that occurs at the late stage of plant development often produces spotted and striped segments in tissues, such as maize seed coat variegation caused by Mu insertion from the hz2 gene [33], [34] and rice leaf color variegation by nDart excision from the OsClpP5 gene [35]. In animals, somatic transposition is seldom transmitted to the next generation because germ cells are set aside from somatic cells at the early stage of embryogenesis. On the other hand, in plants, germ cells are generated from somatic cells at the reproductive stage. In rice, gametes are generated in the SAM; therefore, somatic transposition that occurred in the SAM can be transmitted to the next generation via gametes. In this study, we revealed that most mPing elements transposed in somatic cells of the embryo during the period from 3 to 5 DAP. Being a class II TE that transposes by a cut-and-paste mechanism, mPing is expected to be eliminated from genomic DNA with a certain frequency. However, a previous report demonstrated that the mPing excision sites would be repaired by utilizing a copy of mPing from either the sister chromatid or from the homologous chromosome [29]. The mPing excision site cannot be repaired if mPing transposes in germ cells, which are haploid. It is therefore considered that the somatic transposition of mPing is an important factor for mPing amplification in the genome.

The autonomous elements Ping and Pong mediate mPing transposition in the rice genome. Many japonica cultivars, including EG4 and Nipponbare, have both Ping and Pong. This study demonstrated that Ping plays a predominant role in mPing transposition in EG4. However, a heterologous expression assay using Arabidopsis and yeast showed that Pong had a higher catalytic capacity for mPing transposition than Ping [22], [23]. Furthermore, mPing transposition was observed under stress conditions in several rice cultivars harboring only Pong [14], [17], [19]. In this study, however, we detected very low expression of Pong through the development of rice plants, indicating that Pong would be epigenetically silenced at the transcriptional level in EG4. In contrast, Ping constitutively expressed in all organs including the SAM and embryo. Nevertheless, mPing could be transposing most actively in the embryo during the period from 3 to 5 DAP. Since the stage-specific up-regulation of Ping was observed during the period of mPing transposition, we hypothesized that the expression level of Ping needed to exceed a certain threshold of mPing transposition.

All mPing-active strains (EG4, A119, and A123) showed higher expression of Ping with a peak around 3 DAP than the mPing-inactive strains (Nipponbare, A105, and G190). Although further experiments are needed to elucidate the mechanism of developmental stage-specific up-regulation of Ping expression, we propose two hypotheses: (1) position- and dosage-effect, and (2) effect of SNP. The details of the hypotheses are as follows.

Position- and dosage-effect

Chromosomal position and copy number of TE often affect the transposition activity. The former is known as ‘position effect’ and the latter as ‘dosage effect’. Eight independent Tam3 copies residing in the Antirrhinum majus genome show different transposition activities from each other [36]. In Arabidopsis, germline reversion frequency of Tagl increases in proportion to its copy number [32]. The mPing-inactive strains Nipponbare, A105, and G190 have only one Ping at the same locus, whereas the mPing-active strains EG4, A119, and A123 have respectively seven, six, and ten copies of Ping at different loci except for the Ping-1 locus. Furthermore, the expression pattern of Ping showed slight variation among the mPing-active strains harboring only C-type Ping. These results suggest that the developmental stage-specific up-regulation

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Figure 4. Detection of Ping-ORF1 spatial expression patterns by in situ hybridization analysis. Longitudinal sections through the ovary 3 days after pollination of (A, C) EG4 and (B) Nipponbare; the ovary 5 days after pollination of (D, F) EG4 and (E) Nipponbare; and the shoot apical meristem of (G, I) EG4 and (H) Nipponbare seedlings were hybridized with antisense (A, B, D, E, G, H) or sense (C, F, I) RNA probes. Little staining was obtained with the sense probe (F). Em: embryo, En: endosperm, OW: ovary wall, SAM: shoot apical meristem. Scale bars represent 100 μm.

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Figure 5. SNP in the first intronic region of Ping-ORF1. (A) Determination of the SNP sequence in the first intronic region of Ping-ORF1. The arrowhead indicates the position of the SNP. The number indicates the position of the Ping element. Ping harboring +1261C SNP and +1261T are named ‘C-type’ and ‘T-type’ Ping, respectively. (B) Box plots of mPing copy number in AG lines. The top and bottom of the boxes mark the first and third quartiles, respectively. The center line represents the median, and the whiskers show the range of observed values within 1.5 times the interquartile range from the hinges. Values beyond 1.5 times the interquartile range from the nearest hinge are marked by open circles. ‘No Ping’, ‘C-type Ping’, and ‘T-type Ping’ indicate the groups having no Ping, C-type Ping, and T-type Ping, respectively. Expression of (C) Ping-ORF1 and (D) Ping-ORF2 during embryogenesis in mPing-active strains (A119 and A123) and mPing-inactive strains (A105 and G190). The results are presented as means of three biological replicates. Bars indicate SE. The ratio of (E) Ping-ORF1 and (F) -ORF2 expression level of A105, A119, A123, and G190 to that of Nipponbare. The means in (Fig. 3C) and (Fig. 3D) were used for calculation.
of Ping expression is probably regulated by the position-effect and/or the dosage-effect.

Effect of SNP

Intronic SNPs are known to cause drastic effects on gene expression. In humans, an intronic SNP in SLC22A4 affects transcriptional efficiency in vitro, owing to an allelic difference in affinity to the transcriptional factor RUNX1 [37]. Furthermore, a SNP located in the intronic enhancer region of the thyroid hormone receptor β gene enhances pituitary cell-specific transcriptional activity [38]. In this study, we demonstrated that a SNP is present in the intronic region of Ping-ORF1, and Ping elements in the AG strains were categorized into either T-type or C-type Ping according to the SNP-type. Since all strains that showed a peak in the expression analysis had only C-type Ping, the intronic SNP might influence the developmental stage-specific up-regulation of Ping expression. T-type Ping was present in 14 AG strains as one copy, and its chromosomal location did not differ among strains. In contrast, the copy number of C-type Ping varied from one to ten, and their chromosomal locations, except for Ping-1, differed from each other. These results indicate that T-type Ping has lost its activity, whereas C-type Ping may be still active in the rice genome. Furthermore, we found that the copy number of Ping in strains harboring C-type Ping than in strains harboring T-type Ping. This strongly supports that C-type SNPs in the intronic region of Ping contribute to the amplification of Ping, presumably by the developmental stage-specific up-regulation of Ping expression.

Since the transposition of TE often damages the host genome, TEs with high transposition activity are targeted by the silencing mechanisms. Nevertheless, MITEs amplify to very high copy numbers not only in plant genomes but also in animal genomes. Very little is known about how MITEs attain their high copy numbers by escaping the silencing mechanism. The transposition of Ping is transiently induced by various stresses [14]–[18], indicating that the activity of Ping is suppressed by the silencing mechanisms in many cultivars. Thus, Ping must overcome the silencing mechanism in order to maintain the transposition activity under natural growth conditions. Our results revealed that Ping in EG4 was mobilized by the sufficient supply of Ping transcripts produced only during the period of Ping transposition. This stage-specific activation is thought to be a strategy of the Ping family to amplify Ping by escaping from the silencing mechanism of the host genome. Since no active MITEs other than mPing so far have been identified, it is very difficult to elucidate if the other MITEs also attain their high copy numbers in the same way as Ping amplifies. Given that the other active MITEs are identified, however, our study will help to understand their amplification mechanisms. Our previous study documented the generation of new regulatory networks by a subset of Ping insertions that renders adjacent genes stress inducible [13]. In addition to Ping, other MITEs also contribute to gene and genome evolution via providing new promoter regulatory sequences, transcriptional termination elements, and new alternative exons [39], suggesting that the amplification of MITEs causes gene and genome evolution. Our results provide clues to further understand not only the amplification mechanism of MITEs but also the co-evolution of MITEs and the host genome.

Materials and Methods

Plant materials and sampling

EG4 (cultivar Gimbozu), Nipponbare, and 94 Aikoku/Gimbozu landraces were used in this study (Table S4). Aikoku/Gimbozu landraces were provided from the GenBank project of the National Institute of Agrobiological Science, Ibaraki, Japan. Reciprocal crosses between EG4 and Nipponbare were made in a green house. Before pollination, all anthers were removed from the flowers of maternal plants. The pollinated flowers were covered with protective bags to prevent outcrossing until harvest. After harvesting, success of crosses was checked with the molecular markers. For ontogenical analysis, eight progenies of EG4 (S1) derived from a single parental plant (S0) were grown in a greenhouse, and all S2 seeds were harvested. For S3 plants, each seed was cut into two halves, and the half including the embryo was germinated and the other was sampled. After germination, the radicle and the 1st, 2nd, 3rd, 4th, and 5th leaf blades were sampled. The second leaf was collected from S0 and S2 plants. For estimation of Ping and mPing copy numbers, eight bulked plants were sampled. For RNA extraction, ovaries before pollination and ovaries at 1, 2, 3, 4, 5, and 6 DAP were collected. All samples were immediately frozen in liquid nitrogen and stored at −80℃ until use.

DNA extraction and transposon display

DNA extraction and transposon display was performed following a published protocol [30]. For DNA extraction from endosperm, we used GM quicker 2 (Nippon Gene).

Locus-specific PCR

Sequencing of mPing-flanking fragments excised from transposon display gels and primer design were performed following a published protocol [30]. The genomic locations of the Ping insertion sites were forecasted by a BLAST search in the Rice Annotation Project Database (RAP-DB; http://rapdb.dna.aflrc.go.jp/) [40], [41] using mPing flanking sequences as queries. To prepare enough templates for PCR, whole genome amplification was performed using an illustra GenomiPhi V2 Kit (GE Healthcare). mPing excision was detected by PCR with mPing-sequence characterized amplified region (SCAR) markers [29]. PCR was performed in 10-μl reaction volumes containing 10 ng of the template DNA, 5 μl of GoTaq Green Master mix (Promega), 5% DMSO, and 0.25 μM of each primer (Table S3). PCR conditions were as follows: 94℃ for 3 min; 40 cycles of 98℃ for 10 s, 57℃ for 30 s, and 72℃ for 45 s; and 72℃ for 5 min. To detect the presence of Ping-N, -1, -2, -3, -4, -5, -6, and -7, eight Ping-SCAR markers were used. The genomic locations of the Ping insertion sites were referred from a previous report [42]. For detection of the Ping-1 allele, PCR was performed in 10-μl reaction volumes containing 10 ng of template DNA, 0.2 U of KOD FX Neo (Toyobo), 1×PCR buffer for KOD FX Neo (Toyobo), and 0.2 μM of each primer (Table S5). PCR conditions were as follows: 94℃ for 3 min; 35 cycles of 98℃ for 10 s, 60℃ for 30 s, and 68℃ for 90 s; and 72℃ for 1 min. For detection of Ping-N, -2, -3, -4, -5, -6, and -7 alleles, PCR was performed in 10-μl reaction volumes containing 10 ng of template DNA, 5 μl of GoTaq Green Master mix (Promega), 5% DMSO, and 0.25 μM of each primer (Table S5). PCR conditions were as follows: 94℃ for 3 min; 35 cycles of 98℃ for 10 s, 60℃ for 30 s, and 72℃ for 45 s; and 72℃ for 1 min.

RNA isolation and expression analysis

Total RNA was isolated using TriPure isolation reagent (Roche) and digested using RNase-free DNase (TaKaRa). First strand cDNA was synthesized using a Transcriptor first strand cDNA synthesis kit (Roche). For reverse transcription PCR, PCR was performed in 10 μl reaction volumes containing cDNA generated from 4 ng total RNA, 0.2 U of KOD FX Neo (Toyobo), 1×PCR
buffer for KOD FX Neo (Toyobo), and 0.5 μM of each primer. PCR conditions were as follows: 94°C for 3 min; 35 or 45 cycles of 98°C for 10 s, 60°C for 10 s, and 72°C for 10 s. Relative quantification of Ping-ORF1 and Ping-ORF2 were calculated by the 2^−ΔΔCT method [43] using Light cycler 1.5 (Roche). The UBQ5 gene was used as the calibrator gene. The thermal profile consisted of 10 min at 95°C; and 45 cycles of 4 s at 95°C, 10 s at 60°C, and 1 s at 72°C. Amplification data were collected at the end of each extension step. The primer pairs used in this study are listed in Table S6.

Paraffin sectioning and in situ hybridization
Plate samples were fixed with 4% (w/v) paraformaldehyde and 1% Triton X in 0.1M sodium phosphate buffer for 40 h at 4°C. They were then dehydrated in a graded ethanol series, substituted with 1-butanol, and embedded in Paraplast Plus. The samples were sectioned at 8μm thickness using a rotary microtome. Fragments of Ping-ORF1 (1091 bp) and Ping-ORF2 (1368 bp) were cloned into pBlueScript SK+ (Stratagene) and sequenced. For digoxigenin-labeled antisense/sense RNA probe synthesis, in vitro transcription was performed using T7 RNA polymerase and T3 RNA polymerase. In situ hybridization and immunological detection with alkaline phosphatase were performed according to Kouchi and Hata [44].

SNP detection
PCR was performed in 10-μl reaction volumes containing 10 ng of template of DNA, 5μl of GoTaq Green Master mix (Promega), 5% DMSO, and 0.25 μM of each primer. PCR conditions were as follows: 94°C for 3 min; 35 cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 30 s; and 72°C for 1 min. PCR primers used in this study are listed in Table S6. Because the original sequence contained an AflI restriction site, one mutation was introduced into the reverse primer. The 5-μl PCR products were mixed with 5 μl restriction mixture containing 1 U AflI (TaKaRa), 33 mM Tris-acetate (pH 7.9), 10 mM Mg-acetate, 0.5 mM Dithiothreitol, 66 mM K-acetate, and 0.01% (w/v) bovine serum albumin. After 16 h incubation at 37°C, DNA gel electrophoresis was performed. PCR products (502 bp) including +1261T SNP were not digested, whereas PCR products including +1261C SNP were digested into two fragments (352 bp and 150 bp).

Estimation of Ping and mPing copy number
To determine the copy number of Ping by Southern blot analysis, genomic DNA samples were digested with Eco RI restriction enzyme. These samples were loaded onto an agarose gel, separated by electrophoresis, blotted onto a nylon membrane, and probed with the Ping fragment. The mPing copy number was determined by real-time quantitative PCR as described previously [45] with little modification. Quantitative PCR was performed using the Light-Cycler 480 system (Roche). PCR was performed in 20 μl reaction volumes containing 5 μl genomic DNA (0.4 ng/μl), 1× LightCycler 480 SYBR Green 1 Master mix (Roche), and 0.5 μM of each primer. Specificity of the amplified PCR product was assessed by performing a melting curve analysis on the LightCycler 480 system.

Supporting Information
Figure S1 Verification of the reciprocal crosses between EG4 and Nipponbare. (A) Locus-specific PCR analysis of Ping with locus-specific markers. The maker names and cross combinations are indicated at the top of the profiles. (B) Locus-specific PCR analysis of mPing in Nipponbare genome. The genomic location of the mPing insertions and cross combinations are indicated at the top of the profiles. G and F indicate parental EG4 plants and the F1 plants, respectively. Lane M: DNA size marker (Gene Ladder 100, Nippon Gene), Lane N: Nipponbare. Black and white arrowheads show the bands indicating the presence and absence of Ping/mPing, respectively. (TIF)

Figure S2 Locus-specific PCR analysis of de novo mPing insertions in F1 progenies. Ten representative results are shown. The genomic locations of the mPing insertions and cross combinations are indicated at the top of the profiles. G and F indicate parental EG4 and the F1 plants, respectively. Lane M: DNA size marker (Gene Ladder 100, Nippon Gene), Lane N: Nipponbare. Black and white arrowheads show the bands indicating the presence and absence of mPing, respectively. (TIF)

Figure S3 Ontogenetical analysis of mPing transposition in EG4 by transposon display. Eight progenies (S1) were derived from a single parental EG4 plant (S0). The 2nd leaf blade of the S0 plant and the endosperm, radicle, and leaf blades of each S1 plant were sampled and subjected to transposon display. White, red, and green arrowheads indicate shoot-, radicle-, and leaf-specific insertions, respectively. The rice plant has alternate distichous leaves; therefore, we analyzed the insertion in both [n+1]th and [n+2]th leaves to confirm whether the insertion detected in [n]th leaf is leaf-specific or shoot-specific. But we did not investigate the specificity of the insertions detected in the 4th and 5th leaves using their upper leaves. For this reason, we did not categorize such insertions and marked with the gray arrowhead. E: endosperm; R: radicle; L1–L5: 1st to 5th leaf. For progeny 2-8, samples are applied in the same order as for progeny 1. (TIF)

Figure S4 Locus-specific PCR analysis of de novo mPing insertions in various tissues of a single EG4 plant. Eight representative results are shown. The genomic locations of the mPing insertions and insertion types are indicated at the top of the profiles. Lane M: DNA size marker (Gene Ladder 100, Nippon Gene), Lane N: Nipponbare, Lane E: endosperm, Lane R: radicle, Lane L1–L5: 1st to 5th leaf. Black and white arrowheads show the bands indicating the presence and absence of mPing, respectively. (TIF)

Figure S5 Schematic representation of the relationship between banding patterns obtained in transposon display and the timing of mPing transposition. If mPing transposes in the period indicated by the red bar, the schematic banding patterns indicated by the arrows will be obtained. E: endosperm; R: radicle, L1–L5: 1st to 5th leaf blade, DAP: days after pollination, SAM: shoot apical meristem. (TIF)

Figure S6 Inheritance of de novo mPing insertions in EG4. S2 plants derived from the main culm and the primary tiller of a single S1 plant were assayed. The shoot-specific insertion in the S1 plant (white arrowhead) was inherited by S2 plants, whereas the radicle-specific insertion (red arrowhead) was not. E: endosperm; R: radicle, L1–L5: 1st to 5th leaf. (TIF)

Figure S7 mPing excisions in EG4. mPing excisions were detected by locus-specific PCR using the genomic DNA samples that were used for the ontogenetical analysis of de novo insertion. We analyzed 48 loci. Black and white arrowheads show the bands indicating the presence and absence of mPing, respectively. Figures indicate (A, B) shoot-specific excisions, (C, D) leaf-specific
exonizations, and (E) radicle-specific excision. G: EG4 (S0 plant); N: Nipponbare; E: endosperm; R: radicle; L1–L5: 1st to 5th leaf.

**Figure S8** Detection of mPing-ORF2 spatial expression patterns by in situ hybridization analysis. Longitudinal sections through the ovary 3 days after pollination of (A) EG4 and (B, C) Nipponbare were hybridized with (A, B) antisense or (C) sense RNA probes.

**Table S1** De novo mPing insertion sites detected in F1 progenies. (XLSX)

**Table S2** De novo mPing insertion sites detected in various tissues of a single EG4 plant. (XLSX)

**Author Contributions**

Conceived and designed the experiments: TTs. Performed the experiments: ST. Analyzed the data: ST. Contributed reagents/materials/analysis tools: YO TTs. Wrote the paper: ST TTs.

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