Amplification of the MITE *mPing* with the embryogenesis-specific expression of the transposon *Ping* in rice

2014

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Contents

Chapter I. General 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 (Schnable et al. 2009, Wicker et al. 2005), and they constitute 35% and 14% of the genomes of rice (*Oryza sativa*) and Arabidopsis (*Arabidopsis thaliana*), respectively (International Rice Genome Sequencing Project 2005, Arabidopsis Genome Initiative 2000). 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 (Fedoroff 2012, Cowley and Oakey 2013).

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) (Bureau and Wessler 1992, Bureau and Wessler 1994, Wessler et al. 1995). In the rice genome, MITEs are numerically predominant TEs (Oki et al. 2008), constituting 8.6% of the genome (Chen et al. 2012). 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 *PIF*/*Harbinger* superfamilies, respectively. Because MITEs are mainly deployed in gene-rich regions (Oki et al. 2008, Han et al. 2013) and affect adjacent gene expression (Naito et al. 2009), 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 (Jiang et al. 2003, Kikuchi et al. 2003, Nakazaki et al. 2003). 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 (Jiang et al. 2003). The transposition of *mPing* is suppressed in most rice cultivars, but, like other TEs, *mPing* is activated by exposure to various stress conditions such as gamma-ray irradiation (Nakazaki et al. 2003), hydrostatic pressurization (Lin et al. 2006), cell culture (Jiang et al. 2003), anther culture (Kikuchi et al. 2003), and inhibition of topoisomerase II (Yang et al. 2012). Introgression of distantly related genomes also causes *mPing* transposition (Shan et al. 2005, Yasuda et al. 2012). However, *mPing* is actively transposing without such stresses in the temperate *japonica* rice strain EG4 (cultivar Gimbozu) under natural growth conditions, and its copy number is approximately 1000 copies (Naito et al. 2006). This indicates that *mPing* has overcome the silencing mechanism or established a novel strategy for its amplification in the EG4 genome. In this sense, *mPing* in EG4 is an appropriate material to study the amplification of MITEs in plant genomes.

In this study, I demonstrate that *mPing* is actively transposing in the embryo of EG4

during the period from the regionalization of shoot apical meristem (SAM) and radicle to the formation of the first leaf primordium (3 to 5 days after pollination, DAP) with the aid of developmental stage-specific expression of the autonomous element *Ping*. The results shown in this study provide important evidence for the amplification mechanism not only of *mPing* but also of other MITEs.

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Chapter II. The timing of *mPing* transposition

Introduction

Plant development is divided roughly into three successive phases: embryogenesis, vegetative phase, and reproductive phase. After germination, plants obtain their energy from the sun through photosynthesis and grow during vegetative phase, and they open their flowers and produce their seeds during reproductive phase. Embryogenesis is processed in the pollinated ovary to form the basic structure of plant body. Because angiosperms transmit their genetic information from one generation to the next via their seeds, the period from gametogenesis in reproductive phase to embryogenesis is one of the most important sessions. The process of rice embryogenesis is described in review by Itoh et al. (2005) in detail. Shoot apical meristem (SAM) and radicle were regionalized at 3 days after pollination (DAP) and they morphologically differentiated at 4 DAP. First, second, and third leaf primordial are formed at 5, at 7, and at 8 DAP, respectively. Embryonic organs are matured during the period from 11 DAP to 20 DAP and they would be dormant after 20 DAP.

Plants have acquired the silencing mechanism of TEs in SAM. The maize *Mop1* gene was identified as the differentially expressed gene in SAM (Ohtsu et al. 2007). *Mop1* encodes an RNA-dependent RNA polymerase, which is required for paramutation and transposon silencing (Alleman et al. 2006, Woodhouse et al. 2006), and functions to silence repeat sequences such as transposable elements via production of small interfering RNAs (siRNAs) derived from these repeat sequences (Bender 2004, Chan et al. 2005). Germ cells also employ the silencing mechanisms to repress the

activity of TEs. 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 (Slotkin et al. 2009). On the basis of these results, Slotkin and colleagues proposed that siRNAs derived from TEs activated in the vegetative nucleus silence TEs in the sperm cells. I conceived that *mPing* might overcome such silencing mechanisms in EG4. In this chapter, I investigated the timing of *mPing* transposition during the ontogeny of EG4.

Materials and methods

Plant materials

EG4 (cultivar Gimbozu) and Nipponbare were used in this study. 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 (S_1) derived from a single parental plant (S_0) were grown in a greenhouse, and all S_2 seeds were harvested. For S_1 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 S_0 and S_2 plants. All samples were immediately frozen in liquid nitrogen and stored at –80°C until use.

DNA extraction and transposon display

DNA extraction and transposon display was performed following a published protocol (Tsukiyama et al. 2013). GM quicker 2 (Nippon Gene) was used for DNA extraction from endosperm.

Locus-specific PCR

Sequencing of *mPing*-flanking fragments excised from TD gels and primer design were performed following a published protocol (Tsukiyama et al. 2013). 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 (Monden et al. 2009). 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. PCR conditions were as follows: 94°C for 3 min; 40 cycles of 98°C for 10 s, 57°C for 30 s, and 72°C for 45 s; and 72°C for 5 min. To detect the presence of *Ping-N*, *-1*, *-2*, *-3*, *-4*, *-5*, *-6*, and *-7*, eight *Ping*-SCAR markers were used. 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 2-1). PCR conditions were as follows: 94°C for 3 min; 35 cycles of 98°C for 10 s, 60°C for 30 s, and 68°C for 90 s; and 72°C 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 2-1). 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 45 s;

and 72°C for 1 min.

Results

Transpositions of mPing during gametogenesis

To confirm the hypothesis that, in EG4, *mPing* might overcome the silencing mechanism in germ cells, I developed two F_1 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 2-1). One of the results of TD analysis using two selective bases is shown in Figure 2-2A; all 16 possible primer combinations were analyzed. The banding patterns of F_1 plants were almost the same as those of EG4. The bands that appeared in all F_1 plants but not in the parental EG4 plant were derived from another parental Nipponbare plant (Figure 2-1B). Furthermore, the bands that appeared in only one of eight F_1 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 2-2 and Figure 2-3). I detected 15.5 *de novo* insertions per plant in the selfed progenies of EG4, whereas Nipponbare yielded no *de novo* insertions in the selfed progenies (Figure 2-2B). 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, *de novo* insertions could be obtained only in the F_1 plants from the Nipponbare/EG4 cross. However, *de novo* insertions were obtained in both Nipponbare/EG4 and EG4/Nipponbare populations (Figure 2-2B). Moreover, there

was no significant difference in the number of *de novo* insertions per plant between the two F_1 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

The *mPing* transposition during ontogeny of rice plants was investigated by TD analysis of *mPing* using genomic DNA samples extracted from endosperm, radicle, and leaf blades of eight progenies (S_1) derived from a single parental EG4 plant (S_0) (Figure 2-4). One of the results of TD analysis using two selective bases is shown in Figure 2-5; all 16 possible primer combinations were analyzed. The *de novo* insertions were examined 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 2-6): (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 2-3 and Figure 2-7). Numbers of each insertion obtained in this study are summarized in Figure 2-8A. Plant development is divided roughly into three successive phases: embryogenesis, vegetative phase, and reproductive phase. If *mPing* transposed in the SAM of the S_0 plant during vegetative and/or reproductive phases, the *de novo* insertions would segregate according to Mendel's law among the S_1 progenies.

I obtained no band that appeared in at least two S_1 progenies and was not seen in the S_0 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 (Hamamura et al. 2011). 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 (Itoh et al. 2005). I detected only three radicle/shoot-specific insertions (Figure 2-8A), 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 2-8A). 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 2-8B). 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. I 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 2-8A). Taken together, it was 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. I observed only two endosperm-specific insertions (Figure 2-8A), 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 2-9.

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. I 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 2-10). Both radicle-specific and leaf-specific insertions in the S_1 plants were not detected in the S_2 progenies (0 of 15, 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 (Naito et al. 2006). 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

Our laboratory has already determined the sites of all *mPing* insertions (1163 in total) in the EG4 genome (Naaito et al. 2009), and has investigated *mPing* excisions in a small EG4 population using locus-specific primer pairs (Monden et al. 2009, Tsukiyama et al.

2013). Here the timing of the *mPing* excision was examined with locus-specific PCR using the genomic DNA samples that were used for the ontogenical analysis of the *de novo* insertion. 48 markers were randomly chosen for this study (Table 2-4). The *mPing* excisions were divided into five types with the same criteria as those used for the *de novo* insertions: endosperm-, radicle-, leaf-, shoot-, and radicle/shoot-specific excisions (Figure 2-11). There were no endosperm-specific and radicle/shoot-specific excisions, indicating that no *mPing* transposition occurs during the period from the onset of gametogenesis to the early stage of embryogenesis. I 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 *de novo* insertion, the *mPing* excision also occurs during the period from the regionalization of the SAM and radicle to the formation of the first leaf primordium, although I 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 *de novo* insertion, it was concluded that *mPing* of EG4 was most actively transposing in the 3 to 5 DAP embryo.

Discussion

The transpositions of TEs are categorized into germinal and somatic types according to the type of cells where the transposition takes place. The germinal and somatic transposition respectively represents the transposition occurred in germinal cells, such as pollen and ovary, and somatic cells. Cell specificity of the germinal transposition (either in the female gamete or in the male gamete) is easily determined by estimating

the number of *de novo* TE transposition in reciprocal crosses (TE-active strains and TE-inactive strain). *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 (Fukai et al. 2010). *Tag1* in Arabidopsis shows germinal transposition activity in both male and female germ cells. Consequently, the reciprocal crosses show symmetric transposition of *Tag1* (Liu and Crawford 1998). 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, I obtained only a few *de novo* endosperm-specific and radicle/shoot-specific insertions in the ontogenical analysis, although I detected a number of *de novo* shoot-specific and radicle-specific insertions. It was therefore concluded that most *mPing* transposes not in germ cells but in somatic cells after pollination.

Somatic transposition 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 *Mutator* excision from the *bz2* gene (Levy et al. 1989, Levy and Walbot 1990) and rice leaf color variegation by *nDart1* excision from the *OsClpP5* gene (Tsugane et al. 2006). 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, it was revealed that most *mPing* elements transposed in somatic cells of the embryo at the timing of regionalization of the SAM and radicle. 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 (Monden et al. 2009). 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.

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Table 2-1. *Ping*-SCAR markers used in this study. **Ping-1* in EG4 is inserted into *Pyong* element which is absent at this locus in Nipponbare. When *Pyong* harboring *Ping* is present at this locus, 1817bp PCR product will be obtained. When *Pyong* not harboring *Ping* is present at this locus, 963bp PCR product will be obtained. When *Pyong* is absent at this locus, 420bp product will be obtained.

				Inserted locus			Amplicon Size (bp)	
Marker name	**Forward Primer Sequence (from 5' to 3')**	**Inner Primer Sequence (from 5' to 3')**	**Reverse Primer Sequence (from 5' to 3')**	**Chromosome**	**Position (IRGSP-1.0)**	**Direction**	***Ping* present**	***Ping* absent**
Ping-N	CATCGATCTGTGGAGCAAAC	CCTCTTCAATCCTAGTTGCTTGC	CTATACGCATTCATGCTGGGAAG	6	23,521,638	+	778	298
Ping-1	CCGGTTCGAGCCTTTTAAGCGAAAATC	CCCTCCAGTTCCTAATTTATTGGCGTTTTAGAC AGCATTTGGTGTTCTACAACGTCGATTCTG	AGGGTGTAGTTACAGCTCTGACTTCACCATCC	1	2,640,501	+	*	*
Ping-2	CGTACGTGGCTGTTTAGGTTC	CCTCTTCAATCCTAGTTGCTTGC	ACAAGTAGCTAAGGACCCTACTCCTAC	1	4,220,010	+	634	213
Ping-3	ACTTGCCGCTAGAGCAACTG	CCTCTTCAATCCTAGTTGCTTGC	CTTTGGTCGATCCGACATTG	3	28,019,800	+	735	194
Ping-4	CTCGAGAAATAAACACGAGCATG	CCTCTTCAATCCTAGTTGCTTGC	TTATGGGAAAACACAGGCTTTG	7	26,460,307	-	777	249
Ping-5	ACAGCAAACCAAAGGAGAGAG	CCTCTTCAATCCTAGTTGCTTGC	ATCTTGCTTCACATTTCATTCTTCTAC	9	13,736,143	-	637	242
Ping-6	TCTCAAAACCGGACACACAC	CCTCTTCAATCCTAGTTGCTTGC	TGAGTACTGTTTGGTACTGTGCTG	9	16,690,612	+	650	192
Ping-7	GAGCATTGATCCACAACCATG	CCTCTTCAATCCTAGTTGCTTGC	CTGTTGTGTCCGATCAACAGAAG	9	10,863,118	+	740	258

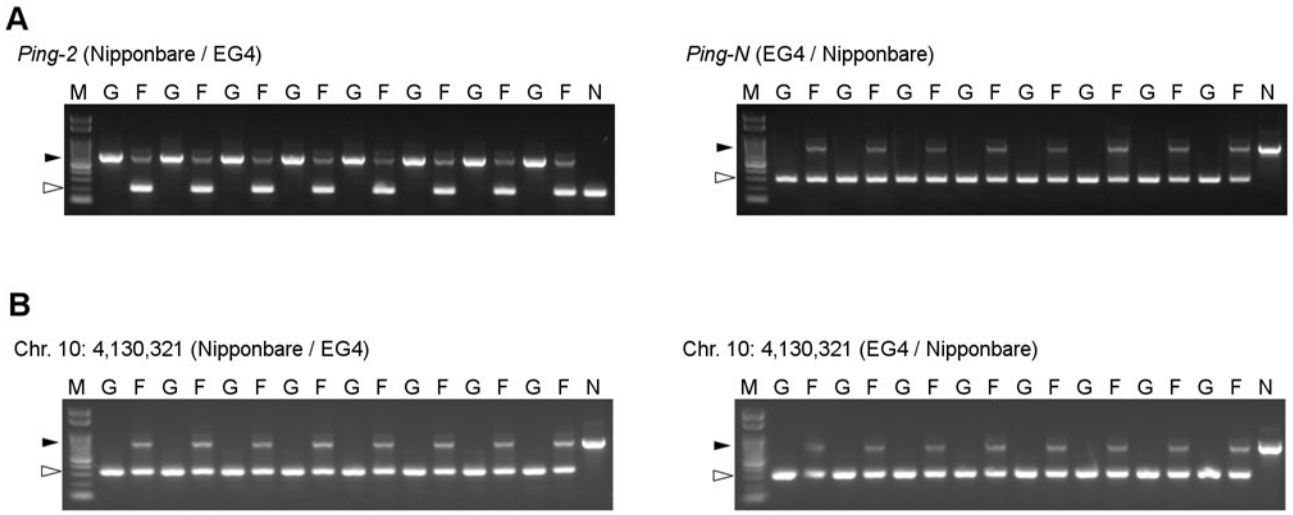

Figure 2-1. Verification of the reciprocal crosses between EG4 and Nipponbare. (A) Locus-specific PCR analysis of *Ping* with locus-specific markers. The maker names and cross combinations are indicated at the top of the profiles. (B) Locus-specific PCR analysis of *mPing* in Nipponbare genome. The genomic 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 F_1 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.

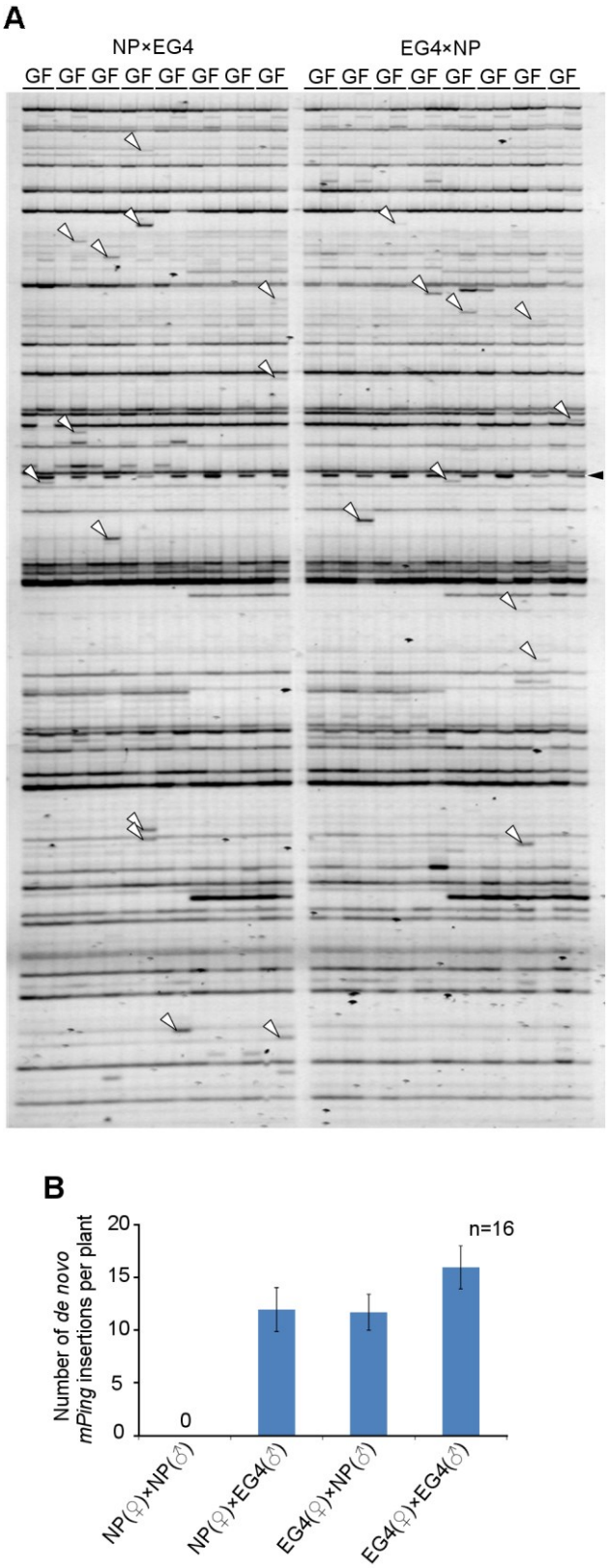

Figure 2-2. Transposition of *mPing* in reciprocal crosses between EG4 and Nipponbare. (A) Transposon display (TD) for *mPing* of the F_1 population from reciprocal crosses between EG4 and Nipponbare. One of the results of TD analysis using two selective bases is shown. The cross combinations are indicated at the top of the profiles, respectively. G and F indicate parental EG4 and the F_1 plants, respectively. White and black arrowheads indicate the bands representing the *de novo mPing* insertion and the band derived from Nipponbare genome, respectively. (B) Mean numbers of *de novo mPing* insertions in a single F_1 plant and in a self-pollinated plant. The cross combinations are indicated at the bottom of the profile. All 16 possible primer combinations were analyzed, and mean values were calculated using 16 individuals (n=16). Bars indicate SE.

Table 2-2. *De novo mPing* insertion sites detected in F_1 progenies.

Inserted locus		Nearest gene			Locus-specific primers		Amplicon Size (bp)	
Chromosome	Position (IRGSP-1.0)	Locus	Position	Distance	Forward primer (5' → 3')	Reverse primer (5' → 3')	*mPing* present	*mPing* absent
5	27,995,065	Os05g0562800	upstream	4400	TCATCGTAGACAGCAGTGGC	ATTGGACACGGAAACTCAGG	757	324
9	12,271,130	Os09g0369500	upstream	3710	CGAGATGTGGGCTAGTGATTAAG	GCCTCAAAGCAAGTAGTCCG	777	344
5	24,649,576	Os05g0501001	upstream	1439	TTGAATGATGAGGTGGAGGAG	AAGCCTGCAGCTTTTTCTTG	750	317
9	14,936,119	Os09g0416900	upstream	2186	TTCTCTTCTGATTGGTGCCC	AATCTCCCCTATCACCCTGG	768	335
1	42,469,355	Os01g0963800	upstream	1007	ATGTGGAGCCATTTCTCTGG	GGCAGCAAATGCATCTTTTC	769	336
12	24,437,496	-	-	>5000	CAGCAGAAGTGAGATCAGAATCAG	TGTGTTGAAACCGGAGTGAC	770	337
8	4,340,753	Os08g0174700	upstream	3418	TGATCCACTGATGATGGTCC	TCCCATAATCCCCTTCCTTC	758	325
12	2,963,500	Os12g0159100	3' UTR intron	-	TGCCCACCTATTGTTTCAGC	ACTTGGAATGACAAAACGCC	778	345
11	22,123,803	-	-	>5000	TTCTGAGAATTTTGCCCTGG	ACACCATCCAAAACATCACG	765	332
6	18,971,562	-	-	>5000	GATCTGTGAACTCAATGATGCC	TAGTTGCAATTTCATCCCCG	778	345

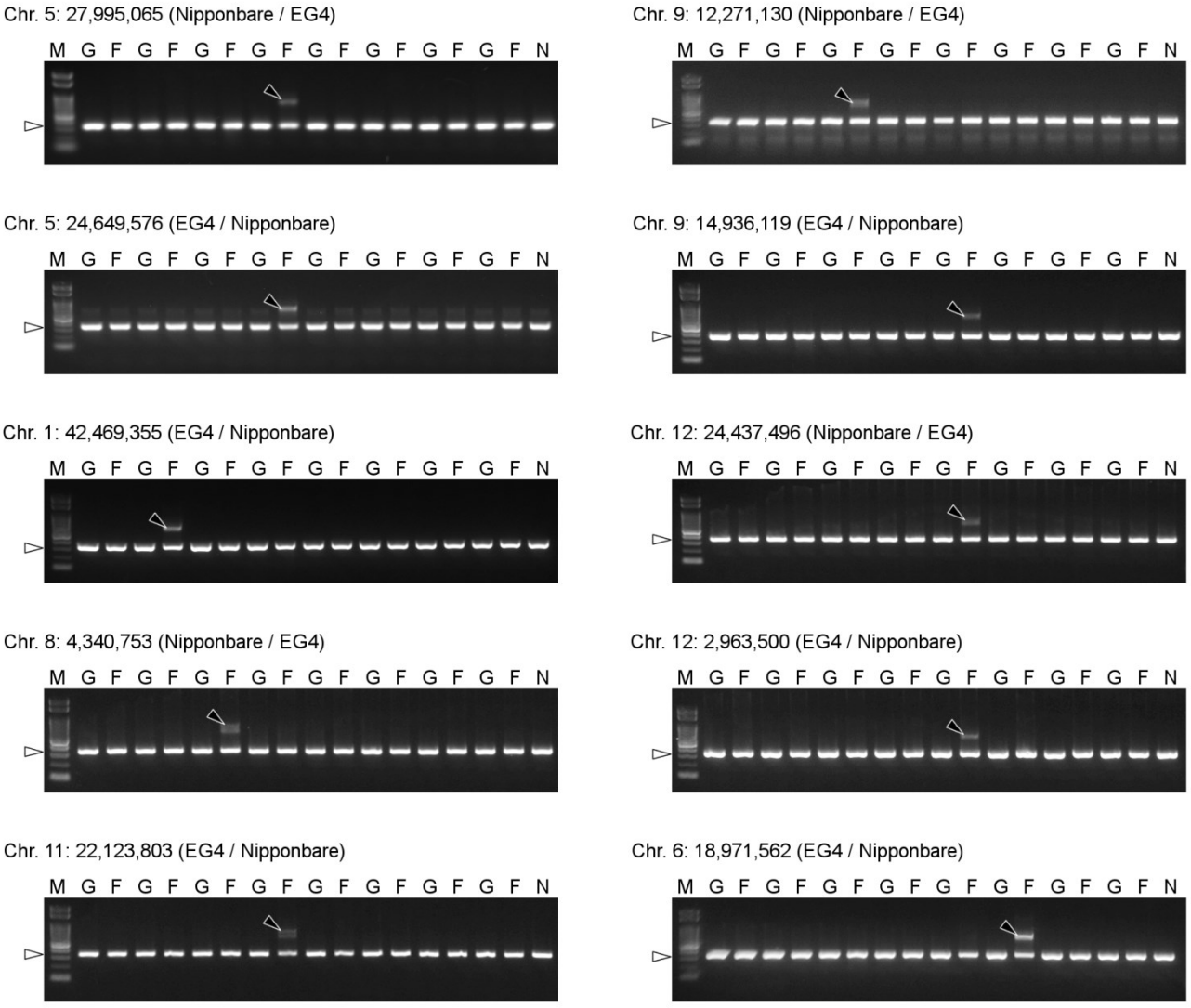

Figure 2-3. Locus-specific PCR analysis of *de novo mPing* insertions in F_1 progenies. Ten representative results are shown. The genomic locations of the *mPing* insertions and cross combinations are indicated at the top of the profiles. G and F indicate parental EG4 and the F_1 plants, respectively. 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.

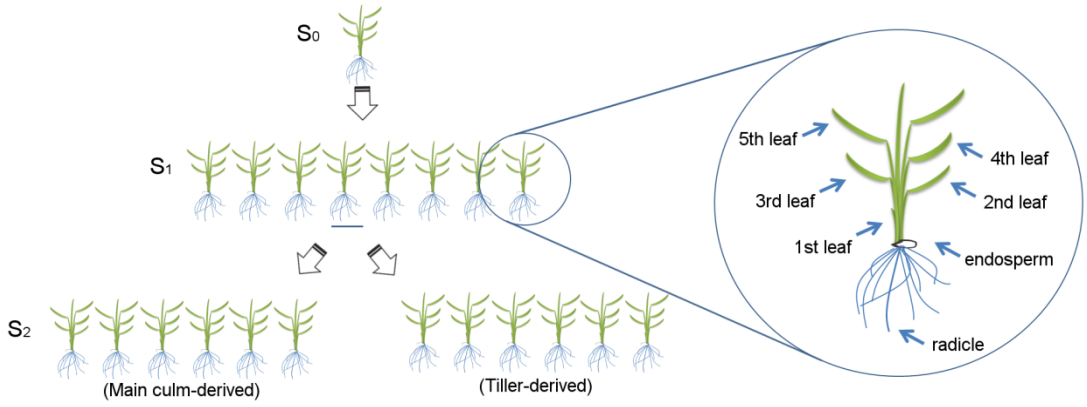

Figure 2-4. Experimental setup for the ontogenical analysis to determine the timing of *mPing* transposition in EG4. Eight progenies (S_1) derived from a single parental EG4 plant (S_0) were grown in a greenhouse. Endosperm, radicle, and leaf blades (1st to 5th) of each S_1 plant were sampled for DNA extraction. S_2 seeds were harvested from the main culm and the primary tiller of each S_1 plant to investigate the inheritance of *de novo mPing* insertions. The 2nd leaf blade of S_0 and S_2 plants was also sampled. Detailed information is provided in the 'Materials and methods'.

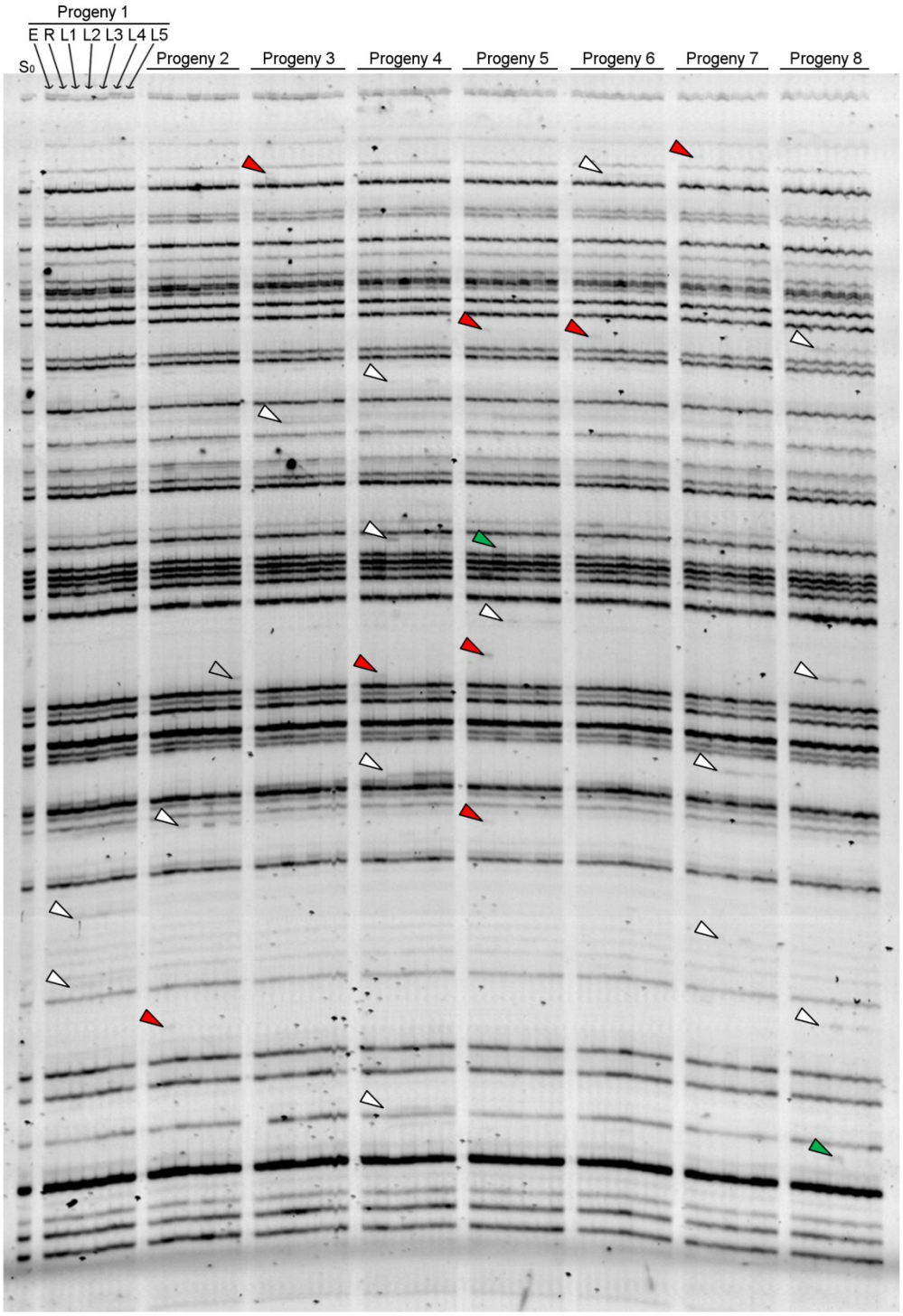

Figure 2-5. Ontogenical analysis of *mPing* transposition in EG4 by transposon display. White, red, and green arrowheads indicate shoot-, radicle-, and leaf-specific insertions, respectively. The rice plant has alternate distichous leaves; therefore, I analyzed the insertion in both [n+1]th and [n+2]th leaves to confirm whether the insertion detected in [n]th leaf is leaf-specific or shoot-specific. But I did not investigate the specificity of the insertions detected in the 4th and 5th leaves using their upper leaves. For this reason, I 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.

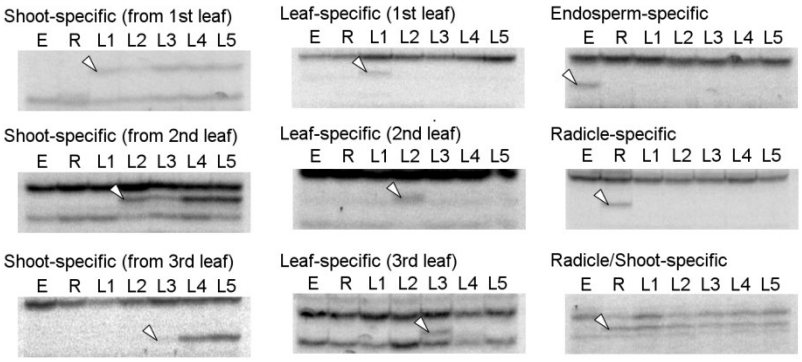

Figure 2-6. *De novo mPing* insertions detected in ontogenical analysis by transposon display. Eight progenies (S_1) derived from a single parental EG4 plant (S_0) were grown in a greenhouse. Endosperm, radicle, and leaf blades (1st to 5th) of each S_1 plant were sampled for DNA extraction. The 2nd leaf blade of S_0 and S_2 plants was also sampled. 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.

Table 2-3. *De novo mPing* insertion sites detected in various tissues of a single EG4 plant.

Inserted locus		Nearest gene			Locus-specific primers		Amplicon Size (bp)	
Chromosome	Position (IRGSP-1.0)	Locus	Position	Distance	Forward primer (5' → 3')	Reverse primer (5' → 3')	*mPing* present	*mPing* absent
6	23,888,837	-	-	>5000	GCGGAAAACGAGAGACAATC	TGAATTGGAGCTGCTAGTTGG	733	300
3	25,286,350	Os03g0651100	5' UTR	-	GCAGGAACTGGAAATTTGGG	CGGAGACGCATTTAATCCAC	769	336
6	26,427,916	Os06g0646700	upstream	86	TAGAGAAGAAGCCGCCAAAG	TACCCGGTCACTGACGTATG	782	349
8	699,354	Os08g0113000	downstream	778	GGCTCCTGACGAAATGAAAG	GCGCACAAAGATAGGTAGGC	745	312
5	18,714,995	Os05g0386400	downstream	392	TGACCTCTTCGTTCAACTGC	CGGGCTAATCTTCCAAACAG	759	326
3	16,194,613	Os03g0399532	upstream	2,816	GACGGACGATTAAAATTGGG	GGCCCACAACTTGAGCTATC	734	301
4	34,590,615	Os04g0677100	upstream	2,383	ATGAATCAGTTGGTTTGGCG	CCCCTTTGTTGTTGATTTGG	778	345
3	9,769,071	Os03g0284100	upstream	382	GTTCTTCCTTCTTCCGGAGC	CGTGGGAATCAAATGAGAGAG	756	323

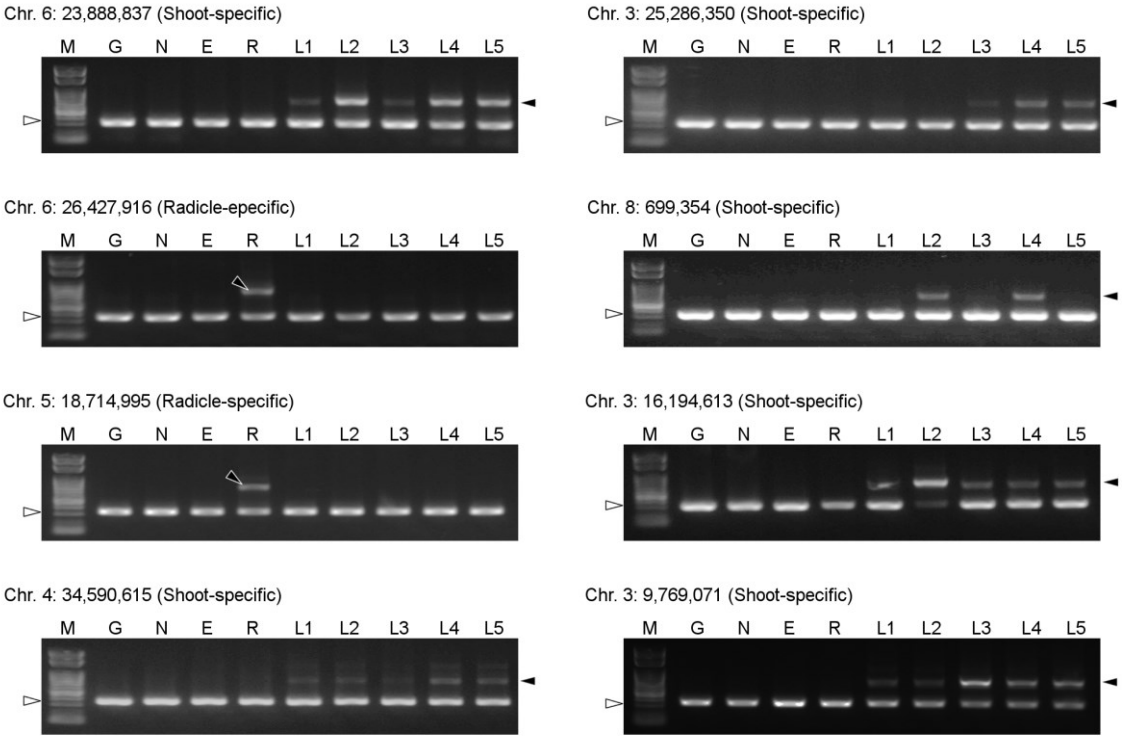

Figure 2-7. Locus-specific PCR analysis of *de novo mPing* insertions in various tissues of a single EG4 plant. Eight representative results are shown. The genomic locations of the *mPing* insertions and insertion types are indicated at the top of the profiles. Lane M: DNA size marker (Gene Ladder 100, 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.

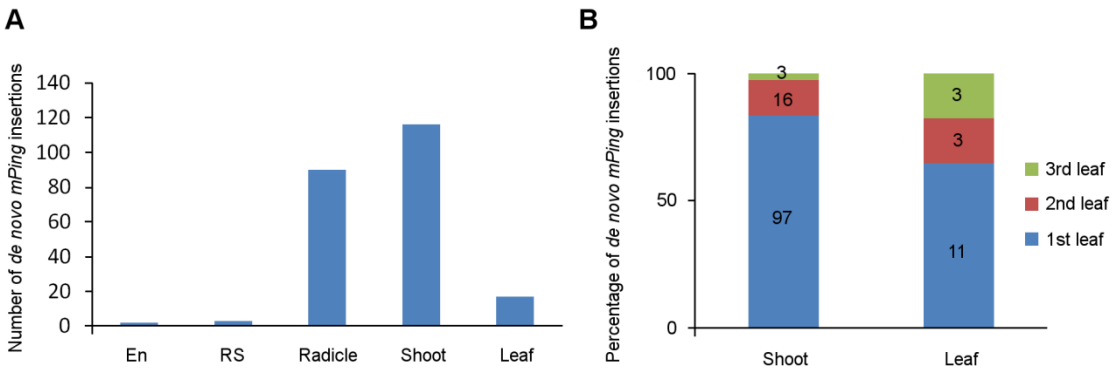

Figure 2-8. *De novo mPing* insertions during rice ontogeny. (A) The number of organ-specific *de novo* insertions in EG4. All 16 possible primer combinations were analyzed. En: endosperm-specific insertion, RS: radicle/shoot-specific insertion, R: radicle-specific insertion, Shoot: shoot-specific insertion, Leaf: leaf-specific insertion. (B) Percentage of leaf positions where the first *de novo mPing* insertion was found. Shoot: shoot-specific insertion, Leaf: leaf-specific insertion.

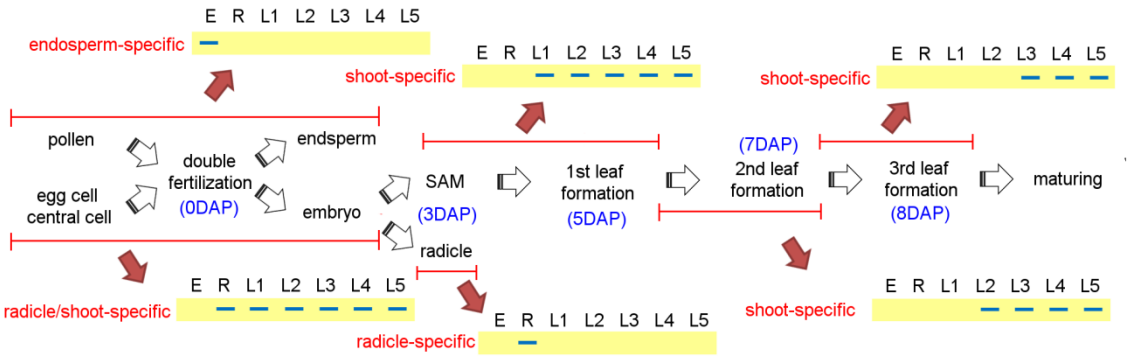

Figure 2-9. Schematic representation of the relationship between banding patterns obtained in transposon display and the timing of *mPing* transposition. If *mPing* transposes in the period indicated by the red bar, the schematic banding patterns indicated by the arrows will be obtained. E: endosperm, R: radicle, L1–L5: 1st to 5th leaf blade, DAP: days after pollination, SAM: shoot apical meristem.

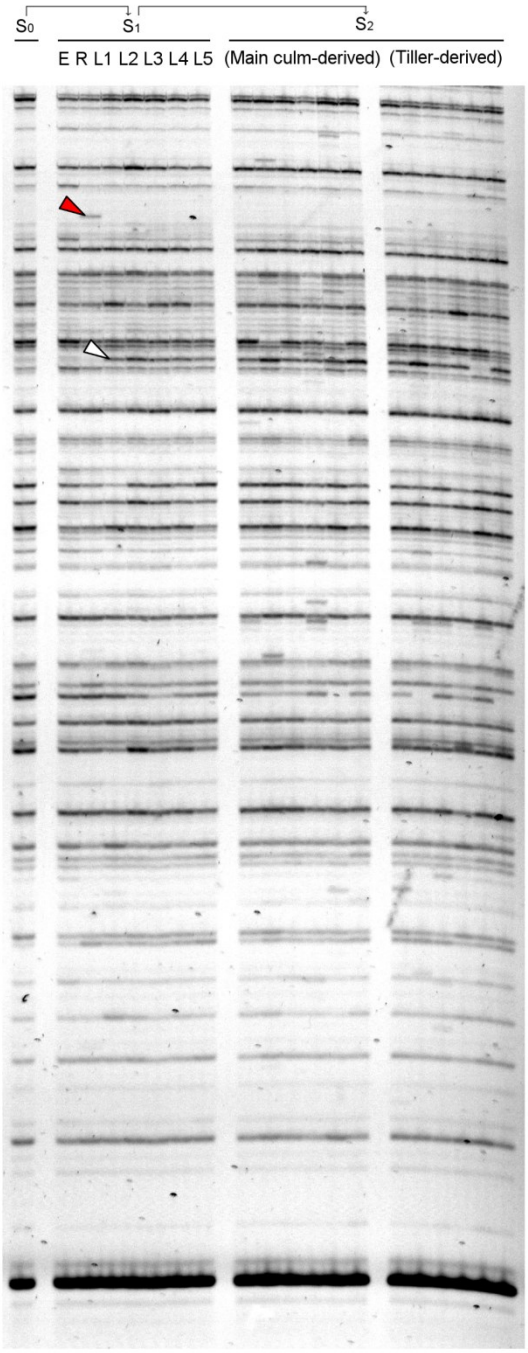

Figure 2-10. Inheritance of *de novo mPing* insertions in EG4. S_2 plants derived from the main culm and the primary tiller of a single S_1 plant were assayed. The shoot-specific insertion in the S_1 plant (white arrowhead) was inherited by S_2 plants, whereas the radicle-specific insertion (red arrowhead) was not. E: endosperm; R: radicle; L1–L5: 1st to 5th leaf.

Table 2-4. *mPing*-SCAR markers used in this study.

			Inserted locus		Amplicon Size (bp)	
Marker name	Forward Primer Sequence (from 5' to 3')	Reverse Primer Sequence (from 5' to 3')	Chromosome	Position (IRGSP-1.0)	*mPing* present	*mPing* absent
MK1_42	TCCCATCCCATTTCCAGTCC	ACCATCTTGCATAGGTCCAC	1	1,132,975	777	344
MK1_75	AGAAGAAGAGGCGCAGATCG	TCCGAATCAAACAAGCCTGC	1	10,987,044	754	321
MK1_103	GAGGTAGTGCTTGGCATTGG	CACAGCAACGATGGTCTCAC	1	30,895,601	670	237
MK1_136	AACAACACTGCACTCGAAGC	TGCATCCAACAGACCAAAGC	1	42,156,581	714	281
MK2_33	GTATCTCCACCCATCCAGGC	TGGAACTAAACTCGGCCTATG	2	223,440	728	295
MK2_63	ACAAACGCGACCAGAAAGTC	CACCTGCAAGGCATGGAAAG	2	13,200,714	799	366
MK2_84	CCCTTCACGATTCAGGAGAAC	CAACCGACCGGATTAAGCAC	2	23,882,557	669	236
MK2_114	TATAGCCCGCTGCTCTTCTC	TTGTGTTTGATGGCAGGTGG	2	32,858,536	678	245
MK3_65	TGCTCTTGTCGTTCACGTTC	GCTCTCAACTCCAACACGTC	3	3,872,618	658	225
MK3_41	GCAGGTCACAGGTTGTGTGA	CCTGTGTTTCTCCTATTCGT	3	11,781,493	735	302
MK3_108	TAACTGCCACCAGGAGTAGC	AAGGAAGAGGTGAGATGGGC	3	24,855,529	671	238
MK3_132	TCAGTTATCCTCTTCCCACTC	ATGGTGGTGGCCGAATAATG	3	31,890,843	719	286
MK4_30	CGTCGATGTGGGCTTGTTTC	GTTGACGATGACGCTGTCC	4	13,285,706	815	382
MK4_40	CTCACTCACTCTCACTGCCG	AGCTAGACAGCCAGACACAC	4	21,600,764	814	381
MK4_61	TATGGAGTGTGGGAATCGGC	CACTTGTAACCGCTGGATGC	4	28,739,872	758	325
MK4_77	GGATGTTGGCTCTTATGCAAG	GTGACTTTGGACTTTCGGGC	4	35,422,419	755	322
MK5_31	AGTGCATAGCTCAAGGCATC	TCTACGGCTCTTCTCTGTGC	5	4,249,078	626	193
MK5_38	CATGTTAATGAGGCCGAGGG	CCACTGTTTCCACAGATCGC	5	14,654,628	709	276
MK5_57	CCGGCTTAGGAACAGGAACC	CACATTGACACCTGGAACCG	5	22,646,609	725	292
MK5_68	ACGTGAATGGTAGGGCAAAC	AATCAGCCAGCATCCAACTG	5	28,151,164	754	321
MK6_29	AAACCCATTTGCTACGCATC	CAGGGAAAGTGTTACCTGGC	6	4,005,008	669	236
MK6_51	TGTCATGGCATCTGCTAAGG	TCTTCAGATTATGGGCTGGG	6	8,316,574	616	183
MK6_74	GTGTTTCGGATGGTGGACAG	TCTTCTCTCCTGCTTGTGGG	6	17,106,339	791	358
MK6_107	TCATGGTTGTGCTGTTGTGC	AAGATTCGTCTCGCGGTTTC	6	30,049,593	770	337
MK7_2	TGCCCTTAGATGGTACTACG	GAGACATCGCTTTGTGTACC	7	3,667,745	559	126
MK7_5	AGGTACAGAAACGCTTGACC	ACCAGTTACACAGCTTAGCC	7	11,852,825	734	301
MK7_45	GCTGCCTTAGCAATCGGTG	CTCAGGTACACGTTTGGTGC	7	22,060,085	668	235
MK7_61	TTCTCCTCCACCTCATCACC	TATCTCTTCCGCTCCGTTCC	7	29,015,072	708	275
MK8_22	TGCATTCACAAGTGGAGTGG	ACATCATAGCCATAGCCGCC	8	458,415	732	299
MK8_38	AGTTAGCCAGTGTTGAATCGC	ACGCACGCATCAGTTTAGTC	8	9,385,127	767	334
MK8_44	GCCATCGGAAGAGAAAGGAG	TTCAACATTCCAACCCATCG	8	19,452,248	668	235
MK8_65	TAGTGGCCTTGCATGTGTTG	GGTCCATGAGCTTGGGTTTG	8	25,881,178	659	226
MK9_16	CCATACCCATCATTCCCATCC	CCACGTAAAGACAAGCGAGG	9	968,867	616	183
MK9_24	TAGGATTCTCTGGGCTCTGC	ACAAGGCTGTATCCGCTGAC	9	7,248,582	664	231
MK9_47	CGTTTGATGTGGCAGGGTG	AGTCGGTGCAATCTTTGTCG	9	15,873,914	747	314
MK9_62	CCTGCAACTGTAACGACGAC	CCCAGCTTTCCGATGAATGG	9	20,845,249	791	358
MK10_3	CTCTTCATTGCACTGCACAA	TTAACCTCGTGTCGGAAGGA	10	4,007,946	604	171
MK10_6	TCTGCTTCCTGCACTGACTT	GATAGTGTGGCCGAGTATAT	10	9,169,250	594	161
MK10_10	GATGATGCTTCTGCATGCAC	AATCGGCAGGCCTGAATTAA	10	13,102,744	604	171
MK10_25	ACCTCGTTCTTGCTCTCAGG	AGTCAAATGGTGGTTCGTGC	10	21,163,118	787	354
MK11_29	GCACCGTTACCAGAAGGTTG	ACGCTTTCGGATACTCATTGG	11	7,503,887	802	369
MK11_38	CACAAACCCAAACATTGCCG	GGTTGGAGCGATGCTAATGG	11	14,670,749	796	363
MK11_47	TGCATGGTATCGTCCTCACC	TCCCTCCAGTCACAAACAGG	11	20,490,425	727	294
MK11_56	AAATGGGCACCAACAAAGGG	ATCTCTTCCTCTTGCTCGCC	11	25,523,876	808	375
MK12_3	CAACATGTAGATGCCAAGGA	ACTGGTTGCAACGATCTTGC	12	3,119,688	711	278
MK12_49	GCAATCCCTCGTCTCTTTCG	CAAGCGAGAACCAAGTCGTC	12	10,612,331	804	371
MK12_77	TGAACTGGTACACTCACAGTC	CAATAAACTCGGGCGGTAGC	12	23,877,483	699	266
MK12_88	CGGCGTTTAGCCCAGTAATC	CTCCAAGCCACCTGCTATATG	12	27,035,708	671	238

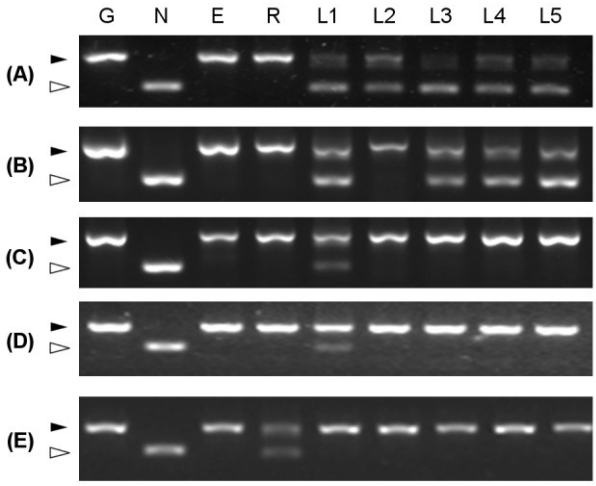

Figure 2-11. *mPing* excisions in EG4. *mPing* excisions were detected by locus-specific PCR using the genomic DNA samples that were used for the ontogenical analysis of the *de novo* insertion. I analyzed 48 loci. Black and white arrowheads show the bands indicating the presence and absence of *mPing*, respectively. Figures indicate (A, B) shoot-specific excisions, (C, D) leaf-specific excisions, and (E) radicle-specific excision. G: EG4 (S_0 plant); N: Nipponbare; E: endosperm; R: radicle; L1–L5: 1st to 5th leaf.

Chapter III. *Ping* expression during embryogenesis in EG4

Introduction

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). The autonomous element *Ping* was identified in the rice genome with *mPing* sequence as a query (Jiang et al. 2003). Sharing 253bp and 177bp of its terminal sequences with *mPing*, *Ping* is thought to be the element that gave rise to the *mPing* transposition (Jiang et al. 2003). Furthermore, the autonomous element *Pong* was also identified in the rice genome by further BLAST searches with *Ping* sequence as a query (Jiang et al. 2003). Indeed, *mPing* transposition was observed under stress conditions in several rice cultivars harboring only *Pong* (Jiang et al. 2003, Lin et al. 2006, Shan et al. 2005). Both *Ping* and *Pong*, which both belong to the *PIF*/*Harbinger* superfamily, have two open reading frames (ORFs). The former encodes a Myb-like DNA-binding protein, and the latter encodes a transposase lacking DNA binding domain. Transposase of most class II elements contains a conserved catalytic domain (DDE motif) and a DNA-binding domain (Hancock et al. 2010, Haren et al. 1999), whereas these domains are encoded separately by two ORFs in both *Ping* and *Pong* (Yang et al. 2007, Hancock et al. 2010). The study of other members of the *PIF*/*Harbinger* superfamily suggested that the Myb-like DNA-binding protein directly binds to the subterminal regions of the transposon in order to recruit the transposase (Sinzelle et al. 2008). Tow heterologous assay systems in Arabidopsis and Yeast revealed that both *Ping* and *Pong* mediate *mPing* transposition and both Myb-like protein and transposase of either *Ping* or *Pong*

or both elements are necessary for *mPing* transposition (Yang et al. 2007, Hancock et al. 2010). In this chapter, I investigated the expression pattern of *Ping* and *Pong* during embryogenesis in EG4.

Materials and methods

Plant materials and sampling

EG4 (cultivar Gimbozu) and Nipponbare were used in this study. 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°C until use.

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 68°C for 10 s. Relative quantification of *Ping*-ORF1 and *Ping*-ORF2 were calculated by the $2^{-\Delta\Delta CT}$ method (Livak et al. 2001) 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.

Paraffin sectioning and in situ hybridization

Plant samples were fixed with 4% (w/v) paraformaldehyde and 1% Triton X in 0.1M sodium phosphate buffer for 48 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 8-μm 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 (Kouchi and Hata 1993).

Results

Expression pattern of Ping in EG4

Both *Ping* and *Pong* provide a Myb-like protein and a transposase, which are encoded by their ORF1 and ORF2, respectively (Figure 3-1), and have been considered as autonomous elements responsible for the *mPing* transposition. I investigated the expression of *Ping*-ORF1, *Ping*-ORF2, *Pong*-ORF1, and *Pong*-ORF2 during embryogenesis to evaluate which autonomous element plays a predominant role in driving the *mPing* transposition in EG4. Reverse transcription-PCR analysis revealed that *Ping*-ORF1 and *Ping*-ORF2 constitutively expressed in the ovary during embryogenesis (Figure 3-2). On the other hand, no transcriptions of *Pong*-ORF1 and *Pong*-ORF2 were observed (Figure 3-2). This strongly suggests that *Ping* predominantly controls the *mPing* transposition in EG4.

The expression level of *Ping*-ORF1 and -ORF2 between EG4 and Nipponbare during embryogenesis was investigated by real-time quantitative PCR (qPCR) analysis. In all developmental stages from 1 to 6 DAP, the expression levels of both *Ping*-ORF1 and -ORF2 were higher in EG4 than in Nipponbare (Figure 3-3A, B). Since EG4 harbors seven copies of *Ping*, whereas Nipponbare has only one copy (Oki et al. 2007), the difference in the expression levels between EG4 and Nipponbare is considered to be attributable to the different copy number of *Ping*. However, it was found that *Ping* of EG4 showed different expression patterns from that of Nipponbare. In Nipponbare, the expression level of *Ping*-ORF1 and -ORF2 gradually declined until 3 DAP, and restored to the basal level at 6 DAP. In contrast, in EG4, the expression levels of both *Ping*-ORF1 and -ORF2 rapidly increased, with a peak at 3 DAP (Figure 3-3A, B). The ratio of relative expression level (EG4/Nipponbare) clearly demonstrated that *Ping* might be up-regulated in a developmental stage-specific manner in the ovary of EG4 (Figure 3-3C). Since *mPing* transposed during the period from 3 to 5 DAP, the rapid increase in *Ping* expression most likely drive the *mPing* transposition.

Accumulation of Ping transcripts in the embryo triggers mPing transposition

The spatial expression pattern of *Ping* was investigated by *in situ* hybridization using *Ping*-specific probes. The probe positions were indicated in Figure 3-1. The *Ping* transcripts were detected in all tissues, *viz.* embryo, endosperm, and ovary wall, in both EG4 and Nipponbare (Figure 3-4A-C, Figure 3-5). Among the tissues, the 3 DAP embryo of EG4 yielded an exceptionally strong signal, indicating a high accumulation of *Ping* transcripts (Figure 3-4A), whereas the 5 DAP embryo showed a much lower accumulation of *Ping* transcripts in EG4 (Figure 3-4D–F). Such a drastic change in

accumulation quantity of *Ping* transcripts with the advance of embryogenesis was consistent with the change in the expression quantity of *Ping* with the advance of embryogenesis, which was investigated by real-time qPCR (Figure 3-3). These results suggest that the tissue- and developmental stage-specific accumulation of the *Ping* transcripts triggers *mPing* transposition at this stage in EG4. To confirm this hypothesis, I evaluated the spatial expression pattern of *Ping* in the SAM during the vegetative phase. As described above, *mPing* hardly transposes in the SAM during this phase. The *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 3-4G–I). Thus the *Ping* transcripts proved to accumulate developmental stage-specifically only in the tissue where *mPing* actively transposes. It was therefore concluded that the high accumulation of *Ping* transcripts triggers the transposition of *mPing* in the 3 DAP embryo of EG4.

Discussion

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* (Yang et al. 2007, Hancock et al. 2010). Furthermore, *mPing* transposition was observed under stress conditions in several rice cultivars harboring only *Pong* (Jiang et al. 2003, Shan et al. 2005, Lin et al. 2006). In this study, however, *Pong* expressed at

very low levels 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, it was hypothesized that the expression level of *Ping* needed to exceed a certain threshold of *mPing* transposition.

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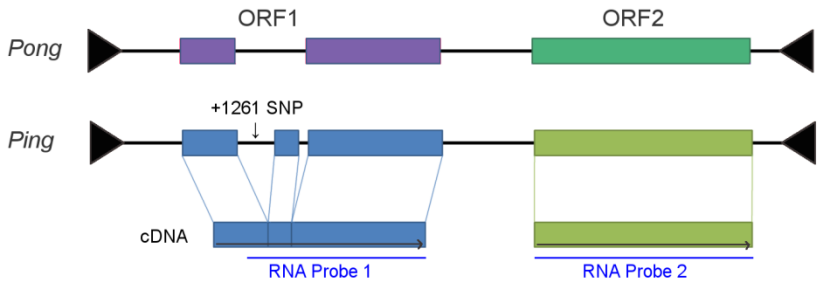

Figure 3-1. Structure of the *Ping* and *Pong* elements. Terminal inverted repeats are indicated by black triangles. Boxes represent ORF1 and ORF2, respectively. Gray horizontal arrows indicate the direction of transcription. RNA probes used are indicated below the ORFs.

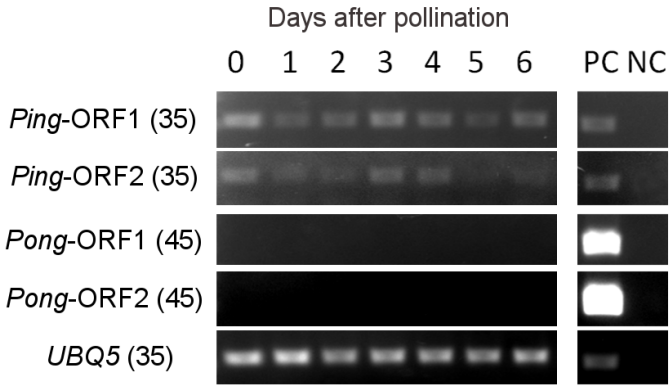

Figure 3-2. Reverse-transcription PCR analysis of *Ping*-ORF1, *Ping*-ORF2, *Pong*-ORF1, and *Pong*-ORF2. Numbers in parentheses are PCR cycle numbers. PC: positive control (0.1 ng genomic DNA), NC: negative control (non-reverse-transcribed RNA).

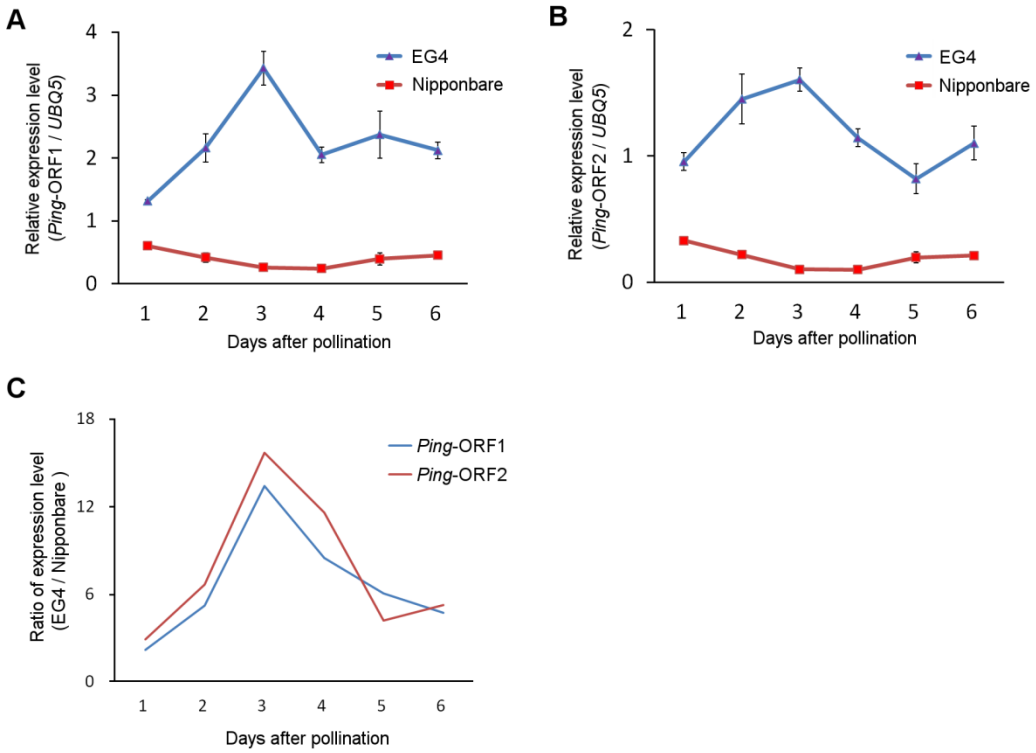

Figure 3-3. *Ping* expression during seed development in EG4. (A) Real-time quantitative PCR of *Ping*-ORF1 and (B) *Ping*-ORF2. The expression level in the Nipponbare ovary just after pollination was set as 1. The results are presented as means of three biological replicates. Bars indicate SE. (C) The ratio of *Ping* expression level of EG4 to that of Nipponbare. The means in (A) and (B) were used for calculation.

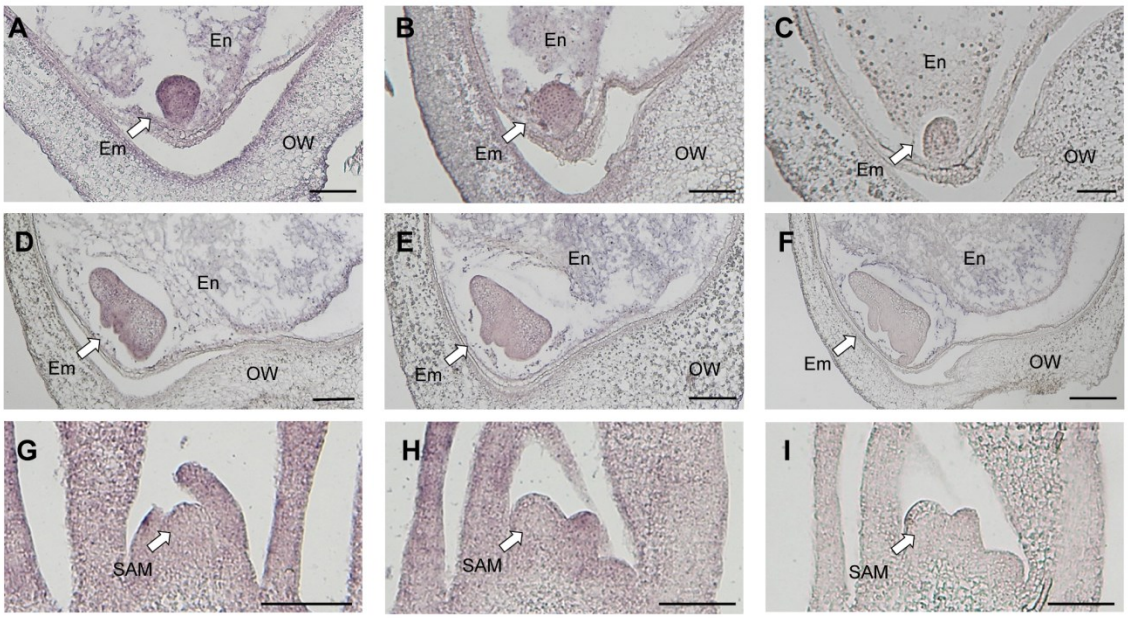

Figure 3-4. Detection of *Ping*-ORF1 spatial expression patterns by *in situ* hybridization analysis. Longitudinal sections through the ovary 3 days after pollination of (A, C) EG4 and (B) Nipponbare; the ovary 5 days after pollination of (D, F) EG4 and (E) Nipponbare; and the shoot apical meristem of (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.

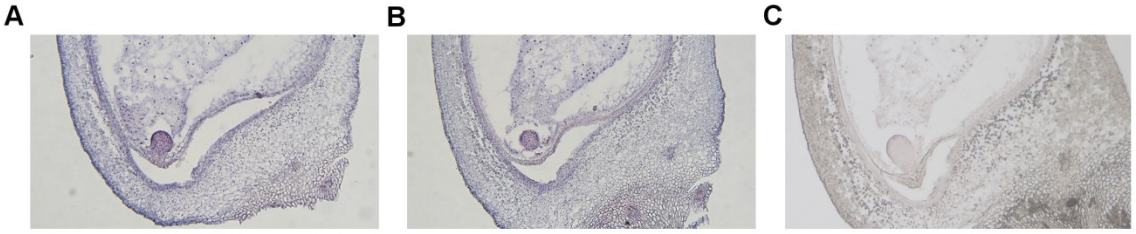

Figure 3-5. Detection of *Ping*-ORF2 spatial expression patterns by *in situ* hybridization analysis. Longitudinal sections through the ovary 3 days after pollination of (A) EG4 and (B, C) Nipponbare were hybridized with (A, B) antisense or (C) sense RNA probes.

Chapter IV. SNP in an intronic region of *Ping*-ORF1

Introduction

EG4 has seven *Ping* elements (*Ping-1* to -*7*), and the *Ping* insertion sites of EG4 have been deployed on 4 chromosomes (Oki et al. 2007). *Ping-1* and *Ping-2* are located on chromosome 1; *Ping-3* on chromosome 3; *Ping-4* on chromosome 7; and *Ping-5*, *Ping-6*, and *Ping-7* on chromosome 9. Nipponbare harbors one *Ping* element (*Ping-N*) on chromosome 6, which was referred from the Rice Annotation Project Database (RAP-DB, Kawahara et al. 2013, Sakai et al. 2013). *Indica* cultivars and some temperate *japonica* cultivars such as Taichung 65 (T65) harbor no *Ping* element.

In addition to EG4, several Aikoku and Gimbozu landraces (hereafter AG strains) are known to exhibit high *mPing* activity (Naito et al. 2006). In the first half of the 20th century, Aikoku and Gimbozu cultivars were popular for Japanese farmers, and used for the breeding of many distinct but closely related strains and landraces. Since AG lines exhibiting high *mPing* activity have not been exposed to mutagenic treatment such as gamma-ray irradiation, these lines have capacity of supporting *mPing* amplification in nature. In this chapter, I focused on the one single nucleotide polymorphism (SNP) between EG4 and Nipponbare in the first intronic region of *Ping*-ORF1 and investigate the effect of SNP on *mPing* activity using 93 AG lines as plant materials.

Materials and methods

Plant materials and sampling

EG4 (cultivar Gimbozu), Nipponbare, T65 (cultivar Taichung 65), and 94 Aikoku/Gimbozu landraces were used in this study. Aikoku/Gimbozu landraces were provided from the GenBank project of the National Institute of Agrobiological Science, Ibaraki, Japan. For DNA extraction, leaf blades were collected. All samples were immediately frozen in liquid nitrogen and stored at –80°C until use.

SNP detection

PCR was performed in 10-µl reaction volumes containing 10 ng of template of DNA, 5 µl 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. Because the original sequence contained an *Afa*I restriction site, one mutation was introduced into the reverse primer. The 5-µl PCR products were mixed with 5 µl restriction mixture containing 1U *Afa* I (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 (Baruch and Kashkush 2012) with little modification. Quantitative PCR was performed using the LightCycler 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 I 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.

Results

SNP in an intronic region of Ping-ORF1

EG4 has seven *Ping* elements (*Ping-1* to *-7*), whereas Nipponbare has only one (*Ping-N*) (Figure 4-1). When all *Ping* elements were sequenced and compared, a SNP in the first intronic region of *Ping*-ORF1 was detected between EG4 and Nipponbare (Figure 4-2). *Ping-N* has a 'T' nucleotide on the SNP region, whereas all *Ping* elements in EG4 have a 'C' nucleotide. I named the former 'T-type *Ping*' and the latter 'C-type *Ping*'.

In addition to EG4, several Aikoku and Gimbozu landraces (hereafter AG strains) are known to exhibit high *mPing* activity (Naito et al. 2006). I investigated the SNP-type of *Ping* and the copy number of *Ping* and *mPing* in 93 AG strains, and evaluated the effect of C-type *Ping* on the *mPing* activity. These 93 AG strains were divided into three groups according to the SNP-type of the *Ping* allele (Table 4-1): strains harboring C-type *Ping*; strains harboring T-type *Ping*; and strains harboring no *Ping*. The strains with C-type *Ping* had more *mPing* copies than those with T-type *Ping*

or no *Ping* (Figure 4-3, Steel–Dwass test, $p < 0.01$). This implies that the C-type *Ping* could drive the *mPing* transposition. I further investigated the expression patterns of *Ping*-ORF1 and -ORF2 in two *mPing*-active strains (A119 and A123) and two *mPing*-inactive strains (A105 and G190) during embryogenesis (from 1 to 6 DAP). A119 and A123 have six and ten copies of C-type *Ping*, respectively, and both A105 and G190 have one copy of T-type *Ping* (Table 4-1). Expression analysis revealed that A105 and G190 kept low expression levels of *Ping*-ORF1 and -ORF2, whereas A119 and A123 showed high expression levels with a peak around 3 DAP (Figure 4-4). This indicates that, in EG4, A119, and A123, the developmental stage-specific expression of *Ping* is controlled by the same factor(s) described in the Discussion.

Discussion

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, I 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 (Kitamura et al. 2001). In Arabidopsis, germinal reversion frequency of *Tag1* increases in proportion to its copy number (Levy et al. 1989). 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 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 (Tokuhiro et al. 2003). Furthermore, a SNP located in the intronic enhancer region of the thyroid hormone receptor β gene enhances pituitary cell-specific transcriptional activity (Alberobello et al. 2011) In this study, it was 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, it was found that the copy number of *mPing* was significantly larger 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 *mPing*, presumably by the developmental stage-specific up-regulation of *Ping* expression.

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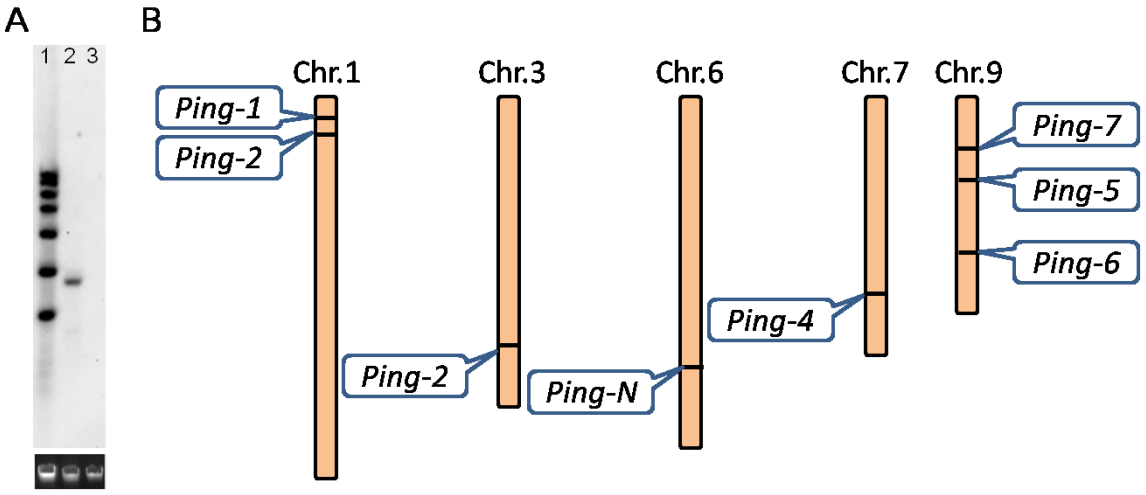

Figure 4-1. *Ping* in EG4 and Nipponbare. (A) Southern blot analysis of *Ping*. The samples of genomic DNA were digested with *Eco*RI restriction enzyme. These samples were loaded onto an agarose gel, separated by electrophoresis, blotted onto a nylon membrane, and probed with the *Ping* fragment. The bottom image shows the loaded genomic DNA before digestion. 1: EG4, 2: Nipponbare, 3: T65. EG4 and Nipponbare respectively harbor 7 and 1 *Ping* elements. T65 harbors no *Ping* elements. (B) The genomic locations of *Ping*-inserted sites. EG4 has seven *Ping* elements (*Ping-1* to *-7*), whereas Nipponbare has only one (*Ping-N*).

Table 4-1. Plant materials used in this study. * +1261 SNP, C: the strains harboring C-type *Ping*, T: the strains harboring T-type *Ping*, -:the strains harboring no *Ping*. ** This strain may be outcrossed. *** *Ping-1* in EG4 is inserted into *Pyong* element which is absent at this locus in Nipponbare. A: Both *Ping-1* and *Pyong* are absent, B: Only *Pyong* is present, C: *Ping-1* is inserted into *Pyong* element.

												Estimated copy number	
ID	JP Number	Cultivar name	+1261 SNP*	*Ping-N*	*Ping-1***	*Ping-2*	*Ping-3*	*Ping-4*	*Ping-5*	*Ping-6*	*Ping-7*	*Ping*	*mPing*
EG4	-	Gimbozu	C	-	C	+	+	+	+	+	+	7	553
Nipponabre	-	Nipponbare	T	+	A	-	-	-	-	-	-	1	51
A101	4510	RIKU AIKOKU	C	-	A	-	-	-	-	-	-	1	9
A102	4713	RIKU AIKOKU	C	-	A	-	-	-	-	-	-	1	8
A103	4774	HAKUBOU AIKOKU	C	-	A	-	-	-	-	-	-	N. D.	20
A104	4982	AIKOKU	C	-	A	-	-	-	-	-	-	1	19
A105	5247	A AIKOKU 2	T	+	B	-	-	-	-	-	-	1	24
A106	5419	AIKOKU	-	-	B	-	-	-	-	-	-	0	18
A107	5777	GOKUWASEAIKOKU	C	-	B/C	-	-	-	-	-	-	1	73
A108	5792	AIKOKUDAIKOKU	C	-	C	-	-	-	-	-	-	2	38
A109	5793	AIKOKUDAIKOKUMOCHI A	-	-	A	-	-	-	-	-	-	0	23
A110	5794	AIKOKUDAIKOKUMOCHI B	-	-	A	-	-	-	-	-	-	0	12
A111	5795	AIKOKUTAREHATOU	C	-	C	-	-	-	-	-	-	2	64
A112	5796	AIKOKUWAITOU	C	-	C	-	-	-	-	-	-	3	243
A113	5810	AIKOKUMITSURYUUTOU	C	-	C	-	-	-	-	-	-	2	277
A114	5998	NAKATEMUBOUAIKOKU	C	-	C	-	-	-	-	-	-	1	70
A115	5999	NAKATEAIKOKU 3	C	-	C	-	-	-	-	-	-	2	110
A116	6044	WAITOUAIKOKU	C	-	C	-	-	-	-	-	-	1	98
A117	6047	AIKOKUTAREHATOU	C	-	C	-	-	-	-	-	-	2	113
A118	6133	BOUZUAIKOKU	C	-	C	-	-	-	-	-	-	1	68
A119	6158	RIKUUAIKOKU 20	C	-	C	-	-	-	-	-	-	6	617
A120	6365	AIKOKU	C	-	C	-	-	-	-	-	-	1	146
A121	6369	AIKOKU	C	-	C	-	-	-	-	-	-	1	83
A122	6442	AIKOKU 70	C	-	C	-	-	-	-	-	-	1	50
A123	6730	AIKOKU	C	-	C	-	-	-	-	-	-	10	372
A124	6744	NAKATEAIKOKU	C	-	C	-	-	-	-	-	-	1	62
A125	6752	NAKATESHINAIKOKU	T	+	B	-	-	-	-	-	-	1	19
A126	6753	WASEAIKOKU	-	-	B	-	-	-	-	-	-	0	19
A127	6757	AIKOKU 1	-	-	A	-	-	-	-	-	-	0	21
A128	6768	AIKOKU	C	-	C	-	-	-	-	-	-	1	14
A129	6771	AIKOKUIBARAKI 1	C	-	C	-	-	-	-	-	-	N. D.	100
A130	6822	AIKOKUIBARAKI 2	C	-	C	-	-	-	-	-	-	2	186

Table 4-1. (continued)

ID	JP Number	Cultivar name	+1261 SNP*	*Ping-N*	*Ping-1***	*Ping-2*	*Ping-3*	*Ping-4*	*Ping-5*	*Ping-6*	*Ping-7*	Estimated copy number	
												Ping	*mPing*
A131	6915	AKITAWASEAIKOKU 5	T	+	B	-	-	-	-	-	-	1	20
A132	6927	WASEAIKOKU	C	-	C	-	-	-	-	-	-	1	43
A133	6928	WASEAIKOKU 2	C	-	C	-	-	-	-	-	-	1	12
A134	7013	AIKOKUHO	C	-	C	-	-	-	-	-	-	4	81
A135	7053	KAIRYOUAIKOKU 17	-	-	B	-	-	-	-	-	-	0	55
A136	7054	WASEAIKOKU 96	T	+	B	-	-	-	-	-	-	1	20
A137	7055	NAKATEAIKOKU 90	C	-	C	-	-	-	-	-	-	1	49
A138	7058	TOUKYOUMUBOUAIKOKU	C	-	C	-	-	-	-	-	-	1	65
A139	7084	AIKOKU 3	C	-	C	-	-	-	-	-	-	1	118
A140	7085	AIKOKUSAI 1	C	-	C	-	-	-	-	-	-	1	31
A141	7095	AIKOKU 20	C	-	C	-	-	-	-	-	-	2	140
A142	7096	AIKOKU 6	C	-	C	-	-	-	-	-	-	1	40
A143	7097	WASEAIKOKU 30	C	-	C	-	-	-	-	-	-	1	45
A144	7189	SHIZUOKAAIKOKU 1	C	-	C	-	-	-	-	-	-	1	23
A145	7198	AIKOKU 5	C	-	C	-	-	-	-	-	-	2	137
A146	7228	A AIKOKU 1	T	+	A	-	-	-	-	-	-	1	27
A147	7332	AIKOKU 3	C	-	C	-	-	-	-	-	-	1	116
A148	7556	AIKOKU A	-	-	B	-	-	-	-	-	-	0	30
A149	7560	CHOUBOUAIKOKU	-	-	B	-	-	-	-	-	-	0	32
A150	7576	SHINKANE/AIKOKU	T	+	B	-	-	-	-	-	-	1	18
A151	7621	KAIRYOUAIKOKUDAIKOKUGATA 1	-	-	B	-	-	-	-	-	-	0	41
A152	7622	KAIRYOUAIKOKUDAIKOKUGATA 2	-	-	B	-	-	-	-	-	-	0	29
A153	9192	BANAIKOKU	-	-	B	-	-	-	-	-	-	0	22
A154	9277	AIKOKU 68	C	-	C	-	-	-	-	-	-	2	21
A155	9285	AIKOKU 1(KYOUTO)	C	-	C	-	-	-	-	-	-	2	82
A156	9310	AIKOKU	-	-	B	-	-	-	-	-	-	0	24
A157	9412	AIKOKUMOCHI	C/T**	+	A	-	-	-	-	-	-	2	119
A158	9513	AIKOKU B	C	-	C	-	-	-	-	-	-	1	69
A159	9514	AIKOKU C	C	-	C	-	-	-	-	-	-	1	15
A160	9515	AIKOKU D	C	-	N.D.	-	-	-	-	-	-	5	61
A161	9516	AIKOKU F	T	+	B	-	-	-	-	-	-	1	44
A162	9517	AIKOKU G	-	-	B	-	-	-	-	-	-	0	17

Table 4-1. (continued)

ID	JP Number	Cultivar name	+1261 SNP*	*Ping-N*	*Ping-1***	*Ping-2*	*Ping-3*	*Ping-4*	*Ping-5*	*Ping-6*	*Ping-7*	Estimated copy number	
												Ping	*mPing*
A163	9650	AIKOKU	C	-	C	-	-	-	-	-	-	1	41
A164	9823	AIKOKU 1	C	-	C	-	-	-	-	-	-	1	67
A165	9824	AIKOKU 2	C	-	C	-	-	-	-	-	-	1	76
A166	10993	AIKOKU	C	-	C	-	-	-	-	-	-	2	65
A167	10994	AIKOKU(4X)	-	-	B	-	-	-	-	-	-	0	12
A168	14931	MUBOUAIKOKU	C	-	C	-	-	-	-	-	-	N. D.	42
A169	15007	KAIRYOUAIKOKU	-	-	B	-	-	-	-	-	-	0	41
A170	15051	WASEAIKOKU 3	C	-	C	-	-	-	-	-	-	1	35
A171	37731	W (WASEAIKOKU)	C	-	C	-	-	-	-	-	-	2	51
G172	6339	SHINGINBOUZU	-	-	B	-	-	-	-	-	-	0	34
G173	6356	GINBOUZU	T	+	A	-	-	-	-	-	-	1	40
G174	6439	GINBOUZUBANSEI	C	-	C	-	-	-	-	-	-	3	111
G175	6440	GINBOUZUCHUUSEI	C	-	C	-	-	-	-	-	-	1	40
G176	6441	WASEGINBOUZU	C	-	C	-	-	-	-	-	-	1	28
G177	6453	GINBOUZU 38(BAN)	C	-	C	-	-	-	-	-	-	1	48
G178	6627	TANGINBOUZU(1)(DS-1439)	C	-	C	-	-	-	-	-	-	1	50
G179	6628	TANGINBOUZU(2)(DS-1440)	C	-	C	-	-	-	-	-	-	1	54
G180	6629	TANGINBOUZU(3)(DS-1441)	-	-	B	-	-	-	-	-	-	0	13
G181	6750	GINBOUZUMIDASHI	C	-	C	-	-	-	-	-	-	1	39
G182	7100	FUKUIGINBOUZU	C	-	C	-	-	-	-	-	-	1	39
G183	7106	GINBOUZU 151	C	-	C	-	-	-	-	-	-	1	48
G184	7230	GINBOUZU 88	C	-	C	-	-	-	-	-	-	1	30
G185	9585	GINBOUZU 1	C	-	C	-	-	-	-	-	-	2	59
G186	9885	WASEGINBOUZU	C	-	C	-	-	-	-	-	-	1	28
G187	9900	GINBOUZU(MAJIRI)	T	+	B	-	-	-	-	-	-	1	33
G188	9955	GINBOUZU(MAJIRI)/0631	T	+	A	-	-	-	-	-	-	1	36
G189	9956	GINBOUZU(MAJIRI)/0632	T	+	B	-	-	-	-	-	-	1	36
G190	9957	GINBOUZU(MAJIRI)/0633	T	+	B	-	-	-	-	-	-	1	36
G191	9958	GINBOUZU(MAJIRI)/0634	T	+	C	-	-	-	-	-	-	1	45
G192	9959	GINBOUZU(MAJIRI)/0635	-	-	C	-	-	-	-	-	-	0	30
G193	15044	TANGINBOUZU	C	-	C	-	-	-	-	-	-	1	35
G194	34032	GINBOZU	-	-	B	-	-	-	-	-	-	0	24

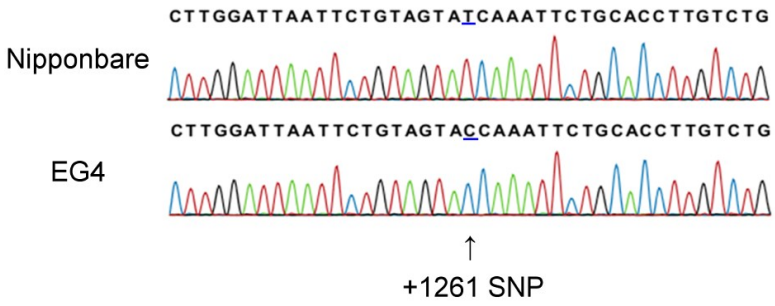

Figure 4-2. Determination of the SNP sequence in the first intronic region of *Ping*-ORF1. EG4 has 7 *Ping* elements and Nipponbare has 1 element. When I sequenced and compared all *Ping* elements, a single nucleotide polymorphism (SNP) in the first intronic region of *Ping*-ORF1 was detected between EG4 and Nipponbare. 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.

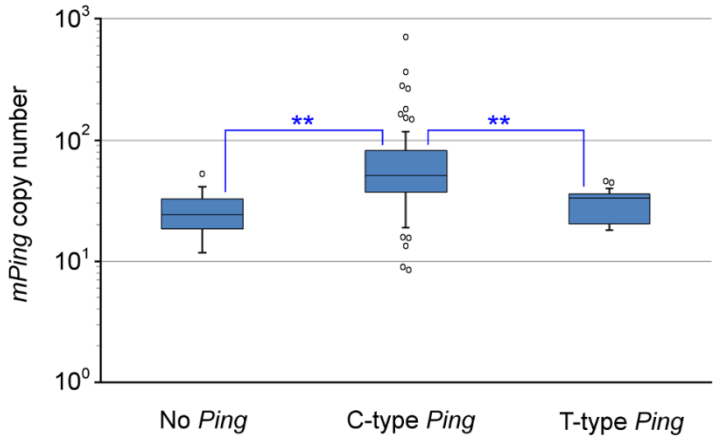

Figure 4-3. Box plots of *mPing* copy number in AG lines. The top and bottom of the boxes mark the first and third quartiles, respectively. The center line represents the median, and the whiskers show the range of observed values within 1.5 times the interquartile range from the hinges. Values beyond 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.

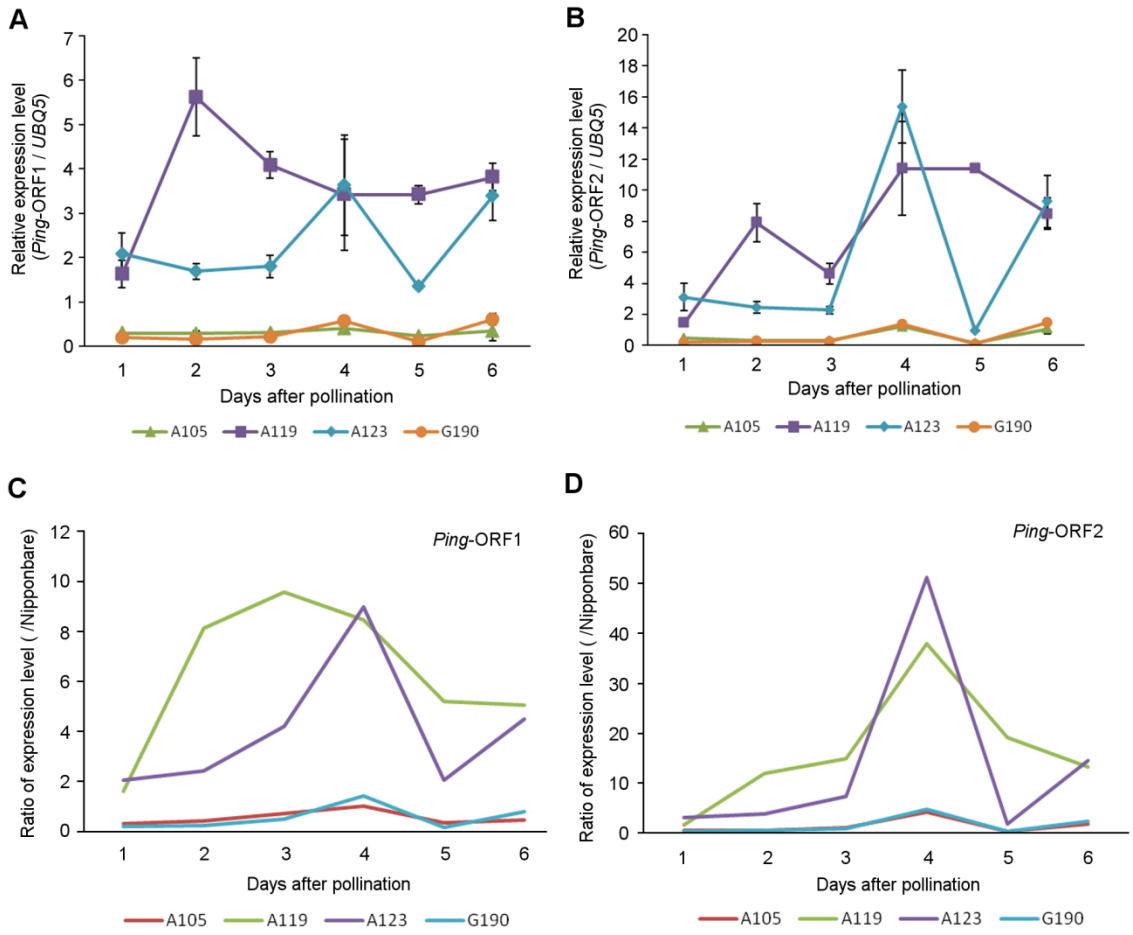

Figure 4-4. *Ping* expression in AG lines. Expression of (A) *Ping*-ORF1 and (B) *Ping*-ORF2 during embryogenesis in *mPing*-active strains (A119 and A123) and *mPing*-inactive strains (A105 and G190). The results are presented as means of three biological replicates. Bars indicate SE. The ratio of (C) *Ping*-ORF1 and (D) -ORF2 expression level of A105, A119, A123, and G190 to that of Nipponbare.

Chapter V. General conclusion

Our laboratory is trying to elucidate how MITEs attain their high copy numbers in the genome. To this end, I chose *mPing*, which is the only active MITE identified in rice, as a material and analyzed the timing of *mPing* transposition in the *mPing*-active strain EG4. Consequently, I successfully found one mechanism of the *mPing* amplification; *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*.

Since the transposition of TEs 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 *mPing* is transiently induced by various stresses (Jiang et al. 2003, Kikuchi et al. 2003, Lin et al. 2006, Nakazaki et al. 2003, Yang et al. 2012), indicating that the activity of *mPing* is suppressed by the silencing mechanisms in many cultivars. Thus, *mPing* must overcome the silencing mechanism in order to maintain the transposition activity under natural growth conditions. This study revealed that *mPing* in EG4 was mobilized by the sufficient supply of *Ping* transcripts produced only during the period of *mPing* transposition. This stage-specific activation is thought to be a strategy of the *mPing* family to amplify *mPing* 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 *mPing*

amplifies. Given that the other active MITEs are identified, however, my study will help to understand their amplification mechanisms. Our previous study documented the generation of new regulatory networks by a subset of *mPing* insertions that render adjacent genes stress inducible (Naito et al. 2009). In addition to *mPing*, other MITEs also contribute to gene and genome evolution via providing new promoter regulatory sequences, transcriptional termination elements, and new alternative exons (Guermonprez et al. 2012), suggesting that the amplification of MITEs causes gene and genome evolution. This study provides clues to further understand not only the amplification mechanism of MITEs but also the co-evolution of MITEs and the host genome.

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Summary

Chapter I. General introduction

Miniature inverted-repeat transposable elements (MITEs) are numerically predominant transposable elements in the rice genome. Because MITEs are mainly deployed in gene-rich regions and affect adjacent gene expression, they are considered to play an important role in genome evolution. However, little is known about how MITEs attain high copy numbers. The rice MITE *mPing* is quiescent in most cultivars under natural growth conditions. However, 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, I provide important findings regarding the transposition and amplification of *mPing* in EG4.

Chapter II. The timing of *mPing* transposition

Plants have acquired the silencing mechanism of transposable elements (TEs) in germ cells. To verify if *mPing* transposes in germ cells in EG4, I developed two F_1 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. If *mPing* was specifically activated in the pollen of EG4, *de novo* insertions could be obtained only in the F_1 plants from the Nipponbare/EG4 cross. However, *de novo* insertions were obtained in both Nipponbare/EG4 and EG4/Nipponbare populations. Moreover, there was no significant

difference in the number of *de novo* insertions per plant between the two F_1 populations. This indicates that the activating factor(s) for the *mPing* transposition is present in both male and female gametes of EG4. To verify if *mPing* transposes in somatic cells in EG4, I performed TD analysis of *mPing* using genomic DNA samples extracted from endosperm, radicle, and leaf blades of eight progenies derived from a single parental EG4 plant, and investigated the *mPing* transposition during ontogeny of rice plants. TD analyses 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. Furthermore, locus-specific PCR showed that *mPing* excisions and insertions arose at the same time (3 to 5 DAP).

Chapter III. *Ping* expression during embryogenesis in EG4

Both *Ping* and *Pong* provide a Myb-like protein and a transposase, which are encoded by their ORF1 and ORF2, respectively, and have been considered as autonomous elements responsible for the *mPing* transposition. I investigated the expression of *Ping*-ORF1, *Ping*-ORF2, *Pong*-ORF1, and *Pong*-ORF2 during embryogenesis to evaluate which autonomous element plays a predominant role in driving the *mPing* transposition in EG4. Reverse transcription-PCR analysis revealed that *Ping* predominantly controls the *mPing* transposition in EG4. Furthermore, real-time quantitative PCR analysis and *in situ* hybridization analysis revealed that the expression levels of both *Ping*-ORF1 and -ORF2 rapidly increased at embryo in EG4, with a peak at 3 DAP. Since *mPing* transposed during the period from 3 to 5 DAP, the rapid increase in *Ping* expression most likely drive the *mPing* transposition.

Chapter IV. SNP in an intronic region of *Ping* -ORF1

EG4 has seven *Ping* elements, whereas Nipponbare has only one. When I sequenced and compared all *Ping* elements, a single nucleotide polymorphism (SNP) in the first intronic region of *Ping*-ORF1 was detected between EG4 and Nipponbare. *Ping* in Nipponbare has a 'T' nucleotide on the SNP region, whereas all *Ping* elements in EG4 have a 'C' nucleotide. I named the former 'T-type *Ping*' and the latter 'C-type *Ping*.' In addition to EG4, several Aikoku and Gimbozu landraces (hereafter AG strains) are known to exhibit high *mPing* activity. I investigated the SNP-type of *Ping* and the copy number of *Ping* and *mPing* in 93 AG strains, and evaluated the effect of C-type *Ping* on the *mPing* activity. Consequently, it was found that the strains with C-type *Ping* had more *mPing* copies than those with T-type *Ping* or no *Ping* (Steel–Dwass test, $p < 0.01$). This implies that the C-type *Ping* could drive the *mPing* transposition.

Chapter V. General conclusion

I successfully found one mechanism of the *mPing* amplification; *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*. In addition to *mPing*, other MITEs also contribute to gene and genome evolution via providing new promoter regulatory sequences, transcriptional termination elements, and new alternative exons, suggesting that the amplification of MITEs causes gene and genome evolution. This study therefore provides clues to further understand not only the amplification mechanism of MITEs but also the co-evolution of MITEs and the host genome.

Acknowledgements

I would like to express my heartfelt gratitude to Dr. Yutaka Okumoto, Dr. Takatoshi Tanisaka, Dr. Takuji Tsukiyama, and all staffs and students in the laboratory of breeding science in Kyoto University for the achievement of my study.

I also would like to express my sincere gratitude to Dr. Hiroshi Hasegawa (The University of Shiga Prefecture) and Dr. Yasuo Nagato (The University of Tokyo). Dr. Hasegawa has invited me to the party for the fellowship between Kyoto University and The University of Shiga Prefecture for 7 years. His many invitations have made me happy and have given me the power for my study. Dr. Nagato has helped my work. He has provided the experimental techniques of *in situ* hybridization analysis. Discussion on the results of *in situ* hybridization analysis with him has been very helpful to my study.

Finally, I also would like to express my sincere gratitude to my family for their love, understanding, encouragement, and support of my study life.

Shota Teramoto

List of publications

Shota Teramoto, Takuji Tsukiyama, Yutaka Okumoto, Takatoshi Tanisaka (2014) Early Embryogenesis-specific Expression of the Rice Transposon *Ping* Enhances Amplification of the MITE *mPing*. PLoS Genet. (in press)

Related papers

Takuji Tsukiyama, Shota Teramoto, Kanako Yasuda, Akira Horibata, Nanako Mori, et al. (2013) Loss-of-function of a ubiquitin-related modifier promotes the mobilization of the active MITE *mPing*. Mol Plant 6: 790-801.

Shota Teramoto, Takuji Tsukiyama, Masayoshi Teraishi, Takatoshi Tanisaka and Yutaka Okumoto (2011) High-fidelity Transposon Display for Rice Active Transposon *mPing*. J Crop Res 56: 63-66.