Note

Distorted genetic segregation of the transposon *mPing* at the long arm of chromosome 12 in rice

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A class II transposable element, *mPing* exists in the rice genome ubiquitously and can transpose even in ordinary cultivation conditions. A copy of *mPing* was identified at the long arm of chromosome 12. In reciprocal backcrossed F_1s between a heterozygote and a homozygote without *mPing*, the male gametes with this *mPing* from heterozygotes were transmitted to the next generation at a lower frequency than those without *mPing*, resulting in distorted genetic segregation in self-fertilized progenies, as well as in F_1s after backcrossing. Pollens with *mPing* tended to germinate on stigma less than those without *mPing*. These results, however, could not explain the lower transmission of male gametes with *mPing*. In addition, no excision of *mPing* was observed in a homozygote. Thus, it was suggested that male gametes with *mPing* were eliminated partly from pollination to fertilization by negative competition against male gametes without *mPing*. Less formation of microspores with *mPing* in meiosis could also be a cause for the distorted segregation, although this could not be examined. At least two ORFs, whose functions have not been identified, are located near this *mPing*. It is plausible that either of these ORFs or both are necessary for the normal functioning of male gametes.

Key Words: male gamete, *mPing*, pollen competition, rice, transposable element.

Introduction

A class II transposable element (TE), *mPing* (*miniature-Ping*) is indigenous to rice (*Oryza sativa* L.) (Jiang *et al.* 2003, Kikuchi *et al.* 2003, Nakazaki *et al.* 2003). With a length of 430 bp and 15-bp terminal inverted repeats, *mPing* makes a specific target site duplication of TAA and/or TTA, and is a non-autonomous TE whose transposition is induced by transposase derived from two related autonomous TEs, *Ping* and *Pong* (Yan *et al.* 2007). The copy number of *mPing* in the rice genome ranges widely from several tens to several hundreds depending on genotype. It is mobilized by tissue culture (Jiang *et al.* 2003, Kikuchi *et al.* 2003), gamma-ray irradiation (Nakazaki *et al.* 2003), inter-specific (Yasuda *et al.* 2013) and inter-generic hybridization (Shan *et al.* 2005), and other stress-inducing treatments (Lin *et al.* 2006). The most pronounced feature of *mPing* is that it is still active in its transposition even under ordinary cultivation conditions, particularly in the genetic background of the *japonica* cultivar 'Gimbozu' (Nakazaki *et al.* 2003).

We have identified several copies of *mPing* which are positioned at different sites in the genome of rice experimental lines derived from 'Gimbozu' (Kakikubo, unpublished data). One of the *mPing* copies positioned near the terminus of the long arm of chromosome 12 {chr12: 24,595,104; Rice Annotation Project-Database (RAP-DB), IRGSP build 5} in a heterozygous state showed strongly distorted segregation in the next generation after self-fertilization, in which homozygotes with *mPing* were statistically fewer than the expected one-quarter of the Mendelian ratio (**Table 1**). It has been suggested that the insertion of *mPing* at this site could influence the expression of genes near the insertion site, particularly in either male or female gametes, resulting in uneven formation between gametes with and without this *mPing* copy. If this assumption is correct, we may be able to identify gene functioning in gametophyte formation in rice. The objective of this study was to investigate the causes of the distorted genetic segregation of *mPing* through genetic analyses, including reciprocal crosses.

Materials and Methods

Plant Materials

In 2005, 2006, and 2008, six segregating populations derived from heterozygotes for different *mPing* copies positioned at different sites in the rice genome were grown (**Table 1**). All plants in these populations were genotyped by the method described below. Segregating ratios were

Table 1. Segregations of the genotypes for *mPing* insertion/non-insertion in six populations derived from heterozygotes showing different insertion positions of *mPing* in the rice genome

Population[a]	Genotype[c]					
	Chromosome	Position[b] (nt)	Hom-n	Het	Hom-i	χ^2 (1:2:1)	P
2008-MLA005	2	19,230,396	25	45	27	0.59	0.745
2008-MLA019	3	13,027,117	24	57	19	2.46	0.292
2008-MLA009	4	31,426,321	25	40	29	2.43	0.297
2008-MLA017	6	7,690,783	30	41	28	3.00	0.223
2006-MLR004	11	24,682,793	25	48	20	0.63	0.728
2005-MLR011	12	24,595,104	24	27	3	16.33	<0.001

[a] The first four digits indicate the year of cultivation for the respective populations.
[b] Referred to RAP-DB, IRGSP build 5.
[c] Hom-n, Het, and Hom-i indicate non-insertion homozygote, heterozygote, and insertion homozygote, respectively, for *mPing* at each position. nt, nucleotide.

tested for goodness of fit to an ordinary Mendelian ratio of 1:2:1 for non-insertion homozygote of *mPing* (Hom-n), heterozygote (Het), and insertion homozygote (Hom-i), respectively.

In 2008, a pair of reciprocal crosses was made between a Het-a (one of the Het) and a Hom-n, both of which were derived from the same original heterozygote, 2005-MLR11-24, in the segregating population of 2005-MLR11. The self-fertilized progenies (S_1) of Het-a, Hom-n, Hom-i, and the F_1s of their reciprocal crosses were grown in 2009. In addition, S_1 of another heterozygote (Het-b) were grown in the same conditions as Het-a, Hom-n, and the F_1s as controls. In 2010, S_1 from two other heterozygotes, Het-c and Het-d, each of which was a segregant in the populations derived from Het-a and Het-b, respectively, in 2009, were grown to confirm the segregation pattern. Hom-n and Hom-i were also grown to examine pollen viability as described below. All of these materials were cultivated in a paddy field of the Faculty of Biology-Oriented Science and Technology, Kinki University, Kinokawa, Wakayama, Japan, at a density of 30-cm inter-row and 15-cm inter-hill with a single plant per hill. A total of 6 g m^{-2} each for nitrogen, phosphate, and potassium were applied as chemical fertilizers at the time of transplanting. Usual cultivation practices were conducted throughout all growing stages. A large number of the S_1 seedlings from a Hom-i were also grown in greenhouse conditions in 2012, in order to confirm the segregation pattern. The numbers of plants of each line used in the analyses are listed in **Table 2**.

Genotyping of the insertion/non-insertion of mPing

For all materials listed above, total DNA was extracted individually from leaf blades at the tillering stage (except in 2012) or seedling stage (in 2012) in accordance with the CTAB method of Doyle and Doyle (1987). Polymerase chain reaction (PCR) was performed with the extracted DNA as a template (10 ng per tube) using a KOD FX Polymerase Kit (TOYOBO, Co. Ltd., Japan), in accordance with the manufacturer's instructions. PCR cycling reactions consisted of one cycle of 94°C for 2 min, 30 cycles of 98°C for 10 sec, appropriate annealing temperature for each primer

Table 2. Segregations of the genotypes for *mPing* insertion/non-insertion at the long arm of rice chromosome 12 in several populations

Population	Genotype[a]				
	Hom-n	Het	Hom-i	χ^2	P
2009					
F_1 of Het-a (♀)/ Hom-n (♂)	19 (17.5)	16 (17.5)	0 (0.0)	0.26	0.612
F_1 of Hom-n (♀)/ Het-a (♂)	43 (25.0)	7 (25.0)	0 (0.0)	25.92	<0.001
S_1 of Het-a	15 (12.5)	31 (25.0)	4 (12.5)	7.72	0.021
S_1 of Het-b	19 (12.5)	26 (25.0)	5 (12.5)	7.92	0.019
S_1 of Hom-n	5	0	0		
S_1 of Hom-i	0	0	5		
2010					
S_1 of Het-c	26 (11.5)	17 (23.0)	3 (11.5)	26.13	<0.001
S_1 of Het-d	13 (8.25)	18 (16.5)	2 (8.25)	7.61	0.022
2012					
S_1 of Hom-i	0	0	160		

The values in parenthesis are expected values from ordinary Mendelian segregation ratios.

[a] Hom-n, Het, and Hom-i indicate non-insertion homozygote, heterozygote, and insertion homozygote, respectively, for *mPing* at chromosome 12. Het-a, Het-b, Het-c, and Het-d are different heterozygotes derived from the original segregating population (2005-MLR011) for *mPing*.

set for 30 sec, and 68°C for 1 min, followed by one cycle of 68°C for 7 min. The primers used were 5′-AGCTTTCTG TTAAAGCCGTGTTAAT-3′ (forward primer) and 5′-AAC TGCATGACCATAAGTACTCAA-3′ (reverse primer) for *mPing* at chromosome 2, 5′-TGTCAAGAAACTTGTAGG GAGAGA-3′ (forward) and 5′-TGCAGATCACAGCTTGT ATAGTTCG-3′ (reverse) for that at chromosome 3, 5′-CAT GCTTAGGTATAAACGACACTC-3′ (forward) and 5′-TGT GCAATCTTAATTTTCTTTCTGG-3′ (reverse) for that at chromosome 4, 5′-AGATTCCCATAATCTGAACTGTTTC-3′ (forward) and 5′-TAGAAGCTAATAACCTCGGGTGC TTT-3′ (reverse) for that at chromosome 6, 5′-TTCCCCT GATCTAATCTTATCGAAT-3′ (forward) and 5′-ACACAA

AGAACACACTACCAGTCAA-3′ (reverse) for that at chromosome 11, and 5′-AGGAGGTAAAAAGTGAGGA GAGAGA-3′ (forward) and 5′-ATTTACTGCTGCTACTCC TATTCCT-3′ (reverse) for that at chromosome 12. The amplified products were resolved by agarose gel (20 g L^{-1} in 1 × TAE) electrophoresis, stained with ethidium bromide and visualized under UV illumination.

Pollen viability, pollen germination on stigma, grain set, and grain germination

Effects of *mPing* insertion on male and female gametophyte formation, and also on germination in the next generation, were examined using the materials in 2010. Furthermore, pollen germination on stigma was also examined using artificially pollinated materials in 2012.

Just before flowering, three panicles were collected from the plants of the Hom-n, Hom-i, and Het lines, and their spikelets were fixed in Carnoy's solution (3:1 ethanol:acetic acid) and stored in 70% ethanol. Pollens from each sample were stained with I-IK solution, and the ratio of well-filled, ordinary pollens was evaluated as an index of pollen viability.

After maturity of plants, several panicles were harvested from the plants of the Hom-n, Hom-i, and Het lines, and their ratios of fertilized grains (grain set) were evaluated by visual inspection. Germinated grains were counted 8 days after sowing on moist paper in a petri dish kept at 30°C.

In 2012, several plants of Hom-n, Het, and Hom-i were grown in pots under ordinary fertilizer conditions. Spikelets of Hom-n plants were emasculated and bugged one day before crossing as a female parent, and pollinated by Hom-n (different plants from female plants), Het, and Hom-i. The reason why Hom-n was selected as female plants was that distorted segregation in the reciprocal crosses was detected only when Hom-n was used as female. After about two hr, all pollinated spikelets were sampled, fixed in Carnoy's solution, and stored in 70% ethanol. After these spikelets were softened by 1 M KOH at 85°C for 30 min, they were stained with 0.1% Aniline blue (Waldeck-Gmbh, Co. KG., Muenster, Germany) in 0.1 M K$_3$PO$_4$ for more than one hr. Stigmata with pollens were placed in 80% glycerol on a slide glass. These stigmata were then squashed gently, and the numbers of total pollens and of germinated pollens on stigma were counted using a fluorescent microscope system (BH2-RFL, Olympus Co. Ltd., Tokyo, Japan) with an excitation wavelength of 490 nm. Germinated pollens were defined as those with apparent pollen tubes.

Significance test was conducted on the assumption that distribution of data could be approximated with a standardized normal distribution.

Agronomic traits

To examine the effects of *mPing* insertion on agronomic traits, plants in the two S$_1$ populations in 2010 derived from Het-c and Het-d (**Table 2**) were measured for their days to heading, panicle number per plant, and longest culm length and panicle length on the longest culm in field conditions.

After genotyping for *mPing* insertion/non-insertion, Hom-n, Het, and Hom-i were compared to each other for the means of the measured traits using *t*-test.

Results

Table 1 shows the segregations for insertion/non-insertion of different *mPing* copies in the respective six different populations. Only the population of 2005-MLR011, in which an *mPing* copy was inserted at chromosome 12, obviously showed a significantly distorted segregation ratio from the expected 1:2:1 ratio, while the other five populations showed expected segregations. Therefore, we examined intensively this *mPing* at chromosome 12.

Four S$_1$ populations derived from four different heterozygotes (Het-a, Het-b, Het-c, and Het-d, all of which were derived from 2005-MLR011) for *mPing* insertion at chromosome 12 consistently showed again a significantly distorted segregation from the expected ratio (**Table 2**). The χ^2 for pooled data across the four populations and for heterogeneity among these four populations were 39.03 ($P < 0.001$) and 10.34 ($P = 0.111$), respectively. Clearly, the shortage of Hom-i, and partly of Het, caused this observed distorted segregation in every S$_1$ population. These results apparently confirmed the primary observation of distorted segregation found in an original population of 2005-MLR011. S$_1$ populations derived from Hom-n and Hom-i showed only Hom-n and Hom-i, respectively, even on a large scale in 2012 for Hom-i (**Table 2**).

The segregation ratios in F$_1$s of the reciprocal crosses between Hom-n and Het-a clearly indicated that a distorted segregation from the expected 1:1 ratio was observed only when the heterozygote was used as the male parent (**Table 2**). This strongly suggests that the male gametes with *mPing*, not the female gametes, fertilized or transmitted to the next generation less frequently than the male gametes without *mPing*. Given the ratio of 43:7 for the ratio of male gametes without *mPing* to those with *mPing* (this means 14% of the fertilized pollens involved *mPing*), and the ratio of 1:1 (no distortion) for the female gametes in a heterozygote, the expected ratio of Hom-n:Het:Hom-i in an S$_1$ should be 43:50:7. This ratio showed a very good fit against the observed results (pooled data of Het-a, Het-b, Het-c, and Het-d in **Table 2**: 73:92:14) ($\chi^2 = 0.45$, $P = 0.800$).

Table 3 shows the differences in the ratios of ordinary pollens, germinated pollens on stigma of Hom-n, fertilized grains, and germinated grains among Hom-i, Het, and Hom-n plants. No significant differences were observed in the ratios of fertilized grains and germinated grains among these three genotypes. This suggests that the insertion of *mPing* at chromosome 12 should not affect these fertilization and germination processes. On the other hand, Hom-n showed the highest ratio of pollen germination on stigma, followed by Het and Hom-i (**Fig. 1**). The difference between the latter two genotypes was significant. This apparently demonstrated that the insertion of the *mPing* at chromosome

Table 3. Ratios of ordinary pollens, germinated pollens on the stigma of non-insertion homozygote for *mPing*, fertilized grains, and germinated grains of the three genotypes for *mPing* insertion/non-insertion at the long arm of rice chromosome 12

Genotype[a]	Ordinary pollens (%)	Germinated pollens on Hom-n stigma (%)	Fertilized grains (%)	Germinated grains (%)
Hom-n	91.1[b] ± 27.9 [8736]	64.5[b] ± 5.9 [152]	77.2 ± 12.8 [933]	99.3 ± 2.0 [600]
Het	89.1[a] ± 27.9 [7124]	57.8[b] ± 6.7 [185]	82.3 ± 10.0 [668]	99.2 ± 2.2 [600]
Hom-i	90.9[b] ± 25.5 [7180]	47.4[a] ± 7.0 [196]	81.3 ± 9.1 [550]	98.8 ± 2.4 [500]

[a] Hom-n, Het, and Hom-i indicate non-insertion homozygote, heterozygote, and insertion homozygote, respectively, for *mPing* at chromosome 12.
All values without [ ] are means ± S.D.
Values in [ ] indicate the total number of samples examined for each trait.
Significant ($P < 0.01$) differences were observed among genotypes with different letters. In the case of no letters, no significant ($P > 0.01$) differences were observed.
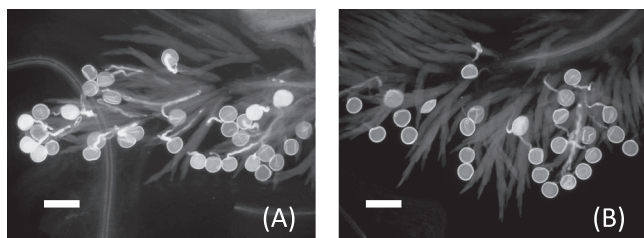
Fig. 1. Pollen germination on stigma of homozygotes for the non-insertion of *mPing* at rice chromosome 12, when pollinated with pollens without *mPing* (A) (by Hom-n) and those with *mPing* (B) (by Hom-i). A scale bar indicates 100 μm.

Table 4. Agronomic characteristics of different genotypes for *mPing* insertion/non-insertion at the long arm of rice chromosome 12 in segregating populations

Genotype[a]	Days to heading	Panicles per plant⁻¹	Culm length (cm)	Panicle length (cm)
Hom-n [38]	120.2 ± 1.9	11.3 ± 4.2	79.4 ± 5.0	18.6 ± 1.7
Het [35]	119.7 ± 1.8	13.0 ± 4.2	79.3 ± 4.6	18.6 ± 1.8
Hom-i [5]	119.2 ± 1.9	8.6 ± 2.9	78.0 ± 4.2	17.3 ± 0.8

[a] Hom-n, Het, and Hom-i indicate non-insertion homozygote, heterozygote, and insertion homozygote, respectively, for *mPing* at chromosome 12.
All values without [ ] are means ± S.D.
Values in [ ] indicate the total number of plants measured.
Non-significant ($P > 0.01$) differences were observed among genotypes for every trait.

12 reduced the pollen germination on stigma, at least of Hom-n. The pollen viability of Het was also significantly lower than the other two genotypes, although the difference was very small.

Table 4 shows the effects of insertion/non-insertion of *mPing* at chromosome 12 on several agronomic traits. No traits examined were affected, at least at the 0.01 probability level, by the insertion of this *mPing*.

Discussion

Rha *et al.* (1994) reported that in an F_2 population from a cross between rice cultivars TCS3 (*indica*-type) and CH106 (*japonica*-type), the segregation of alleles at *Acp1*, an

isozyme locus of acid phosphatase, at the terminus of the long arm of chromosome 12 was significantly distorted from the 1:2:1 ratio; the ratio of homozygotes for the *japonica*-type allele was lower than the expected one-quarter. Moreover, they showed that gametes with a *japonica*-type allele at *Acp1* might be eliminated partly only on the male side, from the results of reciprocal crosses between the F_1 and CH106. Rha *et al.* (1994) assumed the presence of a gametophyte gene, tentatively designated as *ga13*, which appeared to be linked to *Acp1* with a recombination value of $0.10 ± 0.016$, and that might suppress some functions of pollens with the *japonica*-type allele. These results for *Acp1* closely correspond with the present results for the current *mPing* copy. The similarity of positions of *Acp1* and *mPing*, both of which are located at the terminus of the long arm of chromosome 12, should also be noted. Many other loci for male and/or female gametocide (*ga*) and for hybrid sterility (*S*) have been identified in rice (Harushima *et al.* 2001, Koide *et al.* 2008, Xu *et al.* 1997), many of which function as reproductive barriers of intra-specific and inter-specific gene flow.

In the present experiments, fertilization and germination ability after self-fertilization were not significantly different among Hom-i, Het, and Hom-n plants, suggesting that *mPing* insertion could not affect the processes of embryo development after fertilization and germination (**Table 3**). On the other hand, pollen germination on stigma tended to decrease when the pollens involved this *mPing* (**Table 3**). Given equal numbers of pollens with and without *mPing* from heterozygotes, the ratio of germinated pollens with *mPing* in total germinated pollens on stigma was estimated to be 42.4% {=47.4/(47.4 + 64.5)} (**Table 3**). This estimate was not compatible with the estimated ratio of fertilized pollen with *mPing* (14%) as previously indicated. Therefore, it is most plausible that male gametes with this *mPing* copy might be eliminated partly in the processes from pollination to fertilization by negative competition against male gametes without *mPing*. This kind of pollen competition has been reported between cultivated and wild rice species (Song *et al.* 2002). Probably, this insertion of *mPing* might affect only male gametes, and not the agronomic traits examined (**Table 4**). Alternatively, it can be also deduced that the transposon *mPing* at this site specifically excised during
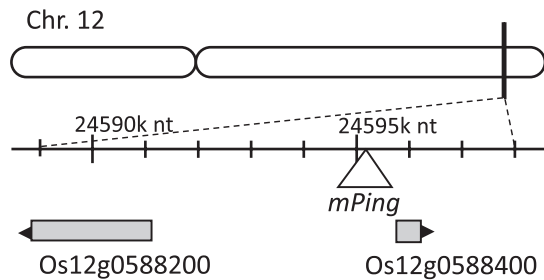
Fig. 2. Relationships between the positions of inserted *mPing* and two ORFs near the long arm of rice chromosome 12. This figure is based on Rice Annotation Project-Database (RAP-DB), IRGSP build 5. nt, nucleotide.

the above-mentioned critical stage only in the male gametes. However, from **Table 2**, no heterozygotes were detected in S_1 populations from Hom-i, even on a large scale in 2012, suggesting that no excision occurred in heterozygotes during this stage. Another possible cause of the distorted segregation could be less formation of microspores with *mPing* compared with those without *mPing* in meiosis. However, this possibility could not be examined definitely, since the individual genotyping of those microspores, as well as gametes, is essential to determining if those have *mPing* or not, which is extremely difficult.

According to the RAP-DB (IRGSP build 5), there are at least two putative ORFs near the inserted *mPing*: Os12g0588400 (24,595,783..24,596,141) and Os12g0588200 (24,589,162..249,591,131), which are located on both sides of *mPing* (**Fig. 2**). So far, the functions of these two ORFs have not been identified from current database searches. Expression analysis should be conducted for these two ORFs. However, this should be done individually in male gametes with and without *mPing* produced from heterozygotes. Although the ORFs located near the insertion site of *mPing* at the long arm of chromosome 12 have not been identified as genes concerned with male gamete functions, the present results demonstrate that *mPing* can be utilized effectively as a tool of gene tagging in rice.

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