RESEARCH PAPER

In planta mobilization of mPing and its putative autonomous element Pong in rice by hydrostatic pressurization

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Abstract

The miniature Ping (mPing) is a recently discovered endogenous miniature inverted repeat transposable element (MITE) in rice, which can be mobilized by tissue culture or irradiation. It is reported here that mPing, together with one of its putative transposase-encoding partners, Pong, was efficiently mobilized in somatic cells of intact rice plants of two distinct cultivars derived from germinating seeds subjected to high hydrostatic pressure, whereas the other autonomous element of mPing, Ping, remained static in the plants studied. mPing excision was detected in several plants of both cultivars in the treated generation (P0), which were selected based on their novel phenotypes. Southern blot analysis and transposon-display assay on selfed progenies (P1 generation) of two selected P0 plants, one from each of the cultivars, revealed polymorphic banding patterns consistent with mobilization of mPing and Pong. Various mPing excisions and de novo insertions, as detected by element-bracketing, locus-specific PCR assays, occurred in the different P1 plants of both cultivars. Pong excision at one locus for each cultivar was also detected by using a Pong internal primer together with locus-specific flanking primers in the P1 plants. In contrast to the pressurized plants, immobility of both mPing and Pong in control plants, and the absence of within-cultivar heterozygosity at the analysed loci were verified by Southern blotting and/or locus-assay. Sequencing at 18 mPing empty donor sites isolated from the pressurized plants indicated properties characteristic of the element excision. Sequence-based mapping of 10 identified mPing de novo insertions from P1 progenies of pressurized plants indicated that all were in unique or low-copy regions, conforming with the targeting propensity of mPing. No evidence for further mPing activity was detected in the P2 plants tested. In spite of the high activity of mPing and Pong in the pressurized plants, amplified fragment length polymorphism (AFLP) analysis denoted their general genomic stability, and several potentially active retrotransposons also remained largely immobile. Further investigation showed that the same hydrostatic pressure treatments also caused mobilization of mPing in the standard laboratory cultivar for japonica rice, Nipponbare. Thus, a simple and robust approach for in planta MITE-mobilization in rice has been established by using high hydrostatic pressure treatment, which may be useful as an alternative for gene-tagging in this important crop plant.

Key words: Hydrostatic pressure, MITEs, rice, tagging, transposon mobilization

Introduction

Transposon-tagging is an important methodology for gene isolation and functional studies in both animals and plants.

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(Klinakis et al., 2000; Bessereau et al., 2001; Kumar et al., 2003). In rice, an active copia-like long-terminal repeat or LTR retrotransposon (Tos17) is currently being employed to generate insertional mutations on a large scale, which has facilitated the isolation of several important genes (Hirochika, 2001; Kumar and Hirochika, 2001; Miyao et al., 2003; Hirochika et al., 2004; Kurusu et al., 2004). However, activation of Tos17 requires tissue culture, a process often-associated with somaclonal variation (Hirochika et al., 1996). Obviously, an in planta transposon-tagging method obviating the tissue culture process is highly desirable in rice.

The miniature inverted transposable elements (MITEs) represent a distinct type of class II DNA transposons that usually transpose via the cut-and-paste mechanism (Feschotte et al., 2002; Casacuberta and Santiago, 2003; Jiang et al., 2004). Miniature-Ping or mPing, the only active MITE so far discovered in any organism, is a 430 bp DNA repeat endogenous to the rice genome, which contains terminal inverted repeats or TIRs (15 bp) and produces target site duplications or TSDs (TAA or TTA) typical of a tourist-like MITE family (Feschotte et al., 2002; Jiang et al., 2003; Kikuchi et al., 2003; Nakazaki et al., 2003). mPing is cryptic under normal conditions (but also see Nakazaki et al., 2003), but can be effectively mobilized by tissue culture (Jiang et al., 2003; Kikuchi et al., 2003) or irradiation (Nakazaki et al., 2003), with mobilized copies preferentially inserting into single-copy, non-coding or genic regions (Jiang et al., 2003). Because mPing has no coding-capacity, and hence is non-autonomous, the transposase (TPase) responsible for its mobilization is provided in trans, probably by either or both of the identified mPing-related, transposase-encoding elements, Ping and Pong, which often showed co-mobilization with mPing under the specific induction conditions (Jiang et al., 2003; Kikuchi et al., 2003; Nakazaki et al., 2003).

The finding that the copy number of mPing varies dramatically among the rice cultivar-groups that are domesticated independently from a common wild species, Oryza ruffipogon (Second, 1982; Ma and Bennettzen, 2004; Zhu and Ge, 2005) suggests that mPing, like transposons in Drosophila and some LTR-retrotransposons in plants, might be mobilized by factors other than tissue culture or irradiation, such as environmental stresses (Capy et al., 1990, 2000; Wessler, 1996; Grandibastien, 1998; Jiang et al., 2003) and interspecific hybridization (Labrador et al., 1994; Shan et al., 2005). Indeed, it was found recently that mPing showed apparent mobility in several introgressed rice lines derived from an interspecific cross between rice and wild rice (Zizania latifolia Griseb.) (Shan et al., 2005).

High hydrostatic pressurization is a physical stress whereby multiple cellular activities, including chromatin structure (Kaamiranta et al., 1998), DNA methylation state (Long et al., 2006), and gene expression (Symington et al., 1991; Wilson et al., 2001; Sironen et al., 2002; Yang et al., 2004), can be affected. Given that activity of transposons is often controlled by epigenetic mechanisms (Okamoto et al., 2001; Miura et al., 2001; Ros and Kunze, 2001; Singer et al., 2001; Lippman et al., 2003; Bender, 2004), and can be closely linked to the general cellular responses to stress (Grandibastien, 1998; Capy et al., 2000), it is speculated that high hydrostatic pressure might constitute a stressful condition whereby some cryptic transposons like mPing might be activated. The present study was aimed at addressing this possibility. It is reported here that mPing, together with one of its putative TPase-encoding partners, Pong, was efficiently mobilized in somatic cells of pressurized rice plants from two distinct cultivars studied. Moreover, multiple mPing insertions had occurred in progeny plants derived from single pressurized parental plants, which were then likely stably inherited. The possible advantages of using hydrostatic pressure-induced mPing mobility, relative to activity induced by tissue culture or irradiation, for gene-tagging in rice is discussed.

Materials and methods

Plant material

Seeds of two established inbred japonica rice cultivars, JL307 and JL01-124, were kindly provided by the Institute of Rice Breeding, Jilin Academy of Agricultural Sciences, Gongzhuling, Jilin, China. The choice of these two rice lines are mainly because of their elite nature as newly released inbred commercial cultivars, and their clear pedigree, such as none of their parental cultivars ever having been subjected to tissue culture and irradiation. In addition to these two cultivars, the standard laboratory cultivar for japonica rice ssp. Nipponbare, was also used.

Hydrostatic pressurization

Rice seeds (soaked in distilled water at ambient temperature for 24 h) were subjected to continuous high hydrostatic pressure (80 MPa, 20 min or 100 MPa, 15 min at ambient temperature), essentially as reported (Fernandes et al., 2004; Long et al., 2006). These conditions were chosen based on previous reports mainly on animal and human cultured cells (Symington et al., 1991; Kaamiranta et al., 1998; Wilson et al., 2001; Sironen et al., 2002; Yang et al., 2004). The germinating seeds were allowed to grow to seedlings in Petri dishes before being transplanted to a conventional paddy field.

PCR-based locus assay on mPing and Pong excision

To detect possible excisions of mPing (AB087615.1) and Pong (BK000586.1), a set of 53 pairs of locus-specific primers each bracketing an intact mPing and five pairs of locus-specific primers each bracketing an intact Pong in the standard laboratory cultivar for japonica rice ssp., Nipponbare (see supplementary Table 1 at JXB online), was designed based on its available whole genome sequence (http://rgp.dna.afrc.go.jp), by the Primer 3 software (http://biocore.unl.edu/cgi-bin/primer3/primer3_www.cgi). Loci containing a mPing or Pong in each of the two cultivars were identified by PCR amplification with this set of primers. It turned out that JL-307 has 25 mPing-containing loci and one Pong-containing locus, while JL01-124 has 11 mPing-containing and one Pong-containing
locus (see supplementary Table 1 at JXB online). For the \textit{mPing}-containing locus assay, a pair of primers flanking \textit{mPing} was used. For the \textit{Pong}-containing locus assay, a \textit{Pong}-internal primer (5'-TGCAATGGAAACGCAGGAT and reverse: 5'-GTCACAATGGGGTTTCACT) was isolated by PCR with primers (Jiang \textit{et al.}, 1996) at positions 5032–5052) was combined with a locus-specific primer at the 3'-flanking region of each locus. All PCR amplifications were performed at annealing temperatures ranging from 58 °C to 62 °C depending on the different pairs of primers. The amplicons were visualized by ethidium bromide staining after electrophoresis through 2% agarose gels. Some of the pairs of primers. The amplicons were visualized by ethidium bromide staining.

\begin{table}[h]
\centering
\begin{tabular}{llll}
\hline
IS locus & Insertion site\textsuperscript{a} & Locus-specific primers (5'-3') & Isolated from \\
\hline
mpISL3 & Ch.2; AP08208.1; position: 18634566; non-coding & For: GGGGAGTTGCAAGTGTTGAT Rev: TCTCTCAAACAGGCATACGA & JL307#1-P1-2 GGCCAGTCACAATGG TAA \\
mpISL4 & Ch.4; AP008210.1; position: 91645; non-coding & For: TGGTTTGCTGGACAGTCAA Rev: GCTCTGACATAAGCGCAACA & JL307#1-P1-2 GGCCAGTCACAATGG TAA \\
mpISL11 & Ch.3; AC146468.1; position: 88418; non-coding & For: ATCCATTCAAAGATGACGAA Rev: AAACACTGACGGTAGGATT & JL307#1-P1-4 GGCCAGTCACAATGG TAA \\
mpISL15 & Ch.1; AP008207.1; position: 23328654; unknown protein (ORF); 7e-23 & For: CGACAGGCTCAAGGACA Rev: AAAACACTGACGGTAGGATT & JL307#1-P1-6 GGCCAGTCACAATGG TAA \\
mpISL18 & Ch.7; AP008213.1; position: 45934; non-coding & For: ATCCATTCAAAGATGACGAA Rev: GGTGCGGAACATGTCCACTA & JL307#1-P1-4 GGCCAGTCACAATGG TAA \\
mpISL22 & Ch.4; AP008210.1; position: 123776; non-coding & For: AGCGCTCGACTACTCCAGAT Rev: CAAATGAGCCCGTAAATCT & JL307#1-P1-1 GGCCAGTCACAATGG TAA \\
mpISL23 & Ch.3; AC121491.2; position: 64666; non-coding & For: TTAGAGCATCCACACAGAA Rev: ATGCCATTAGCACAAGA & JL307#1-P1-3 GGCCAGTCACAATGG TAA \\
mpISL36 & Ch.3; AP008209.1; position: 21161019; non-coding & For: CATGTGCGTGGAAAACAGAG Rev: GCACAGGCTCCAAGACGTA & JL307#1-P1-2 GGCCAGTCACAATGG TAA \\
mpISL43 & Ch.5; AC136219.2; position: 76366; non-coding & For: CAAATGAGCCCGTAAATCT Rev: TTCAGACTCTAGCTTCGAC & JL307#1-P1-3 GGCCAGTCACAATGG TAA \\
mpISL44 & Ch.6; AP008212.1; position: 29583478; for: ATTAAAGCATGCATGGGTGTG Rev: GGTGCGGAACATGTCCACTA & JL307#1-P1-2 GGCCAGTCACAATGG TAA \\
\hline
\end{tabular}
\textsuperscript{a} Based on BLASTN at the NCBI website.
\end{table}

\textbf{Southern blot analysis}

Genomic DNA was isolated from expanded leaves of individual plants by a modified CTAB method and purified by phenol extractions. Genomic DNA (~3 μg per lane) of the various plant lines was digested by \textit{HindIII} or \textit{XhoI} (New England Biolabs, Beverly, Massachusetts). Digested DNA was run through 4% agarose gels by ethidium bromide staining and excisions were gel-purified and labelled with fluorescein-11-dUTP by the Gene Images random primer-labelling module (Amersham Pharmacia Biotech). Hybridization signal was detected by the Gene Images CDP-Star detection module (Amersham Pharmacia Biotech). Hybridization signal was detected by the Gene Images CDP-Star detection module (Amersham Pharmacia Biotech) after washing at a stringency of 0.2× SSC, 0.1% SDS for 2×50 min. The filters were exposed to X-ray films.

\textbf{Isolation of de novo \textit{mPing} insertion sites in progenies of pressurized plants by transposon display}

Transposon display (Van den Broeck et al., 1998; Casa et al., 2000) was performed by either combining the \textit{mPing} subterminal-specific primers (Jiang et al., 2003) with a set of inter-simple sequence repeat or ISSR primers (available upon request) and visualizing the amplicons on 4% agarose gels by ethidium bromide staining.
Examples of established japonica 4000 germinating seeds were pressurized for each of two cultivars (Schulman et al., 2004; Shan et al., 2005), or by the protocol exactly as reported by Jiang et al. (2003) except using silver-staining for band visualization, as in the AFLP analysis (see below). Novel bands that appeared in progenies of pressurized plants in the mPing-SSR amplifications but were absent in the corresponding ISSR-alone amplifications, were considered as putative mPing de novo insertions and isolated for sequencing. The insertions were then confirmed by PCR amplifications using mPing-flanking primers, as described above for the excision analysis.

Amplified fragment length polymorphism (AFLP) analysis
The standard AFLP technique employing EcoRI and MseI enzymes was used (Vos et al., 1995), and the experimental conditions are exactly as described (Wang et al., 2004).

Results and discussion
4000 germinating seeds were pressurized for each of two established japonica rice cultivars, JL307 and JL01-124, under the conditions specified (see Materials and methods). More than 90% of the 4000 treated seeds of each cultivar produced normal-looking adult rice plants in the field, and no difference was observed between the two pressurization conditions. At the time of maturity, four plants in each cultivar (designated as #1 to #4 for cultivars JL307 and JL01-124, respectively) were selected out because they showed apparent phenotypic novelty compared with their respective control plants. The changed traits included spike morphology (varied number and distribution of spikelets per spike), kernel shape (ratio of length to width) and fertility (X Lin, unpublished data). Such morphologically aberrant plants were not observed in the two cultivars over years of field tests and cultivation. To test for possible mPing mobility in these eight (four of each cultivar) phenotypically changed individuals, genomic DNA was isolated from flag-leaves of the plants and from seedlings of >1200 randomly chosen seeds of the control plants (in 128 pools, each containing 10 or 11 individual seedlings) for each cultivar, and the samples were subjected to PCR-based locus analysis for possible mPing excisions. Of the 25 and 11 mPing-containing loci analysed, respectively, for cultivar JL307 and JL01-124 (see supplementary Tables 1 and 2 at JXB online), 12 and two loci showed evidence for possible element excision in one or more of the pressurized plants, being reflected by simultaneous amplification of both an upper and a lower band from a given sample, with the size difference between the two bands being consistent with deletion of an mPing copy (Fig. 1a, and data not shown). This indicates that somatic cells of flag leaves from the pressurized, phenotypically novel rice plants contained heterogeneous cells with regard to the presence or absence of members of mPing at specific loci. In contrast to pressurized plants, all the >1200 control seedlings randomly collected from each cultivar showed singular upper bands at all 36 loci analysed (25 and 11, respectively, for cultivar JL307 and JL01-124) (see supplementary Fig. 1 at JXB online, and data not shown). This strongly suggests that potential allelic heterozygosity at these mPing-containing loci within a cultivar was not a contributory factor to the appearance of the lower bands in the pressurized plants (Fig. 1a). The second potential possibility is that the selected phenotypically novel plants happened to be plants, or descendants thereof, of either mechanically (other rice cultivars) or biologically (pollination by other rice cultivars) contaminated seeds. To address this concern, the eight selected plants of both cultivars were subjected to locus-specific PCR analysis at all the rest (28 and 42, respectively, for JL307 and JL01-124) of the 53 loci, each of these loci does not contain an mPing in either of the two cultivars. Consequently, exactly identical amplification patterns were found at all the loci analysed, which either produced a small-sized band coinciding with lack of an mPing for most of the loci, or no amplification product for several other loci, probably due to divergence at the primers designed according to the Nipponbare genomic sequence (data not shown). Because each japonica rice cultivar has distinct mPing-containing loci (Jiang et al., 2004; X Lin, unpublished data), the above analysis thus confidently ruled out contamination (mechanical or biological) as a cause for the appearance of novel, small-sized bands in the pressurized plants relative to the control plants in both cultivars. This result also indicated that, in the pressurized

![Fig. 1](attachment:image.jpg) Examples of mPing excision in flag-leaf somatic cells of pressurized rice plants (P₀ generation) from two distinct cultivars, JL307 and JL01-124, as revealed by PCR amplification with locus-specific primers: (a) locus mpL7 for JL307 and (b) locus mpL10 for JL01-124. Sizes of the lower bands coincide with deletion of a full-length mPing copy at each locus, as confirmed by sequencing. The PCR products were visualized by ethidium bromide-stained agarose-gels. M is the 100 bp molecular size marker (the Fermentas Biol., Maryland). Similar results were obtained at several other mPing-containing loci.
plants, the reinsertion of mobilized mPing did not occur within the loci analysed; this is as expected given the genome-wide insertion propensity of mPing except for a strong preference towards single-copy, non-coding or genic regions (Jiang et al., 2003, 2004). A final concern is that some unknown elicitors may have caused mobility of mPing in the selected plants. Nonetheless, the fact that both the pressurized and the control plants were grown together under the same normal field conditions, and, furthermore, the >1200 tested control seedlings in each cultivar did not show even a single excision event (see supplementary Fig. 1 at JXB online, and data not shown), render this possibility extremely remote.

To validate the mobilization of mPing and possibly also of its putative transposase-donors, Pong and Ping (Jiang et al., 2003, 2004; Kikuchi et al., 2003; Nakazaki et al., 2003), as well as to test if the somatic cells wherein mPing mobilization occurred would have formed gametes or germ line, one plant from each cultivar (#1 for cultivar JL307, designated as JL307P#1, and #3 for cultivar JL01-124, designated as JL01-124#3), was selected to produce selfed progenies (designated as P1 plants). These two P0 plants were chosen because they showed the most mPing excisions based on the locus analysis. Eight and 14 P1 plants, respectively, derived from JL307P#1 and JL01-124#3 were used for Southern blot analysis, together with their respective P0 and untreated parental lines, by using the full-length mPing and fragments of Pong and Ping as probes (see Materials and methods). It was found that loss of parental bands and (or) the appearance of novel bands occurred in either or both of the mPing and Pong hybridization profiles in P0 and all eight P1 plants of JL307P#1, and in P0 and five of the 14 P1 plants in JL01-124#3 (Fig. 2a, b, d, e), whereas the hybridization patterns of Ping remained constant in the pressurized plants studied of both cultivars (Fig. 2c, f). This latter result suggested that although Ping existed in these two rice cultivars, it was stable under the high pressurization conditions, at least in the plants studied, and hence, probably did not contribute to the mobilization of mPing in these plants. Nevertheless, the possibility can not be ruled out that Ping may have been transcriptionally activated, and hence, provided some of the TPase, which had facilitated mPing mobility (Jiang et al., 2003). On the other hand, the loss and gain of hybridizing bands in the Southern blotting patterns of mPing and Pong is consistent with the ‘cut-and-paste’ model of mobilization of mPing and Pong (Feschotte et al., 2002; Casacuberta and Santiago, 2003; Jiang et al., 2004). The variable Southern blotting patterns of mPing in the pressurized plants were also fully supported by the well-established mPing-specific transposon-display analysis (Jiang et al., 2003), where loss and gain of mPing-anchored amplicons was easily recognized (see supplementary Fig. 2 at JXB online). Taken together, these data not only implicated concomitant mobilization of mPing and Pong in somatic cells of pressurized plants but also pointed to meiotic inheritance of insertions to the next generation, i.e. the affected somatic cells had given rise to gamet cells. Further, because the analysed P1 progenies were derived from single P0 parental individuals, but they produced apparent different banding patterns in both mPing and Pong hybridization profiles (Fig. 2), it is likely that each P0 gamete had harboured more than one transposition event and different gametes contained different transpositions, a property required for efficient tagging (Bessereau et al., 2001; Klinakis et al., 2000; Kumar et al., 2003; Hirochika et al., 2004). To test if parental heterozygosity might have been a contributory factor to the observed variable hybridization patterns of mPing and Pong in P0 as well as among P1 progenies of pressurized plants (Fig. 2), eight randomly chosen, untreated control plants for each cultivar were analysed by the same Southern blotting, and identical banding patterns appeared for all eight plants in each cultivar for both mPing and Pong (Fig. 3). This is consistent with the results of the PCR-based locus-assay at both the P0 generation (see supplementary Fig. 1 at JXB online, and data not shown) and P1 generation (see below). Collectively, complete stability of mPing in control plants for each cultivar was evident, and hence, strongly suggests that within-cultivar heterozygosity was not a cause for the observed variation in mPing and Pong in the pressurized plants and their progenies (Figs 1, 2, and see below).

To analyse the P1 plants that showed variable Southern blot patterns for mPing and Pong (Fig. 2) further, a PCR-based locus assay was performed at all identified 36 mPing-bracketing loci (25 and 11, respectively, for JL307 and JL01-104) and two Pong-bracketing loci (one and one, respectively, for JL307 and JL01-104) (see supplementary Table 1 at JXB online). It was found that 18 and three mPing-bracketing loci, respectively, for JL307 and JL01-124 produced a lower-band in one or more of the studied P1 plants (Fig. 4a, b; data not shown). Notably, in some plants only a lower-band was amplified (Fig. 4a, b), indicating homozygosity of the plants at the loci. In other words, both the paternal and maternal gametes forming the given zygote must have had mPing at the specific loci excised. Of the Pong-bracketing loci analysed, one in each cultivar (PonL5 in LJ307 and PonL4 in JL01-124) showed clear evidence for excision (Fig. 4c, d). Moreover, due to nature of the Pong-bracketing locus-assay (using a Pong-internal primer in combination with a flanking primer, see Materials and methods), only homozygous Pong-devoid loci can be identified. Therefore, as in the case of mPing, many of the P1 plants were actually homozygous for Pong excisions (Fig. 4c, d). To test for possible heterozygosity at the assayed loci (mPing or Pong-containing) in control plants, 123 randomly chosen individuals (in 41 pools) for each cultivar were analysed, and at all loci a monomorphic pattern denoting the absence of an mPing or Pong was observed (Fig. 4e, f, g, h; data not shown). This analysis
thus testified again that the observed mPing/Pong excisions in the pressured P1 plants were not due to original heterozygosity, and the presence of homologous mPing- or Pong-devoid loci was resultant from concomitant element excision at the specific loci in both male and female gametes.

It has been shown that MITEs often, but not always, excise imprecisely, i.e. leave various excision footprints...
from the P1 plants of both cultivars was isolated. It was lower bands together with their corresponding upper ones hydrostatic pressure would leave footprints or not, a set of cultivar JL01-124. For a rationale of this Southern blot analysis see Fig. 2. the ORF2 region to the same blot as in (a). (c, d) The same analysis for (Kikuchi et al., 2003; Nakazaki et al., 2003; Shan et al., 2005). To test if the excision of mPing and Pong induced by hydrostatic pressure would leave footprints or not, a set of lower bands together with their corresponding upper ones from the P1 plants of both cultivars was isolated. It was found that of the 21 mPing excisions sequenced (18 and three, respectively, for JL307 and JL01-124), only one (mPL46 of JL307) left footprints (with a stretch of 14 extra-nucleotides at the excision site), whereas the rest (20) had excised precisely (see supplementary Table 2 at JXB online). Of the two Pong excisions sequenced, one (PonL4 of JL01-124) had left footprint (with three nucleotides in front of the target site duplication, TAA, being deleted) and the other had not (see supplementary Table 2 at JXB online). This suggests that for the hydrostatic pressurization-mobilized mPing/Pong to leave an excision footprint or not is probably dependent on both the host genotype and genomic positions of the elements, or is simply stochastic.

A hint for mPing and Pong de novo insertions was implicated by the appearance of novel bands in the two pressurized P0 plants and their P1 progenies of both cultivars in Southern blot analysis (Fig. 2). To verify this, a modified version (Shan et al., 2005) of the transpose-display technique (Van den Broeck et al., 1998; Casa et al., 2000), namely combining mPing or Pong subterminal-specific primers with one of a set of inter-simple sequence repeat (ISSR) primers, was used to visualize and isolate several novel bands present in P1 progenies of the hydrostatic pressurized plants relative to their control plants. Sequence analysis on the isolated fragments enabled identification of respectively eight and five clones containing, at their 5’ end, the stretch of nucleotides of mPing or Pong (Table 1). The contiguous upstream sequences putatively flanking complete members of mPing at these loci were deduced from the public genome sequence for japonica rice, cv. Nipponbare (http://rgp.dna.affrc.go.jp), and hence, enabled the design of locus-specific primers (see Materials and methods). PCR amplification using these putative mPing-containing primers in untreated control plants produced only lower bands of the sizes expected for the absence of mPing at all the identified loci, whereas the upper bands of sizes expected to contain a member of mPing or Pong, either alone or together with a lower band, were amplified from some P1 progenies of the pressurized P0 plants (Fig. 5a, b; data not shown). Upon cloning and sequencing the full-length (mPing-containing) of the isolated upper bands, it was found that all products indeed contained the complete TIRs (GGCCAGTCACAATGG) and the TSDs (TAA or TTA) characteristic of newly transposed mPing (Jiang et al., 2003; Kikuchi et al., 2003; Nakazaki et al., 2003). Thus, little doubt remains that the isolated mPing-containing loci represent bona fide de novo insertions of the elements present only in P1 progenies of the hydrostatically pressurized P0 plants. Allelic heterozygosity in untreated control plants of both cultivars was again not found in any of these loci, as shown by the amplification of a singular lower band donating the absence of an mPing member at each of the loci in 63 analysed random individual plants (in 21 pools) for each cultivar (Fig. 5c, d; and data not shown).

For efficient gene-tagging by a specific transposon, it is important that the host genome is not simultaneously undergoing large-scale random rearrangements or being mutagenized by other transposons or retrotransposons as well. To address this issue, the general genomic composition of the pressurized plants relative to that of the control plants was investigated first by the amplified fragment length polymorphism (AFLP) technique (Vos et al., 1995; Wang et al., 2005). Of the total of 396 and 402 clear bands scored, that were generated by 20 EcoRI+MseI primer combinations (each generating from 15 to 25 clear bands in the middle-part of the gels), and that were completely reproducible between the two duplicates for each sample (starting from restriction and ligation, i.e. the first step of AFLP), respectively, for the two cultivars, JL307 and JL01-124, none showed evidence of genomic change in the

![Image](image_url)
pressurized plants as compared with their controls (Fig. 6; and data not shown). Although this analysis can not rule out
the occurrence of random genomic rearrangements, it clearly suggests that high pressurization has not induced
a general genomic instability in the two rice cultivars. Next, Southern blots containing the same pressurized P1 plants of
the two cultivars were probed with three copia-like retrotransposons, Tos10, Tos17, and Tos19, known to be
active under stress conditions like tissue culture (Hirochika et al., 1996). Consequently, it was found that only Tos17
showed one possible retrotransposition event in a single P1 plant of cultivar JL01-124, whereas Tos10 and Tos19
remained stable in all plants studied of both cultivars (see supplementary Fig. 3 at JXB online). Thus, absence of the bands in some
of the P2 plants denotes excision of Pong at the specific loci. The asterisk refers to a non-specific band. Stability of mPing and Pong and lack of heterozygosity at the analysed loci in untreated control plants of both cultivars were revealed by the same analysis on 123 randomly chosen plants
(in 41 pools, each containing three plants). The PCR products were visualized by ethidium bromide-stained agarose-gels. Identical results were obtained for all analysed mPing- or Pong-containing loci. M is the 100 bp molecular size marker (the Fermentas Biol., Maryland).

For gene-tagging purpose, it is also important that the element insertions are not only heritable but also stable in
the progenies (Bessereau et al., 2001; Klinakis et al., 2000; Kumar et al., 2003; Hirochika et al., 2004). To test this, 30 randomly chosen P2 progenies of each of the P1 plants were analysed by PCR amplification with several identified mPing de novo insertions that were already homozygous in the identified P1 plants (producing only an upper band). No evidence was found for excision of these inserted mPing copies in any of the P2 plants studied (data not shown).

An ideal property of mPing to be used for tagging is its preference for unique or low-copy regions in the rice
genome (Jiang et al., 2003, 2004). It was found that the hydrostatic pressure-mobilized mPing members also showed this targeting propensity for single or low-copy genomic regions, as all 11 de novo insertions isolated mapped to low-copy, genic or non-coding regions involving different chromosomes (Table 1).

Given the dramatic genotypic difference in rice with regard to the activity of mPing under a specific stress
condition (Jiang et al., 2003; Kikuchi et al., 2003; Nakazaki et al., 2003; Shan et al., 2005), it is probably premature to extrapolate the present results to other rice
cultivars. Nonetheless, preliminary data indicated that one of the hydrostatic pressurization conditions reported here
(80 MPa, 20 min) also caused mPing activation in the

Fig. 4. Examples of PCR-based locus assay for mPing [loci mpL27 (a), mpL41 (b)], and Pong [loci ponL5 (c), ponL4 (d)] excisions in flag-leaf somatic
cells of P1 (selfed progenies of a single pressurized P0 plant) individuals of the two cultivars, which showed variable mPing and Pong patterns in
Southern blot analysis (Fig. 2). In (a) and (b), sizes of the lower bands coincide with deletion of a full-length mPing copy at each locus (determined by
sequencing). In (c) and (d), the presence of the expected bands (238 bp for locus ponL5, 248 bp for locus ponL4; determined by sequencing) was resulted
from amplifications by using a Pong-internal primer (5'-TCATGACGCTAGTA at positions of 5032 to 5052) together with a locus-specific primer located in the 3' flanking region for each Pong-containing locus (see supplementary Table 2 at JXB online). Thus, absence of the bands in some
of the P2 plants denotes excision of Pong at the specific loci. The asterisk refers to a non-specific band. Stability of mPing and Pong and lack of heterozygosity at the analysed loci in untreated control plants of both cultivars were revealed by the same analysis on 123 randomly chosen plants
(in 41 pools, each containing three plants). The PCR products were visualized by ethidium bromide-stained agarose-gels. Identical results were obtained for all analysed mPing- or Pong-containing loci. M is the 100 bp molecular size marker (the Fermentas Biol., Maryland).
of the 23 randomly selected P0 individual plants germinated from pressure-treated Nipponbare seeds, four showed apparent new mPing insertions, although both Ping and Pong remained stationary (Fig. 7). One possible explanation for this observation is that there might have been transcriptional activation of either or both of the TPase-donors (Ping and Pong), which although catalysed...
occasional mPing transposition, was nonetheless insufficient to catalyse their own movement, probably due to the larger sizes of Ping and Pong (Jiang et al., 2004). In contrast to mPing activity in pressurized plants, no change in the mPing pattern was detected in 24 randomly chosen Nipponbare plants (data not shown). Because the 23 pressurized plants were randomly chosen, this experiment allows an estimation on frequency of de novo mPing insertions in four plants (marked by asterisks) were indicated by filled triangles. In contrast to mPing, no changes were detected in Pong or Pong.

Supplementary material

Detailed information regarding the following contents is available at the Journal of Experimental Botany website, http://jxb.oxfordjournals.org/ (i) 53 pairs of the mPing- and Pong-harbouring, locus-specific primers; (ii) characteristics of mPing and Pong excisions in P1 progenies of pressurized rice plants; (iii) stability of mPing in untreated control plants; (iv) examples of mPing-specific transposon display; and (v) near complete immobility of the copia-like LTR retrotransposon Tos17 in the pressurized rice plants.

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