DOI: 10.1007/s11427-006-0097-3

Alterations in DNA methylation and genome structure in two rice mutant lines induced by high pressure

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Received May 6, 2005; accepted September 19, 2005

Abstract By using high-pressure treatment, two mutant lines were obtained from a genetically stable *japonica* rice cultivar Bijing38. Genomic DNA of the mutant lines, together with the original line (Bijing38), was either undigested or digested by *Hpa* II/*Msp* I, and then subjected to molecular analysis using two markers, ISSR and RAPD. Results indicated that changes in the PCR amplification profiles of both markers are apparent in the two mutant lines compared with the original rice cultivar, suggesting that there had been both sequence changes and DNA methylation modifications in the mutant lines. Southern blot analysis using diverse sequences, including two cellular genes (S2 and S3), a set of retrotransposons (*Osr7*, *Osr36*, *Tos19* and more), and a MITE transposon family (*mPing* and *Pong*), confirmed the results, and indicated that changes in DNA methylation pattern, genomic structure, and possible activation of some transposons indeed occurred in the mutant lines. Moreover, these changes are stably maintained through selfed generations and in different organs. Thus, our results indicate that it is possible to obtain stable mutants in rice by high pressure treatments, and the molecular basis of the mutants may include both genetic and epigenetic changes. Therefore, high hydrostatic pressure seems a promising approach for plant mutagenesis.

Keywords: DNA methylation, genomic changes, high pressure, mutagenesis, rice.

Pressure is expected to be an important parameter to affect characteristics of matters and control rate and equilibrium of chemical reactions. As a fundamental thermodynamic variable, it also has effects on biomacromolecules and a lot of physiological and biochemical reactions in an organism. High-pressure biology has become a new field with the rapid development of theory and technology of high-pressure since the 1980s, and has extended to many fields of biological sciences[1−7].

Treating plant seeds by high pressure was first attempted in the 1920s. But only the effects of high pressure on seed germination were reported[8]. Until now, it is not clear at the molecular level whether high-pressure treatment can induce heritable genetic variations in plants.

Xu *et al.*[9,10] first reported that rice seeds treated by high-pressure showed obvious physiological changes

and stable and hereditable morphological variations. We recently characterized two rice mutants derived from high pressure-treated seeds by two molecular markers, RAPD (random amplified polymorphic DNA) and ISSR (inter simple sequence repeat), and found that the polymorphism on their DNA is clearly distinct[11]. To our knowledge, this was the first demonstration that high-pressure can indeed induce variation at the DNA sequence level.

The stable morphological variation in an organism may be caused by physical changes of its DNA sequence or by heritable epigenetic modifications on its chromatin such as chromatin remodeling and DNA methylation[12]. To further study the molecular basis of morphological variation in the two rice mutant lines, we conducted the present study. Specifically, we aimed to (1) test if there has been epigenetic changes in the mutant lines, and for this purpose, we analyzed genomic DNA digested by a pair of isoschizomers, *Hpa* II and *Msp* I, that has differential sensitivity to cytosine methylation at the CCGG sites by ISSR and RAPD markers, followed by Southern blot analysis; (2) study possible changes in genomic structure, and for this purpose, we used as probes, various DNA fragments, including selected genes, a MITE (miniature inverted-repeat transposable element) transposon family[13−15]. and a set of LTR (long-terminal repeat) type of retrotransposons[17,18]

1 Materials and Methods

1.1 Plant materials and high-pressure treatment

A stable *japonica* rice cultivar Bijing38 and two mutant lines derived from this cultivar after high-pressure treatment were used in this study. Morphological deciphers of these three rice lines can be summarized as follows: (1) Bijing38: with an average plant height of *ca.* 85 cm, an overall erect statue and ellipsoid-shaped kernels with long awn. (2) Mutant line 1: with an apparently reduced number of tillers, shortened growth period by 20 d and awnless kernels. (3) Mutant line 2: with a significantly increased number of tillers and *indica* rice-like, slender awnless-kernels.

High pressure apparatus used to produce the two mutants is a 3X15MN oil-pressure apparatus with a high pressure container up to 400 MPa (manufactured by Institute of Physics, Chinese Academy of Sciences). The diameter of the up piston is 200 mm with a driving force of 3 MN. The diameter of down piston is 130 mm with a driving force of 1.5 MN. The inner diameter of the pressure container is 69 mm, and the depth is 134 mm. The container is pressurized through O-shape circles, which are made of nitrile-rubber. The highest pressure can reach up to 400 MPa.

Treatment: About 600 seeds of Bijing38 were treated by continuous high pressure at 100 MPa for 24 h. Mutant line 1 was selected among 30 survived plants, which showed apparent phenotypic variations at the first generation. About 600 seeds of Bijing38 were treated by continuous high pressure at 300 MPa for 20 min. Mutant line 2 was selected among 30 survived plants, which showed apparent phenotypic variations at the first generation.

1.2 Maintenance and propagation of plant material

The two mutants were obtained in Guangzhou, Guangdong Province, China. The mutants had been maintained along with their exact parental lines by strict selfing for 3—6 generations to develop into mutant lines. For the present study, the original cultivar and two stabilized mutant lines were further planted for one more generation in Jilin Academy of Agricultural Sciences, Gongzhuling, Jilin Province, China.

1.3 DNA isolation and molecular marker analysis

Genomic DNA was isolated from expanded leaves of Bijing38 and the two mutant lines by a modified CTAB method, then purified by phenol extractions. The DNA was either undigested or digested by a pair of isoschizomers, *Hpa* II and *Msp* I (New England Biolabs Inc.) for 24—48 h. Complete digestion was ensured by using an excess amount of enzymes and by gel-inspection. The DNA samples were then subjected to analysis by two molecular markers, ISSR and RAPD. Detailed conditions for ISSR and RAPD analysis were as reported earlier[11].

1.4 PCR amplification and assay for transposon mPing

To detect possible *mPing* excisions, a set of 53 pairs of locus-specific primers each bracketing an intact

mPing in the standard laboratory cultivar for the *japonica* rice ssp., Nipponbare (http://rgp.dna.affrc.go.jp), was designed by the Primer 3 software (http://biocore.unl.edu/cgi-bin/primer3/primer3_www.cgi)[19]. The 20 μL PCR amplification reaction contains 2 μL 10× buffer, 2.5 μL 25 mmol/L $MgCl_2$, 2.5 μL dNTP (2.5 mmol/L), 10 μmol/L primer 1, 10 μmol/L primer 2, 1 U *Taq* DNA polymerase, and 50 ng template DNA. Conditions for amplification are: 94℃ for 3 min, 94℃ for 1 min, 58℃ for 1 min, 72℃ for 1 min, 30 cycles from the second step, and a final extension of 72℃ for 7 min. Amplified products were examined by electrophoresis of 1% agarose gels in TAE buffer.

1.5 Gel-purification and sequencing

Bands of possible *mPing* excisions representing putative empty donor sites were isolated and purified with an UNIQ-10 DNA-purification kit (Shanghai Sangon Biotech. Co., Ltd), and sequenced by automatic sequencing (Shanghai Sangon Biotech. Co., Ltd).

1.6 Southern blot analysis

Genomic DNA (3 μg per lane) was digested by *Eco*R I, *Hin*d III, *Hpa* II or *Msp* I (New England Biolabs Inc.) for 24—48 h. An excess amount of enzyme was used and samples of DNA were monitored by agarose gel electrophoresis to ensure the digestion was complete. Digested DNA was fractionated by running through 1% agarose gels for 24—36 h, and transferred onto Hybond N^+ nylon membranes (Amersham Pharmacia Biotech) by the alkaline transfer recommended by the supplier. Probes S2 (AB042550) and S3 (AY328087) were specific fragments of two drought-resistant genes in rice, which had been amplified in our laboratory. Probes of *Osr7*, *Osr36* and *Tos19* are fragments of the RT (reverse transcriptase) regions of the respective LTR-type retrotransposons. Probes for *mPing* and *Pong* were PCR amplified specific fragments of the MITE transposon family *mPing* and *Pong*[19]. All probes were labeled with fluorescein-11-dUTP by the Gene Images random prime-labeling module (Amersham Pharmacia Biotech). Hybridization signals were 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 film for 1—3 h depending on signal intensity.

2 Results and analysis

2.1 The pressure-induced morphological variations are highly stable across different growing environments

The original cultivar, Bijing38 and the two mutant lines were obtained by high-pressure treatments in Guangzhou[11]. The mutant lines were propagated by selfing for 3—6 generations, and the changed traits (see sec. 1) were stable. For this study, the two mutant lines were grown in Gongzhuling, Jilin Province, China, and we found that all the changed morphological traits except one were faithfully maintained. The only changed trait that was not faithfully inherited was early maturity of mutant line 1, which actually became late-maturing (about 8—10 d later than the original cultivar, Fig. 1). All other changed traits including the most conspicuous one, the seed-shape, showed obvious true-to-type inheritance (Fig. 2).

2.2 Changes in DNA methylation patterns in the two lines as revealed by methylation-sensitive ISSR, RAPD and Southern blot analysis

Genomic DNA, undigested or digested by *Hpa* II/*Msp* I, was analyzed by ISSR and RAPD markers. The amplification patterns of both undigested and digested DNA showed distinct polymorphism between the original cultivar and each of the two mutant lines (Fig. 3). Meanwhile, Southern blot analysis using two stress (drought)-responsive cellular genes, S2 and S3, as probes, also revealed obvious changes in the DNA methylation patterns between the original cultivar and the two mutants. Thus, the coupled changes in ISSR and RAPD markers, as well as in gel-hybridization patterns in the two rice mutant lines relative to their original cultivar have provided unequivocal evidence that extensive and heritable alteration in DNA methylation had occurred in rice as a result of high pressure treatment.

2.3 Changes in genomic structure as revealed by cellular genes, a set of LTR retrotransposons and a MITE transposon family

To study if there had been changes in genome

Fig. 1. Plant morphology of the two pressure-induced mutant lines and the wild-type cultivar Bijing38, at the stage of maturity.

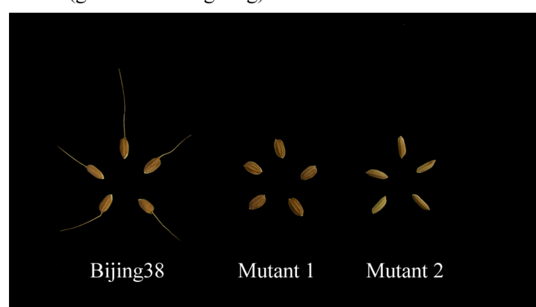

Fig. 2. Kernel morphology of the two pressure-induced mutant lines and the wild-type cultivar Bijing38. These phenotypic novelties were stably inherited upon selfing, and under different growing areas, in Guangdong and Jilin provinces

structure in the two mutant lines, we prepared DNA gel-blots carrying genomic DNAs of the two lines and their original cultivar, which were digested by two methylation-insensitive enzymes, *Eco*R I and *Hin*d III. We used as probes, the two stress-responsive cellular genes (S2 and S3), a set of LTR retrotransposons (including *Tos10*, *Tos19*, *Osr7*, *Osr23*, *Osr36*, *Osr42* and *Osr43*), and a MITE transposon family (*mPing* and *Pong*). Results indicated that 7 of the 11 probes studied, including S2, S3, *Osr7*, *Osr36*, *Tos19*, *mPing* and *Pong*, showed apparent genomic changes in both mutant lines (Fig. 4). The changed patterns included increase or decrease of copy numbers, and alterations in restriction site(s) of the enzymes. We consider that at least some of the changes in retrotransposns and the MITE transposon family were likely due to transpositional activity of these elements after the high pressure treatments.

2.4 Loss of mPing copies may suggest their transpositional excision

We used a set of previously designed locus-specific primers bracketing *mPing* (53 pairs) on the *japonica* rice standard laboratory cultivar, Nipponbare[19], to analyze the two mutant lines. PCR amplifications were performed on genomic DNA of the two mutant lines and their original cultivar Bijing38. We identified 16 *mPing*-containing loci in this cultivar, although three loci, *mPL*15, *mPL*18 and *mPL*44, are heterozygous. The *mPing* of 8 loci were absent in mutant line 1, and of 12 loci were absent in mutant line 2. Some typical results are presented in Fig. 5. Sequence analysis on these PCR products indicated that they most probably resulted from transpositional excisions of the element, hence representing empty donor sites (data not shown).

2.5 The genetic and epigenetic alterations in DNA molecular structure and modification are stable over generations and in different organs

Although the morphological variations were highly stable across environments (in Guangdong and Jilin), a further confirmation for the stability of the genetic and epigenetic variations at the DNA molecular level still needed to be investigated. For this purpose, genomic DNAs were isolated from the seedlings and radicles of

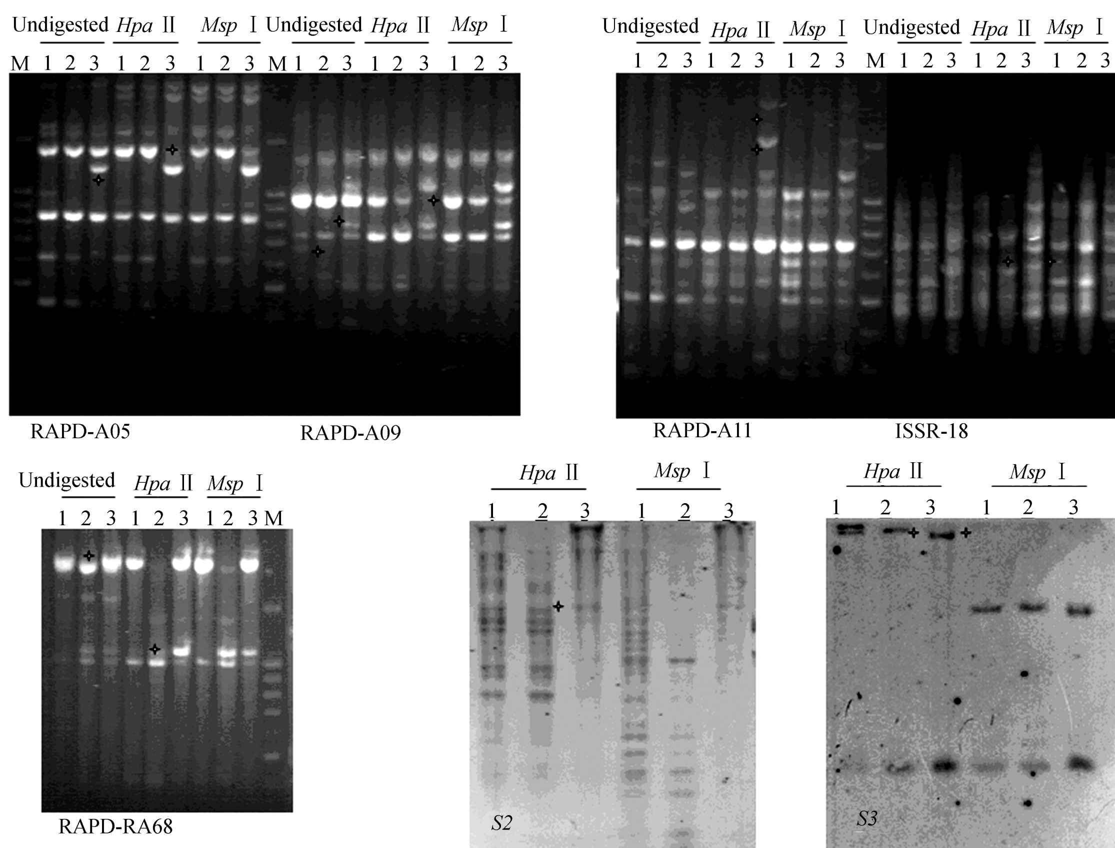

Fig. 3. Methylation-sensitive ISSR and RAPD amplification profiles and Southern blot analysis with two stress-responsive genes as probes on *Hpa* II or *Msp* I digested genomic DNA of the two mutant lines (lanes 2 and 3) and the original wild-type cultivar Bijing38 (lane 1). Primers and enzymes used are indicated. The asterisks denote changed bands due to methylation alterations in the mutant lines relative to the wild-type cultivar. M, the 100 bp DNA size marker.

different seeds in 2002, 2003 and 2004 (grown in Jilin), and adult leaves at the milking stage (grown in Jilin in 2004). The DNAs were subjected to both *mPing* amplification-assay and Southern blot analysis with selected probes. In all cases the same results as observed earlier were obtained, indicating that both the genetic and epigenetic variations are stable over mitotic cell divisions, in the course of development, and even in meiotic transmission (Fig. 6 and data not shown).

3 Discussion

All but one distinct phenotypic novelty in the two rice mutant lines (derived from a genetically stable cultivar Bijing38 after high-pressure treatment) is stable in different growing areas, Guangdong and Jilin provinces. The only exception being the date of maturity, which is understandable, gives that rice is an extremely photoperiod-sensitive plant.

Generally speaking, the heritable morphological variations in an organism can be caused by genetic changes in DNA sequence or by epigenetic modification in DNA and/or chromatin proteins[12]. Of course, not all genomic changes (genetic or epigenetic) can be translated into morphological variations. For example, a mutation in non-transcribed genomic regions (i.e. introns) and DNA methylation in heterochromatic areas usually do not have phenotypic consequences. In addition, synonymous mutations that do not cause a change in the encoded amino acid will not produce morphological mutations. In this regard, the fact that both mutant lines expressed multiple novel traits strongly suggests that the genetic and/or epigenetic changes occurring in these two lines were extensive, as was indeed verified by molecular studies.

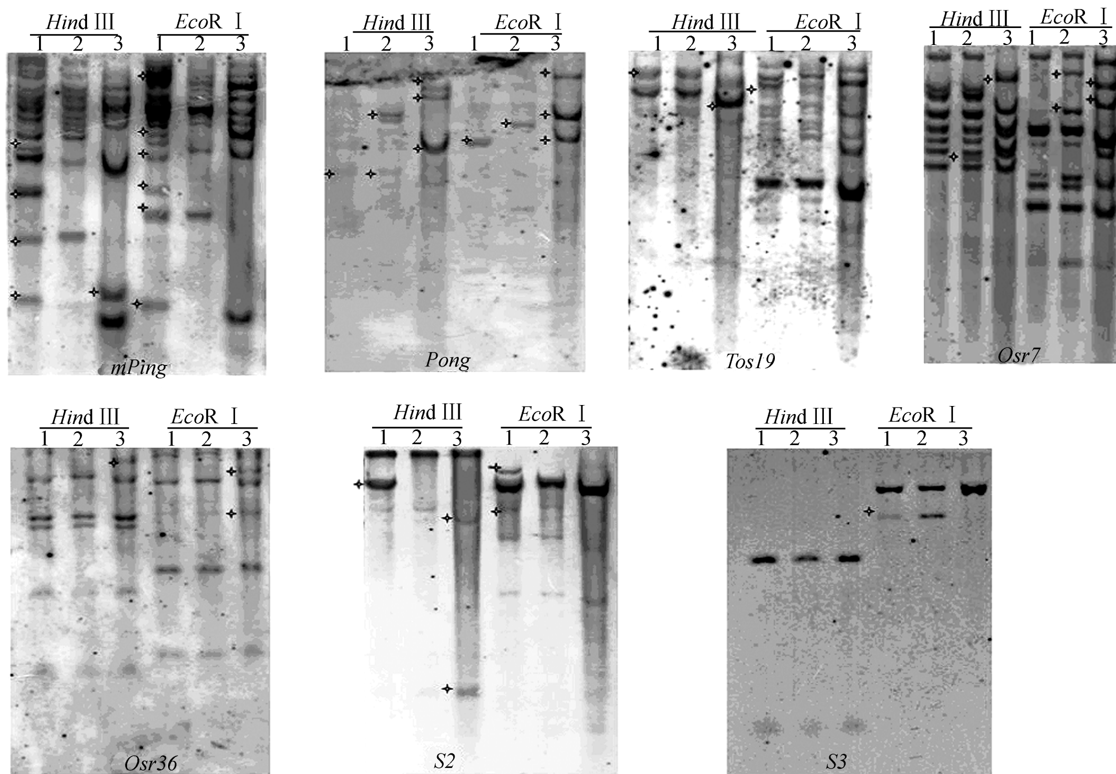

Fig. 4. Changes in genomic structure in the two mutant lines (lanes 2 and 3) as compared with their wild-type cultivar (lane 1), as detected by Southern blot analysis using methylation-insensitive enzymes, *Eco*R I and *Hin*d III, and probed by a set of LTR-retrotransposons, a MITE transposon family and the two stress-responsive genes. Enzymes used and probe names are indicated. The asterisks denote changed bands due to structural mutations in the mutant lines relative to the wild-type cultivar.

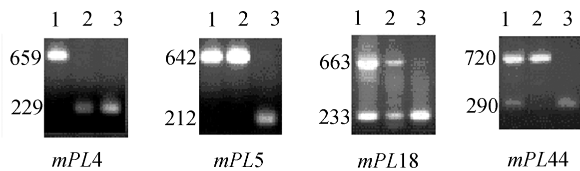

Fig. 5. Results of PCR-based *mPing* amplification analysis. Lanes 1—3, The wild-type rice cultivar Bijing38, and the two mutant lines, respectively. Primers used are marked at the bottom. Sizes of the amplification products are indicated.

3.1 High pressure has caused alterations in DNA methylation pattern in rice

Hpa II and *Msp* I are a pair of isoschizomers recognizing the same tetranucleotide site, CCGG, but having different sensitivities to methylation at the inner and outer cytosines. Thus, by incorporating restriction by the enzyme pairs with a detection method based either on PCR amplification like ISSR and RAPD, or on gel blot hybridization, will enable the detection of DNA methylation changes of the cytosines at the CCGG sites. By using these methods, we found that alterations in DNA methylation pattern apparently occurred as a result of high-pressure treatment. Moreover, the affected sequences appeared diverse, as both the two stress-responsive cellular genes, S2, S3, and transposable elements detected the alterations.

3.2 High pressure has caused changes in DNA structure in rice

Based on the appearance of novel bands and disappearance of original bands in undigested genomic DNA of the two mutant lines in ISSR and RAPD amplification profiles, it was suspected that there had been wide-spread changes at the DNA level as a result of high-pressure treatment (see sec. 2 and ref. [11]). Nevertheless, because PCR-based analysis may introduce artifacts, the detected changes need to be validated by an independent approach. Thus, the results on Southern blot analysis also showing extensive changes in various sequences including cellular genes, the LTR-type retrotransposons and a MITE transposon family have unequivocally verified the issue.

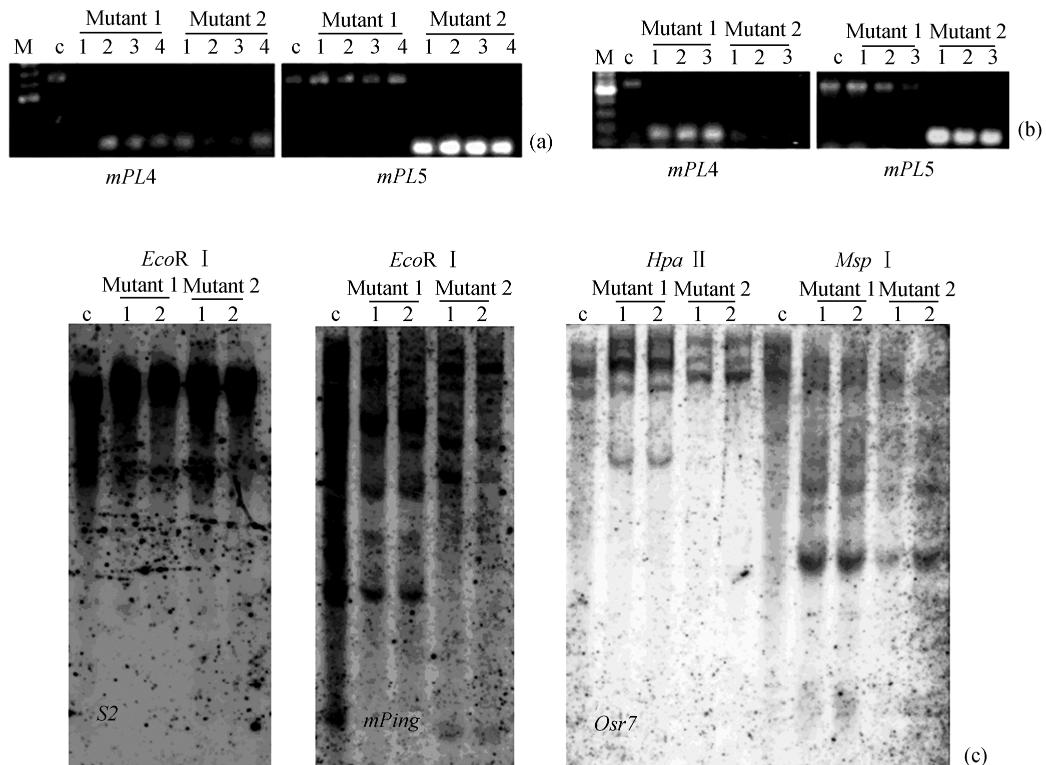

Fig. 6. Examples of the stable inheritance of the genetic and epigenetic changes in the two mutant lines across selfed generations and in different organs. (a) Results of PCR-based *mPing* amplification analysis. Lane c, The wild-type rice cultivar Bijing38; lanes 1—4, the seedlings germinated from seeds harvested in three consecutive years, 2002, 2003 and 2004 as well as the adult leaves at the milking stage in plants growing in 2004, respectively; M, the 100 bp DNA size marker. Primers used are marked at the bottom. (b) Results of PCR-based *mPing* amplification analysis. Lane c, The wild-type rice cultivar Bijing38; lanes 1—3, radicles of seeds harvested in 2002, 2003 and 2004, respectively; M, the 100 bp DNA size marker. Primers used are marked at the bottom. (c) Results of Southern blot analysis. Lanes 1 and 2, Seedlings germinated from seeds harvested in 2003 and 2004. Enzyme used and probes are indicated at the bottom.

It is particularly interesting that we found most of the genomic changes in retrotransposons and the MITE transposon, *mPing* and *Pong*, involved increase and/or decrease in copy numbers, as this may imply transpositional activation of the elements. It is well documented that these elements are largely quiescent under normal conditions, but can be activated by various stresses, like tissue culture[13,14], pathogen infection[12,19], and wide hybridization[19]. In this sense, it can be envisioned that high pressure may also represent a kind of abiotic stress, whereby dormant elements can be activated. In the case of *mPing*, we screened 53 pairs of primers designed based on the complete genome sequence of Nipponbare[19]. As a result, we found 16 loci in Bijing38, which contained *mPing*. Of these 16 loci, 8 and 12 loci were respectively absent from the two mutant lines compared with the original cultivar Bijing38 (see sec. 2). We assume that these changes were caused by transpositional excision of *mPing*. Indeed, sequence analysis confirmed the possibility, as there were no other changes except the clean loss of *mPing*, which has been documented earlier in a set of rice introgression lines as one of the canonical transpositional characteristics of *mPing*[19]. Because *mPing* has no coding capacity, the transposase required to catalyze its transposition needs to be provided by a related autonomous element[13,16]. Based on sequence homology and co-mobilization with *mPing*, an element called *Pong* is implicated as the possible autonomous element responsible for *mPing* mobilization[13,16]. In accord with this, Southern blot analysis with *mPing* and *Pong* as probes showed that both elements detected changes in the two mutant lines, thus further supporting the possibility of co-ac-

tivation of *mPing* and *Pong* by high pressure.

Transposable elements are ubiquitous genomic components of all eukaryotes. They comprise two main types based on the model and mechanism of their transposition: class I elements (involving a reverse transcription process in transposition) and class II elements (transpose directly by DNA). The LTR-type retrotransposons belong to class I elements, whereas the MITE type belongs to class II elements. Because transposable elements play important roles in the structure and function of their host genomes, they have been widely studied[13−21]. In this respect, the possible activation and extensive changes in these elements after high pressure treatment should bear significant biological and practical implications.

Although there have been numerous reports on the effects of high pressure on microorganisms[3], mechanistic issues are largely unclear. It is believed that the double helix of DNA molecular is more stable than protein molecules under high-pressure conditions, as for example, the covalent bond of DNA is stable under 1200 MPa[5]. Therefore, we assume that the changes in DNA sequence, alterations in DNA methylatiom pattern and possible activation of transposable elements observed in this study are probably not directly caused by the pressurization on DNA molecules. Instead, it is more likely that the fidelity of some cellular control systems, like the mismatch repair and systems controlling chromatin transcription, was impaired by the high-pressure treatments. In addition, pressure may act as a stress signal that can also induce alterations in DNA modification (e.g. methylation) and RNA transcription.

Acknowledgements This study was supported by the National Emphases Research Foundation of China (Grant No. 2001CB711201), the Jilin University Innovation Foundation, and the National Science Award for Distinguished Young Investigators in China (Grant No. 30225003)

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