Transgenerational memory of gene expression changes induced by heavy metal stress in rice (Oryza sativa L.)

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Abstract

Background: Heavy metal toxicity has become a major threat to sustainable crop production worldwide. Thus, considerable interest has been placed on deciphering the mechanisms that allow plants to combat heavy metal stress. Strategies to deal with heavy metals are largely focused on detoxification, transport and/or sequestration. The P1B subfamily of the Heavy Metal-transporting P-type ATPases (HMAs) was shown to play a crucial role in the uptake and translocation of heavy metals in plants. Here, we report the locus-specific expression changes in the rice HMA genes together with several low-copy cellular genes and transposable elements upon the heavy metal treatment and monitored the transgenerational inheritance of the altered expression states. We reveal that plants cope with heavy metal stress by making heritable changes in gene expression and further determined gene-specific responses to heavy metal stress.

Results: We found most HMA genes were upregulated in response to heavy metal stress, and furthermore found evidence of transgenerational memory via changes in gene regulation even after the removal of heavy metals. To explore whether DNA methylation was also altered in response to the heavy metal stress, we selected a Tos17 retrotransposon for bisulfite sequencing and studied its methylation state across three generations. We found the DNA methylation state of Tos17 was altered in response to the heavy metal stress and showed transgenerational inheritance.

Conclusions: Collectively, the present study elucidates heritable changes in gene expression and DNA methylation in rice upon exposure to heavy metal stress and discusses implications of this knowledge in breeding for heavy metal tolerant crops.

Keywords: Heavy metal stress, Transgenerational memory, Gene expression, DNA methylation

Background

Plants are sessile organisms and are often confronted with a variety of stress factors simultaneously, which can dramatically decrease their yield and quality. In the recent years, heavy metal pollution, i.e., contamination of the natural environment with cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), and zinc (Zn) has become a global problem, affecting about 235 million hectares of the arable land worldwide [1]. Heavy metals compromise crop productivity and pose a threat to human health via heavy metal accumulation in the food chain [2]. In plants, heavy metals interfere with several metabolic processes including photosynthesis, water relations, and nutrient uptake, resulting in reduced plant growth, stunting, and in some instances, death [3, 4]. Cu is an essential micronutrient; however, if present in excess it also causes toxicity to plants [5]. Cr is also a common metal contaminant in the Earth’s crust. While

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Metal-transporting P-type ATPases (HMAs) play a role in heavy metal homeostasis [18]. In the Cd/lead (Pb) group and a copper (Cu)/silver (Ag) group, these ATPases can be divided into two subgroups: a zinc (Zn)/cobalt (Co)/cadmium (Cd) metal-substrate specificity and regulation, which all indicate unique features in expression sub-cellular localization, and metal specificity and regulation, which all indicate unique functions within the gene family. For instance, AtHMA1, AtHMA5-AtHMA8 were reported to play a role in Cu transport [19–22]. AtHMA2-AtHMA4 were involved in Cd translocation and sequestration [23–25]. In contrast, the rice HMA transporter family is not as well characterized. For instance, OsHMA1 and OsHMA9 were postulated to play a role in Zn transport [26, 27]. OsHMA2 and OsHMA3 were reported to be involved in the transportation of Cd [28–30], OsHMA4 and OsHMA5 have a function in Cu transport, loading, and detoxification [31, 32]. However, little research has been performed on OsHMA6, OsHMA7, and OsHMA8.

Modulation of gene expression is one rapid strategy to respond to environmental stresses. It has been repeatedly shown that heavy metal stress induces changes in gene expression. For instance, transcript profiling of the Cd-tolerant cultivar of Chinese flowing cabbage revealed numerous changes in gene expression in response to Cd treatment including upregulation of HMA3 and HMA4 [33]. Research in Sedum plumbeum showed elevated expression of the SphMA3 gene in response to Cd stress suggesting a role in Cd detoxification and normal growth of young leaves under Cd stress [34]. Similarly, in Lycopersicum esculentum, heavy metal transporters COPT1 and COPT2 could be induced to express under Cu stress [35]. Functional genomics tools have been extensively used to examine mechanisms conferring tolerance to various heavy metal stresses. In a recent report, genome-wide transcriptome analysis in rice showed dose-dependent changes in expression of metal ion transporter genes in response to Cd stress [36].

One way to maintain changes in gene expression is via epigenetic modification. Indeed, epigenetic variation contributes to phenotypic plasticity in response to the environmental changes [37]. In particular, DNA methylation is an important epigenetic marker, which regulates gene expression as an adaptive mechanism for survival under stress. In a recent study, genome-wide single-base resolution maps of methylated cytosines and transcript profile of Cd-treated rice was reported [38]. The study showed that most of the epigenetically regulated genes were transcriptionally activated under Cd stress, and many of these genes represent formerly characterized stress responders, metal transporters and transcription factors [38]. Despite initial progress, implementation of these epigenetic markers in plant breeding has stalled because the heritability of these makers has not yet been tested [37].

Since rice (O. sativa L.) is one of the major staple grains worldwide, increasing its productivity and nutritional quality is one of the foremost priorities. In the interest of ensuring food security and better nutritional quality, it is important to reduce the accumulation of toxic elements in rice grains [39, 40]. A deep understanding of the genes responsible for the sequestration of toxic elements can enable the development of crop varieties with reduced content of these elements in the edible plant parts. Our previously, work has shown that heavy metal stress (Cd, Cr, Cu, and Hg) could inhibit...
further shoot and root development of the ten-day-old rice seedlings and induce transgenerational changes in their DNA methylation pattern at specific loci [41]. Rice plants were treated with two different concentrations of Cd, Cr, Cu, or Hg to determine dose-dependent responses to these heavy metals. As expected, more hypomethylations were observed at specific-loci on the higher doses of Cd, Cr, and Cu but no change in DNA methylation pattern was witnessed upon Hg treatment. Remarkably, the progeny of the stressed plants exhibited enhanced tolerance to the same stress their progenitors experienced and showed the transgenerational inheritance of changes in the DNA methylation patterns [41]. The aim of this study was to address whether locus-specific changes in gene expression also take place in response to the heavy metal stress and whether different classes of genes have common or specific responses to heavy metal stress.

Results
Heavy metal stress induced locus-specific gene expression changes in the S0 plants
We previously showed that heavy metals elicit epigenetic changes in DNA methylation patterns of specific loci and in a transgenerational manner [41]. In the present study, we addressed whether locus-specific changes in gene expression also take place in response to the heavy metal stress and whether different classes of genes have common or specific responses to the heavy metal stress. To test this possibility, we assessed the expression of 18 randomly-distributed and functionally diverse genes by reverse transcription (RT)-PCR in the heavy-metal stressed rice seedlings (Fig. 1). Out of these 18 genes, two (Tos17 and Osr42) were formerly tested by us to respond epigenetically to the heavy metal stress, seven (Homeobox gene, DNA-binding protein, Elongation factor, HSP70, SNF-FZ14, S3, and YF25) were randomly distributed cellular genes, and nine genes (OsHMA1-OsHMA9) were known to be heavy metal transporters. This panel of genes allows testing if global or specific transcriptional changes are involved in heavy-metal stress avoidance or mitigation in rice. In the S0 generation, plants for expression analysis were selected on the basis of the gel-blot analysis. Specifically, S0 plants that showed the most conspicuous modifications in DNA methylation patterns under Cu2+ (1000 μM), Cd2+ (1000 μM), Cr3+ (1000 μM) and Hg2+ (50 μM) treatments were selected for the expression analysis [41].

Interestingly, we found two rice TE (transposable element) genes, the Tos17 and Osr42 that showed significantly up-regulated expression under all or three of the four heavy metal treatments (Fig. 1 and Table 1). Specifically, for Tos17, there are two copies in wild-type rice cv. Nipponbare, one located on chromosome 10 dubbed Tos17A, and the other located on chromosome 7 called Tos17B. The two Tos17 copies are identical except for a 90 bp insertion in Tos17A [42]. We designed gene-specific primers to study expression changes in the two copies under heavy metal stress. The results showed that the two copies of Tos17 seldom exhibit activation of gene expression under all four (100%) heavy-metal treatments (S0 plants), particularly under Cd stress. Similarly,
Osr42 showed a significantly up-regulated expression under three (Cu, Cr, and Hg) of the four (75%) heavy metal treatments. The two TE genes exhibited contrasting expression patterns in Cd-treated plants, while Tos17 showed the most conspicuous activation of gene expression, Osr42 exhibited no change in expression.

In addition, among seven low-copy cellular genes (Homeobox gene, DNA-binding protein, Elongation factor, HSP70, SNF-FZ14, S3, and YF25), five of the genes (Homeobox gene, DNA-binding protein, Elongation factor, HSP70, and SNF-FZ14) showed transcriptional upregulation in all (100%) heavy metal treated plants (Fig. 1 and Table 1). Whereas, YF25 showed significant down-regulation under Cd treatment to complete suppression under other heavy metal treatments (Cu, Cr, and Hg), and S3 exhibited no change in expression under any of the tested heavy metal treatments.

We also tested the nine rice HMAs (OsHMA1-OsHMA9) and found that 7 HMAs showed significant up-regulation under at least one of the four heavy metal treatments (Fig. 1 and Table 1). Specifically, OsHMA1 showed up-regulated expression in Cd and Hg-treated plants (two of the four heavy metal treatments; 50%). Similarly, OsHMA2 showed significantly up-regulated expression in Cu-treated plants (one of the four heavy metal treatments; 25%). OsHMA5 showed significant transcriptional activation under Cu, Cd, and Hg treatments (three of the four heavy metal treatments; 75%). OsHMA6 and OsHMA7 showed transcriptional activation under all four (100%) heavy metal treatments. OsHMA8 showed significant transcriptional activation in Hg and Cr treated plants (two of the four heavy metal treatments; 50%), whereas OsHMA9 showed significant transcriptional activation in Cd and Hg treated plants.

**Table 1** Gene expression changes observed for the 18 functionally diverse random genes in heavy metal treated seedlings of rice cv. Matsumae (S0 generation)

| Gene name | Genbank acc.a | Chr.b | Gene expression changes observed in heavy metal treated plants of S0 generationc |
|-----------|---------------|-------|---------------------------------------------------------------------------------|
|           |               |       | Cu2+ (1000 μM L−1) | Cd2+ (1000 μM L−1) | Cr3+ (1000 μM L−1) | Hg2+ (50 μM L−1) | Freq. (%)  |
| Transposable elements (TEs) |               |       | U | U | U | U | 100 |
| Tos17     | AC087545(Tos17A) | 10    | U | U | U | U | 100 |
| Osr42     | AF458768       | 4     | N | U | U | U | 75  |
| Low copy protein-coding genes |               |       | U | U | U | U | 100 |
| Homeobox gene | AB007627       | 2     | U | U | U | U | 100 |
| DNA-binding protein | X88798       | 5     | U | U | U | U | 100 |
| Elongation factor | D12821       | 7     | U | U | U | U | 100 |
| HSP70     | X67711         | 11    | U | U | U | U | 100 |
| SNF-FZ14  | DQ239432       | 7     | U | U | U | U | 100 |
| S3        | AY328087       | 12    | N | N | N | N | 0   |
| YF25      | DQ239435       | 11    | D | D | D | D | 100 |

Rice P18 subfamily of Heavy Metal-transporting P-type ATPases (HMAs)

| Genbank acc. | Chr. | Cu2+(1000 μM L−1) | Cd2+(1000 μM L−1) | Cr3+(1000 μM L−1) | Hg2+(50 μM L−1) | Freq. (%)  |
|--------------|------|------------------|-------------------|-------------------|-----------------|-------------|
| OsHMA1       | AP003935       | 6     | N | U | N | U | 50  |
| OsHMA2       | AP004278       | 6     | U | N | N | N | 25  |
| OsHMA3       | AP005246       | 7     | – | – | – | – | –   |
| OsHMA4       | AP004184       | 2     | N | N | N | N | 0   |
| OsHMA5       | AL606647       | 4     | U | U | N | U | 75  |
| OsHMA6       | AP004836       | 2     | U | U | U | U | 100 |
| OsHMA7       | AP004376       | 8     | U | U | U | U | 100 |
| OsHMA8       | AC125472       | 3     | N | N | N | U | 50  |
| OsHMA9       | AP008212       | 6     | N | U | N | U | 50  |

Totald (%) | 72.2 | 72.2 | 66.7 | 83.3 |

Note: *Determined by BlastN searches performed at NCBI

bChanges in gene expression pattern were defined as: N = No change in gene expression; U = Significantly up-regulated expression; D = Significant down-regulated expression; and - = No expression

cNumber of times a gene responded similarly to different heavy metal stresses; represented as percentage in the table

dNumber of times a heavy metal stress affected different genes in a similar fashion; represented as percentage in the table. For calculations the two copies of the Tos17 were treated separately.
(two of the four heavy metal treatments; 50%). OsHMA4 did not show significant transcriptional changes under any of the four heavy metal treatments, and OsHMA3 showed no expression either in plants treated with any of the heavy metals or mock plants.

Taking the results of all four heavy metal treatments together, (i) different genes responded from none (0%) to all (100%) studied heavy metal treatments by exhibiting alterations in their respective expression patterns. Specifically, 10 of the 18 genes responded to all four heavy metal treatments by transcriptional upregulation. Interestingly, TEs and the low-copy number protein-coding genes showed more transcriptional plasticity than HMAs under heavy metal stress. (ii) With respect to the number of genes that showed transcriptional changes in response to heavy metal stress, Hg treatment induced changes in expression patterns of the maximum (83.3%) number of genes followed by Cu/Cd (72.2%), and Cr (66.7%) treatments. (iii) With respect to type (up- or down-regulation) of the gene expression changes occurring in response to the heavy metal treatment, all genes responded by up-regulation of expression, except YF25 that showed transcriptional downregulation and S3, which exhibited no change in expression pattern (Table 1).

The altered gene expression patterns were transgenerationally inherited, coupled with additional alterations in the S₁ generation

To test if the altered gene expression state of the S₀ plants would be maintained in the next generation, we selfed a single Hg²⁺ (50 μM) treated plant, as this treatment induced gene expression changes in the majority of the studied genes (83.3%) (Table 1). Later, the leaf-tissue collected from the S₁ seedlings growing under optimal conditions was subjected to transcript profiling of 14 genes including two transposable element genes, four cellular genes, and eight OsHMAs. All fourteen genes tested here showed transcriptional changes in Hg-treated S₀ plants. We divided the expression state of S₁ progeny into three patterns of expression: inheritance of Hg-treated S₀ pattern, reversion to the mock pattern, and a differential expression pattern. The last category was further divided into two sub-categories: transgenerational memory (further up-regulated expression pattern) and other (cf. Fig. 2 and Table 2).

Specifically, for the two copies of Tos17 (Tos17A and Tos17B), the S₁ progeny either exhibited inheritance of the S₀ expression pattern (62.5% for Tos17A and 12.5% for Tos17B) or further up-regulation of it (37.5% for Tos17A and 87.5% for Tos17B) (Fig. 2 and Table 2). Similarly, for Osr42, 100% S₁ progeny showed further up-regulation of the S₀ expression pattern.

Out of four low-copy number protein-coding genes (Fig. 2 and Table 2), for Homeobox gene and HSP70, the majority of S₁ progeny (75% for Homeobox gene and 87.5% for HSP70) exhibited stable inheritance of the S₀ expression pattern, and the remainder (25% for Homeobox gene and 12.5% for HSP70) showed reversal to the mock expression pattern. On the other hand, YF25 which showed significant down-regulation in the S₀ generation, exhibited inheritance of the altered expression state, reversal and novel gene expression pattern in the S₁ progeny at frequencies of 25, 37.5, and 37.5%, respectively. For SNF-FZ14, which showed transcriptional activation in S₀ generation exhibited further up-regulated expression pattern in the majority (75%) of the S₁ plants and exhibited inheritance of the altered expression state in the remaining 25% of the progeny.

For the eight OsHMAs tested (Fig. 2 and Table 2), all showed up-regulated expression in S₀ plants compared to the mock-treated plants, but differences were found in the S₁ generation: OsHMA1 showed further up-regulated expression in 100% progeny. OsHMA2 showed 50% inheritance of up-regulated expression and reversal to the basal expression state in 50% of the progeny. OsHMA4 showed the inheritance of the S₀ expression state in 37.5% of the progeny and reversal to the basal expression state in 62.5% of the progeny. OsHMA5 showed inheritance, reversal and further up-regulated expression patterns in 50, 25, and 25% of the S₁ plants, respectively; OsHMA6, OsHMA7, and OsHMA8 showed inheritance of the altered expression state in 25, 12.5, and 62.5% of the S₁ progeny, and further up-regulated expression in 75, 87.5, and 37.5% of the progeny. OsHMA9 showed significantly up-regulated expression in the S₀ plants, and all S₁ progeny (100%) inherited the expression pattern.

In summary, we found that for those genes that showed changes in expression in the S₀, two major gene expression patterns were manifest in the S₁ progeny: either inheritance of the S₀ expression pattern (41.7%) or adaptation to a new expression pattern (51.7%). However, the maintenance of change in gene expression varied among the genes tested. For instance, some genes (Tos17A, Homeobox gene, HSP70, OsHMA2, OsHMA5, OsHMA8 and OsHMA9) exhibited inheritance of the expressed state from S₀ to S₁ generations in ≥50% progeny plants, whereas other genes (Tos17B, Osr42, SNF-FZ14, OsHMA1, OsHMA2, OsHMA6, and OsHMA7) showed a further up-regulated expression in ≥50% progeny plants suggesting genetic memory of the altered expression pattern gained in response to the heavy metal treatment that is transmitted to the next generation.
The altered gene expression states were transgenerationally persistent, coupled with the genetic memory in the S\textsubscript{2} generation

To further test if the altered expression states are transgenerationally persistent, we selected one S\textsubscript{1} plant (plant #3) that exhibited all three expression patterns for several of the tested genes, i.e., inheritance of the S\textsubscript{0} expression pattern, reversal to the basal expression pattern and adaption of a new expression pattern, to obtain S\textsubscript{2} progeny. To study the expression pattern, we performed the RT-PCR analysis of seven genes (\textit{Tos17}, \textit{SNF-FZ14}, \textit{OsHMA1}, \textit{OsHMA2}, \textit{OsHMA6}, \textit{OsHMA7}, and \textit{OsHMA9}) in the leaf-tissue of 14 randomly selected S\textsubscript{2} individuals grown under optimal conditions. The seven genes selected for RT-PCR analysis showed increased expression in the S\textsubscript{0} generation and exhibited different expression patterns in the S\textsubscript{1} generation. Of the seven genes tested, we identified four gene expression patterns in the S\textsubscript{2} progeny, i.e., the inheritance of the S\textsubscript{1} expression state, reversion to the S\textsubscript{0} expression state, reversion to the mock expression state, and a novel expression pattern (Fig. 3 and Table 3). We observed the majority of S\textsubscript{2} progeny inherited the expression state of the S\textsubscript{1} progenitor, 36.6% progeny showed inheritance of the S\textsubscript{1} expression state, 22.3% progeny reverted to the S\textsubscript{0} expression state, 22.3% progeny showed reversal to the basal expression state (similar to mock), and the remaining 18.8% progeny adopted a new expression pattern.

On gene by gene basis, the proportions of S\textsubscript{2} progeny following one of the four expression patterns (see above) also varied, for instance, in case of \textit{Tos17A}, \textit{OsHMA7}, and \textit{OsHMA9}, $\geq$50% S\textsubscript{2} progeny exhibited inheritance of the S\textsubscript{1} expressed state. For \textit{OsHMA1} and \textit{OsHMA7}, $\geq$50% S\textsubscript{2} progeny showed reversal to the expression state of the S\textsubscript{0} progenitor. Similarly, for \textit{SNF-FZ14} 64.3% S\textsubscript{2} progeny showed a reversal to the basal expression state. Whereas, in the case of \textit{Tos17B} and \textit{OsHMA6}

![Fig. 2 Transgenerational inheritance of altered expression states of 14 genes in a single Hg-treated S\textsubscript{0} rice plant. The mock-treated plant serves as a control, and the S\textsubscript{0} parental line is the reference for changes in the gene expression in response to Hg-treatment. RNA was isolated from eight S\textsubscript{1} individual progeny derived from the S\textsubscript{0} parent. The results were highly reproducible among the three independent RNA batches, and hence, only one was presented. Gene names are listed to the left and amplification cycles are labeled to the right of the gel. Relative band intensities were used to calculate the percent progeny falling in either of the three gene expression categories: i) inheritance of Hg-treated S\textsubscript{0} pattern, ii) reversion to the mock pattern, and iii) a differential expression pattern (predominately up-regulated expression compared to the S\textsubscript{0} progenitor). The rice \textit{Actin} gene (Genbank accession # X79378) was used as a control for normalization of RNA input. Lack of genomic DNA was validated by the \textit{Actin} gene on the template without RT](image-url)
### Table 2 Transgenerational alteration and inheritance of gene expression patterns in 8 randomly chosen S₁ plants derived from a Hg²⁺(50 μm L⁻¹)-treated S₀ individual

| Gene name | Alteration of gene expression pattern in the S₀ plant and its S₁ progenies | Type and Freq. (%) of pattern |
|-----------|-------------------------------------------------|-------------------------------|
|           | Ho -Hg²⁺ 1 2 3 4 5 6 7 8 | Inherit. of S₀ pat. | Rev. to mock Pat. | New pat. (Trans. memory/other) |
| Tos17A    | U i i +U i +U i +U i +U i | 62.5 | 0.0 | 37.5 (37.5/0.0) |
| Tos17B    | U i +U +U i +U +U +U +U +U +U | 12.5 | 0.0 | 87.5 (87.5/0.0) |
| Osr42     | U +U +U +U +U +U +U +U +U +U | 0.0 | 0.0 | 100.0 (100.0/0.0) |
| Homeobox gene | U i r r r r r r r r | 75.0 | 25.0 | 0.0 (0.0/0.0) |
| HSP70     | U r i i i i i i i i i | 87.5 | 12.5 | 0.0 (0.0/0.0) |
| YF25      | D i i r r r r r r | 25.0 | 37.5 | 37.5 (0.0/37.5) |
| SNF-FZ14  | U i i +U +U +U +U +U +U | 25.0 | 0.0 | 75.0 (75.0/0.0) |
| OsHMA1    | U +U +U +U +U +U +U +U +U +U | 0.0 | 0.0 | 100.0 (100.0/0.0) |
| OsHMA2    | U i D D i D i D i D i | 50.0 | 0.0 | 50.0 (0.0/50.0) |
| OsHMA4    | U r r i i i i r r r r | 37.5 | 0.0 | 62.5 (0.0/62.5) |
| OsHMA5    | U i i r +U i +U r i | 50.0 | 25.0 | 25.0 (25.0/0.0) |
| OsHMA6    | U i i +U +U +U +U +U +U | 25.0 | 0.0 | 75.0 (75.0/0.0) |
| OsHMA7    | U i +U +U +U +U +U +U +U +U | 12.5 | 0.0 | 87.5 (87.5/0.0) |
| OsHMA8    | U i i i i +U +U +U +U +U +U | 62.5 | 0.0 | 37.5 (37.5/0.0) |
| OsHMA9    | U i i i i i i i i | 100.0 | 0.0 | 0.0 (0.0/0.0) |
| Average Freq. (%) | | | | 41.7/100 |

Explanation of symbols: U = up-regulated gene expression in the S₀ plant; +U denotes further up-regulated gene expression in the S₁ progeny plant; D denotes down-regulated gene expression in the S₁ progeny plants compared to the mock control; i denotes inheritance of S₀ expression pattern in the S₁ progeny; r denotes reversal to Mock control expression pattern in the S₁ progeny.

Note: The average frequency of the specified pattern in S₁ progeny plants. For calculations the two copies of Tos17 were treated separately.

Relative band intensities were used to calculate the percent progeny following in either of the three gene expression categories: i) inheritance of Hg⁺-treated S₀ pattern, ii) reversion to the mock pattern, and iii) a differential expression pattern (cf. Fig. 2).
respectively 50 and 28.6% S2 progeny showed a further up-regulation of the S1 expression pattern.

Collectively, these results suggested that the altered gene expression states induced by heavy metal stress are heritable (11.6%; Table 3), and hence indicates transgenerational memory is involved. Additionally, the progeny also appears to maintain the upward trend of induced expression in response to heavy metal stress.

### DNA methylation changes of Tos17 and its transgenerational effect

To further explore whether DNA methylation was also altered due to heavy metal stress and to explain its inheritance across generations, we chose Hg-treated S0 plants, one S1 individual (#3) and one S2 individual (#11) to investigate the methylation state and its transmission. We chose Tos17 as a representative gene to test because both copies of Tos17 showed induced expression in the S0 and the progeny kept the trend through two successive generations. We analyzed cytokine methylation patterns of Tos17A and Tos17B by bisulfite sequencing (Fig. 4). Specifically, we inspected the 5′-LTR and its immediate upstream and downstream regions as well as the 3′-LTR and its immediate upstream and downstream regions for Tos17A and Tos17B located on chromosomes 7 and 10, respectively. The results of bisulfite sequencing are presented in Fig. 4, and some salient observations are described: (i) The region immediately upstream of 5′-LTR in Tos17A showed no change in DNA methylation in the S0 plants and the S1/S2 progeny; the LTR region was slightly methylated at CG and CNG regions in the mock-treated plants and showed CG hypermethylation in S0 plants, further hypermethylation in S1 progeny and inheritance of methylation state in S2 plants. (ii) The 3′-LTR and its flanking regions in Tos17A showed CG hypermethylation and partial methylation for CNG and CNN sequences in the mock plants. However, the CG methylation pattern remained unchanged in the S0, S1, and S2 plants. A slight loss of CNG methylation was observed in the body and LTR regions in S0 plants, but increased methylation levels were observed in the S1 progeny. In the S2 progeny, a slight decrease in methylation pattern in the body region and hypermethylation in the LTR region was observed (Fig. 4a). (iii) The flanking region upstream of the 5′-LTR of Tos17B was unmethylated in the mock plants and showed slight de novo methylation in CNG sequences in the S0 plants, a pattern which disappeared in the S1 progeny. In contrast, the 5′-LTR and the downstream body regions of Tos17B showed heavy methylation in CG sequences, and slight to moderate increases in CNN and CNG methylation compared to the mock control. A decrease of CG methylation was observed in the S1, as well as a decrease in CNG methylation in both S0 and S1, but an increase in CNN methylation was found in the S2 progeny (Fig. 4b). Taken together, the results of bisulfite sequencing at Tos17A and Tos17B
confirmed that DNA methylation changes occur in response to the heavy metal treatment and also showed transgenerational inheritance. Furthermore, the major pattern of DNA methylation changes is CNG hypomethylation in the S₀, which showed different transgenerational effects in either the 3′-region of Tos17A or 5′-region of Tos17B.

The gene expression and DNA methylation of two copies of Tos17 changed under heavy metal stress and showed transgenerational memory of the stress. In addition, under certain circumstances, some of the epigenetically silenced TEs are known to become activated and then transpose. TE activity is often causally linked to the compromised repressive epigenetic state in which cytosine DNA methylation is a critical component. We, therefore, analyzed Tos17 mobility in the S₀, S₁, and S₂ generations by Southern blotting. The results showed that Tos17 stayed inactive, which is evident from the consistent copy number maintained in individuals from the S₀, S₁, and S₂ generations (Fig. 5).
Discussion and conclusions

In this study, locus-specific gene expression changes and the transgenerational effect of heavy metal stress in rice were analyzed. For this purpose, we chose two retrotransposons, seven protein-coding genes, and nine rice OsHMAs, most of them except seven OsHMAs were analyzed in a previous study of the transgenerational inheritance of modified DNA methylation patterns in response to heavy metal stress [41]. In the present study, we addressed whether the altered expression state of the target genes in response to heavy metal stress is transgenerationally inherited and whether different kinds of genes have common or specific responses to the same heavy metal stress. Based on our previous findings, we chose a single dose of each heavy metal that induced maximum DNA methylation changes [41], and also in a previous study of the transgenerational effect of heavy metal stress in rice [41]. The results showed that 16 of 18 genes exhibited up-regulated expression in response to at least one kind of heavy metal treatment, which suggested that these might all be involved in the heavy metal transport. Previous reports suggested OsHMA1 to be exclusively involve in Zn transport [26], however, in the present study, it showed significantly up-regulated expression in Hg treated rice plants, implicating that it might be also involved in transporting Hg. Similarly, OsHMA2 was formerly reported to be expressed in the root maturation zone and to function in the root-shoot translocation of Zn and Cadmium (Cd) [28, 44]. In the present study, OsHMA2 showed transcriptional activation in Cu treated rice plants, suggesting its potential role in copper (Cu) transport. OsHMA3 was localized to tonoplast in the root cells and was found to be responsible for Cd sequestration in vacuoles [29, 30, 45]. In the present study, OsHMA3 showed no expression in rice shoots or induction after Cu, Cd, Cr or Hg treatment, which is consistent with a recent report that it was not induced in roots and shoots of Cr-treated rice plants [46]. However, over-expression of OsHMA3 was shown to enhance Cd tolerance in rice [47], and a loss-of-function allele was shown to accumulate Cd in grains and shoots [48]. Interestingly, it was recently shown that OsHMA3 driven under the control of the OsHMA2 promoter was successful at reducing Cd accumulation in rice grains [28]. OsHMA4 is localized to the vacuolar membrane, and its expression was shown to be induced by long-term Cu treatment and suppressed by Cu deficiency [31] suggesting its role in Cu sequestration in vacuoles and consequently Cu tolerance. In the present study, OsHMA4 was only slightly induced by Cu treatment, which is in conformity with the previous reports where OsHMA4 was only shown to be induced by long-term Cu treatment [27, 31]. OsHMA5 was mainly expressed in the roots at the vegetative stage, and its expression was shown to be up-regulated by the excess of Cu and other metals such as Zn, Fe, and Mn [32]. Here, we report that OsHMA5 is not expressed in the shoots of mock-treated plants, but is induced in the presence of Cu, which is consistent with a previous study [32]. Additionally, we noticed that OsHMA5 exhibits induced expression in the presence of Cd and Hg as well. There are few reports on the function of OsHMA6, OsHMA7, and OsHMA8. These genes are largely silent in the shoots and only exhibited transcriptional activation under heavy metal stress. Although detailed functions are not known for these genes, our data suggest they may also play a role in heavy metal detoxification. Previous reports showed that OsHMA9 is mainly expressed in vascular tissues and its expression could be induced by high concentrations of Cu, Zn or
Cd [27]. In the present study, OsHMA9 showed significant transcriptional activation in Cd and Hg treated plants, and a slight up-regulation in Cu treated plants. Our data support an additional role for OsHMA9 in Hg efflux.

To confirm and extend our findings, we tested whether the altered gene expression state of S0 plants was transgenerationally inherited by the S1 and S2 progeny. We reported an average inheritance rate of 41.7% in the S1 and 36.6% in the S2 (Figs. 2, 3 and Tables 2, 3). However, the rate of inheritance varied depending on gene in question. A majority of the genes tested showed up-regulated expression in the S1 (41.7%) and about 11.6% maintained the trend of up-regulated expression and exhibited further up-regulation in the S2. It indicates that the progeny maintained a memory of the altered expression state of the progenitors even after removal of the heavy metal. Recently, some studies showed a clear connection between the ethylene signaling and response to heavy metal stress in diverse plant species [49–51].

We have not evaluated this aspect in the present study, but believe it is worthy of checking the transcriptional pattern of ethylene biosynthesis and signaling genes in heavy metal treated plants and study the transgenerational inheritance of the expression pattern.

The traditional concept of epigenetics refers to heritable changes in gene expression without an accompanying change in the DNA sequence. Recent research advocates inclusion of the ‘memory concept’ in the formal definition of epigenetics, as even after the disappearance of the initial stress signal, the DNA and/or chromatin modifications are transmitted to maintain the altered transcriptional state from one generation to another [52, 53]. Several studies showed that epigenome is remodeled in plants upon exposure to diverse stresses and DNA methylation pattern is most likely to respond [54–59]. It has been proposed that the DNA methylation state is only partially transmitted to the immediate offspring, as part of it resets during sexual reproduction, which in turn limits the transmission of the acquired epigenetic alterations from parents to offspring [60, 61]. However, our previous research demonstrated that the heavy metal-induced DNA methylation changes in rice are inheritable for at least two successive generations [41]. Here, we monitored the DNA methylation changes under heavy-metal stress in two copies of Tos17 and studied the transgenerational inheritance of epigenetic changes by bisulfite sequencing (Fig. 4). We observed that the major DNA methylation change in Tos17 is CNG hypomethylation, which showed variable inheritance patterns in the 3′- and 5′-regions of the two genomic copies of Tos17 (Tos17A and Tos17B). These observations conform with our previous findings where CNG hypomethylation was most prevalent in response to heavy metal stress and showed at least partial inheritance of the epigenetic changes [41, 43]. DNA methylation changes are associated with changes in gene expression. For instance, A. thaliana mutants defective in DNA methylation showed that regulation of phosphate-starvation-responsive genes requires changes in the DNA methylation pattern [59]. Thus, we set out to find the relationship between DNA methylation and gene expression. Our data suggest that there is no direct correlation between the methylation status and gene expression for Tos17. Moreover, Tos17 stayed silent over three generations, which indicates that the methylation changes in Tos17 are not sufficient for its activation followed by transposition. However, it is unclear whether the heritable change in gene expression is related to methylation changes as there can be locus-specific changes in methylation. Moreover, our study was limited to Tos17A and Tos17B.

Interestingly, recent research has proposed a key role for dynamic changes in chromatin substructure in transgenerational memory of gene expression changes in response to various stresses [62–64]. In line with this research, maize researchers showed that stress-induced changes in chromatin structure activate transposable elements, and new transposition events contribute to altered phenotypes observed in the progeny [65]. Several studies indicated that DNA methylation and small interfering (si) RNAs might play a role in transgenerational epigenetic memory, i.e., modification in gene expression patterns that are transmittable across generations via the germline [37, 66–69]. Therefore, we expect a role for siRNA in the observed transgenerational memory of heavy-metal induced transcriptional and epigenetic changes in the rice genome. However, as noted by Probst and Mittelsten [63], while the concept of transgenerational memory is attractive, it is difficult to determine the actual mechanism contributing to it and the number of generations in which it persists.

Methods

Plant material

O. sativa L. ssp. japonica, cv. Matsumae, a cultivated rice, used in the present study was initially obtained from Japan and has since been propagated for more than twenty generations in our laboratory. For the experiments elaborated here, seeds were thoroughly washed with distilled water and germinated in the dark at 28°C in Petri dishes containing distilled water. After two days incubation, seedlings were transferred to a greenhouse maintained at 26°C under a 12 h photoperiod.

Heavy metal treatment

The ten-day-old, seedlings were subjected to different heavy metal treatments: Cu^{2+} (50 μM or 1000 μM...
CuSO₄), Cd²⁺ (50 µM or 1000 µM CdCl₂), Cr³⁺ (50 µM or 1000 µM CrCl₃) or Hg²⁺ (50 µM or 1000 µM HgCl₂) in Hoagland nutrient solution for a week. As several microelements in Hoagland nutrient solution are either used as sulfates or chlorides, and the pH of the solution is also adjusted using sulfuric acid, so we made no attempts to balance the sulfate and chloride ions in the Hoagland solution. Additionally, the treatments are similar to the one reported in our previous work [41]. Mock controls were grown in parallel in the Hoagland nutrient solution. After treatment, seedlings were transplanted to the field. Leaf samples were harvested at different time points in liquid nitrogen and stored at −80 °C until used. The plants were marked “stressed S0”. Panicles of several selected stressed and mock plants were bagged for self-pollination and seeds were collected to produce the next generation of plants, which were labeled as S1. In a similar way, S2 generation plants were produced, and the seeds were harvested.

**Reverse-transcription PCR (RT-PCR) analysis**

RT-PCR was performed essentially as reported in Liu et al. [70]. In brief, total RNA was isolated from expanded young leaves using Trizol reagent (Invitrogen) following the manufacturer’s instructions. RNA was converted to cDNA using Super ScriptTM RNAse H reverse transcriptase kit (Invitrogen), and subjected to RT-PCR analysis using gene-specific primers (Additional file 1: Table S1). The rice Actin gene (Genbank accession # KX79378) was used as the control for normalization of RNA input. Gene-specific primers were designed using Primer 3 (http://bioinfo.ut.ee/primer3/) and are listed in Additional file 1: Table S1. Different cycle numbers were used for different genes to ensure amplifications stay within the linear range for each gene. For S₀ samples, we pooled seedlings and used three technical replications to check the gene expression changes. Whereas, for the S₁ and S₂ individuals, three batches of independently prepared total RNAs were used as technical replications. The amplified products were visualized via agarose gel electrophoresis and ethidium bromide staining.

**Bisulfite sequencing of the Tos17 loci**

Genomic DNA was extracted from fully expanded rice leaves and was given a bisulfite treatment [71]. Briefly, an EZ DNA Methylation-Gold Kit from Zymo Research was used to treat 5 µg of genomic DNA. The PCR primers, which were used to amplify bisulfite-converted genomic DNA for the two copies of the Tos17 (Transposon of Oryza sativa 17), are listed in Additional file 2: Table S2. From 10 to 15 clones for each sample were sequence analyzed. The methylation level was expressed as the percentage (%) per site for each of the three cytosine contexts (CG, CHG, and CHH). Methylation level was calculated by dividing the number of non-converted (methylated) cytosines with the total number of cytosines underlying a sequenced region. The sequences were analyzed by the Kismeth program (http://katahdin.mssm.edu/kismeth/revpage.pl), and the results were presented as histograms.

**Southern blotting**

Genomic DNA was isolated from fully expanded leaves of heavy metal-stressed and mock control rice plants by a modified CTAB method [72] and purified by phenol extraction. For the transposon activity analysis, 5 µg of genomic DNA was digested with Hin_dIII (NEB) and resolved on 1% agarose gel. Subsequently, DNA was transferred to Hybond N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, New Jersey) via alkaline transfer, as recommended by the manufacturer. Only one Tos17 copy was used as a probe in the present study (see Additional file 1: Table S1). For probe preparation, the Tos17 fragments were amplified via PCR at annealing temperature 59 °C. The authenticity of the PCR products was confirmed by DNA sequencing. The fragments were gel-purified and labeled with fluorescein-11-dUTP using the Gene Images random prime-labeling module from Amersham Pharmacia Biotech. Hybridization signal was detected by the Gene Images CD²⁺-P-Star detection module (Amersham Pharmacia Biotech) after two stringent washes with 0.2 × SSC and 0.1% SDS for 50 min each. Subsequently, the membrane was exposed to X-ray film.

**Additional files**

Additional file 1: Table S1. List of gene-specific primers used for RT-PCR analysis and amplification of probe used for Southern blotting. (DOC 50 kb)

Additional file 2: Table S2. List of primers used for bisulfite sequencing of Tos17. (DOC 39 kb)

**Abbreviations**

Cd: Cadmium; Co: Cobalt; Cr: Chromium; CTAB: Cetyltrimethylammonium bromide; Cu: Copper; Hg: Mercury; HMA: Heavy Metal-transporting P-type ATPases; NO: Nitric oxide; Pb: Lead; RT-PCR: Reverse transcription-polymerase chain reaction; SDS: Sodium dodecyl sulfate; SSC: Saline sodium citrate; TE: Transposable element; Zn: Zinc

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**Authors’ contributions**

CWX, XL, MVY performed RT-PCR analysis and they contributed equally to this work; ZYH performed bisulfite sequencing; ZYH, YCL, WJM, LCH, ZR are responsible for the heavy metal treatment of rice plants; ZTT, LXY, JLL, VNN, MJ are responsible for rice filed propagation; LB participated in the design, SR and OXF conceived the study, participated in the design and wrote the manuscript with help from KAS. All authors have read and approved the final manuscript.
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Availability of data and materials
All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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Not applicable.

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Competing interests
Author Bao Liu is a Section Editor of BMC Plant Biology and no other authors have competing interests.

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