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ORIGINAL PAPER

Rapid generation of rice mutants via the dominant negative suppression of the mismatch repair protein OsPMS1

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Abstract Mismatch repair (MMR) is a conservative pathway for maintaining the genome integrity of different organisms. Although suppression of MMR has resulted in various mutation phenotypes in *Arabidopsis*, the use of this strategy for mutation breeding in major crops has not been reported. Here, we overexpressed a truncated version of the OsPMS1 protein in rice; this approach is expected to suppress the rice MMR system through a dominant negative mechanism. We observed a wide spectrum of mutation phenotypes in the progeny of the transgenic plants during seed germination and the plant growth stages. Genomic variations were detected with inter-simple sequence repeat (ISSR), and sequencing of the differential ISSR bands revealed that the mutation occurred as a point mutation or as microsatellite instability at high frequencies. Plant lines with agronomically important traits, such as salt and drought tolerance, various tiller number, and early flowering, were obtained. Furthermore, we obtained mutants with important traits that are free of the transgene. Together, these results demonstrate that MMR suppression can be used as an efficient strategy for mutation breeding in rice.

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Introduction

With the increase of the world population and the reduction in arable land caused by environmental problems, the agricultural industry is now facing the challenge of providing more calories for the globe on restricted arable land areas. Thus, the breeding of new crop cultivars with a high-yield potential and stress tolerance is becoming more important (Miflin 2000). In the past, different strategies have been adopted for crop breeding and considerable progress was made (Phillips 1993). Mutation breeding, which introduces heritable change in the DNA level through chemical and radiation treatment, has resulted in large population of mutants in different plant species. Until now, more than 2,700 varieties including major crops such as rice, wheat and maize lines derived from mutagenesis programs have been released (http://www-infocris.iaea.org/MVD/). Crossing, another powerful method for bringing together targeted traits from different individuals, has been used by plant breeders and has produced a lot of cultivars (Yuan 2001). Genetic engineering has also been used for plant breeding by introducing targeted traits into plants, thus contributing to the rapid generation of desired cultivars (Lemaux 2008, 2009; Rommens et al. 2006). However, all these methods have their limits (Chao et al. 2005). The mutants resulting from chemical or radiation mutagenesis are highly toxic and are recovered with difficulty (Kodym and Afza 2003). Crossing is based on accumulating traits from different cultivars and is limited by the availability of genetic resources. Genetic engineering is still limited by the lack of genes that can improve the traits without exhibiting side effects (Huang

et al. 2008; Park et al. 2009; Zou et al. 2008). In addition, the selectable marker present in transgenic materials is another concern regarding the safety of the transgenic approach (Celec et al. 2005; Halford and Shewry 2000; Hare and Chua 2002; Miki and McHugh 2004; Ramessar et al. 2007).

Instead of introducing DNA damage by chemical and radiation treatments, negative regulation of the DNA repair system is expected to have the similar effects on mutant production (Bronner et al. 1994; Fishel et al. 1993; Will et al. 2007). Thus, the DNA mismatch repair system (MMR) might be a good target for establishing an in vivo mutation system (Chao et al. 2005). MMR is a conservative pathway responsible for maintaining the genome integrity of different organisms. This system is responsible for recognizing and repairing erroneous insertions, deletions and misincorporation of bases, events which can arise during DNA replication and recombination, as well as during the repair of some forms of DNA damage (Felton et al. 2007; Plotz et al. 2006). The major players in the MMR system include MutS, MutH, and MutL. MutS forms a dimer (MutS2) that recognizes the mismatched base on the daughter strand and binds the mutated DNA. MutH binds the hemimethylated sites along the daughter DNA, but its action is latent, because it is only activated upon contact by a MutL dimer (MutL2); the latter binds the MutS-DNA complex and acts as a mediator between MutS2 and MutH, resulting in MutH activation. Activation of MutH cleaves the unmethylated strand at the GATC site. Subsequently, the segment from the cleavage site to the mismatch is removed by exonuclease (with assistance from helicase II and SSB proteins). The gap is filled by DNA polymerase III and DNA ligase (Kolodner and Marsischky 1999; Modrich 1994).

Eukaryotes have MutL homologs designated Mlh1 and Pms1 (mammalian PMS2 corresponds to PMS1 in yeast, plants, and nematodes). They form a heterodimer that mimics MutL in *E. coli*. The human homolog of prokaryotic MutL has three forms designated MutLα, MutLβ, and MutLγ. The MutLα complex consists of two subunits, MLH1 and PMS2; the MutLβ heterodimer comprises MLH1 and PMS1, whereas MutLγ is composed of MLH1 and MLH3. MutLα acts as the matchmaker or facilitator, coordinating events in mismatch repair (Constantin et al. 2005; Drummond et al. 1995; Flores-Rozas and Kolodner 1998; Harfe et al. 2000; Modrich 1991, 2006; Wang et al. 1999). The truncating mutation of the PMS2 gene at codon 134 was first found in a HNPCC patient; the results showed that this mutant could indeed exert a dominant negative effect, resulting in biochemical and genetic manifestations of MMR deficiency (Nicolaides et al. 1998). Studies have demonstrated that MMR deficient cells or whole organisms exhibit a significantly increased frequency of point mutations and microsatellite instability (MSI) characterized by 1–4 base pair insertions/deletions within repetitive nucleotide tracts (Agostini et al. 2010; Hoffman et al. 2004; Poynter et al. 2008). Using the same strategy, Chao et al. (2005) demonstrated the feasibility of using reversible MMR deficiency via the expression of human *PMS2-134* in *Arabidopsis* to enhance genetic diversity in the transgenic plants.

Rice is one of the most important food crops in the world, and mutation breeding using new methods could improve the yield and quality of this crop (Yuan 2001). In this report, we cloned the rice MutL homolog *OsPMS1* and overexpressed the truncated version of this protein OsPMS1-136 (1-136 amino acids of OsPMS1) in the rice cultivars "Zhonghua 11 (*Oryza sativa* L. spp. *japonica*)" and "Shuangsixiangzhan (*Oryza sativa* L. spp. *indica*)" (Fig. 1). We studied the mutant phenotypes of the transgenic plants and analyzed the mutations at the nucleotide level. Finally, we selected some mutants with agronomically important traits from the transgenic progeny that did not contain the transgene.

Materials and methods

Construction of the *OsPMS1-136* expression vector

The initial 408 nucleotides of the *OsPMS1* coding region were amplified by RT-PCR from rice total RNA with primers (Fw-PMS1-136: TTTGGATCCATGGCCGGCGG CTCGT, Re-PMS1-136: TTTGAATTCTTACACGCCCG AGTGCGCGA); a stop codon (TAA) was introduced by the reverse primer. The amplified fragment (*OsPMS1-136*) was restricted with *Bam*HI and *Eco*RI and inserted into the same enzyme sites in the binary vector pHQSN (Fig. 2a).

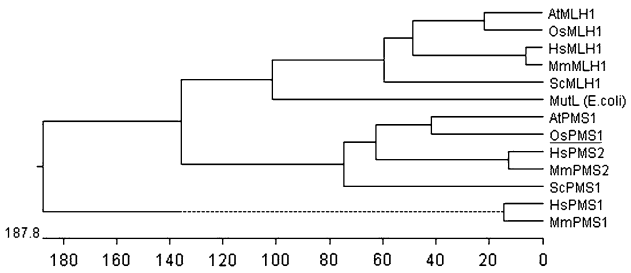

Fig. 1 Phylogenetic tree analysis of MutL homolog proteins from some model organisms. The MutL homolog proteins used were: *Escherichia coli* MutL (ZP_03059130.1); *Saccharomyces cerevisiae* PMS1 (AAM00563.1) and MLH1(ABC86950.1); *Mus musculus* MmPMS1 (NP_705784.1), MmPMS2 (NP_032912.2) and MmMLH1 (AAF64514.1); *Homo sapiens* HsPMS1 (NP_000525.1), HsPMS2 (AAH93921.1), and HsMLH1 (AAA17374.1); *Arabidopsis thaliana* AtPMS1 (AT4G02460, NP_567236.1), and AtMLH1 (AT4G09140, NP_567345.2); *Oryza sativa* OsPMS1 (Os02g0592300, NP_001 047292.1) and OsMLH1 (Os01g0958900, NP_001045457.1). OsPMS1 is *underlined*

This binary vector contained the *OsPMS1-136* cDNA, which was under the control of the maize *Ubiquitin* promoter and terminated with the NOS terminator, and was fully sequenced to verify the integrity of the promoter and the *OsPMS1-136* cDNA. The binary vector was then transferred into *Agrobacterium tumefaciens* EHA105 by the freeze–thaw method (Chen et al. 1994).

Rice transformation and plant growth

The two rice cultivars "Zhonghua 11 *(Oryza sativa* L. spp. *japonica)*" and "Shuangsixiangzhan *(Oryza sativa* L. spp. *indica)*" were used for the experiments. Rice transformation was performed as previously described (Li and Li 2003). The transgenic plants were grown in the greenhouse and the mature seeds were harvested. To screen the mutants, T2 or T3 seeds from the transgenic plants (including seeds of homozygous transgenic plants, seeds of hemizygous transgenic plants, and seeds of the wild type) were germinated and grown on 1/2 MS medium (Murashige and Skoog 1962) at 25 °C, with a 16/8 h day/night light regime. After 2 weeks, the germinated seedlings were transplanted to the greenhouse. Salt-tolerant rice plants were selected at the seed-germinating stage, by growing the seeds on 1/2 MS medium containing 250 mM of sodium chloride for 30 days. Drought-tolerant rice plants were selected by growing germinated seedlings of 10 cm in height in soil without supplying water.

Southern blot analysis

For Southern blot analysis, 10 μg of each genomic DNA sample was digested with *Bam*HI and *Eco*RI at 37 °C overnight, separated on a 0.8 % (w/v) agarose gel, and transferred to Hybond N+ membranes. Digoxigenin (DIG)-labeled *OsPMS1-136* cDNA fragment and *hptII* fragment were synthesized by PCR and used as the probe. The primers for amplification of *OsPMS1-136* probe are Fw-PMS1-136 and Re-PMS1-136, and the primers for *HPT* probe are hptFw GCTGGG GCGTCGGTTTCCACTA TCGG and hptRe CGCATAACAGCGCTCATTGAC TG GAGC, which amplify a 375 bp fragment in the coding

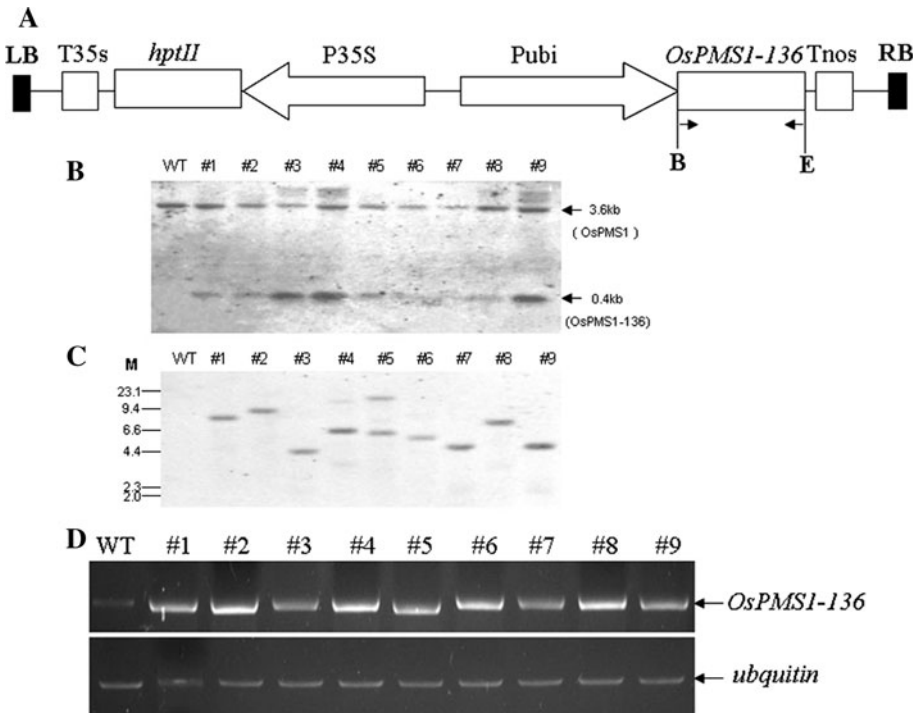

Fig. 2 Generation of OsPMS2-136-overexpressing transgenic rice plants. Map of the overexpression vector for *OsPMS1-136*, the truncated version of *OsPMS1* (**a**). The coding region (amino acids 1 to136) of *OsPMS1* was placed under the control of the maize *ubiquitin* promoter and terminated by the nopaline synthase terminator (NosT). The *OsPMS1-136* expression cassette was cloned in the binary vector pCAMBIA 1390. Southern blot analysis of the transgenic lines with DIG labeled *OsPMS1-136* probe (**b**). Genomic DNA from different lines about 5 μg was restricted with *Eco*RI and *Bam*HI. WT, the wild-type plant was used as a negative control; #1–5, individual *OsPMS1-136* transgenic plants of the *indica* rice "Shuangsixiangzhan"; #6–9, individual *OsPMS1-136* transgenic plants of the *Japonica* rice "Zhonghua 11". The same filter as in **b** was hybridized with DIG labeled *HptII* probe to show the transgene copies in different lines (**c**). RT-PCR analysis of *OsPMS1-136* transcripts in the transgenic lines (**d**). WT, wild-type plant; #1–5, individual OsPMS*1-136* transgenic plants of the *indica* rice "Shuangsixiangzhan"; #6–9, individual *OsPMS1-136* transgenic plants of the *Japonica* rice "Zhonghua 11". *Ubiquitin* transcripts were used as the internal control

region of *Hpt*. Prehybridization, washing, and chemiluminescent detection of the blots was performed according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

Semi-quantitative reverse transcription PCR (RT-PCR)

Total RNA was extracted from 200-mg tissue samples using the Trizol reagent. All samples were treated with DNase I. First-strand cDNA synthesis was performed using 5 μg of total RNA and Moloney murine leukemia virus reverse transcriptase (Promega). Expression of the target genes was compared to the housekeeping gene *ubiquitin*, and the cycling conditions were as follows: 5 min at 94 °C; 25 cycles of 30 s at 94 °C, 30 s at 55 °C, and 40 s at 72 °C, and a final extension step for 10 min at 72 °C. The RT-PCR products were analyzed by electrophoresis on a 2 % agarose gel.

Amplification and analysis of the genome mutations

Genomic DNA was isolated from freshly harvested young leaves of 12 plants of each cultivar. A total of 16 inter-simple sequence repeat (ISSR) primers were used in the study. The ISSR primers (UBC set 9) 807–817 and 821–827 were synthesized based on the sequences from the Biotechnology Laboratory at the University of British Columbia, Canada. ISSR-PCR was conducted in a reaction volume of 15 μl containing 30 ng of template DNA, 0.2 μmol L^{-1} primers, 200 μmol L^{-1} each dNTP, 10 mmol L^{-1} Tris–Cl (pH 8.3), 50 mmol L^{-1} KCl, 2.0 mmol L^{-1} MgCl2, and 1 U of Taq polymerase. PCR amplification conditions were set as follows: initial denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min. The PCR was performed in 96-well plate thermal cycler (Eppendorf, Germany). The amplified products were mixed with loading dye (0.4 g ml^{-1} sucrose and 2.5 mg ml^{-1} bromophenol blue), resolved on 18 mg ml^{-1} agarose gel in 0.5× Tris borate EDTA (TBE) buffer at room temperature at constant voltage of 100 V, and detected by ethidium bromide staining (0.5 mg ml^{-1}). After electrophoresis, the differential PCR bands were cloned and sequenced. Finally, three primer pairs were designed for sequencing the three loci that showed sequence variation by ISSR between the transgenic *OsPMS1-136* plant and the wild type.

Results

Generation of *OsPMS1-136* transgenic plants

The human PMS2 protein (AAH93921.1) was used as a query to search for homologous proteins against the rice database in NCBI (National Center for Biotechnology Information), and two proteins named OsMLH1 and OsPMS2 were found in the rice genome. Of these, OsPMS2 showed higher sequence homology to hPMS2. Here, we name the protein OsPMS1, because it shows high sequence homology with the *Arabidopsis* AtPMS1. *OsPMS1* is predicted to encode a putative protein consisting of 923 amino acid residues. Our search for conserved domains revealed that amino acids 9–329 and amino acids 716–865 in the OsPMS1 protein represent two conserved domains, namely mutL (DNA mismatch repair protein MutL, CDD129673) and MutL_C (MutL C terminal dimerization domain, CDD141970). A total of 17 ATP-binding sites and one Mg^{2+} binding site were present in the mutL domain. Phylogenetic analyses revealed that OsPMS1 shares a high level of identity to AtPMS1 from *Arabidopsis thaliana*; In fact, the identity in the conserved domain region is as high as 90 %. The identity reached about 50 % when comparing OsPMS1 with *Homo sapiens* PMS2 (Fig. 1).

As the expression of the truncated version of *HsPMS2* (*PMS2-134*) in *Arabidopsis* showed strong mutation effects (Chao et al. 2005), we speculate that a truncated version of *OsPMS1* (*OsPMS1-136*) will produce the same effects in rice. Consequently, we cloned the cDNA sequence coding for the first 136 amino acids of OsPMS1 and designated it *OsPMS1-136*. An expression vector containing *OsPMS1-136* under the control of the maize *ubiquitin* promoter was constructed for the negative regulation of the rice DNA MMR (Fig. 2a). A total of nine independent transgenic lines were obtained through *Agrobacterium tumefaciens*-mediated transformation. Five of the nine transgenic lines were of the *indica* rice "shuangsixiangzhan" and four lines were of the *japonica* rice "Zhonghua 11". Southern blot analysis of genomic DNA (restricted with *Bam*HI and *Eco*RI) from the transgenic plants with *OsPMS1-136* probe showed that two bands with a molecular weight of 3.6 and 0.4 kb appeared in the blot, corresponding to the endogenous *OsPMS1* and the transgene *OsPMS1-136* (Fig. 2b). The same blot was used for examine the transgene copies with a *Hpt*II probe, and the results showed that the transgene copies in different lines are 1 or 2 (Fig. 2c). RT-PCR analysis was performed to examine the expression levels of *OsPMS1-136* in these lines, and a much higher level of OsPMS*1-136* expression was detected compared with that in the wild-type plant (Fig. 2d). The transgenic plants were transferred to greenhouse and grown to maturity to harvest their seeds. We did not find obvious phenotypic differences in the T0 generation between the control lines (transformed with pCAMBIA 1390) and the regenerated *OsPMS1-136* transgenic plants during the growth period. The seeds from different lines were harvested and germinated on hygromycin containing medium, we selected three lines from each cultivar, which showed a germination ratio of 3:1 for

resistant seeds to sensitive ones for further studies. These lines also showed single copy transgene integration in their genome.

The MMR deficiency in rice leads to phenotypic variations

The mutation phenotypes including lethal, dwarf, male sterility, various tiller number and flowering time were observed during the germination of T1 seeds and the growth of T1 plants in the greenhouse. These mutation phenotypes were expected, because the interference of the MMR system begins when the transgene enters into the rice cell. Due to the deficiency of MMR, some mutated loci may arise during the period of selection of transgenic calli and the latter regeneration process. The mutation loci will become heterozygous or homozygous state in the T1 progeny. To strictly estimate the mutation frequency and the phenotypic effects, we germinated the T2 progeny seeds homozygous for the transgene on 1/2 MS medium solidified with agar under non-selective conditions. At the same time, we used a wild type and a homozygous transgenic pCAMBIA1390 line as controls. During the germination stage, a lethal phenotype and dwarf seedlings were observed at much higher frequency in both *indica* rice and *japonica* rice after 2 weeks (Fig. 3a, b). In the *indica* rice "Shuangsixiangzhan", the lethal mutants represented about 13.0–14.3 % of all the seeds, and the dwarf mutants were noted at a frequency as high as 44.3 % (Table 1). In the *japonica* rice "Zhonghua 11", lethal mutants represented about 4–6 % of all the seeds, and the dwarf mutants were noted at a frequency of about 23–30.3 % (Table 2). During the growth of the plants in the greenhouse, more interesting mutant phenotypes, such as less or more tillers, early flowering and dwarf, were observed (Fig. 3c–e).

Interference of the MMR system is expected to randomly cause mutations in the whole genome. Therefore, the mutant phenotype will segregate with the transgene in the progeny of hemizygous transgenic plants. We further screened some progeny of the T2 lines that were derived from hemizygous T1 plant. These T2 lines are consisted of homozygous, hemizygous and wild type progeny for the transgene. During seed germination and the plant growth stages, we observed a similar mutant phenotype spectrum, with slightly lower frequencies for the lethal and dwarf phenotypes compared to the homozygous transgenic plants (Tables 1, 2). This indicates that the presence of the transgene in a hemizygous state is sufficient for inducing mutations, and also most of the mutation phenotypes were not caused by the T-DNA insertion.

To further confirm that interference of the MMR in rice can cause mutation and the transgene can segregate with the mutant phenotype, we examined the germination and growth of the segregated T2 wild type lines. We also find the lethal and dwarf seedling phenotype in both *indica* and *japonica* rice, the lethal mutants represented about 7.0–8.7 %, and the dwarf mutants were scored at a frequency of 33.0–35.3 % in the *indica* rice (Table 1). These results confirmed that mutation phenotypes can segregate with the transgene. However, the mutation frequency is lower than the lines with the transgene. In the *japonica* rice "Zhonghua 11", a similar lower mutation frequency was found for the segregated T2 wild type lines (Table 2).

PCR and RT-PCR analyses were performed to examine the expression level of *OsPMS1-136* in independent descendants such as dwarf and normal plants. We found that plants with *OsPMS1-136* integration showed both the normal and mutant phenotypes, whereas plants without *OsPMS1-136* also presented normal and mutant phenotypes (Fig. 3f). These results demonstrate that the mutant phenotype caused by the interference of the MMR system can be stably maintained in the absence of *OsPMS1-136*. Thus, mutants free of the transgene can be obtained by this method.

Negative regulation of MMR causes genetic diversity

Different organisms require MMR for repairing the errors produced during DNA replication. DNA polymerase errors in the replication of microsatellites are highly prone to slippage and produce small insertions and deletions without the MMR system. In addition, the absence of MMR also causes single base mutations. These have been reported in the *Arabidopsis* mutant MSH2 (Hoffman et al. 2004) and during the expression of *PMS2-134* in *Arabidopsis* plants (Chao et al. 2005).

To determine the effects of the negative regulation of MMR on rice genome sequences, 16 ISSR primers were used to examine the amplified fragment length polymorphism in the wild-type "Zhonghua 11" plants and eight T2 offspring from the *OsPMS1-136* transgenic line #8 (*japonica* rice "Zhonghua 11"). Of the 16 ISSR primers used, eight showed clearly amplified bands (Fig. 4a). Further use of these primers for the amplification of genomic DNA from the transgenic progeny resulted in distinct bands in some of the offspring.

Next, the bands showing differences between the wild-type and the *OsPMS1-136* plants were cloned and sequenced. These sequences were localized in the rice genome by the BLAST algorithm of the NCBI database. We further studied three fragments that were located on chromosomes 1 and 6; two of them were in the intergenic region, and one was in the coding region of a glycosyl transferase gene (Table 3). To detect the changes in all the sequences including the ISSR primer region, specific primer pairs were designed to include the sequences upstream and downstream of the ISSR primers. PCR was performed again with

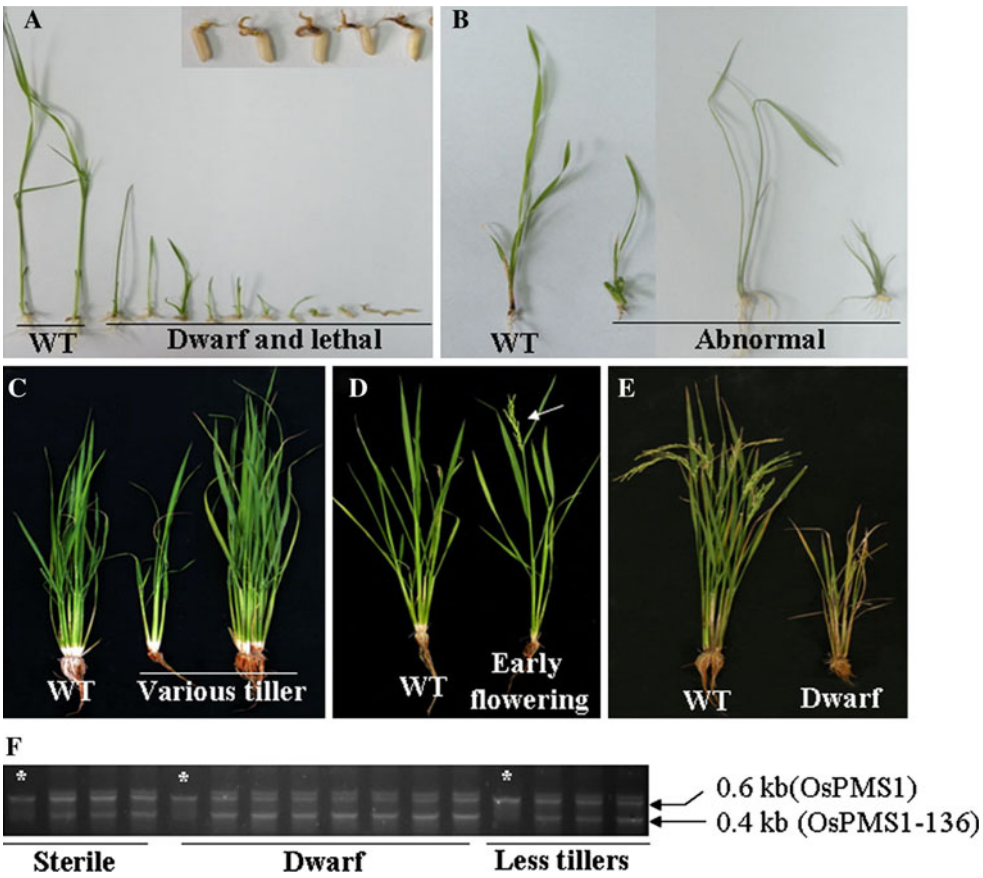

Fig. 3 Representative mutation phenotypes observed during the germination and growth of the *OsPMS1-136* transgenic progenies, and PCR examing of the segregation of transgene in mutants (#1 transgenic plants of *indica* rice "Shuangsixiangzhan"). Dwarf and lethal phenotypes during the seed germination stage of the transgenic seeds. The seeds showed a lethal phenotype shortly after germination (**a**; Insert; #1). Abnormal seedlings from the transgenic seeds (**b**; #6). Adult plants showed variation in the number of tillers, early flowering and dwarf phenotypes, respectively (**c–e**). Out segregation of the transgene in some of the mutant plants (**f**). *Asterisks* indicate the plants without the transgene

Table 1 Mutation frequencies of phenotypic mutants in OsPMS1-136-expressing single-seed descendent populations (Indica cultivar "Shuangsixiangzhan")

Shuangsixiangzhan	Number of T2 progenies with mutant phenotype		
	Normal (%)	Dwarf (%)	Lethal (%)
Wild type	99.0 ± 1.0	1.0 ± 1.0	0
p1390	98.0 ± 1.0	2.0 ± 1.0	0
#1 WT	57.3 ± 3.1	34.3 ± 2.1	8.3 ± 1.5
#1 He	46.7 ± 4.5	41.3 ± 3.2	12.0 ± 2.6
#1 Ho	45.3 ± 4.5	41.3 ± 4.2	13.3 ± 1.5
#2 WT	60.0 ± 3.0	33.0 ± 2.0	7.0 ± 2.6
#2 He	47.3 ± 4.6	42.3 ± 4.2	10.3 ± 1.2
#2 Ho	43.7 ± 3.2	43.3 ± 2.1	13.0 ± 2.6
#3 WT	52.7 ± 2.1	35.3 ± 3.1	8.7 ± 1.5
#3 He	44.3 ± 2.3	43.0 ± 4.0	12.7 ± 2.3
#3 Ho	41.3 ± 3.1	44.3 ± 4.0	14.3 ± 1.6

100 seeds of T2 generation derived from individual lines were used for each germination treatment, and each treatment was repeated three times. #1, #2, #3 represent different transgenic lines, and WT, He, Ho represent seeds derived from segregated wild type, hemizygous or homozygous plants, respectively. p1390, transgenic plants with pCAMBIA1390

the specific primers and the amplified fragments were sequenced.

The sequence results confirmed that both MSI and mononucleotide mismatches occurred in the progeny of the *OsPMS1-136* transgenic lines (Table 3 and Fig. 4b–d). We sequenced three fragments of 717, 569, and 619 bp in length, respectively; they represented three loci in the rice genome. For each locus, we obtained the sequence information from 8 to 11 T2 progeny. The results showed that both mononucleotide mismatches and MSI occurred frequently in the

Table 2 Mutation frequencies of phenotypic mutants in OsPMS1-136-expressing single-seed descendent populations (Japonica cultivar "Zhonghua 11")

Zhonghua 11	Number of T2 progenies with mutant phenotype		
	Normal (%)	Dwarf (%)	Lethal (%)
Wild type	99.0 ± 1.0	1 .0 ± 1.0	0
p1390	99.0 ± 1.0	1 .0 ± 1.0	0
#6 WT	75.3 ± 1.5	22.7 ± 2.3	2.0 ± 1.0
#6 He	73.0 ± 2.65	25.0 ± 2.65	2.0 ± 1.7
#6 Ho	67.0 ± 4.0	29.0 ± 3.0	4.0 ± 1.0
#7 WT	72.3 ± 3.2	25.7 ± 3.2	2.0 ± 1.0
#7 He	72.3 ± 3.1	24.0 ± 2.6	3.7 ± 1.5
#7 Ho	71.0 ± 3.0	23.0 ± 2.0	6.0 ± 1.0
#8 WT	72.3 ± 2.1	26.3 ± 2.3	1.3 ± 0.6
#8 He	68.3 ± 4.5	30.0 ± 3.6	2.0 ± 1.0
#8 Ho	65.7 ± 2.5	30.3 ± 4.0	4.0 ± 2.6

100 seeds of T2 generation derived from individual lines were used for each germination treatment, and each treatment was repeated three times. #6, #7, #8 represent different transgenic lines, and WT, He and Ho represent segregated wild type, hemizygous and homozygous lines, respectively. p1390, transgenic plants with pCAMBIA1390

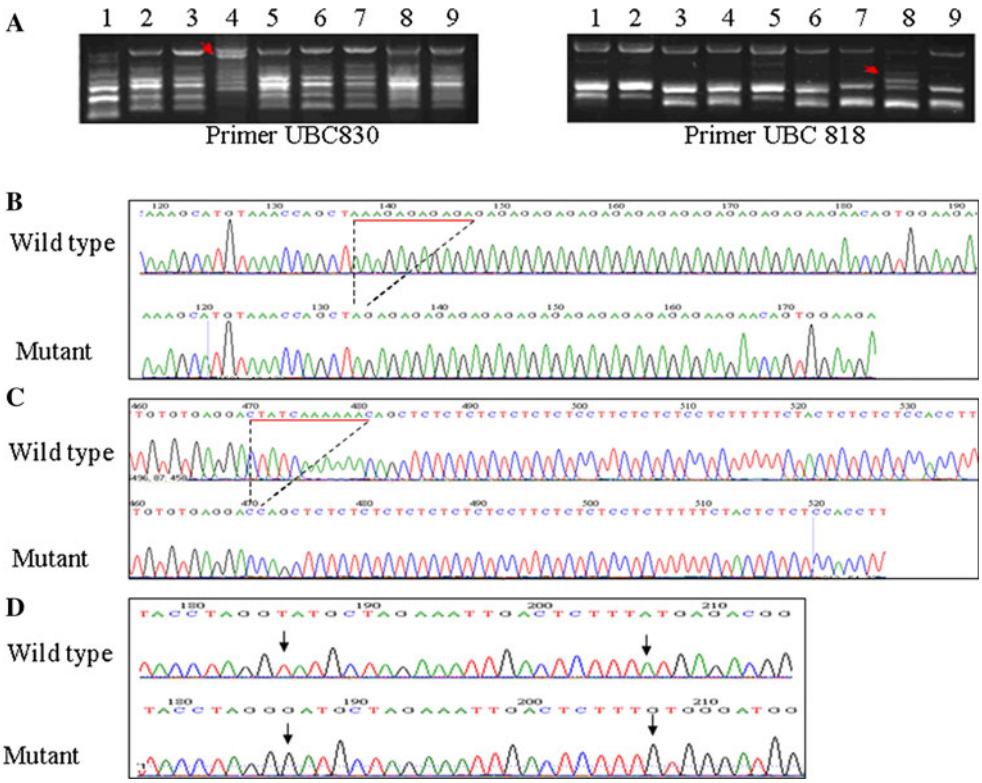

Fig. 4 Expression of *OsPMS1-136* induces microsatellite instability and sequence variation in the rice genome (#8 transgenic plants of *japonica* rice "Zhonghua 11"). Representative pictures showing the screening of sequence variation in transgenic T2 progeny with ISSR primers as indicated in the figures (**a**). *1* wild-type, *2–9* individual transgenic progeny. *Arrows* indicate the differential bands compared with the wild type. Analysis of the sequences around the ISSR differential bands between wild-type and transgenic rice (**b–d**). The GA repeats were deleted in the transgenic progeny (**b**). Single A repeats were deleted (**c**). Single nucleotide mutations were present in the transgenic progeny (**d**). The *red lines* and *arrows* indicate the deletion of oligonucleotides and mononucleotide substitution, respectively

sequenced region (Table 3). We found six kinds of base-pair substitution events, including A:T–G:C, A:T–C:G, G:C–A:T, G:C–T:A, A:T–T:A, G:C–C:G. However, the mutation rates for these substitutions were not the same. Among these substitutions, there are 13 A:T–G:C, 3 A:T–C:G, 3 G:C–T:A, 2 A:T–T:A and only 1 G:C–A:T and none G:C–C:G (Fig. 4d). The results demonstrated clearly that the rate of A:T–G:C mutation is substantially higher in the *transgenic OsPMS1-136 progeny* than the rates for the other types of base-pair substitution events. We also detected the occurrence of oligonucleotide deletion in the sequenced region frequently (Fig. 4b, c). Together, the calculated mutation rates in these three regions reached 1.1–2.7 per kilobase for all the sequences.

Table 3 Mutation types in OsPMS1-136-expressing single-seed descendent populations

Sequences	1	2	3
No. of sequences	9	11	8
Location	Chromosome 6 28956591-28957307	Chromosome 1 39953336-39953907	Chromosome 6 29907473-29908090
Length	717 bp	569 bp	619 bp
A:T–G:C	2	7	4
A:T–C:G	1		2
G:C–A:T	1		
G:C–T:A		1	2
A:T–T:A	1		1
G:C–C:G			
Oligonucleotide deletion	2	5	3
Oligonucleotide insertion	1	4	1
Total mismatches	8	17	13
Mutation frequency	1.2/kb	2.7/kb	2.6/kb

Three fragments from the differential bands amplified with ISSR primers were used in this study. Mutation frequencies were calculated as total mismatches/no. of sequenced fragments × fragment length. Data obtained from transgenic #1

Screening and obtaining important agricultural traits

Based on the above results, the mutation effects occurring via the negative regulation of the MMR system in rice is comparable to that in the similar experiments performed in *Arabidopsis* (Chao et al. 2005). To screen mutants that have important agronomic traits, we germinated T2 seeds of the transgenic *OsPMS1-136* (#1) line on half-strength MS medium supplemented with 250 mM NaCl for 2 weeks; the seedlings were then transferred to half-strength MS medium for recovery (Fig. 5a). A total of 33 salinity-tolerant individuals were selected from 400 offspring; some of them grew to maturity and set seeds (Fig. 5b). For drought-tolerant mutant selection, we germinated T2 seeds of the transgenic *OsPMS1-136* (#1) plants in soil. After the seedling reached a height of about 10 cm, the water supply was withheld and the growth of the seedlings was monitored for 3 weeks. Eight individuals survived (Fig. 5c), and some of these plants grew to maturity and set seeds (Fig. 5d).

To obtain salt tolerant mutants free of transgene, we germinated T3 wild type seeds derived from transgenic *OsPMS1-136* (#1) on 1/2 MS medium supplemented with 250 mM NaCl. On this medium, the control seeds transformed with p1390 can not germinate, while about 60 % of the seeds (243 of 400) segregated from *OsPMS1-136* (#1) can germinate and grow to a height of 3–5 cm, which showed significant salt tolerance. However, we also observed about 30 % of the seedlings showed a dwarf phenotype and about 10 % of the seeds can not germinate (Fig. 6). These results indicate that the mutants obtained by the interference of MMR might contain mutations in addition to salt tolerance in other loci in the genome.

Discussion

In this study, we demonstrate the feasibility of an in vivo mutation method in rice through the negative regulation of the MMR system. We show that overexpression of the truncated protein of the *OsPMS1* gene (*OsPMS1-136*) can produce genomic mutations, such as MSI and mononucleotide substitution, thus generating mutant phenotypes in rice. Screening of the transgenic progeny resulted in mutants that have important agronomical traits such as less tillers, or tolerance to salt and drought stress. Finally, transgene-free mutants were obtained for developing a breeding program further.

MMR is a conservative DNA repair pathway present in different organisms. The mechanism of this pathway for DNA repair has been studied in detail, and important components such as MutS and MutL have been identified and cloned from *A. thaliana* (Ade et al. 1999; Culligan and Hays 1997; Jean et al. 1999). MMR deficiency in *Arabidopsis* causes genome instability and increases mutation rates (Leonard et al. 2003; Hoffman et al. 2004). Chao et al. (2005) overexpressed the human *PMS2-134* (a truncated version of *HsPMS2*) in *Arabidopsis* and reported a high frequency of mutation rates in plants. Here, we demonstrate that suppression of the MMR system in rice was as efficient in producing mutant phenotypes as that in *Arabidopsis*. In *indica* rice, overexpression of the OsPMS1-136 resulted a lethal mutation rate of 14.3 % and a dwarf mutation rate of 44.3 % in the progeny, which are much higher than that was found in the progeny from the EMS (1.6 %) treated IR64 seeds (with lethal mutation rate of 4.51 % and dwarf mutation rate of 0.55 % in M3 population (Wu et al. 2005)). As the truncated OsPMS1 can be

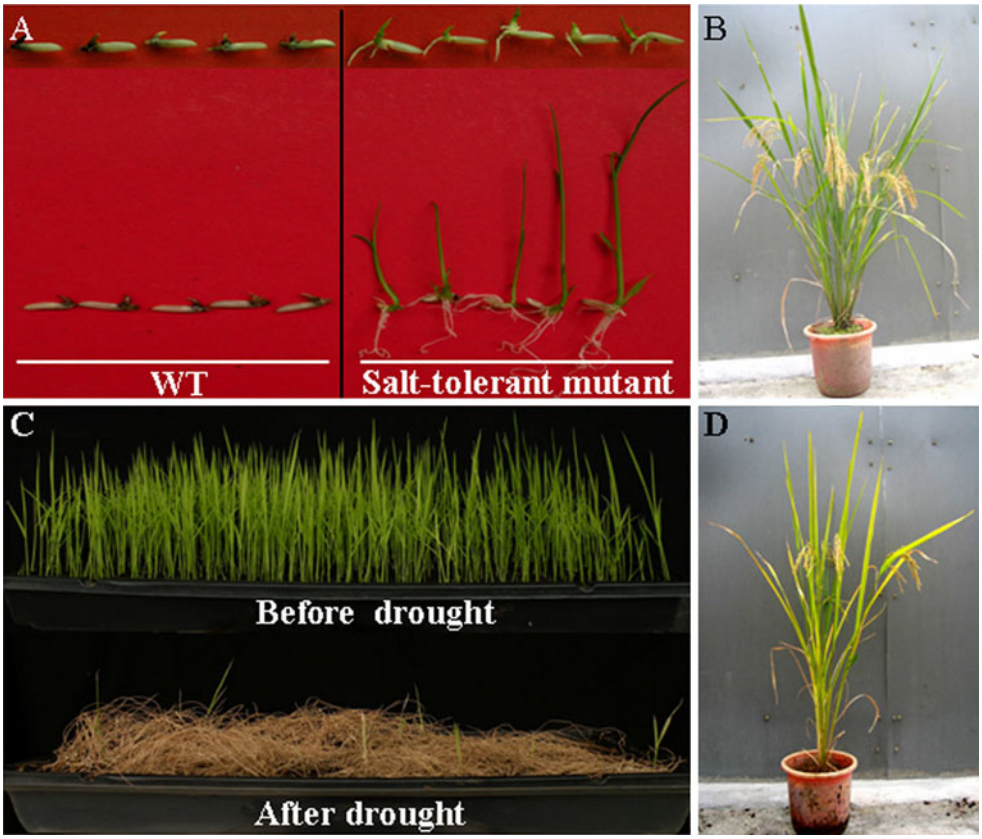

Fig. 5 Screening of mutants that are tolerant to salt and drought stress (#1 transgenic plants of *indica* rice "Shuangsixiangzhan"). Screening of mutants that are tolerant to salt stress (**a**). Top, wild-type and transgenic seeds were germinated and grown on 1/2 MS medium supplemented with 250 mM NaCl for 3 weeks; bottom, The seedlings were transferred to 1/2 MS and allowed to recover for 5 days. Salt tolerant seedlings were transferred to green house and grown to maturity (**b**). Screening of mutants that are tolerant to drought stress (**c**). Transgenic seeds were germinated and grown in soil for 2 weeks (*up*). And the plants were grown without water supply for additional 3 weeks (*bottom*). Eight individuals plants were obtained. Drought tolerant seedlings were transferred to green house and grown to maturity (**d**)

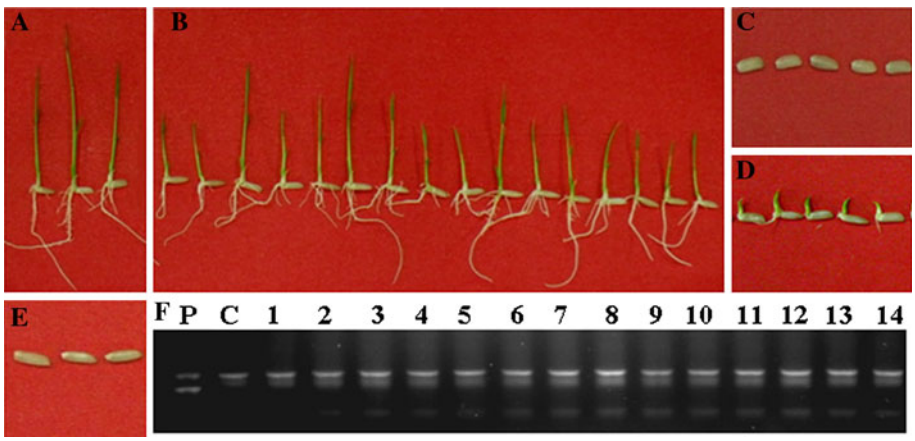

Fig. 6 Obtaining of transgene free salt tolerant plant. **a** Control seeds of pCAMBIA1390 germinated on 1/2 MS medium for 1 week. **b–d** Transgene free progeny segregated from *OsPMS1-136* (#1) germinated on 1/2 MS medium supplemented with 250 mM NaCl for 2 weeks. **e** Control seeds of pCAMBIA1390 germinated on the salt containing medium for 2 weeks. **f** PCR confirmation of the absence of transgene in salt tolerant plants. *P* Template DNA from *OsPMS1-136* (#1) plants. *Upper band* represents amplification of the endogenous DNA, *lower band* represents transgene fragment

regarded as an endogenous mutagen which persisted in different cell types and during the life cycle of a plant, the stronger mutation effects can be expected compared with the exogenous mutagen treatment. The higher mutation ratios we observed in hemizygous and homozygous T2 populations than in wild-type indicates that dominant negative suppression of the endogenous MMR system is active during gametophyte, fertilized egg, embryo and seedling.

In addition to the high mutation efficiency, suppression of the MMR in rice resulted different mutation phenotypes from that of EMS treatment. In OsPMS1-136 transgenic progeny, dwarf and lethal phenotypes are the major mutation phenotypes, while in EMS treated seeds, albino phenotype appeared frequently [about 8 % of M2 progeny from the EMS treated IR64 seeds are albino (Wu et al. 2005)]. Furthermore, suppression of the MMR in rice induced both single nucleotide mutation and micro- satellite instability, while EMS treatment mainly induced single nucleotide mutation. In addition, about 60 % (13 out of 22) A:T–G:C transitions were found in OsPMS1-136 transgenic plants, while EMS treatment produced 70 % G:C–A:T (Till et al. 2007). These indicate that the manipulation of DNA damage or DNA repair process might produce different mutant types. The combination of these two methods will have the potential to obtain higher mutation frequency.

Although MMR is a conservative pathway in different organisms, its contribution to mutation correction could be different in various species. In the two rice subspecies, we found that negative regulation of MMR in the *indica* rice "Shuangsixiangzhan" caused a more severe mutation effect than that in *japonica* rice "Zhonghua 11". This effect was indicated by the high frequency of dwarf and lethal phenotypes, as well as more variations in the field-grown plants. This indicates that *indica* rice either produces more errors during DNA metabolism or that the MMR system is more important for maintaining the genome integrity in this subspecies. Rice varieties are traditionally classified into two major subspecies, *Indica* and *Japonica*, which differ in their adaptation to different climatic, ecogeographic and cultural conditions (Chang 2003). A former comparative genomic study by Ma and Bennetzen (2004) demonstrated that *indica* exhibits higher sequence variation than *japonica.* They found that the rate of site substitution mutations detected in *indica* was higher than detected in *japonica*, a higher ratio of solo LTRs to intact LTR retrotransposons exists in *indica* than in *japonica* and more deletions in the *indica* genome than in the *japonica* genome. Further studies of the efficiency of DNA MMR system in the two subspecies may explain the adaptation of different rice ecotypes to environment.

The present work demonstrates a new method for rice mutagenesis, and it can be applied at least in two aspects. First, it can be used for mutation breeding in rice. This has been proved in our experiments by obtaining agronomically important mutants, such as male sterile, flowering time, stress tolerance etc. Importantly, the mutation phenotype can be maintained without the transgene. Once a desired phenotype is obtained, the transgene can be segregated out by breeding strategies, which will not cause bio-safety concerns. At least two methods for segregating out the transgene can be adopted. If a desired mutant is hemizygous for the transgene, the mutant can be found in its self-pollinated null transgenic progeny. For a mutant that is homozygous for the transgene, the mutant needs to be backcrossed with the wild-type plant, and the mutants with the transgene segregated out could be found in the progeny. Second, it can be used independently or together with the EMS mutation method for obtaining high mutation rates for TILLING (Targeting Induced Local Lesions IN Genomes) (McCallum et al. 2000, Colbert et al. 2001). Although TILLING has been used to identify point mutations in different species (Perry et al. 2003; Slade et al. 2005; Till et al. 2004; Wu et al. 2005), the application of this method to rice, however, has been hampered by the difficulty in obtaining a population with a sufficiently high mutation density, a relatively low number of EMS induced mutations were reported for the *indica* rice variety IR64 when treated with 0.8 and 1 % EMS (Wu et al. 2005). Alternative mutagens and methods were also used to improve the mutation frequency in rice. For example, treatment of single zygotes of *Oryza sativa japonica* cv. Taichung 6 with *N-methyl-nitrosourea* (MNU) resulted in one mutation in every 135 kb genome sequence (Suzuki et al. 2008). The Nipponbare populations treated either with 1.5 % EMS or by sequential soaking in 1 mM sodium azide and 15 mM MNU showed a similar density of putative mutations detected by TILLING, $\sim$1/300 kb (Till et al. 2007). These mutation rates are still low; furthermore, treatment with mutagen often resulted in insufficient seed germination due to toxicity. Thus, our present method might also be an alternative choice for the TILLING purpose.

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