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A new AOX homologous gene OsIM1 from rice (*Oryza sativa* L.) with an alternative splicing mechanism under salt stress

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Abstract A differentially expressed *OsIM1* gene was isolated from rice salt-tolerant mutant M-20 by differential display. Sequence analysis revealed that the amino-acid sequence of OsIM1 showed 66% and 62% identity with PTOX from tomato (*Capsicum annuum*) and AtIM from Arabidopsis, both of which encoded chloroplast-orientated terminal oxidase. Comparison of the nucleotide sequence of the *OsIM1* cDNA with its genomic sequence revealed that *OsIM1* genomic DNA contained nine exons and eight introns. A pseudo-transcript (*OsIM2*), which probably resulted from the abnormal splicing of the *OsIM1* pre-mRNA, was also identified. Southern-blot analysis showed that there existed only one copy of the *OsIM1* gene in the rice genome. RFLP analysis located it on rice chromosome 3. The Northern blot revealed that *OsIM1* was up-regulated by NaCl and ABA treatment. RT-PCR analysis indicated that *OsIM*1 and *OsIM*2 co-existed in the *OsIM* transcript pool, and the ratio of *OsIM1*/*OsIM2* was differentially regulated by salt stress in the salt-sensitive variety and the salt-tolerant varieties.

Keywords DDRT-PCR · *OsIM* gene · Alternative splicing · Salt stress · Rice (*Oryza sativa* L.)

Introduction

High salinity, as the most severe abiotic stress, does great harm to agriculture by affecting crop growth and production. The damage caused by salt stress is often related to the production of reactive oxygen species (ROS). ROS is a collective term that includes several oxygen radicals such as superoxide, hydrogen peroxide and hydroxyl radicals. Both transgenic plants overexpressing ROS scavengers and mutants with higher ROS scavenging ability showed increased tolerance to environmental stresses (reviewed by Xiong et al. 2002).

Alternative Oxidase (AOX) is an inner mitochondrial membrane protein that functions as terminal oxidase in the alternative (cyanide-resistant) pathway of respiration where it generates water from ubiquinol (Cournac et al. 2002). AOX serves to relieve oxidative stress originating from environmental stresses, especially salt and dehydration stress by limiting mitochondrial ROS formation via preventing specific components of the respiration chain from over-reduction (Purvis and Shewfelt 1993; Wagner 1995; Popov et al. 1997; Purvis 1997). In recent years, many *AOX* genes have been characterized in plants (Kumar and Soll 1992; Ito et al. 1997; Saisho et al. 1997) and two genes, *AtIM* from *Arabidopsis thaliana* (Carol et al. 1999; Wu et al. 1999) and *PTOX* from tomato (*Lycopersicum esculentum*) (Josse et al. 2000), have been found. These two genes encoded proteins that functioned as a plastid terminal oxidase for carotenoid synthesis in the phytoene de-saturation step. Carotenoids were not only directly involved in photoprotection by eliminating singlet oxygen and other reactive species but were also related to the synthesis of ABA, which indirectly prevent the damage of ROS.

We are interested in the abiotic stress-tolerant relative genes and have isolated a series of salt-responsive genes, e.g. the *OsZFP1* gene and the *SAMDC* gene from rice (Li and Chen 2000, 2001), the *NTHK1* gene from tobacco (Zhang et al. 2001; Xie et al. 2003), and the *AhCMO* gene from *Atriplex hortensis* (Shen et al. 2002). In the present study, a salt-responsive gene was identified from rice by using the differential-display (DDRT) method. The gene showed high homology to the *AtIM* gene from Arabidopsis and thus was designed as *OsIM1*. The genomic organization and expression of *OsIM1* in rice were investigated. Its possible function in stress responses was also discussed.

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Materials and methods

Plant materials and treatments

The seeds of the rice variety 77-170 (*Oryza sativa* L. var. 77-170), its salt-tolerant mutant M-20 and another salt-tolerant variety Lansheng, were imbibed in water at 37 °C for 2 days and then germinated at 26 °C with a photoperiod of 12 h. At the three-leaf stage, the M-20 and 77-170 seedlings were grown for 2 days in the solution containing 120 mM of NaCl. Shoots were harvested and total RNAs were extracted for differential display analysis.

The rice seedlings at the three-leaf stage of rice variety 77-170, M-20 and Lansheng were transferred to a solution containing 150 mM of NaCl for various times or transferred to different concentrations of ABA (10 μM, 50 μM, 100 μM) for 6 h. The shoots were harvested, frozen in liquid nitrogen and stored at –70 °C. Total RNAs were extracted for RT-PCR.

Differential display RT-PCR (DDRT-PCR)

Total RNA extraction was conducted as described by Zhang et al. (1996). First-strand cDNAs were synthesized from 400 ng of total RNA (pre-digested by DNase I) for 60 min at 37 °C in a 40-μl reaction volume with 400 U of MMLV reverse transcriptase (Promega). DDRT-PCR was performed according to procedures described previously (Li and Chen 2000). PCR analysis was conducted in a 25-μl volume containing 5 ul of first-strand cDNAs, 1 × PCR buffer, 2.5 μM of $T_{12}MN$, 0.5 μM of arbitrary primer (Operon), 2 μM of dNTPs, 5 μCi of α-[^{32}P]-dCTP (Amersham) and 1 U of *Taq* DNA polymerase. The amplification profile was 3 min at 94 °C for pre-denaturation, 40 cycles of 15 s at 94 °C, 2 min at 40 °C, 1 min at 72 °C and an additional extension for 10 min at 72 °C. The isotope-labeled PCR products were separated on a 6% non-denaturing polyacrylamide gel and exposed to the X-ray film. The cDNA fragments showing differences between salt-stressed and control seedlings were retrieved from the sequencing gel and eluted in 100 μl of water by boiling for 10 min. One twentieth of the elution was used as templates for PCR re-amplification. The re-amplification condition was the same as that in the initial DDRT-PCR except that the dNTPs concentration was increased to 20 μM without isotope. The re-amplified cDNA fragments were recovered from the agarose gel and cloned into pGEM-T easy vector (Promega) as described in the manufacturer's instruction.

Construction and screening of a salt-stressed cDNA library

Total RNA was extracted from salt-treated M-20 seedlings and then PolyA$^+$ RNA was purified with oligo(dT) cellulose. A λ-ZAPII cDNA library was constructed with a λ-ZAPII cDNA library kit (Stratagene) and screened following the manufacturer's protocol.

Rapid amplification of the cDNA end (RACE)

5'-RACE was performed with the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. The full-length cDNA was obtained by RT-PCR and cloned into pGEM-T easy vector for sequencing. The gene-specific sequences of primers for RACE are as follows:

GST1: 5'-CGGCACCCTAGCAATCGTCTCCAATACG-3'
GST2: 5'-GTATCGTGATGACTGACTCCGTGAGG-3'.

Genomic Southern analysis and restriction fragment length polymorphism (RFLP) mapping

DNA isolation followed the method described by McCouch et al. (1988). Total DNA (5 μg) was digested overnight, separated on 0.8% agarose gel and transferred onto Hybond N$^+$ nylon membranes for Southern-blot analysis. Southern hybridization was carried out overnight at 65 °C using α-[^{32}P]-dCTP-labeled OsIM cDNA as a probe. The filters were washed at 65 °C with 2 × SSC, 0.1% SDS; 1 × SSC, 0.1% SDS and 0.5 × SSC and 0.1% SDS each for 15 min.

A segregation double-haploid (DH) population of 127 lines by anther culture of an F1 hybrid between an indica variety ZYQ8 and Japonica variety JX17 was used as a mapping population (Shen et al. 1998). Total DNAs of the two parents were digested completely by *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Sca*I and *Xba*l. The *Dra*I and *Eco*RI were chosen to digest DNA of DH lines and separated on a 0.8% agarose gel and transferred onto nylon membranes for Southern hybridization using *OsIM* cDNA as a probe. Linkage analysis was conducted using the MAPMAKER/EXP. 3.0 software.

Northern-blot analysis

Total RNA (30 μg) was separated on a 1% formaldehyde agarose gel and blotted onto the Hybond N$^+$ membranes in 20 × SSC. Pre-hybridization, hybridization and washing procedures were conducted according to standard protocols. After stripping the membranes, radiolabeled 18S rDNA was used to detect the quality and quantity of RNA.

DNA sequencing and data analysis

DNA sequencing was conducted on an ABI 373A automatic sequencer. The amino-acid sequence was compared with those released in GenBank databases using the BLAST analysis program. The full-length sequence of *OsIM1* has been deposited in GenBank under the accession No. AF085174.

Reverse transcription PCR (RT-PCR) analysis

To detect the relative abundance of *OsIM1* and *OsIM2* in the *OsIM* transcript pool, two pairs of transcript-specific primers were designed according to the sequences of *OsIM1* and *OsIM2*. The sense primer IM1f (5'-GGGAGAAGGAGCAGACCGA-3') and antisense primer IM1r (5'-GCCACCCAATTCTTCCATG-3') are *OsIM1*-specific, while IM2f (5'-TTTTTACTCGGTCTTTCTTG-3') and IM2r (5'-ACAGGCCTTCATCGTCT-3') are *OsIM2*-specific. Primers of an actin gene Rac1 (sense: 5'-GGAACTGGTATGGTCAAGGC-3'; anti-sense: 5'-AGTCTCATGGATAACCGCAG-3') were also designed and used to amplify a control. Templates for RT-PCR were reverse-transcribed cDNAs from total RNA of 77-170, M-20 and Lansheng, which have been subjected to varying durations of salt treatment (150 mM of NaCl). The PCR reaction was performed at 94 °C, 3 min for one cycle; followed by 40 cycles of 94 °C, for 50 s, at 50 °C (for *OsIM2*) or 55 °C (for *OsIM1*), 1.5 min, at 72 °C, 1.5 min with an extension of 10 min at 72 °C. The corresponding PCR products were separated on 1% agarose gel.

Results

Differential display

The differential display method was conducted to identify differentially expressed genes between salt-stressed and non-stressed seedlings of rice variety M-20 and 77-170 respectively. Two anchor primers ($T_{12}CA$, $T_{12}GA$) and 20 arbitrary primers (10 mer, Operon) were combined to perform DDRT-PCR reactions. Among the 2,600 cDNA

Fig. 1 Comparison of the amino-acid sequence of OsIM1 with PTOX and AtIM. The amino-acid sequence of OsIM1 was compared with those of tomato PTOX and Arabidopsis AtIM for maximum alignment. T1 and T2 represent putative trans-membrane domains; B1 and B2 represent putative iron-binding motifs. A putative transit peptide was also indicated. *Dashes* were included for maximum alignment

fragments displayed on the sequencing gel, 83 fragments showed differential signals between two varieties and four of them were proved to be salt-inducible by Northern analysis. One fragment, which was amplified by $T_{12}CA/OPS_{17}$, was chosen for further analysis. Sequence analysis of this clone revealed that it was 452 bp in length and had similarity with Arabidopsis *AtIM* gene. Thus the corresponding gene was named *OsIM*.

Isolation and structural analysis of the full-length *OsIM* gene

To obtain the full-length sequence of this *OsIM* gene, a salt-stressed cDNA library of rice was constructed and screened with the 452-bp *OsIM* gene fragment. A total of 12 clones were obtained. According to the restriction enzymatic digestion (*Eco*RI + *Xho*I) patterns, the 12 clones were classified into three different groups. Sequencing analysis showed that each group represented a distinct but still related sequence of *OsIM* gene. The three corresponding *OsIM* genes were designated respectively as *OsIM1* (Accession No. AF085174), *OsIM1a* and *OsIM2* (Accession No. AF288401). *OsIM1* was 1.4 kb in length and was still partial in its 5'-coding region. *OsIM1a* is the same as *OsIM1* except that its 3'-UTR lacked a 26-bp nucleotide sequence. *OsIM2* didn't have a complete ORF and seemed to be a pseudogene. The 5'-RACE method was further adopted and the 5' coding region of the *OsIM1* gene was obtained. The full-length of the *OsIM1* gene was 1,512 bp and encoded a protein of 337 amino acids (Fig. 1), with a molecular mass of 39 kDa and a pI of 5.76.

The amino-acid sequence of the *OsIM1* protein was compared with other AOX proteins (Fig.1). The result revealed that OsIM1 showed high amino-acid identity with the new AOX members AtIM (62%) from *Arabidopsis thaliana* and PTOX (66%) from *Capsicum annuum*, and also showed weak but significant homology with all the other AOXs members (25–29%). By comparison with AtIM, two putative transmembrane domains (T1, T2) and two putitive iron-binding motifs (EXXH) B1 and B2 were identified in OsIM1(Fig. 1). A transit peptide of 35 amino acids was also predicted by ChloroP1.1 software. This peptide probably functioned during the entry of OsIM1 into the chloroplast.

Genomic organization and chromosome localization of the *OsIM1* gene

To investigate the genomic organization of the *OsIM1* gene, a commercial rice-genomic DNA library (Clontech) was screened and the genomic-DNA sequence (approximately 3.0 kb) of the *OsIM* gene was obtained and sequenced. By aligning the cDNA sequence of *OsIM1* with the genomic DNA sequence, we found that *OsIM1* genomic DNA had nine exons and eight introns (Fig. 2). All the introns had a GT at the 5'-end and an AG at the 3'-end, consistent with the GT/AG splicing rule. The correct splicing resulted in *OsIM1* mRNA, whereas incorrect splicing probably led to the formation of a pseudo-transcript *OsIM2*, which contained the last four exons plus the intron between the sixth and the seventh exons (Fig. 2). The other sequence of the 5'-region of the *OsIM2* was not known.

To map the *OsIM* gene onto the rice chromosome, RFLP analysis was performed. The genomic DNAs from two parents JX17 and JYQ8 were digested with various restriction enzymes (*Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Sca*I and *Xba*I) and subjected to hybridization with the *OsIM1* gene as a probe. Polymorphism was then detected in the digestions with *Dra*I and *Eco*RI (Fig. 3A). The polymophic band in the *Dra*I digestion was used to

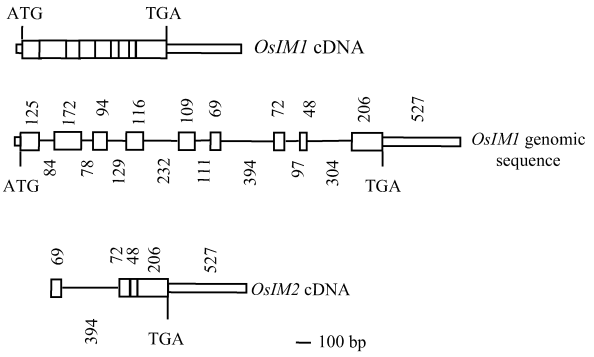

Fig. 2 Genomic organization of the *OsIM1* gene. Schematic representation of cDNA and the genomic sequence of the *OsIM1* gene were presented and compared. Exons are indicated by *boxes*, and introns by *lines*. *Numbers* indicate the length (bp) of exons (above each box) or introns (under each line). The translation initiation site (ATG) and stop codon (TGA) are indicated. The partial cDNA of the abnormal spliced *OsIM2* gene are also presented. Please note the presence of intron 6 in this pseudo *OsIM* cDNA

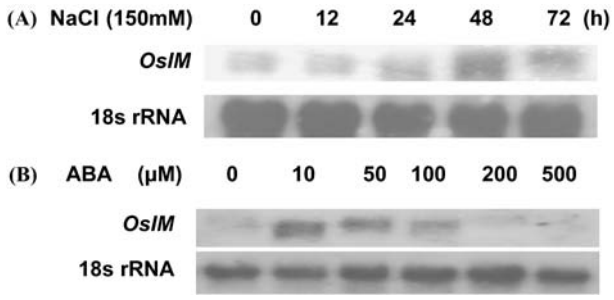

Fig. 4A, B Expression of *OsIM1* of rice seedlings in response to salt stresses and ABA treatment. (**A**) The rice seedlings were treated with 150 mM of NaCl. Thirty micrograms of total RNA was loaded for each sample and the blot was hybridized with the *OsIM1* cDNA probe. The same blot was stripped of the probe, and re-hybridized with 18S rDNA to verify the integrity and equal loading of RNA samples. (**B**) The rice seedlings were treated with a different concentration of ABA for 6 h. Other procedures were the same as in (**A**)

analyze the segregation in the individuals of the DH population derived from JX17 and JYQ8. The *OsIM* gene was then mapped onto chromosome 3 between markers G164 and RG756 (Fig. 3B) by using the constructed linkage map.

Differential accumulation of *OsIM* transcripts under environmental stresses

The expression of the *OsIM* gene under stresses was investigated in response to salt stress in rice seedlings using the *OsIM1* cDNA as a probe. Considering the homology of *OsIM1* with *OsIM1a* and *OsIM2*, it is likely that all the three transcripts were detected. The results showed that salt stress caused a steady accumulation of *OsIM* transcripts, and the expression peak was observed at 48 h after initiation of the stress (Fig. 4A).

Since the plant hormone ABA is also involved in many stress responses, the expression of *OsIM* was analyzed in response to different concentrations of ABA treatment. The results (Fig. 4B) showed that *OsIM* was up-regulated by ABA and the strongest induction was observed at a concentration of 10 μM, which was 4-fold higher than that at 0 μM. At higher ABA concentrations, the expression of *OsIM* was repressed. The possible feedback inhibition by ABA, which is the final product of carotenoid biosynthesis pathway (Cunningham and Gantt 1998), suggested that *OsIM* might regulate the biosynthesis of ABA, a universal plant hormone to relieve environmental stresses.

Because three *OsIM* transcripts were found, and *OsIM1* (plus *OsIM1a*) probably represented the functional ones and *OsIM2* represented the pseudo-one, two sets of specific primers were designed to amplify the gene

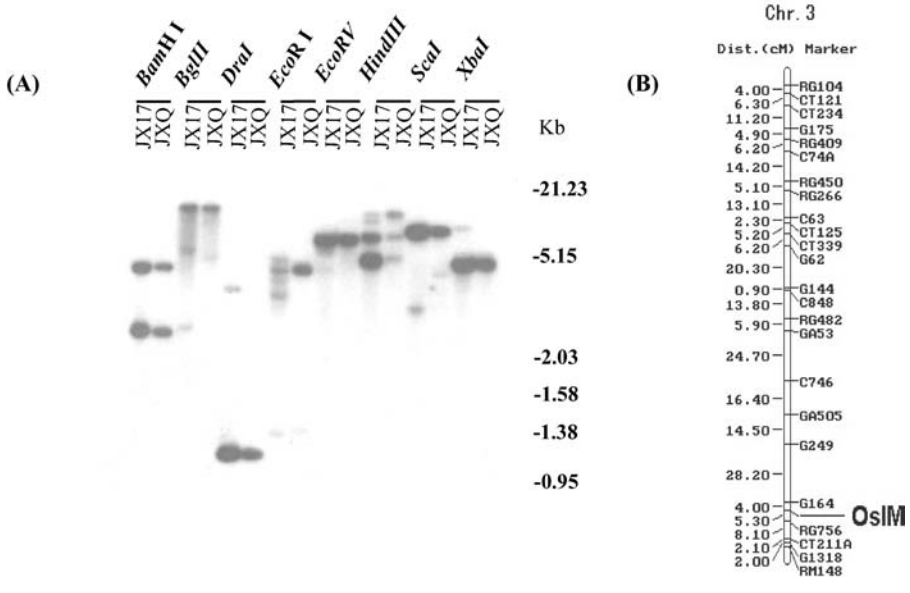

Fig. 3A, B Southern analysis and chromosome localization of the *OsIM1* gene. Rice genomic DNA was digested with *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Sca*I and *Xba*I (**A**) and hybridized with the cDNA of *OsIM1*. The polymorphic fragment was obtained in *Dra*I-digested DNA and this RFLP band was mapped onto rice chromosome 3 (**B**) between G164–RG756 using JX17/JXQ DH population and its linkage map

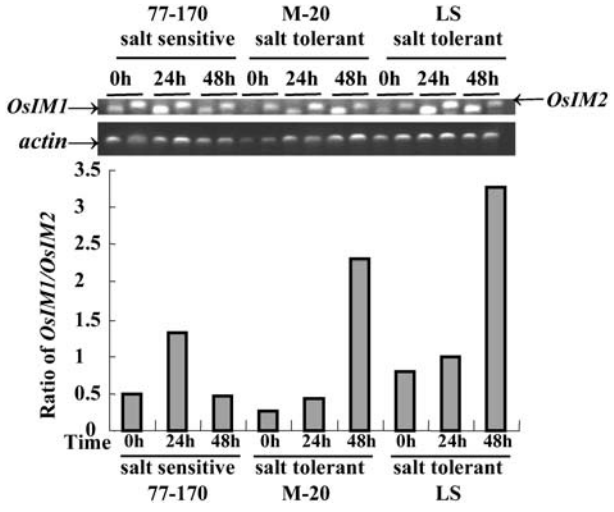

Fig. 5 RT-PCR analysis of the relative abundance of *OsIM1* and *OsIM2* in salt-tolerant and salt-sensitive varieties under salt stress. cDNA from each sample was used as a template for RT-PCR analysis. Two sets of specific primers were used to amplify the *OsIM1* and *OsIM2* fragments. Actin was amplified as a control. The intensity of each band was quantitated by an Imaging Densitometer (BioRad) and the ratio of *OsIM1*/*OsIM2* was calculated

fragment corresponding to *OsIM1* (313 bp) and *OsIM2* (352 bp) respectively. The ratio of *OsIM1* to *OsIM2* was determined under salt-stress conditions (Fig. 5). The results showed that *OsIM1* and *OsIM2* co-existed in the transcript pool of the *OsIM* gene in all the three rice varieties under normal conditions. Upon salt stress, the ratio of *OsIM1*/*OsIM2* went up steadily in salt-tolerant varieties M-20 and Lan Sheng (LS). In salt-sensitive 77-170, the ratio reached a peak at 24 h after the stress was initiated and then declined (Fig. 5).

Discussion

Environmental stresses, especially salt and dehydration stress, will result in oxidative stress in plants (Dahan et al. 1997). AOXs serve as key enzymes in relieving oxidative stress in mitochondria by quenching ROS (Day et al. 1995). In the present study the *OsIM1* gene was isolated from rice and shared homology with the new *AOX* members *AtIM* and *PTOX*. Because AtIM and PTOX behaved like a quinol: oxygen oxidoreductase in the chloroplast, we thus proposed that OsIM1 might also function as a similar enzyme. However, the exact function of OsIM1 remains to be elucidated.

Studies have shown that both AtIM and PTOX were involved in carotenoid synthesis. Mutation of these genes impaired phytoene de-saturase, which caused accumulation of phytoene and a block in carotenoid synthesis. Carotenoid can prevent the plant cells from oxidative stress by reacting with ROS directly or indirectly (Britton 1995; Demming-Adams et al. 1996; Frank and Cogdell 1996). Due to the fact that *OsIM* was induced by salt stress, it was possible that OsIM functioned to decrease the ROS content generated under salt-stress conditions. The rice *OsIM1* was also induced by ABA treatment, indicating its possible roles in plant responses to ABA by facilitating the production of carotenoids.

The genomic organization of the present *OsIM1* gene was also compared with *PTOX* and *AtIM* (Josse et al. 2000). They all had nine exons and the length of all the three genes was highly conserved in the exons. The length of the exons (from exon 3 to exon 8) are the same in the three genes, i.e. 94 bp in exon3, 116 bp in exon4, 109 bp in exon5, 69 bp in exon6, 72 bp in exon7 and 48 bp in exon8. For exon 9, its length was 206 bp in both *OsIM* and *AtIM*, but not in *PTOX*. The conserved genomic organization indicated the importance of these genes in the house-keeping aspects of plant life.

Comparison of the *OsIM* cDNA and genomic DNA sequence revealed that normal splicing resulted in transcript *OsIM1*, and the abnormal splicing led to the pseudo-transcript *OsIM2*. Under normal conditions both transcripts were present, indicating that two splicing mechanisms existed even without stress. The reason for this phenomenon was not clear. Upon salt-stress, *OsIM1* was induced in both salt-tolerant varieties and maintained at a high level, while the increase of the ratio of *OsIM1*/*OsIM2* mainly resulted from the decrease of *OsIM2*; whereas in the salt-sensitive variety, the quantity of *OsIM1* transcript went up in 24 h and decreased more quickly than *OsIM2*, which resulted in a decrease of the ratio of *OsIM1*/*OsIM2*. The difference in the induction pattern of *OsIM1*/*OsIM2* may reflect the competition between the two splicing mechanisms during salt stress, and the salt-tolerance probably not only resulted from an increased expression level of *OsIM1*, but also due to the ability to regulate the ratio of *OsIM1*/*OsIM2* in plants. A similar post-transcriptional mechanism was also observed in other plants (Marrs and Walbot 1997; Ranson et al. 1998; Dinesh-Kumar and Baker 2000), except that the alternative transcripts were functional in the authors research. Further investigation should elucidate more about the function and the regulatory mechanism of *OsIM* in plant-stress responses.

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