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Molecular Characterization of Two Rice cDNAs Encoding GDP-Mannose-3′, 5′-Epimerase and Their Expression Patterns*

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Abstract GDP-mannose-3′, 5′-epimerase (GME), which converts GDP-mannose into GDP-L-galactose, is essential for the biosynthesis of L-ascorbic acid in higher plants. The molecular characterization of two GME genes from rice has been reported. Firstly, both cDNAs were isolated from the rice mature leaves using RT-PCR technique. By comparing their sequences with homologues from other plants, it was found that GME genes were highly conserved among plant species, though phylogenetic study showed that all known GMEs could be divided into two distinct groups corresponding to monocots and dicots. Secondly, the genomic organization of rice OsGME genes was investigated, and a similarity of splice patterns was revealed. Finally, the expression patterns of the two cDNAs have been studied in various tissues and under different stress conditions by semiquantitative RT-PCR assay. The results showed that the *OsGME1* transcript was up-regulated in response to cold stress, and gibberellin might regulate L-ascorbic acid levels by affecting transcription of both *OsGME* genes.

Key words L-ascorbic acid biosynthesis, gene expression, semiquantitative RT-PCR assay.

L-ascorbic acid (vitamin C, abbreviate to AA in this paper) is not an unfamiliar antioxidant to us all. AA was isolated from plant and mammalian sources in 1932 [1]. Since then, numerous reports on its physiological and metabolic roles in both plants and animals have been published. Unfortunately, humans, together with other primates, are unable to synthesize AA by themselves [2]. Consequently, plant yielding ascorbic acid provides the major source of dietary vitamin C for humans.

All higher plant species contain high concentrations of AA that range from 10 to 300 mmol/L in many different cell compartments, e.g., mitochondria, chloroplast, and cytosol[3]. In plants, AA are involved in many physiological processes and has important functions in growth and metabolism[3~5]. In its antioxidant role, AA can directly scavenge reactive oxygen species (ROS), such as superoxide, singlet oxygen, and H_2O_2[4,6]. Through this regulation of ROS level, AA is implicated in the control of cell growth, cell division and programmed cell death[7]. Meanwhile, as a cofactor to a number of iron and copper-containing oxygenases, AA is also required in the biosynthesis of some phytohormone, such as ethylene and gibberellic acid [8]. Furthermore, recent study indicated that AA content was correlated with expression of some genes in transcription level, including some pathogenesis related genes and genes involved in the biosynthesis of abscisic acid[9].

Although the biosynthesis of L-ascorbic acid in animals was well understood many years ago, the plant pathway remained quite unknown to scientists until 1998, in which year our understanding of AA biosynthesis in plants underwent a breakthrough when Smirnoff and Wheeler proposed a functional pathway of AA biosynthesis. In Smirnoff-Wheeler pathway, AA was synthesized from GDP-D-mannose via GDP-L-galactose, L-galactose and L-galactono-glactone as intermediates [10]. Subsequently, several cDNAs encoding enzymes required in this hypothetical

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pathway were cloned from various plant species. One of these enzymes, GDP - mannose - 3′, 5′ - epimerase (GME), catalyzing the conversion of GDP-D-mannose to GDP-L-galactose, has been purified and cloned from *Arabidopsis thaliana* [11]. The enzyme contains a modified version of the NAD binding motif and is simulated by the oxidized nicotinamide nucleotides (NAD^+ and $NADP^+$) and inhibited by their reduced forms (NADH and NADPH), which associates the enzyme activity with the redox state of cell. Enzymatic characterization study showed that this enzyme catalyzed two distinct epimerization reactions that produce either GDP-L-galactose or GDP-L-gulose, and the type of reactions seems to be depended on the molecular form of the enzyme. Further, both recombinant and native enzymes co-purified with a Hsp70 heat-shock protein (*Escherichia coli* DnaK and *A. thaliana* Hsc70.3, respectively), suggesting the Hsp70 molecular chaperones might be required in folding and/or regulation of this enzyme[11,12].

The multiple roles played by AA mean that the concentration of the compound must be tightly controlled. This is accomplished through various mechanisms, including the expression of genes involved in the biosynthesis pathway, the regulation of enzyme activity, the transportation of the molecule, and the regeneration of its oxidized form[13]. As a vital enzyme in AA biosynthesis, the expression of GME genes would be a good candidate for the regulation of AA levels. Recently, a proteomic study performed by our laboratory, which attracted our attention intensively, has showed that the enzyme was accumulated in rice seedlings exposed to cold stress[14]. However, no published paper has so far been concerned about the transcription pattern of this enzyme in plants. In the present study, the characterization of two rice GME genes, designated *OsGME1* and *OsGME2* respectively, was reported. A phylogenetic study of all known GME proteins was performed, and the effects of various hormones and stress treatments on OsGME genes expression were examined and discussed. To our knowledge, this is the first study in the transcriptional level of GME genes.

1 Materials and methods

1.1 Plant materials and treatments with exogenous factors

Rice (*Oryza sativa* L. ssp. japonica) was used in this study. All the seeds were surface-sterilized with 5% javel water for 1 h, then germinated on wet filter paper at 30℃ for 24 h in dark condition. The plants were grown in the field in normal season, sample of roots, stems, leaves, glumes before pollination, 2-day glumes after pollination, and 15-day-old seeds were harvested in adult age and immediately frozen in liquid nitrogen and then stored at −80℃ until use.

Moreover, for various stress treatment, rice seedlings were grown in the green house with an 16 h light (28℃) and 8 h dark (23℃) regime for 10 days, and the humidity was maintained at 30%. Hogland solution was supplied every two days to provide whole nutrition to the seedlings. Then cold stress treatments were performed in the same way as previous study by incubating the seedlings in a gradually decreasing temperatures form 15℃, 10℃ and 5℃, 24 h for each treatments. Besides, the roots of some seedlings were submerged separately in aqueous solutions containing H_2O_2, ethephon (an ethylene-releasing compound, ET), abscisic acid (ABA), salicylic acid (SA), indole-3-acetic acid (IAA), jasmonic acid (JA), gibberellin (GA_3) for 3 h and in different concentration aqueous solutions of NaCl, $CuSO_4$, $CdSO_4$, $ZnSO_4$ for 6 h, respectively. Other seedlings were also treated separately with heat shock (42℃) for 6 h, drought for 0.5 h and 1 h, wounding for 0.5 h and 1 h. All these prepared materials were also immediately frozen in liquid nitrogen and stored at −80℃ until use.

1.2 Cloning and sequencing of the full-length cDNA

Total RNA of mature leave was extracted using RNeasy Plant Mini Kit (Qiagen) according to the instructions provided by the manufacturer. The first strand cDNA was synthesized with 1 μg purified total RNA using RT-PCR system (Promega) according to the manufacturer's protocol. The resulting first strand cDNA was used as a template for PCR amplification using the following primers synthesized by Sangon (Shanghai) based on the NCBI query screen result. GME1-1: 5′ TCATCATCCTCCTCCTCCCTATC 3′ (sense), GME1-2: 5′ TGACAAAATGTAATCCATA-CGAAG 3′ (antisense) for OsGME1 and GME2-1: 5′ CTCTTCCTTCCGACTTGTGA 3′ (sense), GME2-2: 5′ AATTGTATAGATGGTGGTGGC 3′ (antisense) for OsGME2, respectively. PCR was performed on a DNA amplication machine (PTC-200, MJ Research) for an initial denaturation at 94℃ for 5 min, 28 cycles at 94℃ for 30 s, 60℃ for 30 s, 72℃ for 1 min and a final 72℃ for 10 min. The amplified fragments were

isolated, gel-purified using the GeneClean Ⅲ kit (Bio 101), cloned into a pMD18-T vector (TaKaRa) and sequenced by Bioasia.

1.3 Database searching and sequence analysis

The sequence data used in this study were collected from a query search in the BLAST program at NCBI (http://www.ncbi.nlm.nih.gov/blast) and TIGR (http://tigrblast.tigr.org/cmr-blast/) using the amino acid sequence of AtGME protein. The genome organization and map location were investigated by the corresponding genome sequence with the map viewer at NCBI (http://www.ncbi.nlm.nih.gov/mapview/). About 1 000 bp upstream of the translation initiation codon ATG of two target genes were used as the upstream regulatory region to search possible *cis*-regulatory elements in the PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE/). The molecular mass and p*I* was calculated by Compute p*I*/*Mw* (http://us.expasy.org/tools/pi_tool.html). The deduced amino acid sequences of ORFs were initially aligned using the program ClustalX (ver 1.81) with the default gap penalties. The unrooted phylogenetic tree was constructed by neighbor-joining method provided by MEGA program (ver 3.0).

1.4 Semiquantitative RT-PCR assay

Total RNA of various samples were extracted using the same method mentioned above. To remove genomic DNA, RNA samples were treated with RNase-free DNase Ⅰ (Sigma). These DNA-free RNA samples were quantified spectrophotometrically, carefully diluted to equal concentrations and quality checked by agarose gel electrophoresis. The first strand cDNA was synthesized with 1 μg purified total RNA using RT-PCR system (Promega) mentioned above. One-thirtieth of the first strand cDNA products were used for amplification. A 500 bp fragment of the rice constitutively expressed actin gene *rac1* was used as a control. Three pairs of specific primers were used in the semiquantitative RT-PCR assay. GME1-3: 5′ GGGTTCATTCAGTCCAACCACT 3′, GME1-4: 5′ CAGCCATCTCGTTCATGCTTAC 3′ for OsGME1, GME2-3: 5′ CTGATATGGGAGGGATGGGATTC 3′, GME2-4: 5′ ATGAGCGTGTTGTCGGAGTTGC 3′ for OsGME2, Actin-1: 5′ GTCTGCGATAATG-GAACTG 3′, Actin-2: 5′ TACCACCACTGAGAA-CGATG 3′ for *rac1*, respectively. To discriminate the PCR product of the synthesized one-strand cDNA from the product of contaminating genomic DNA in electrophoresis, all of primer pairs were designed to overspan at least one intron in the target genes. The primer pair GME1-3/GME1-4 overspans two introns in the OsGME1 gene, GME2-3/GME2-4 overspans three introns in the OsGME2 gene, and the Actin1/Actin2 overspans two introns in the *rac1* gene. We also performed experiments to determine the appropriate number of cycles so that not only the amplification product was clearly visible on the agarose gel and can be quantified, but also the amplification was in the exponential range and has not reached a plateau (data not shown). Therefore, we can guarantee the linearity between the amount of input RNA and the final RT-PCR products. All RT-PCR expression assays were performed and analyzed at least twice in independent experiments.

2 Results and discussion

2.1 Molecular cloning and sequence analysis of *OsGMEs*

Previously, in the proteomic study of rice in respond to cold stress, 41 up-regulated proteins were identified under gradual low temperature stress. One of these proteins, which migrated at about 43 ku and p*I* 5.8, was up-regulated obviously. Searching the peptide mass fingerprint of this protein in the MASCOT database, it was found to be a homology of *Arabidopsis* GDP-mannose 3′,5′-epimerase[14]. To comprehensively understand the function of this protein, the ORF of *Arabidopsis* GDP-mannose 3′, 5′-epimerase gene was used as query sequence to screen the NCBI database. Finally, two full-length cDNAs from rice deposited by the rice full-length cDNA consortium were identified. These two cDNAs, designated as *OsGME1* and *OsGME2*, were isolated from the rice mature leave by RT-PCR and cloned to the pMD18-T vector and sequenced as described in **Materials and methods**. The sequencing results were in accordance with the sequence data in NCBI.

An alignment of all known GME proteins from plant species was shown in Figure 1a. The sequences of different GME proteins vary from 371 to 380 amino acids, with a high level of identity except the N terminal. A postulated NAD binding site (GAGGFI)[12] was underlined and existed throughout all the GMEs from plants. Additionally, the amino acid sequences of GMPase and GalLDH, another two AA biosynthetic enzymes, were also highly conserved in various plant species[15,16]. These results should be regarded as strong evidences for the conservation of Smirnoff-Wheeler

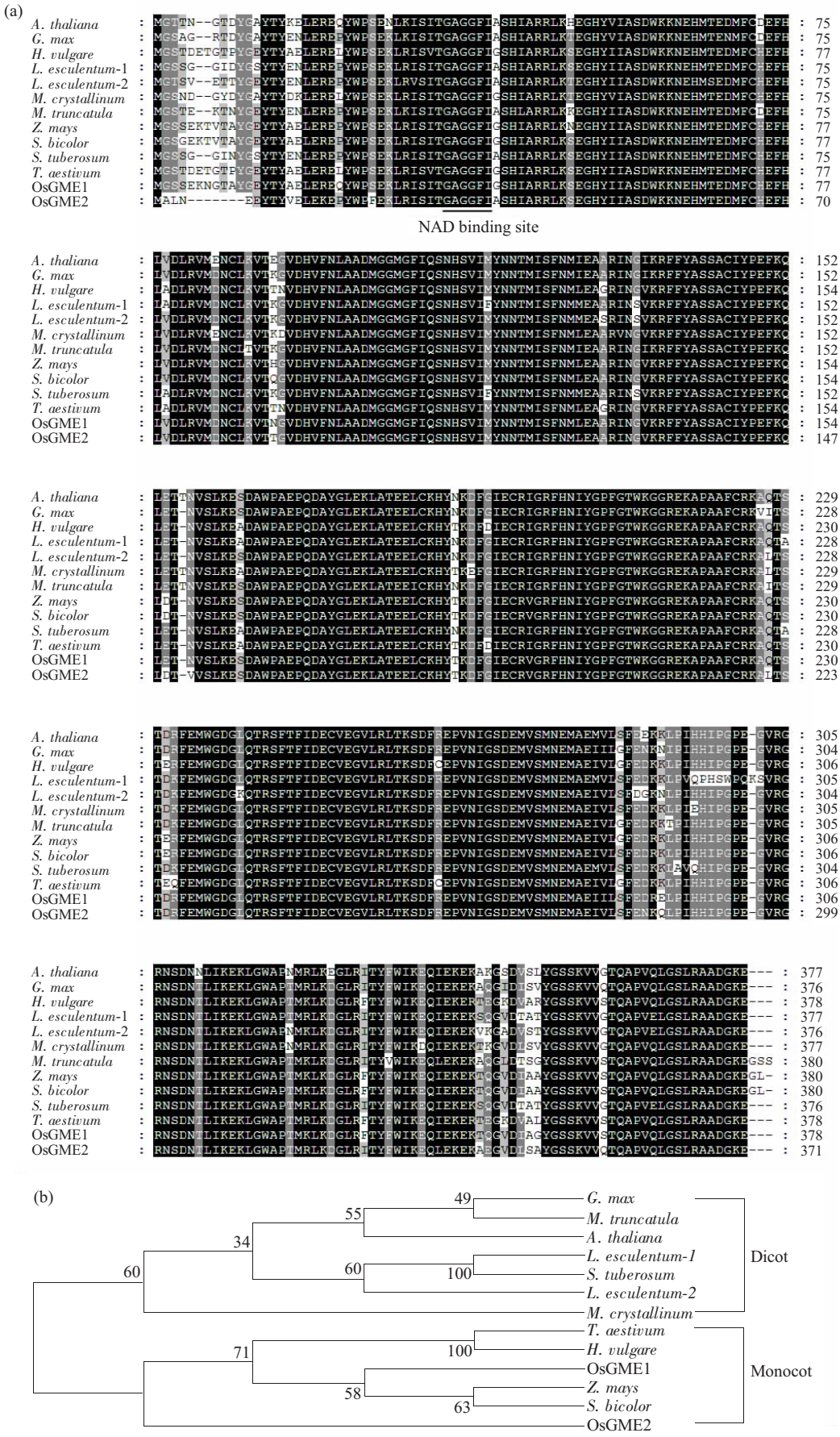

Fig. 1　Comparison of deduced amino acid sequence form rice OsGMEs with known GMEs

pathway in plant kingdom. Based on the alignment, an unrooted phylogenetic tree was constructed by maximum parsimony method with MEGA 3.0 software (Figure 1b). The tree could be clearly divided into two distinct groups corresponding to monocots and dicots, suggesting that duplications and diversifications for GME genes might occur after the divergence of the monocots and dicots. Interestingly, OsGME1 and OsGME2 were not grouped into one branch and the latter was separated from other GMEs in monocots, which indicated that the two proteins might play different roles in rice.

Protein sequence alignment (Figure 1a) was constructed by the ClustalX program. The amino acid residues identical among the sequences are indicated in black box, while similar residues are shown in grey boxes. Dashes indicated gaps in the sequence to allow for maximal alignment. Suggested NAD binding site was underlined. Unrooted phylogenetic tree (Figure 1b) was constructed using MEGA program. All plant sequences analyzed were clustered in two groups. The accession numbers of various organism are: *G. max* (CAD70060), *M. truncatula* (CAD70062), *A. thaliana* (AAM51587), *L. exculentum*-1 (deduced from BT013590), *S. tuberosum*(CAD70061), *L. exculentum*-2 (CAD70057), *M. crystallinum* (CAD70059), *T. aestivum* (CAD70064), *H. vulgare* (cad70065), OsGEM1 (AAP53779), *Z. mays* (CAD70058), *S. bicolor* (CAD70063), OsGEM2 (deduced from AK102348).

2.2 Genomic organization of rice *OsGME* genes

A BLAST search among the complete rice genome sequence was performed using the *OsGME1* and *OsGME2* cDNA sequence respectively. The result showed that *OsGME1* is located in the rice chromosome 10 and *OsGME2* is existed in the chromosome 11. Boundaries between exons and introns were determined by aligning rice GME cDNAs with the corresponding genomic sequences. As shown in Figure 2, there are some similarities in the splice patterns of *OsGME1* and *OsGME2*: they all contain seven exons separated by six introns; all exon/intron junctions match the consensus GT-AG rule; the first exon consists of the 5′-UTR and the second exon starts with A residue, followed by the ATG translation start codon. In fact, these similarities in splice pattern also exist in the *Arabidopsis AtGME*. However, *OsGME2* contains three more large introns compared with their counterparts in *OsGME1* and *AtGME*.

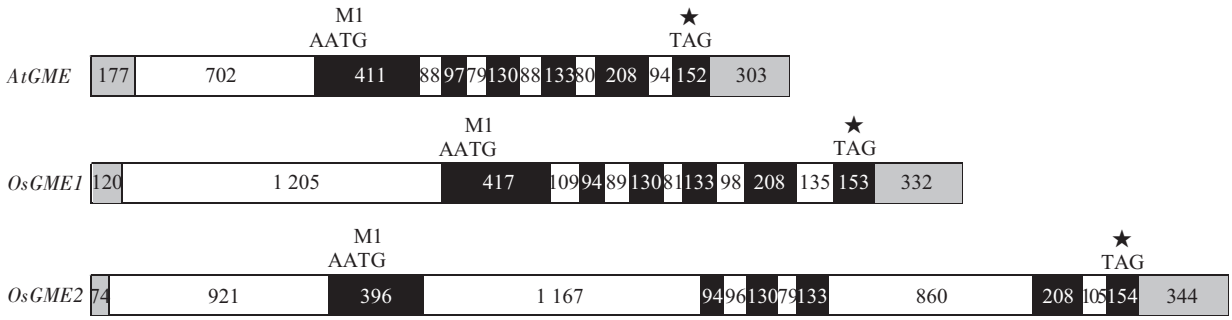

Fig. 2 Gene maps of rice and *Arabidopsis* GMEs

Exons are indicated as black boxes and introns as white boxes and untranslated regions as grey boxes. Exon, intron and untranslated region sizes are indicated with the number of the bases within each box, and the length of each box is in scale with the corresponding number of nucleotide residues. Start codons and stop codons are indicated using three-letter codes above the corresponding gene. Structural maps of other plant GME genes are absent from this study because of lacking genomic sequences.

2.3 The tissue expression patterns of rice *OsGME* genes

To investigate the expression pattern of the rice *OsGME* genes in various tissues and at different developmental stages, total RNA from the roots, stems, mature leaves of adult rice, and the roots, shoots of 10-day-old rice seedlings, and glumes before pollination, 2-day glumes after pollination, 15-day-old developing seeds were subjected to semiquantitative RT-PCR assay using primers specific for each gene. All the RT-PCR experiments were repeated twice independently and the similar results were obtained. As shown in Figure 3, both *OsGME1* and *OsGME2* mRNA were abundant in vegetative tissues including leaves, shoots, stems and roots, indicating the indispensable roles played by AA in growth and metabolism. However, their expressions were at a relative low level in developing seeds. It is known that

rice seed contains little or no vitamin C, which is a defect of this staple food. Therefore, we suggest the absent of AA in developed rice seeds may result, at least partly, from the decrease of *OsGME* transcripts in seed developmental stage. Moreover, though *OsGME1* mRNA was low in glumes, *OsGME2* transcripts were accumulated in 2-day glumes after pollination (Figure 3), indicating that the latter might play a special role in rice embryogenesis.

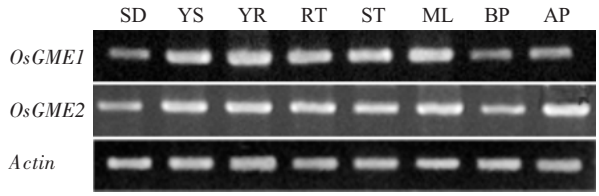

Fig. 3 RT-PCR analysis of *OsGME* transcripts in various tissues

Total RNA isolated from 15-day-old seeds (SD), shoots of 10-day-old seedlings (YS), roots of 10-day-old seedlings (YR), roots of adult rice (RT), stems of adult rice (ST), mature leaves of adult rice (ML), glumes before pollination (BP), 2-day glumes after pollination (AP) were subjected to semiquantitative RT-PCR assay. The PCR products were separated in 1% agarose gel and stained with ethidium bromide. The constitutively expressed rice actin-1 gene (*Actin*) was chosen as a control and equal amounts of *Actin* were detected in all lanes.

2.4 *OsGME* genes expression in response to gradual low temperature stress treatments

Because the deduced proteins of OsGME1 and OsGME2 possess the same calculated p*I*s and similar molecular mass, the PepideMass tool was used to calculate theoretical peptide mass values of the two proteins and the result showed that the values of OsGME1 meet the experimental MALDI-TOF data, indicating that OsGME1, but not OsGME2, was the up-regulated spot in the previous proteomic analysis (Figure 4b) [14]. As expected, the gradual low temperature stress treatments increased the expression of *OsGME1* (Figure 4a). Three low-temperature-responsive elements (LTREs, two of GCCGAA and one of GCCGC) are found in the 1 000 bp sequence of *OsGME1* upstream region. It is possible that these LTREs might play roles in response to cold stress in rice seedlings. However, merely 1.5-fold increase in transcription level of *OsGME1* was not a convincible explanation to the high accumulation of OsGME1 protein, which is 2.5 to 3-fold in the proteomic study (Figure 4b)[14]. Based on the fact that rice Hsc70.3 was also up-regulated in protein level under cold stress[14], we speculated that the increase of OsGME1 might partly ascribe to the Hsc70.3 molecular chaperone's help of stimulating the enzyme folding correctly [12], which reduced the degradation of false-folding OsGME1 and thus resulted in an accumulation in protein level. In contrast, only negligible effect on *OsGME2* can be seen in these treatments. This result was coincident with our speculation above that OsGME1 was the up-regulated protein spot in the proteomic analysis. Considering this dissimilarity together with their different expression pattern in various tissues and different locations in the phylogenetic tree, despite the overlapping of their functions could not be denied, we suggest that these two rice GME genes may possess some special traits respectively or under different regulation.

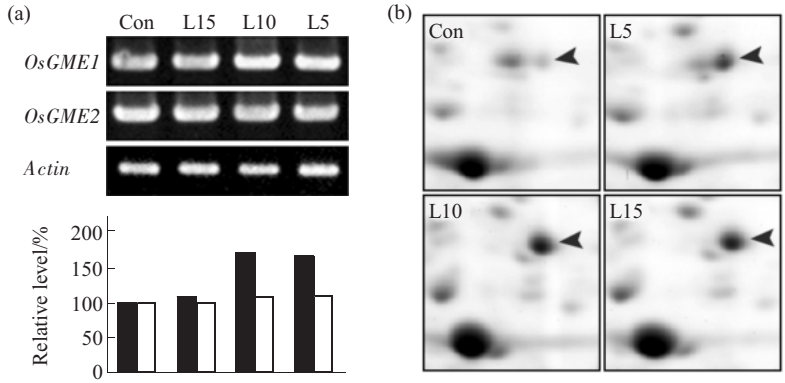

Fig. 4 Expression of *OsGME* genes exposed to gradual low temperature stress and change of OsGME1 protein in previous study

(a) Expression of *OsGME* genes exposed to gradual low temperature stress. 10-day-old rice seedlings were incubated in a growth chamber with declined temperatures from 15℃, 10℃, and 5℃, 24 h for each treatment. (b) Change of OsGME1 protein in response to the gradual low temperature process from normal temperature to 15℃,10℃, and 5℃ (marked as con, LT15, LT10, LT5) in previous report of our paper[14]. It is used here only for the purpose of discussion.

2.5 *OsGME* genes expression in response to plant hormones, signaling agents, wounding and drought stress conditions

To examine effects of exogenous factors on *OsGMEs* expression, semiquantitative RT-PCR assay was also performed using rice seedlings treated with various stresses described in the materials and methods. All the RT-PCR experiments were also repeated twice independently. It should be emphasized that all the hormone and signaling agent treatments did not cause any visible physiological effects on the treated seedlings, while seedlings subjected to wounding or drought stress were wilted obviously.

In hormone treatments, GA_3 resulted in a decrease in both *OsGME1* and *OsGME2* transcripts (Figure 5a). Possible link between AA and gibberellin synthesis has been suggested: AA seems to function as enzyme cofactor of 2-oxoglutarate-dependent dehydrogenase, an enzyme involved in the biosynthesis of GA_3[9]. In the *Arabidopsis* ascorbate deficient mutant *vtc1*, two transcripts encoding 2-oxoglutarate-dependent dehydrogenases increased, perhaps in order to compensate for the decreased cofactor availability [9]. Thus AA content may influence GA_3 biosynthesis. Interestingly, our data suggested that GA_3 may also regulate AA levels by affecting transcription of OsGME genes. We speculated that exogenous GA_3 lessened the necessity of *in vivo* biosynthesis, since excess GA_3 would result in some unpredictable effects on plant development. Therefore, the cofactor availability for the 2-oxoglutarate-dependent dehydrogenase was reduced through down-regulating GME transcription. Albeit attractive, this hypothesis needs more evidences in future investigation.

Apoplastic AA is the major antioxidant buffer in plants and has been suggested to function as the first defense system against oxidative damage caused by environmental stress[17,18]. AA requirement to cope with stress conditions was directly supported by the study of *Arabidopsis* ascorbate deficient mutant *vtc1*. This mutant has only 30% of the leaf ascorbate found in the wild type (Col0) and shows constitutive induce of a number of pathogenesis-relate (PR) transcripts [9,19]. However, no significant change of *OsGME* genes was detected in the rice seedlings exposed to IAA, ET, ABA, SA, JA treatments (Figure 5a), high temperature, heavy metal, H_2O_2 treatment (Figure 5b), and drought, wounding stress (Figure 5c) in our study. In addition, study on *CmGalLDH* (the enzyme catalyzing the last step of AA biosynthesis in melon) showed that the mRNA level of this gene keep a steady state under IAA, ABA, SA, JA treatments or exposed

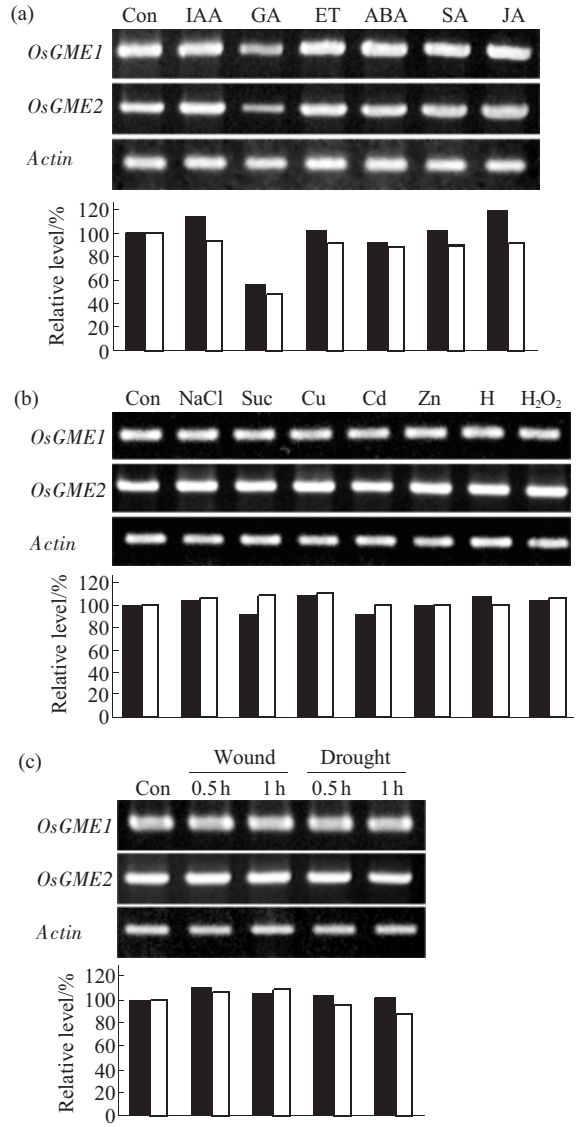

Fig. 5 Expression of *OsGME* genes exposed to various exogenous factors

(a) 10-day-old rice seedlings were treated with different hormones: 10 μmol/L indole-3-acetic acid (IAA), 10 μmol/L gibberellin (GA_3), 2 mmol/L ethephon (ET), 100 μmol/L abscisic acid (ABA), 5 mmol/L salicylic acid (SA) 100 μmol/L jasmonic acid for 3 h. (b) 10-day-old rice seedlings were exposed to wound or drought for 0.5 h and 1 h, respectively. (c) 10-day-old rice seedlings were treated with 200 mmol/L NaCl (NaCl), 200 mmol/L sucrose (Suc), 100 μmol/L $CdCl_2$ (Cd), 100 μmol/L $CuSO_4$ (Cu), 1 mmol/L $ZnSO_4$, 42℃ heat shock (H), 10 mmol/L H_2O_2 for 6 h. The control (Con) was treated with distilled water. Total RNA were isolated from shoots of the seedlings and subjected to RT-RCR assay, the loading mRNA amounts were standardized by comparison with PCR product of rice actin-1 gene, resulting relative values of expression intensity are shown at the bottom, black column indicate the relative expression level of *OsGME1*, white column indicate the level of *OsGME2*.

to wounding and high temperature [20], which was similar to our results. It was reasonably assumed that the regulation of AA levels under these stress conditions did not, at least not mainly depended on *in vivo* synthesis of *OsGME* transcripts.

In this study, we described the molecular characterization of two *OsGME* genes and their expression patterns. Firstly, an alignment of all known GME proteins from plant species was performed, which showed that GME genes were highly conserved among plant species and all known GMEs can be divided into two distinct groups corresponding to monocots and dicots. Secondly, through investigation on the genomic organization of rice *OsGME* genes, a similarity of splice patterns was revealed. Finally, the expression of *OsGME* genes have been studied in various tissues and under different stress conditions and the results were examined and discussed. Although the properties of two genes were to most extent identical with each other, there were still some evidences implying their possible functional differences. Moreover, our results suggest that both transcriptional and post-translational mechanisms exist in regulation of *OsGME* genes. As we known, this is the first report on detailed description of GME gene structure and expression in plants. It may be expected that this work will provide a framework for continued studies on the exact mechanism of AA regulation and the biological functions of GME genes in plants.

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两个水稻 GDP-甘露糖-3′, 5′-异构酶基因特征及表达模式的研究 *

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摘要 GDP- 甘露糖 -3',5'- 异构酶（GME）可以催化 GDP- 甘露糖转化为左旋 GDP- 半乳糖，该反应对于高等植物体内抗坏血酸的合成是非常重要的. 但目前在分子水平上还没有对 GME 基因进行研究的报道. 通过逆转录 PCR（RT-RCR）技术从水稻成熟叶片中克隆到两个 GME 基因的 cDNA 序列，并与其他植物物种中的 GMEs 进行比对，结果显示，GME 基因在所有植物物种中高度保守，尽管进化树分析表明单子叶植物 GMEs 和双子叶植物 GMEs 在进化上相互独立. 同时，分析这两个水稻 GME 基因的剪切模式揭示了二者也存在高度相似性. 采用半定量 RT-PCR 技术对两个 GME 基因在不同组织和不同胁迫条件下的表达模式进行研究表明，*OsGME1* 基因在冷胁迫条件下表达水平上调，这和先前水稻冷胁迫蛋白质组学研究的结果是一致的. 而 *OsGME2* 和 *OsGME1* 基因在用赤霉素处理条件下表达水平均下调，暗示赤霉素可能通过调节 GME 基因的表达来调控植物体内的抗坏血酸合成.

关键词 植物抗坏血酸合成，基因表达，半定量 RT-PCR 技术

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