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Gm1-MMP is involved in growth and development of leaf and seed, and enhances tolerance to high temperature and humidity stress in transgenic *Arabidopsis*

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ABSTRACT

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent endopeptidases. *Gm1-MMP* was found to play an important role in soybean tissue remodeling during leaf expansion. In this study, *Gm1-MMP* was isolated and characterized. Its encoding protein had a relatively low phylogenetic relationship with the MMPs in other plant species. Subcellular localization indicated that Gm1-MMP was a plasma membrane protein. *Gm1-MMP* showed higher expression levels in mature leaves, old leaves, pods, and mature seeds, as well as was involved in the development of soybean seed. Additionally, it was involved in response to high temperature and humidity (HTH) stress in R7 leaves and seeds in soybean. The analysis of promoter of *Gm1-MMP* suggested that the fragment from −399 to −299 was essential for its promoter activity in response to HTH stress. The overexpression of *Gm1-MMP* in *Arabidopsis* affected the growth and development of leaves, enhanced leaf and developing seed tolerance to HTH stress and improved seed vitality. The levels of hydrogen peroxide (H_2O_2) and ROS in transgenic *Arabidopsis* seeds were lower than those in wild type seeds under HTH stress. Gm1-MMP could interact with soybean metallothionein-II (GmMT-II), which was confirmed by analysis of yeast two-hybrid assay and BiFC assays. All the results indicated that *Gm1-MMP* plays an important role in the growth and development of leaves and seeds as well as in tolerance to HTH stress. It will be helpful for us understanding the functions of Gm1-MMP in plant growth and development, and in response to abiotic stresses.

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent proteases according to the MEROPS database [1]. They have a common domain structure with a signal peptide, a propeptide, a catalytic domain that contains an HEXXHXXGXX(H/D) zinc-binding sequence characteristics of the metzincin superfamily of proteinases [2], a hinge region, and a C-terminal hemopexin-like domain. The hemopexin-like domain has been found to play a functional role in substrate binding [3]. However, plant and nematode MMPs lack a hemopexin-like C-terminal domain [4]. It has been shown that MMPs in human play key roles in many physiological and pathological processes [5,6]. In plants, some MMPs have been identified, but only a few of them have been characterized. The first plant MMP was identified in soybean and named as soybean metalloendoproteinase-1 (SMEP1) [7], but several previous researchers considered that the soybean SMEP1 should be renamed to Gm1-MMP [8]. *Gm1-MMP* was suggested to perform a role in tissue remodeling during leaf expansion [9]. In cucumber, *Cs1-MMP* was found to be associated with senescence and cell death in cotyledon development by processing corpses and digesting away cell residues [10]. In Loblolly pine, *Pta1-MMP* was suggested a positive function in the completion of seed germination and subsequent seedling elongation and establishment [11]. In *Arabidopsis*, *At1-MMP* and *At4-MMP* were increased in expressing levels during seed/silique development; *At4-MMP* was also highly expressed during seed germination. *At2-MMP* was expressed in

Abbreviations: 4MU, 4-methylumbelliferone; BiFC, Bimolecular fluorescence complementation; BF, Bright field; CV, Cultivar; ECM, Extracellular matrix; GFP, Green fluorescent protein; HTH, High temperature and humidity; MMPs, Matrix metalloproteinases; MT, Metallothionein; ORF, Open reading frame; qRT-PCR, Quantitative reverse transcription PCR; RH, Relative humidity; ROS, Reactive oxygen species; YFP, Yellow fluorescent protein.

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young rosettes, young flowers and mature siliques [12]. Additional roles for MMPs in biotic and abiotic stress responses have been suggested in higher plants. For example, in soybean, *GmMMP2* was activated in response to pathogenic infections, wounding and dehydration [13], and the expression of *Slti114* was induced by low temperature or wounding [14]. In the tobacco suspension line BY-2, *Nt1-MMP* was expressed constitutively at low level, but was induced immediately after treatment with *Pseudomonas syringae* [15]. In *Medicago truncatula*, *Mt1-MMP* expression was induced in young nodules, specifically in association with *Sinorhizobium meliloti* infection [16]. In *Arabidopsis*, the expression of *At2-MMP* was tightly controlled in a tissue-responsive way during stress conditions. In 4-week-old plants, *At2-MMP* was induced in leaves by cadmium or methyl jasmonate, and in roots by sodium chloride [17]. In tomato, *Sl3-MMP* worked as a positive regulator of defense response against *Botrytis cinerea* and *Pseudomonas syringae* pv. tomato DC3000 [18].

Soybean [*Glycine max* (L.) Merr] is the largest oilseed crop produced and consumed, accounting for 58% of the world oilseed production [19]. Due to high protein (about 40%) and oil contents (about 20%), soybean seeds are prone to deterioration before harvest and during its processing and storage [20,21]. Soybean seed deterioration usually results in the decrease in yield, germination vigor and storability [20,22,23]. High temperature and humidity (HTH) stress during the development and maturity of soybean seed can lead to pre-harvest deterioration in the field [20]. Previously, we conducted a comparative proteomics study to reveal the proteome profiles of developing seeds under HTH stress using a pre-harvest seed deterioration-resistant soybean cv. Xiangdou No. 3. Among 45 identified proteins in developing seeds of cv. Xiangdou No. 3, a matrix metalloproteinases, Gm1-MMP (GenBank Accession No. U63725) was found to be accumulated in abundance under the HTH stress.

In the present study, we isolated and characterized the soybean MMP gene *Gm1-MMP*. Our results suggested that *Gm1-MMP* presented different expression patterns among diverse soybean tissues and was involved in response to HTH stress in leaves and developing seeds. The *Gm1-MMP* promoter had many stress-responsive *cis*-regulatory elements. Its 5′-deletion fragment from −399 to −299 was essential for its promoter activity in response to HTH stress. Overexpression of *Gm1-MMP* could affect the growth and development of *Arabidopsis* leaves and enhance leaf and developing seed tolerance to HTH stress. Gm1-MMP could interact with GmMT-II. Overexpression of *Gm1-MMP* could reduce the concentration of reactive oxygen species (ROS) in *Arabidopsis* under HTH stress.

2. Materials and methods

2.1. Plant materials and various treatments

Two soybean cultivars (pre-harvest seed deterioration-sensitive cv. Ningzhen No. 1 and -resistant cv. Xiangdou No. 3) were used in this study [24]. Seeds were sown in plastic pots and managed under the normal condition [30 °C/20 °C, 70% RH, and 10 h/14 h (light/dark)]. For the tissue specific expression assay, roots, stems and young leaves were collected at V1 stage; mature leaves and flowers were collected at R2 stage; older leaves and pods were collected at R4 stage and harvest mature seeds were collected at R8 stage. HTH stress on potted soybean plants (R_7 stage) was performed as previously described [24]. Treated plants were transferred to growth cabinet at 40 °C/30 °C, 100%/70% humidity (RH) and 10 h/14 h (light/dark) light cycle for 7 d. Control plants with the same developmental progression were cultivated under the normal condition. The leaves and seeds from the middle portion of the treated and control plants were collected at 0, 12, 24, 48, 96, and 168 h during the treatment, respectively. All samples were immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Isolation and sequence analysis of Gm1-MMP

Total RNA was extracted from soybean leaves by Plant Total RNA Isolation Kit (Biosci Biotech, China). cDNA was synthesized using M-MLV Reverse Transcriptase (Takara, Japan). The full-length opening reading frames (ORF) of *Gm1-MMP* were obtained by reverse transcription PCR (RT-PCR). The gene-specific primers Gm1-MMP-F and Gm1-MMP-R were used to isolate *Gm1-MMP* gene (Supplementary Table 1). PCR amplification was carried out for 5 min at 95 °C, followed by 30 cycles of 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 90 s with a final extension at 72 °C for 10 min.

21 homologous proteins of Gm1-MMP were collected from NCBI (http://www.ncbi.nlm.nih.gov/blast/). The accession numbers of all these MMP family proteins are as follows: At2-MMP (At1g70170), At3-MMP (At1g24140), At5-MMP (At1g59970), Rc2-MMP (XM_002520165), Nt1-MMP (DQ508374), StMMP3-like (XM_006363472), Vr2-MMP (XM_014651914), Ai2-MMP (XM_016347138), Car1-MMP (XM_004504446), Pv1-MMP (XM_007157457), At4-MMP (At2g45040), Sl1-MMP (XP_010325488), At1-MMP (At4g16640), Os2-MMP (NP_001057259), ZmMMP1 (NP_001151749), Os1-MMP (NP_001048075), Gs1-MMP (KN652558), Cs1-MMP (AJ133371), Mt1-MMP (Y18249), Gm2-MMP (AY057902), and GmSlti114 (ABW96008). Sequences alignment and phylogenetic tree analysis were clustered and constructed by DNAman and MEGA7.0, respectively. Bootstrap values were based on 1000 replicates.

2.3. Subcellular localization of Gm1-MMP protein

The coding sequence of *Gm1-MMP* was amplified from pMD19-Gm1-MMP using a pair of primers pA7-Gm1-MMP-F and pA7-Gm1-MMP-R (Supplementary Table 1) and inserted into pA7-GFP entry vector. The recombinant plasmid pA7-Gm1-MMP and the empty vector pA7-GFP were introduced into onion epidermal cells and tobacco leaf cells by particle bombardment method. The pm-ck CD3-1001 was used as PM (plasma membrane) maker [25]. The onion epidermal cells and tobacco leaf cells were then bombarded by PDS-1000/He (Bio-Rad, USA), with an 1100 psi split membrane and gold particles coated with the plasmid DNA. After bombardment, the onion epidermal cells and tobacco leaf cells were incubated on MS medium in a dark chamber at 25 °C for 24 h, and plasmolysis was achieved by treating the onion epidermal cells with 30% sucrose solution for 10 min. Fluorescence was observed using a LSM780 confocal microscopy imaging system (Zeiss, Germany).

2.4. Gene expression analysis

Total RNA from different soybean tissues samples was extracted and used as templates for cDNA synthesis by Superscript II reverse transcriptase (Takara, Japan). Real time quantitative RT-PCR (*q*RT-PCR) was performed according to the application manual. The primers are listed in Supplementary Table 1 (RT-Gm1-MMP-F and RT-Gm1-MMP-R); the soybean *Actin* gene (accession No. V00450) was used as a standard control in RT-PCR reactions. The following thermal cycle conditions were used: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 58 °C for 20 s, and 72 °C for 20 s. All reactions were performed in triplicate. Following the PCR, a melting curve analysis was performed. Ct or threshold cycle was used for relative quantification of the input target number. The relative gene expression levels were calculated using the $2^{-D\Delta CT}$ method.