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Plasma membrane localization of soybean matrix metalloproteinase differentially induced by senescence and abiotic stress

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Abstract

We isolated and characterized a stress-inducible gene, designated as *Slti114*, encoding matrix metalloproteinase (MMP) in soybean. The derived amino acid sequences of Slti114 show the 69 % homology with MMP2 from *Glycine max* (AAL27029). The size of the full-length cDNA of *Slti114* is 1194 bp with open reading frame comprised of 394 amino acids. RNA expression of *Slti114* was induced by low temperature or wounding. During early stage, *Slti114* RNA level was extremely high, but *Slti114* RNA was not detectable just after cotyledons became yellowish. Green fluorescent protein fusion expression system confirmed that Slti114-smGFP and H^+-ATPase-RFP were co-localized to the plasma membrane. Purified glutathione-S-transferase (GST)-Slti114 protein was shown to digest myelin basic protein (MBP) *in vitro*, but not gelatin. This report provides strong evidence that plasma membrane MMP, Slti114 protein may play a critical role during abiotic stress and senescence in plant.

Additional key words: abiotic stress, *Glycine max*, low temperature, RNA expression.

Introduction

Among a plethora of proteins present in extracellular matrix (ECM), the matrix metalloproteinases (MMPs) are capable of degrading the extracellular matrix substrates and remodeling the extracellular matrix. The MMPs have a common domain structure with a signal peptide, a propeptide, a catalytic domain, a hinge region, and a C-terminal hemopexin-like domain. The Cys switch mechanism is involved in the coordination of Cys residue in the conserved sequence PRCGVPDV to the active center zinc atom. The catalytic domain requires two zinc ions and at least one calcium ion for enzyme activity and contains an HEXGHXXGXXH zinc-binding sequence characteristic of the metzincin superfamily of proteinases (Das *et al.* 2003). The hemopexin-like domain has been shown to play a functional role in substrate binding (Gomis-Rüth *et al.* 1997). However, plant and nematode MMPs lack a hemopexin-like C-terminal domain (Das *et al.* 2003, Massova *et al.* 1998).

There are many reports about molecular cloning of plant MMPs (Delorme *et al.* 2000, Macedo *et al.* 1999, Maidment *et al.* 1999, Pak *et al.* 1997). Soybean *SEMP1* was first cloned in plant (Graham *et al.* 1991) and subsequently was shown to be expressed on in adult leaves but not in other plant tissues or in young developing leaves (Pak *et al.* 1997). Soybean SMEP1 was demonstrated to extracellular tightly bound to the cell wall (Pak *et al.* 1997). The 60 kDa native metallo-proteinase isolated from soybean was shown to be present only during the first 15 d post-emergence and may play a crucial role in rapid cell growth and leaf expansion (Huangpu and Graham 1995). Cucumber *Cs1-MMP* was proposed to play a crucial role in programmed cell death (PCD) (Delorme *et al.* 2000). *In vivo* function of plant MMP was first reported using *Arabidopsis* T-DNA mutant analysis and mutation of *Arabidopsis At2-MMP* was demonstrated to cause late flowering and accelerated senescence (Golldack *et al.* 2002). Recent research has been concentrated on the identification of the role

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Abbreviations: ECM, extracellular matrix; smGFP - soluble-modified green fluorescent protein; GST - glutathione-S-transferase; MBP - myelin basic protein; MMP - matrix metalloproteinase; PCD - programmed cell death; RFP - red fluorescent protein.

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of MMPs during developmental and environmental signals. It is interesting to reveal the way how the multigene family of the MMPs plays a different role in responsive to PCD and environmental stresses and how MMPs recognize and degrade the specific types of the substrates present in ECM. This report is about the molecular and biochemical analysis of *Slti114* induced during abiotic stresses and cotyledon development. To our knowledge, this is the first report of subcellular localization of MMP-GFP in the plant cell.

Materials and methods

Plants and treatments: Soybean [*Glycine max* (L.) Merr. cv. Sinpaldal 2] plants were grown in a growth chamber with 16-h photoperiod with irradiance of 250 μmol m^{-2} s^{-1} at 28 °C for 3 - 4 weeks. To induce low temperature stress, plants were placed in a refrigerator at 4 °C upto 48 h as previously described (Cho *et al.* 2007). Soybean leaves from the whole plant were cut with scissors as a wounding stress. Non-stressed soybean leaves were used for control. Cotyledons were sampled at different developmental stages after soybean seeds were germinated. Plant samples were immediately frozen in liquid nitrogen, and stored at -75 °C.

RNA isolation and Northern blot analysis: Total RNAs were isolated using a modified hot-phenol method (Verwoerd *et al.* 1989). Total RNA (20 μg) was fractionated on a 1 % formaldehyde agarose gel, and transferred to nylon membrane. The membrane was hybridized to a ^{32}P-dCTP labeled probe using DNA fragments encompassing *Slti114* cDNA. The probe was labeled by a random priming method using the *Ladderman* labeling kit (*Takara,* Otsu, Japan) (Chung *et al.* 2007). Hybridization and washing procedure was carried out as described by Church and Gilbert (Church and Gilbert 1984).

Cloning of *Slti114* gene using 5' rapid amplification cDNA ends (5' RACE): cDNA synthesis was carried out using reverse transcription kit (*Promega,* Madison, USA). The full-length *Slti114* cDNA was obtained using SMART RACE cDNA amplification kit (*Clontech,* Mountain View, USA) according to the manufacturer's instruction. Gene-specific primer SP1-Slti114 (5'-CAC TTGGAAGTAACCAGCCCATGTTACCGT-3') and nested primer SP2-Slti114 (5'-ACCTGGATGTCGGCG TTGTCGTAG-GTTGTC-3') were synthesized (*Bioneer,* Daejeon, Korea) for the RACE.

Construction of Slti114-GFP: For the GFP localization study, the entire cDNA region of *Slti114* was amplified by PCR using Ex Taq (*Takara*) with a sense primer containing a unique *Xba*I restriction enzyme site and an ATG initiation codon (5'-TCTAGAATGAAACCGT ACCTACGTC-3') and an antisense primer with a unique *BamH* I restriction site (5'-GGATCCGATACA AAAGGAGCAATACGT-3'). PCR amplified DNA fragments (1 190 bp) were cloned into *pGEM-T Easy* (*Promega*) vector yielding pGEM-T-Slti114 and the nucleotide sequences of the inserted fragment were confirmed by DNA sequencing. The *Xba*I / *BamH*I DNA fragments of pGEM-T-Slti114 were introduced into the corresponding sites of smGFP plasmids resulting in Slti114-smGFP. Thus, the start codon of smGFP is translationally fused to the C-terminal region of *Slti114* (David and Vierstra 1996).

Plasma membrane localization of Slti114-GFP: The smGFP, H^+-ATPase-RFP (Jin *et al.* 2001) and Slti114-smGFP constructs were transformed into *Agrobacterium tumefaciens* sp. strain C58c1, respectively. Infiltration was carried out as described by Chung *et al.* (2004b). Generally, leaves of 4- to 5-week-old *Nicotiana benthamiana* plants were syringe-infiltrated with the acetosyringone-induced *Agrobacterium* culture.

The subcellular distribution patterns GFP, RFP- and GFP-fused proteins expressed in *N. benthamiana* were observed with a *Leica SP2-AOBS* (Mnnheim, Germany) laser confocal microscope. Expression of the fusion protein and vector control was monitored 36 h after infiltration. Excitation wavelength of 488 nm was used, and fluorescence of GFP (green) and chloroplasts and RFP (red) was observed simultaneously. Leaf samples were observed under a 63× magnification.

Production of GST-Slti114 protein in *E. coli*: For the GST-fusion system, N-terminus of Slti114 (1-160) was not included for the efficient protein expression and the remaining region about 700 bp size covering open reading frame region (161-394) was amplified by PCR using *Ex Taq* (*Takara*, Japan) with a sense primer containing a unique *BamH*I restriction site and an ATG initiation codon (5'-GGATCCATGTTGTCCATACG ATG-3') and an antisense primer with a unique *EcoR*I restriction site (5'-AATTCCTAATACAAAAGGAGC AAT-3'). PCR amplified DNA fragments (700 bp) were cloned into *pGEM-T Easy* vector (*Promega*) and the nucleotide sequences of the inserted fragment were confirmed by DNA sequencing. The *BamH*I/*EcoR*I fragments of pGEM-T-Slti114 were introduced into the corresponding sites of pGEX-4T-1 plasmids resulting in GST-Slti114. Thus ORF of *Slti114* is translationally fused to the C-terminal region of GST. GST-fusion protein induction and purification was carried out as previously described (Chung *et al.* 2004a).

Zymography and myelin basic protein (MBP) assay: Gelatin (1 mg cm^{-3}) was copolymerized into 15 % (m/v) polyacrylamide-SDS gels. Purified GST-Slti114 samples and culture supernatant from human hepatocellular

carcinoma cell line (Hep3B) as positive control (Chung *et al.* 2002) were added to unreduced Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2 % SDS, 10 % glycerol, 5 % mercaptoethanol, and 0.001 % bromophenol blue). After electrophoresis, the gel was washed in Triton X-100 before incubation overnight at 37 °C in an assay buffer (100 mM Tris-HCl, pH 7.5, 30 mM $CaCl_2$, and 0.02 % NaN_3). Subsequently, the gel was stained with Coomassie blue to detect bands of gelatin digestion.

Degradation of myelin basic protein (MBP) (*Sigma*, St. Louis, USA) was assayed to monitor the proteinase activity of the purified GST-Slti114 protein as previously described by Maidment *et al.* (1999). MBP (0.2 µg) was incubated for 0.5, 1, 4 h at 37 °C without or with the purified GST-Slti114 protein. The resulting reactions were analyzed on SDS-PAGE (Laemmli 1970).

Results

The full length cDNA clone of *Slti114* was obtained by 5' RACE from the low temperature-stressed soybean. The full length cDNA sequences of *Slti114* are composed of 1 194 bp (Fig. 1; Genbank number EU131091). The *Slti114* has an open reading frame (ORF) of 1 182 nucleotides encoding an acidic polypeptide (M_r 44 kDa) with 394 amino acid residues (pI 5.5). Based on multiple alignments of protein sequences (Fig. 1*A*), plant MMPs share very low sequence homology from 29 to 69 % with other plant MMPs. Slti114 contains the representative MMP signature such as N-terminal signal peptide (SP), propeptide motif (PP) with Cys-switch motif (Cys),

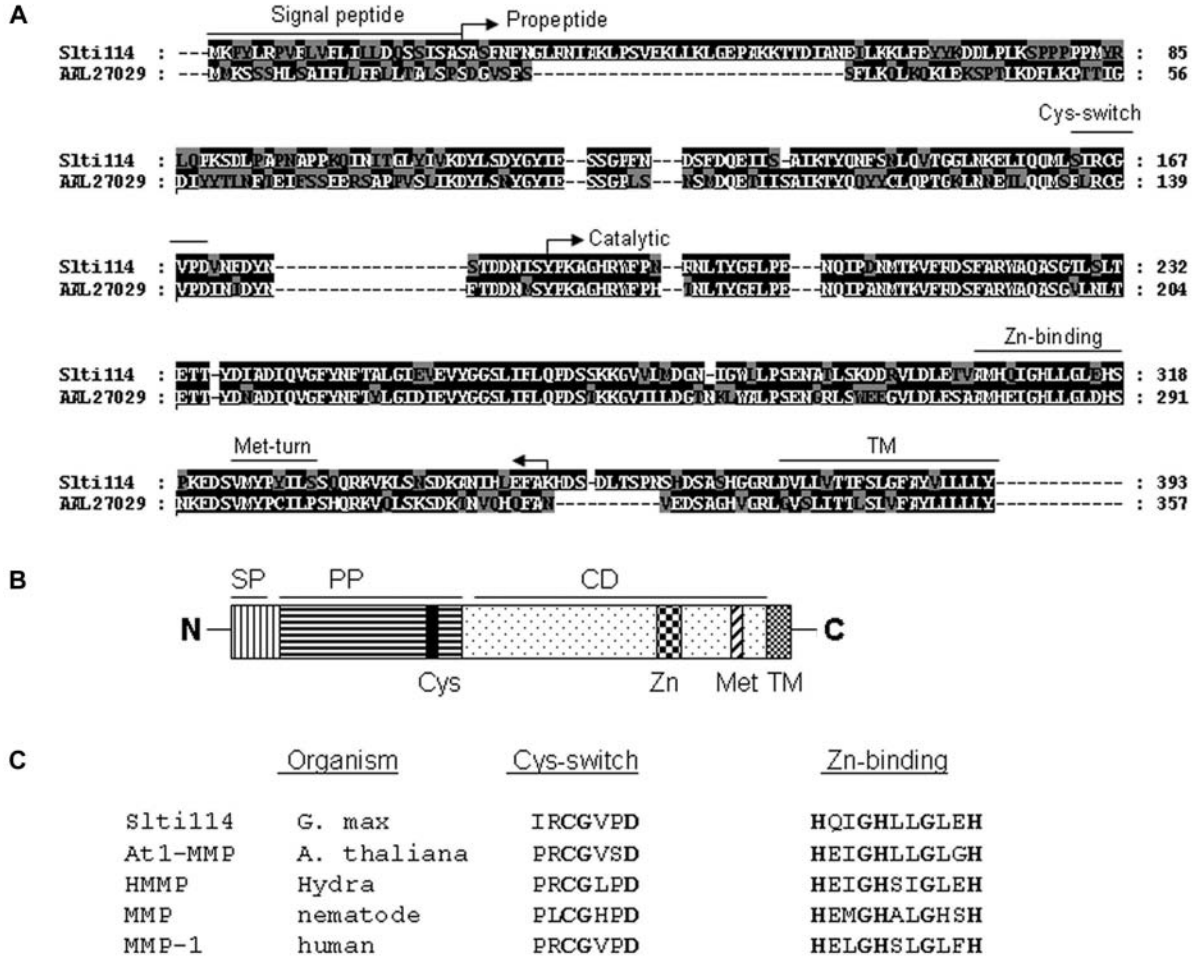

Fig. 1. Alignment of the deduced amino acid sequences of Slti114 with plant MMPs and representative signature of MMP protein with conserved regions. *A* - Protein alignment was generated using *CLUSTALW* at the T-coffee website (Notredame *et al.* 2000), and displayed with *GENDOC*. Each N-terminal signal peptide (SP), cys-switch (Cys), Zn-binding (Zn) and Met-turn (Met) domain is indicated in a box, the propeptide (PP) and catalytic domain (CD) was denoted as an *arrow*, the C-terminal transmembrane domain (TM) is underlined. *B* - Structural signature of Slti114 is representative as MMP characteristic of SP, PP, CD, TM, Cys and Zn motifs. *C* - Conserved cys-switch and Zn-binding domains of Slti114 are compared with those of MMPs from *Arabidopsis*, nematode, hydra and human.

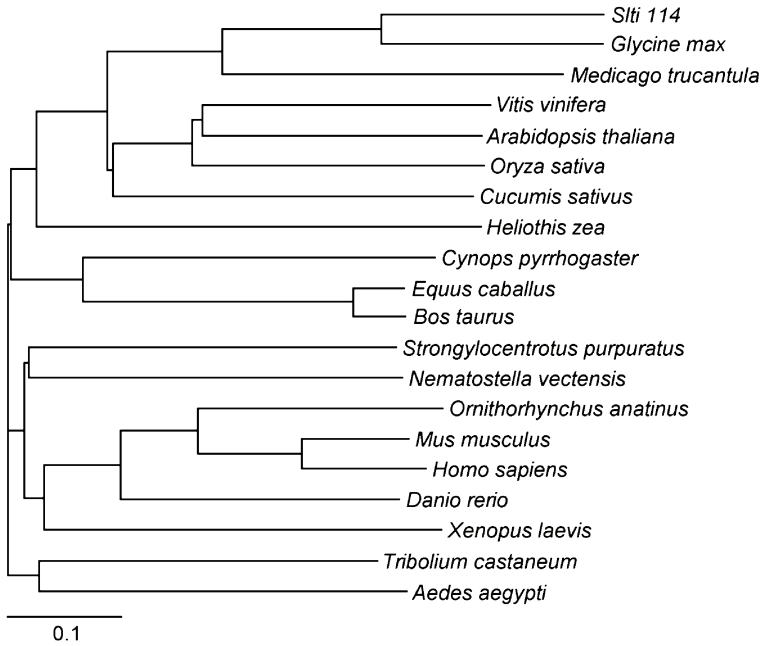

Fig. 2. Molecular phylogenetic tree for the aligned amino acid sequences of Slti114 and the MMPs. Proteins are from the following species, *Glycine max* (AAL27029), *M. sativa* (CAA770), *A. thaliana* (ABF19013), *V. vinifera* (CAN76268), *O. sativa* (EAY87473), *C. sativus* (CAB76364), *H. zea* (NP_690489), *C. pyrrhogaster* (BAC66372), *E. caballas* (XP_001499275), *B. taurus* (NP_776814), *S. purpuratus* (XP_785149), *N. vectensis* (XP_001640669), *O. anatinus* (XP_001512754), *M. musculus* (AAI12380), *H. sapiens* (AAG41981), *D. rerio* (XP_001332289), *X. laevis* (NP_001083604), *T. castaneum* (XP_969495), *A. aegypti* (EAT45766). *ClustalX* (ver. *1.18*) and *Treeview* (ver. *1.6.6*) programs were used to analyze phylogenetic relationships. The phylogenetic relationships showed that those MMP sequences were clustered on the level of orders (100 % of bootstrap value).

enzyme catalytic domain (CD) with the highly conserved Zn-binding motif (Zn), Met-turn motif (Met) followed by C-terminal transmembrane domain (TM) (Fig. 1*B*). A Cys-switch, PRCGVPDV-like sequence and a Zn-binding consensus sequence HEXXHXXGXXH are highly conserved among MMPs from diverse organism (Fig. 1*C*)

An extensive phylogenetic analysis using the deduced amino acid sequences of *Slti114* and nineteen metallo-proteinase protein sequences was inferred on the basis of the multiple sequence alignment (Fig. 2). Slti114 showed the highest protein sequence identity as 69 % to the *Glycine max* MMP2 (AAL27029) and the lowest protein sequence identity as 29 % to the *Cucumis sativus* (CAB76364). Compared to that of soybean GmMMP2, Slti114 shares 34 % homology with soybean SMEP1 (Pak *et al.* 1997).

Northern blot was carried out to confirm that *Slti114* is responsive to low temperature stress (Fig. 3*A*). RNA expression of *Slti114* gradually increased and reached maximum at 48 h by low temperature stress (Fig. 3*A*). *Slti114* RNA level reached maximum 3 h just after wounding stress and then its level declined gradually (Fig. 3*B*). *Slti114* gene expression may be related to signal transduction pathway during abiotic stresses exposed to soybean.

We monitored *Slti114* RNA expression in the soybean cotyledon under development (Fig. 3*C*). RNA expression of *Slti114* increased after germination and was highest at day 9 (Fig. 3*C*). From that time, there was a decline in this RNA content in the yellowish cotyledons (Fig. 3*C*). This indicates that *Slti114* may play an important role in the beginning senescence process of soybean cotyledons.

We investigated the subcellular localization of Slti114-smGFP fusion protein in the *N. benthamiana* leaves infiltrated with *Agrobacterium* containing Slti114-smGFP binary constructs (Fig. 4). Soybean is not easy to transform and furthermore in the case of co-expression of Slti114-smGFP/H^+-ATPase-RFP, transformation efficiency may be much lower. Thus, we used tobacco trans-formation system for targeting experiment (Fig. 4). The control smGFP protein was observed in the cytosol and nucleus of the epidermal cells (Fig. 4). Red fluorescence is derived from the chloroplast autofluorescence. We performed the co-expression of Slti114-smGFP and H^+-ATPase-RFP as a plasma membrane marker (Jin *et al.* 2001) in the *N. benthamiana*. There are red signals from the chloroplast autofluorescence and the RFP signal (Fig. 4). Compared to the chloroplast autofluorescence signal, the RFP shows brighter and smaller red signal. Slti114-smGFP was exactly co-localized to the clear RFP signal but not to the chloroplast (Fig. 4). Since H^+-ATPase-RFP is the marker for the localization of plasma membrane (Jin *et al.* 2001), it can be concluded that Slti114-smGFP is localized to the plasma membrane. This observation suggests that Slti114 be localized in the plasma membrane as a potential proteinase during signal transduction in plants.

Function of *Slti114* was examined using GST-Slti114

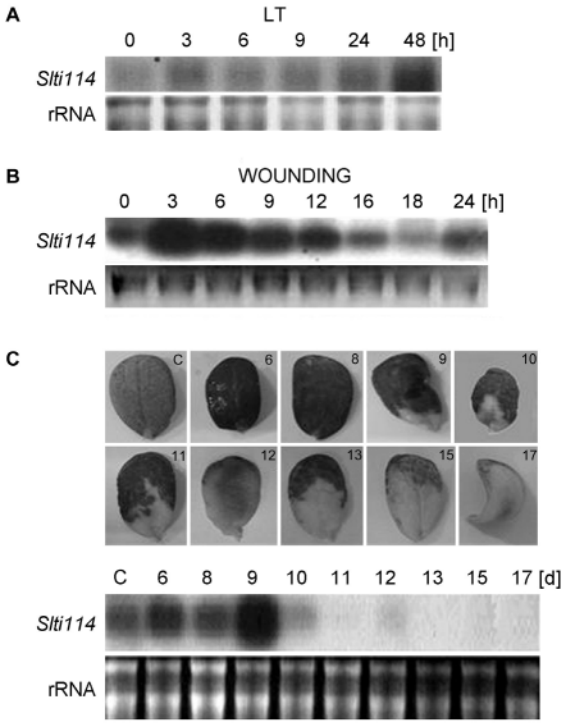

Fig. 3. Expression of *Slti114* gene during low temperature, wounding stresses or in developing soybean cotyledons. *A* - time course of *Slti114* expression in soybean leaves treated with low temperature (4 °C). *B* - expression of *Slti114* in wounding stressed leaves. *C* - RNA expression of *Slti114* in soybean cotyledon under developmental stage from early expanding stage to cell death. Following electrophoresis, RNA was transferred to nylon membrane and hybridized with a probe specific for *Slti114*. 20 μg of total RNAs was loaded on each lane. RNA blot analysis was carried out as described in materials and methods. This experiment was repeated three times with the same results.

fusion protein expressed in *E. coli* (Fig. 5). Purified GST-Slti114 protein (50 kDa) was applied for zymography for the detection of direct MMP activity in SDS-PAGE (data not shown). MMP-9 and MMP-2 showed strong activity of gelatin degradation (data not shown), but GST-Slti114 MMP could not degrade gelatin (data not shown). Slti114 protein without GST moiety also was employed for zymography, but the protein could not degrade gelatin (data not shown). It might be due to the possibility that gelatin is not the substrate by Slti114 in plants.

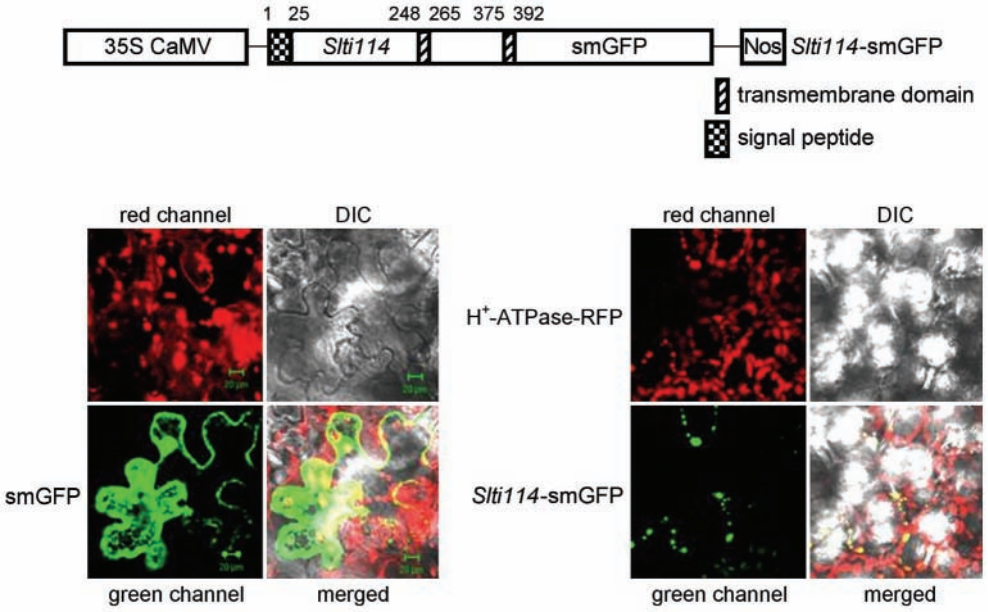

Fig. 4. Slti114-smGFP construct and confocal images of smGFP, H^+-ATPase-RFP and Slti114-smGFP protein in *N. benthamiana*. As a control vector, single smGFP was localized to anywhere (*left panel*). The Slti114-smGFP and H^+-ATPase-RFP were co-localized to plasma membrane (*right panel*). For each panel, DIC shows the actual image, red channel shows chloroplast fluorescence and green channel shows GFP fluorescence. Merged image shows red and green channel at once. The bar size is indicated as 10 μm. smGFP H^+-ATPase-RFP or Slti114-smGFP vector was transiently expressed in *N. benthamiana* using *Agrobacterium*-mediated transformation method. Localization of the introduced genes was examined after 36 h using a laser confocal microscope. The same results were produced from the repeated experiment.

We tested if GST-Slti114 protein degrades MBP protein *in vitro* (Fig. 5). Enhanced MBP protein degradation was observed in the presence of the purified GST-Slti114 protein compared to that of the control (Fig. 5). This provides that Slti114 function as a protease, but do not degrade gelatin *in vitro*.

Discussion

In this report, we present the molecular characterization of the novel plant *Slti114* gene related to abiotic and senescence signals. Plant MMPs have been known to be induced by diverse stresses and senescence (Delorme *et al.* 2000, Golldack *et al.* 2002). It was reported that *Arabidopsis At2-MMP* RNA expression was induced by cadmium, jasmonic acid or NaCl treatment in both leaf and roots (Golldack *et al.* 2002). Soybean *MMP2* was shown to be induced by pathogen infection and alfalfa *MtN9* is differentially expressed during root nodulation according to NCBI database (http://www.ncbi.nlm.nih.gov/). This indicates that plant *MMP*s including *Slti114* are implicated in defense response to abiotic and biotic stresses.

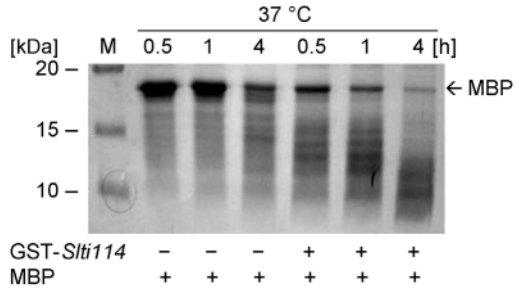

Fig. 5. MBP digestion assay of GST-Slti114 protein. MBP (0.2 μg) was incubated for 0.5, 1, 4 h at 37 °C without or with the purified GST-Slti114 protein (2 μg). The resulting reactions were analyzed on SDS-PAGE and the protein gels were visualized with Coomassie-staining (Laemmli 1970).

Plant MMPs have been demonstrated to be implicated in senescence (Delorme *et al.* 2000, Maidment *et al.* 1999, Pak *et al.* 1997). *Slti114* RNA expression was induced during soybean cotyledon development (Fig. 3C). RNA expression pattern of *Slti114* was different from that of cucumber *Cs1-MMP* (Delorme *et al.* 2000). *Slti114* RNA expression was abundant prior to senescence, but *Cs1-MMP* RNA expression was strong in the senescent cotyledon just prior to cell death stage (Delorme *et al.* 2000). During cotyledon senescence process, *Cs1-MMP* and *Slti114* induction pattern may be derived from the difference in the senescence mechanism between soybean and cucumber. The role of *Slti114* is wide open. *Slti114* may function as a signal inducer in cotyledon senescence, or Slti114 may serve as a defensive role in plant leaves against low temperature and/or wounding in soybean.

Plasma membrane localization of Slti114 protein was predicted from the protein sequence targeting signal. Based on the PSORT (http://www.psort.org/) and localization prediction for protein sorting signals (http://www.cbs.dtu.dk/servies/SignalP/), Slti114 contains a cleavable signal peptide at the N terminus, along with two predicted transmembrane domains at the C-terminus (Figs. 1*A* and 4). This structure is typical type Ia plasma membrane proteins, and may alternatively indicate a glycosylphosphatidylinositol anchor linkage to the plasma membrane. At1-, At2-, At3-, and At5-MMPs all contain N-terminal signal peptide and C-terminal transmembrane domain (Maidment *et al.* 1999). Previously, Pak *et al.* (1997) reported that soybean SMEP1 was found to be tightly bound to the cell wall. This is the first *in vivo* report of plant MMP localization using GFP-fusion expression in plants (Fig. 4).

Compared to the inability of gelatin digestion by GST-Slti114, MBP protein was efficiently digested by GST-Slti114 as early as 30 min after incubation initiation (Fig. 5). It was reported that recombinant *Arabidopsis* At1-MMP, nematode MMP-H19 and MMP-Y19 was not able to digest gelatin (Maidment *et al.* 1999, Wada *et al.* 1998). Like GST-Slti114, At1-MMP was shown to digest MBP, but the digestion pattern by At1-MMP is quite different from that by stromelysin-1 (MMP-3) (Maidment *et al.* 1999). In contrast, cucumber Cs1-MMP and soybean SMEP1 were demonstrated to have *in vitro* gelatin degradation activity (Delorme *et al.* 2000, Huangpu and Graham 1995). It can be deduced that the target substrate by each MMP is different. The cell wall is crucial to growth and development of the plant, along with its response to environmental stresses and attack by pathogens or insects (Cassab 1998). The plant cell wall contains a number of ECM macromolecules, including hydroxyproline-rich glycoproteins, arabinogalactan proteins, glycine-rich proteins, and proline-rich proteins, some of which have homology to vertebrate ECM molecules (Maidment *et al.* 1999). It is possible that there may be other substrates than gelatin used by Slti114 in plant ECM.

In this paper, we report the molecular cloning of the full-length cDNA sequence of soybean *MMP*, *Slti114* induced by low temperature, wounding stresses and in the developing cotyledons prior to PCD during senescence process. We demonstrate that Slti114-smGFP fusion protein was co-localized to the plasma membrane with a marker protein, H^+-ATPAse-RFP. *In vitro* protease activity of GST-Slti114 fusion protein provided its potential degrading enzyme in the plant cell during abiotic stresses and senescence in soybean plants. The *in vivo* role of *Slti114* should be elucidated further related to senescence, biotic, or abiotic stresses in plants.

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