Molecular cloning and characterization of a novel stress responsive gene in alfalfa

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Abstract

A suppression subtraction hybridization (SSH) cDNA library of alfalfa (*Medicago sativa* L.) cv. Zhongmu NO.1 had been constructed to identify differentially expressed genes under stress. Based on the sequence of a 460 bp expressed sequence tags (ESTs), a cDNA of 1652 bp was cloned by rapid amplification of cDNA ends (RACE) method. This gene (*MsPBL*) was predicted to encode a 434-amino-acid protein, which contained a Phox and Bem1 (PB1) domain. PB1 domain is a functional domain comprising about 80 amino acid residues, which exists in many signal transduction proteins and mediates dimerization in the proteins. PB1 domain is mostly involved in two cell signal transduction pathways: MAPK and NF-KB. When fused to the green fluorescent protein, we found MsPBL localization in the nucleus of onion (*Allium cepa* L.) epidermal cells. The transcripts of *MsPBL* rose significantly when alfalfa was treated with 300 mM NaCl, 0.1 mM ABA, and 20 % polyethylene glycol (PEG-6000). These results indicated that *MsPBL* may be functional within the nucleus as a signal transduction protein to allow alfalfa to rapidly respond to the environmental stress signals.

Additional key words: *Allium cepa*, gene expression, *Medicago sativa*, PB1 domain, phylogenetic tree, RACE, subcellular localization.

Introduction

Environmental stresses, such as cold, salinity, and drought have an enormous impact on crop productivity throughout the world (Boyer 1982, Long *et al*. 2006). A common feature across several of these stresses seems to be their osmotic nature. Compared with many other plants, alfala is relatively salt tolerant (Munns *et al*. 2008). Recently we cultivated a new salt tolerant cultivar of alfalfa, named Zhongmu NO.1. In contrast to many other cultivars of alfalfa, the yield of Zhongmu NO.1 increased under moderate salinity. In order to study the molecular mechanism of salt resistance in Zhongmu NO.1, we constructed a salt stress SSH cDNA library, and got 82 uni-ESTs comprised of 16 contigs and 66 singletons (Jin *et al*. 2010). Based on a 460 bp EST (*GenBank* accession No. ABD32345.1), we cloned a new gene encoding a 434-amino-acid protein which contained a Phox and Bem1 (PB1) domain. This gene was tentatively named *MsPBL*. PB1 domain is a functional domain comprising about 80 amino acid residues which exists in many signal transduction proteins and mediates dimerization in the proteins (Ito *et al*. 2001). PB1 domain is mostly involved in two signal transduction pathways: mitogen activated protein kinase (MAPK) and nuclear transcription factor (NF-kB).

A common element in many eukaryotic regulatory pathways is a three-kinase cascade, known as a MAP kinase module. In this module some kinases contain a PB1domain. The module consists of three protein kinases that act sequentially within a pathway: a MAP kinase kinase kinase or a MEK activator kinase kinase (MEKK), a MAP kinase/ extracellular signal-regulated protein kinase (ERK) or a MAP kinase activator (MEK), and a

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Abbreviations: DEPC - diethylpyrocarbonate; EST - expressed sequence tag; MAPK - mitogen activated protein kinase; RACE - rapid amplification of cDNA ends; SSH - suppression subtraction hybridization.
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MAP kinase or ERK homolog (English *et al*. 1995). MAP kinases are critical participants in major cellular events including proliferation, differentiation and stress responses (Hunter *et al*. 1995). MAP kinase pathways are found in all eukaryotes and are involved in transducing a variety of extracellular signals including growth regulators, UV radiation and osmotic stress (Waskiewicz *et al*. 1995). MAPK cascades are usually composed of three protein kinases that upon activation undergo sequential phosphorylation (Robinson *et al*. 1997). By phosphorylation of conserved threonine and tyrosine residues, a MAPK becomes activated by a specific MAPK kinase (MAPKK). A MAPKK kinase (MAPKKK) activates MAPKK through phosphorylation of conserved threonine and/or serine residues (Munnik *et al*. 1999).

PB1 domain plays a very important role in the nuclear factor kB (NF-kB) signal transduction pathway (Moscat *et al*. 2006). NF-kB is a nuclear transcription factor that regulates expression of a large number of genes. PB1 domain form heterodimers using a β-grasp topology in a "front-to-back" arrangement (Terasawa *et al*. 2001, Ponting *et al*. 2002). A canonical PB1-PB1 interaction, which involves heterodimerization of two PB1 domains, is required for the formation of macromolecular signaling complexes ensuring specificity and fidelity during cellular signaling. The interaction between two PB1 domains depends on the type of PB1. The PB1 domain is classified into three types, type I, type II, and type I/II (Hirano *et al*. 2004). Type I contains a motif of 28 amino acid residues with highly conserved acidic and hydrophobic residues named the OPCA motif. Type II contains a conserved lysine residue on the side opposite to the OPCA motif. Type I/II contains the OPCA motif and the conserved lysine residue, and thus can selfinteract in a front-to-back topology (Saio *et al*. 2009).

Until now, many MAPK genes have been isolated from plants, and some of them are relevant to abiotic stress signal transduction. In this paper, we report the cloning and characterization of a new salt induced gene from alfalfa, designated as *MsPBL*. Bioinformatics analysis of this gene indicated the putative protein MsPBL contained a PB1 domain, but no significant kinase domain. The phylogenetic tree analysis indicated that MsPBL was homologous with some proteins of some other plants. But until now the function of these proteins are still unknown. For investigating the subcellular localization of *MsPBL*, *MsPBL-GFP* fusion gene construct was transformed into epidermal cells of onion using particle gun. It is a fast and easy method to investigate subcellular localization of some proteins. The expression of *MsPBL* was characterized in alfalfa cv. Zhongmu NO.1 under osmotic stress induced by NaCl and polyethylene glycol (PEG-6000) or under ABA application.

Materials and methods

Alfalfa (*Medicago sativa* L.) cv. Zhongmu NO.1 seedlings were grown in Hoagland's nutrient solution. The solution was changed every 5 d. Two weeks after sowing, the seedlings were transferred into Hoagland's solution with 300 mM NaCl, 0.1 mM ABA, or 20 % PEG-6000. Epidermis of onion (*Allium cepa* L.) was incubated on ½ MS agar plates containing 1 % sucrose (pH 5.7).

Total RNA was extracted by using *Trizol* reagent (*Invitrogen*, USA) and then dissolved in ddH_2O which was treated with diethylpyrocarbonate (DEPC). Before reverse transcription, the total RNA was treated with RNase-free DNase I (*Fermentas-MBI*, Canada) for 30 min at 37°C. The cDNA was synthesized by *PrimeScript* reverse transcriptase (*Takara*, Japan). The harvested complementary cDNA was deposited at -20°C until use.

A SSH cDNA library of cv. Zhongmu NO.1 induced by NaCl had been constructed and screened before, and an EST (*GenBank* accession No. ABD32345.1) of a new gene was isolated. Rapid amplification of cDNA ends (RACE) was performed to amplify its unknown 3′ and 5′ ends. Total RNA was isolated from 2-weeks-old alfalfa seedlings by *Trizol* extract method. Any contaminated genomic DNA was removed by incubating the total RNA with RNase-free DNase I (*Fermentas-MBI*) at 37 °C for 30 min. And total RNA was used to synthesize 5′-RACE-ready-cDNA and 3′-RACE-ready-cDNA according to the manufacturer's recommendation of *BD SMARTTM RACE* cDNA amplification kit (*Clontech*, USA). Based on the EST sequence already obtained, we designed gene-specific primers P1 (5′-CTGGACCGGAGTTGTTTTGC ACAGCTTG-3′) and P1-net (5′-CCACCGTGGTAG GACTGTAGGGCTGAAC-3′) to amplify the 5′-cDNA end, and designed primer P2 (5′-AGTGTATGGTGC GGGGATGAGGCATGTGGC-3′) to amplify the 3′-cDNA end. PCR reaction conditions were as follows: pre-denaturalization at 94 °C for 5 min, thermocycling was performed at 30 cycles with 94 °C for 30 s, 68 °C for 2 min, and an additional polymerization step at 72 °C for 10 min. A pair of primers P-F (5′-ACAAGTTAACGT GTCTCCTCCAAAT-3′) and P-R (5′-ACGCAAATG GAGTAATTAAATTACCT-3′) was designed to amplify the full-length of the cDNA. PCR product was separated by electrophoresis on a 1 % agarose gel stained with ethidium bromide, purified by using the DNA gel extraction kit (*Takara*). The products were cloned into the pMD-19T vector (*Takara*) and then transformed into *E. coli* DH5α. And recombinant plasmids were sequenced by *Huada Gene Company* (BGI, China).

By comparing and aligning the sequences of the

known EST, 5′RACE, and 3′RACE sequences, the full-length cDNA sequence of *MsPBL* was obtained. *MsPBL* and its putative encoding protein was subsequently analyzed for molecular characterization, such as the conserved motifs, sequence homology, secondary structure, phylogenetic analysis, subcellular localization, expression analysis under different environmental stresses.

The secondary structure of the putative protein was analyzed by *HNN* secondary structure prediction method (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html). The specifically functional sites of the putative protein were analyzed by *SMART* (http://smart.embl-heidelberg.de/). Sequences homology analysis was against nucleotide and protein database of *GenBank* by using *BLAST* (*NCBI*) program. A multiple alignment of the putative PB1 domain with some other known PB1 domains was performed by *SMART*. The phylogenetic relationship between the amino acid sequences was analyzed through *Clustal X 1.8* and *MEGA 4.1* programs by using Neighbor Joining method. Potential signal peptide cleavage site was identified using *SignalP 3.0* (http://www.cbs.dtu.dk/ services/SignalP/). The pI/M_r of the putative protein was predicted on *ExPaSy* website (http://us.expasy. org/tools/pi.tool.htm).

To observe the subcellular localization of MsPBL, a *MsPBL-GFP* fusion was constructed. Primer P3 (5′-TAC TCGAGATGGCGTCGTCACTCC-3′) and P4 (5′-CGA CTAGTATTATGCGGCGAGTTGG-3′) were designed to amplify the coding sequence of *MsPBL*. The PCR product was cloned in PMD19-T vector. The recombinant vector PMD19-T-*MsPBL* and transient expression vector PA7-*GFP* were digested with *Xho I* and *Spe I* restriction endonucleases. Then digested PA7-*GFP* fragment and *MsPBL* gene fragment were linked with T4-ligase, and then, the fusion construct PA7-*MsPBL-GFP* was obtained. *MsPBL-GFP* was controlled by a 35S promoter. The PA7-*MsPBL-GFP* vector was then transformed into onion inner-epidermis by using particle gun (*Bio-Rad PDS-1000,* USA), and visualized under laser scanning confocal microscope (*Nikon C1 Plus*, Japan).

The 2-week-old seedlings were transferred into Hoagland's solution with salt (300 mM), ABA (0.1 mM), and PEG-6000 (20 %) for 10 min, 30 min, 2 h, 4 h and 8 h. The seedlings grown on Hoagland's solution without additives were used as control. The roots and shoots were harvested separately and total RNA was reverse transcribed, and the synthesized cDNA was used as template in real-time PCR. The real-time fluorescent quantitative PCR was analyzed by *ABI-7500* (*ABI*, USA). P5 (5′-TCAGCCCTACAGTCCTACCA CGG-3′) and P6 (5′-GTTCCACCTCTCTGCAGCTC ATC-3′) were used for amplification of *MsPBL*. Actin gene was used as housekeeping gene to normalize the target gene quantities. The amplified fragment of *MsPBL* was 147 bp. P7 (5′-GATACTCTTTCACCACAA CAGCCG-3′) and P8 (5′-ACTTCAGGACAACGGA AACGCT-3′) were used for amplification of actin gene. The amplified fragment of actin gene was 190 bp. The PCR annealing temperature of all primers was 60 °C.

Results and discussion

Cloning of the full-length cDNA of *MsPBL*: In the first round of 5′ RACE PCR reaction, there was no significant fragment by using P1 and UPM (UPM was contained in the *BD SMARTTM RACE* cDNA amplification kit). Then a nested PCR reaction was applied by using P1-net and nested universal primer (contained in the *BD SMARTTM RACE* kit), and a 1250 bp fragment was obtained (Fig. 1*A*). The product of the first round 5′ RACE PCR reaction was used as the template of the nested PCR reaction. A 451 bp fragment (Fig. 1*B*) was isolated by using 3′ RACE primers P2 and UPM. The 460 bp known EST sequence, 451 bp 3′ end sequence and 1 250 bp 5′ end sequence were assembled to be the full-length of the cDNA. The full-length of the cDNA was 1 639 bp (Fig. 1*C*), which contained a 1 302 bp open reading frame (ORF) that encoded a protein of 434 amino acids (Fig. 2). The molecular mass of the putative protein was about 47.56 kDa, and the pI is about 5.85. This putative protein contained a PB1 domain, and was homologous to a putative protein of *Populus trichocarpa* (XM002305609). It was tentatively named *MsPBL* (*GenBank* accession No. GQ868709).

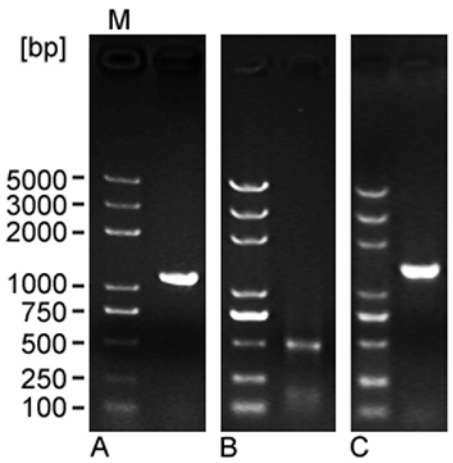

Fig. 1. Electrophoresis gels of the *MsPBL*: *A* - 5′ RACE nested PCR reaction fragment by using primers P1-net and nested universal primer; *B* - 3′ RACE fragment using primers P2 and UPM; *C* - full-length fragment of *MsPBL* by using primers P-F and P-R. M - DNA marker.

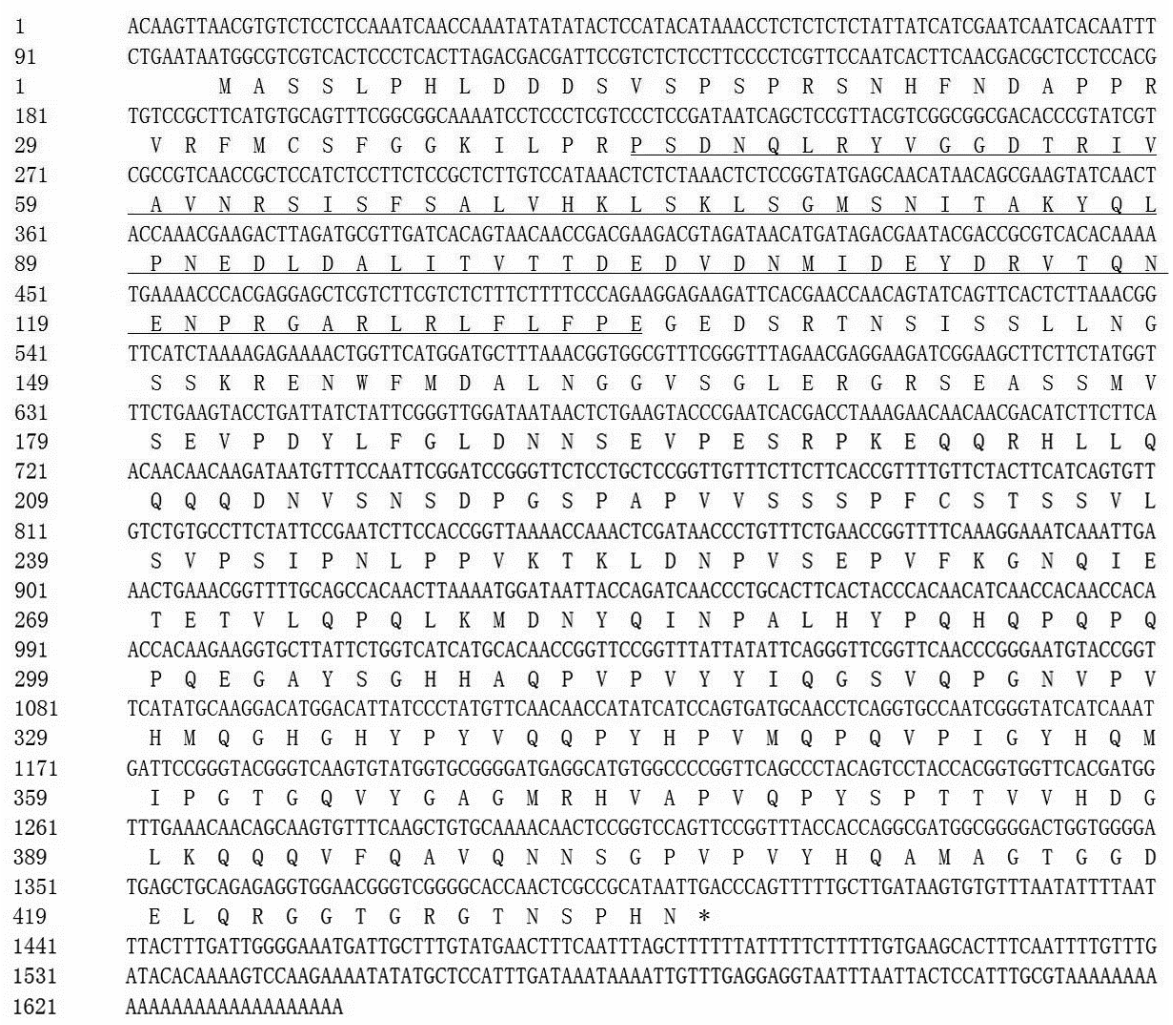

Fig. 2. Nucleotide and putative encoding amino acid sequence of *MsPBL*. The amino acids underlined indicated the putative PB1 domain.

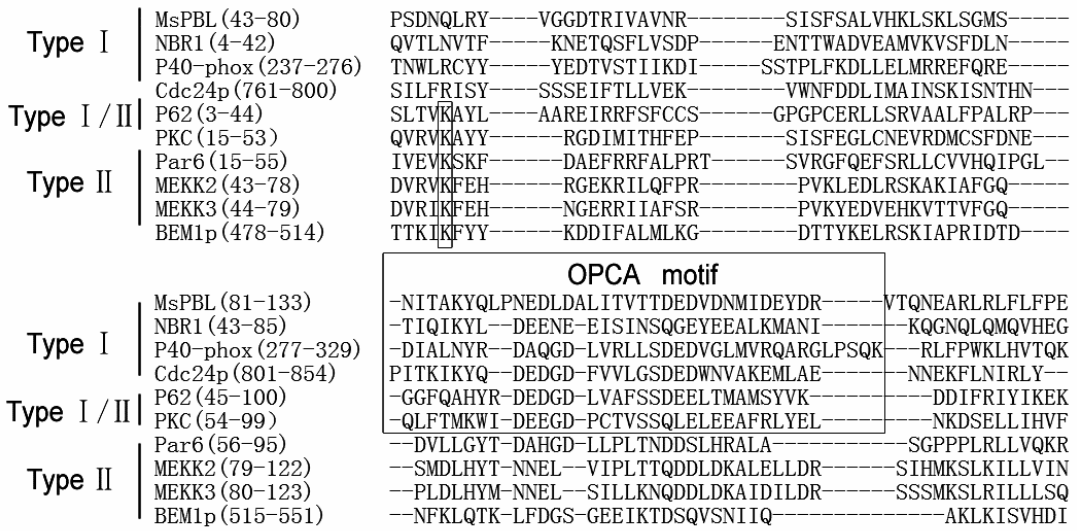

Fig. 3. Multiple alignment of the putative PB1 domain of MsPBL and some other proteins. The other proteins have been characterized to contain three types of PB1 domains (Saio *et al*. 2009).

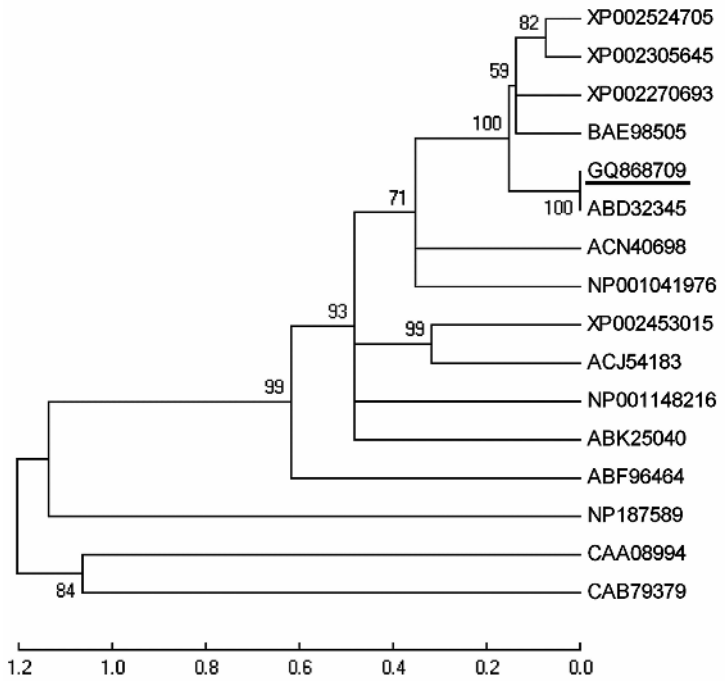

Fig. 4. The phylogenetic tree analysis of MsPBL and some other proteins which contain PB1 domain. The phylogenetic tree was constructed by Neighbor-Joining method using amino acid sequences. The amino acid sequences were obtained from *NCBI* with the accession numbers as below: GQ868709 from *Medicago sativa*; ABD32345 from *Medicago truncatula*; XP002524705 from *Ricinus communis*; XP002305645 from *Populus trichocarpa*; XP002270693 from *Vitis vinifera*; BAE98505, NP187589, CAA08994 and CAB79379 from *Arabidopsis thaliana*; ACN40698 and ABK25040 from *Picea sitchensis*; NP001041976 and ABF96464 from *Oryza sativa*; NP001148216 from *Zea mays*; XP002453015 from *Sorghum bicolor*; ACJ54183 from *Nicotiana benthamiana*. The reliability of the tree is measured by bootstrap analysis with 1000 replicates.

The secondary structure of the putative protein MsPBL: It was predicted that MsPBL consisted of α-helix (15.0 %), extended strand (14.3 %), and random coil (70.7 %). The functional site prediction result showed that there was a PB1 domain from Pro^{43} to Glu^{133} (Fig. 2). According to the alignment result performed by *SMART*, the amino acid sequence of MsPBL was homologous at the conservative region with $P40^{phox}$ which contained a type I PB1 domain (Saio *et al*. 2009). The PB1 domain of MsPBL contained a motif of 28 amino acid residues with highly conserved acidic and hydrophobic residues, but did not contain a conserved lysine residue on the side opposite to the OPCA motif (Fig. 3). So the PB1 domain of MsPBL might belong to type I PB1 domain. Based on the amino acid sequence, a phylogenetic tree was constructed by using Neighbor-Joining method. The result indicated that MsPBL was homologous with some proteins of some other plants (Fig. 4). Until now the function of these proteins are unknown.

Subcellular localization of MsPBL: To observe the subcellular localization of MsPBL, the *MsPBL-GFP* fusion was transformed into onion inner-epidermis by using a gene gun. In cells where the PA7-GFP vector was introduced alone (Fig. 5), fluorescence was visualized throughout the cell with no clear preference for localization. However, in cells expressing the *MsPBL-GFP* fusion, fluorescence was found to be clearly localized in the nucleus (Fig. 5). The position of the nucleus was visualized under laser scanning confocal microscope.

In addition, we also used *ProtComp v. 9.0* program online (www.softberry.com) to predict the subcellular localization, and the result showed that *MsPBL* most probably localized in nucleus.

Expression of *MsPBL* under different environmental stresses: A real-time PCR analysis was carried out to investigate the expression of *MsPBL* gene in different environmental stresses (300 mM NaCl, 0.1 mM ABA and 20 % PEG-6000). The relative expression of *MsPBL* was calculated according to Pfaffl (2001).

Compared to control, the transcripts of *MsPBL* in roots and shoots increased significantly after NaCl treatment for 2 h (Fig. 6*A,B*). Under the treatment of 0.1 mM ABA, the relative level of *MsPBL* mRNA in roots began to increase significantly after 10 min (Fig. 6*C*), while in shoots after 30 min (Fig. 6*D*). Under the PEG treatment, the relative level of *MsPBL* mRNA did not increased significantly in roots (Fig. 6*E*), but increased in shoots after 10 min (Fig. 6*F*).

Many signal transduction genes in plants have been isolated so far, but identified signal transduction genes in

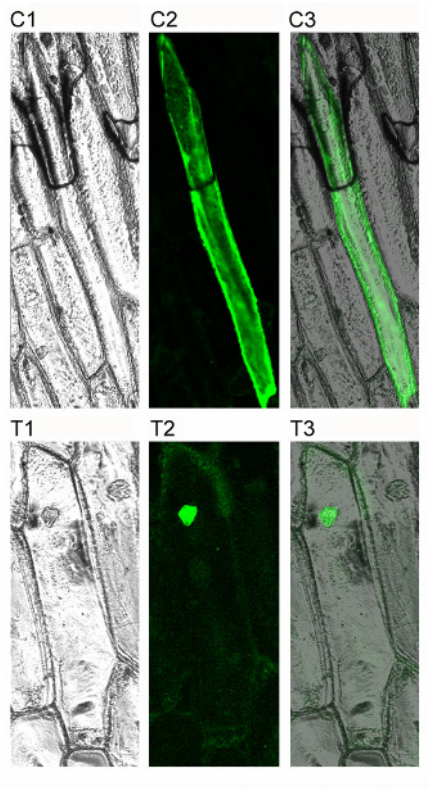

Fig. 5. The subcellular localization of MsPBL in onion inner-epidermis. The PA7-*MsPBL-GFP* vector and the pA7-GFP empty vector were transformed into onion epidermal cells by using a gene gun. The fluorescence signals were examined by a confocal laser scanning microscope. The GFP fluorescence from cells expressing MsPBL-GFP fusion protein was localized in the nucleus (T1 - T3). The GFP fluorescence was distributed throughout the entire cells expressing pA7-GFP vector (C1 - C3). The photographs were taken in superposition of bright light vision (C1 and T1), dark field vision (C2 and T2), and bright and dark vision (C3 and T3).

alfalfa are still little. In this study, we reported a salt-stress induced gene *MsPBL* from alfalfa on the basis of 460 bp EST in a SSH cDNA library. This gene encoded a putative protein of 434 amino acids. Sequence analysis of MsPBL indicated that it contained a PB1 domain. Analysis and multiple alignment of the putative PB1 domain and some other proteins indicated that the PB1 domain of MsPBL belonged to type I PB1 domain. The PB1 domain is a modular domain mediating specific protein-protein interactions. PB1 domain is contained in many proteins, especially many signal transduction MAP kinases. So *MsPBL* was predicted as singal transduction gene, but currently the precise role of this gene is unknown and further study is needed.

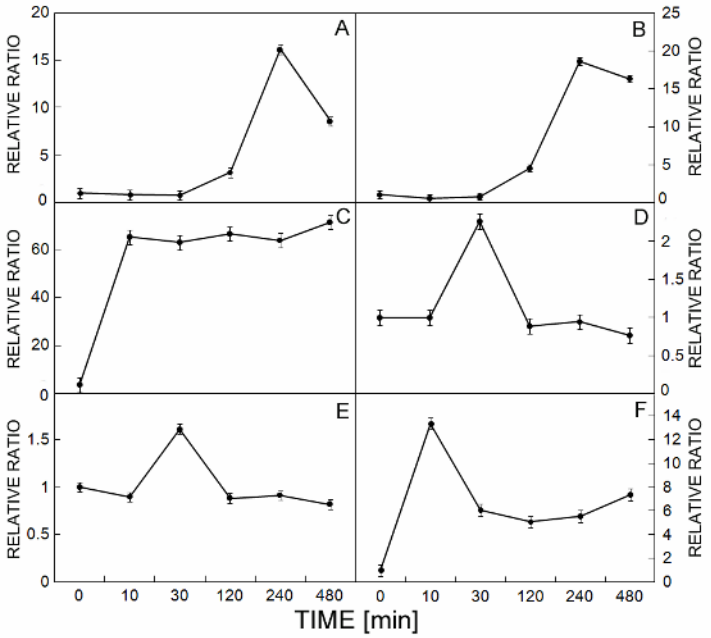

Fig. 6. The relative mRNA levels of *MsPBL* in alfalfa under different stresses: *A* - root treated by NaCl; *B* - shoot treated by NaCl; *C* - root treated by ABA; *D* - shoot treated by ABA; *E* - root treated by PEG-6000; *F* - shoot treated by PEG-6000.

The subcellular localization analysis indicated that *MsPBL* is localized in the nucleus. *MsPBL* could be induced in alfalfa leaves and roots by NaCl, ABA and PEG-6000. These results also indicated that *MsPBL* might participate in signal transduction pathways under drought and salt stress conditions. Previous results showed that salt-induced gene expression is ABA-dependent (Agarwal and Jha 2010). Moreover, the ABA-deficient mutants of *Arabidopsis* (Zhu 2000) such as *aba3* (Leon-Kloosterziel *et al*. 1996, Heidenreich *et al*. 2005), *sre2-1* (Gonzalez-Guzman *et al*. 2004), *sto1/nced3* (Ruggiero *et al*. 2004), and ABA insensitive mutants may

have similar phenotype but they are different in salt tolerance (Finkelstein *et al*. 2005). Our results show that *MsPBL* was induced more quickly by ABA than NaCl. So we predict that *MsPBL* induction by NaCl is ABA-dependent. But the mechanism is unknown, so further studies are required.

Plants are sessile organisms, and must adapt to a number of environmental factors they face continuously. For example, drought and high salinity could change the metabolism of cells directly, but before these changes happened, drought and salinity signals must be recognized and transmitted by signal proteins in membrane or cytoplasm. Our results showed that the *MsPBL* is localized in nucleus. This implied that, when the alfalfa encountered the drought or high-salinity stress, these signals might be transmitted by *MsPBL* in cytoplasm, and then the *MsPBL* goes to the nucleus to regulate some defensive genes expression by coaction with the transcript factors. However, further studies are required to confirm that *MsPBL* is functional in nucleus as a signal transmitter.

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