Molecular cloning and characterization of a subtilisin-like protease from *Arabidopsis thaliana*

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ABSTRACT. The *Arabidopsis thaliana* genome encodes 56 subtilisin-like serine proteases (subtilases). In order to evaluate the protease activity of a previously uncharacterized subtilase, designated as AtSBT1.9, we cloned its full-length cDNA from *A. thaliana* seedlings. An AtSBT1.9 mature peptide coding sequence was inserted into the bacterial expression vector, pMAL-c2x, and the recombinant vector was transformed into *Escherichia coli* BL21 (DE3). The recombinant AtSBT1.9 tagged by maltose binding protein (MBP) was induced as a 117.5-kDa protein in the soluble form in *E. coli* BL21 (DE3). MBP-AtSBT1.9 was expressed at a level of 11% (w/w) of the bacterial total protein. Protein purification using Amylose Resin revealed a recombinant AtSBT1.9 protease activity of 9.23 U/mg protein at pH 7 and 25°C. Maximal activity occurred over a broad pH (7-8) and temperature (25°-42°C) optimal range. Validation of AtSBT1.9 protease activity would help in characterizing its *in vivo* function in *A. thaliana*.

Key words: *Arabidopsis thaliana*; Bacterial expression; Protease activity; Serine protease; Subtilase

INTRODUCTION

Subtilisin-like proteases (subtilases) are a type of serine proteases and constitute the largest group of peptidases (Dodson and Wlodawer, 1998; Figueiredo et al., 2014). Eukaryotic subtilases are encoded by a variety of organisms, including animals, plants, and fungi, and form the SB subfamily within the S8 serine protease family (Rautengarten et al., 2005).

Several subtilases have been identified in plants. For example, 63, 56, and 15 subtilase genes are known in *Oryza sativa*, *Arabidopsis thaliana*, and *Lycopersicon esculentum*, respectively (Meichtry et al., 1999; Rautengarten et al., 2005; Tripathi and Sowdhamini, 2006; Figueiredo et al., 2014). Therefore, it is likely that subtilases contribute significantly to the developmental processes and signaling cascades in plants (Rautengarten et al., 2005). An *A. thaliana* subtilase, AtSBT3.3, seems to be involved in activating downstream immune signaling during plant-pathogen interactions (Ramírez et al., 2013). The loss-of-function gene mutations SDD1 (AtSBT1.2) and ALE1 (AtSBT2.4) lead to abnormal stomatal density and leaf shape, respectively. These results indicate that some subtilases may play a role in developmental processes through the generation of peptide signals (von Groll et al., 2002; Matos et al., 2008). Overexpression of another subtilase (AtSBT5.4) produces a clavata-like phenotype with fasciated inflorescence stems and compounded terminal buds. Appearance of this phenotype depends on AtSBT5.4 activity, because substitution of the active-site serine abolished the overexpression phenotype (Liu et al., 2009). Other specific functions performed by plant subtilases include root development (Sénéchal et al., 2014) and seed germination (Rautengarten et al., 2008). Although several subtilases have been characterized, most of their functions in plants remain unknown.

AtSBT1.9 (GenBank accession No. NP_569044) is an *A. thaliana* subtilase ubiquitously expressed in leaves, roots, stems, flowers, inflorescences, and seedlings (Rautengarten et al., 2005). However, the natural AtSBT1.9 protein hasnever been purified from *A. thaliana*, and no reports to date describe its function or provide its biochemical characterization. We expressed the AtSBT1.9 protein in *E. coli* by cloning its cDNA and constructing a bacterial expression system. This provided a convenient and abundant source of recombinant AtSBT1.9 protein, which allowed for an *in vitro* protease activity assay and enzyme characterization. Recombinant AtSBT1.9 will enable further functional studies to establish its putative role in *A. thaliana*.

MATERIAL AND METHODS

Plant material, bacterial strains, andvectors

Arabidopsis thaliana plants (ecotypes Col-0) were used to clone cDNA of AtSBT1.9. Sterilized seeds were plated on 1/2 MS solid medium and kept at 4°C for three days, and then transferred to growth chambers at 22°C under 16 h light/8 h dark regime. *E. coli* DH5α and vector pMD18-T (TaKaRa, Japan) were used for DNA manipulation. *E. coli* BL21 (DE3) and vector pMAL-c2x (New England Biolabs, USA) were used for expression of AtSBT1.9 gene.

Cloning of AtSBT1.9 cDNA

Total RNA was isolated from 10-day-old *A. thaliana* seedlings using an RNeasy Mini Kit (Qiagen, USA). We designed a specific sense primer, ForP1, containing a *Bam*HI site (5'-CGA

GGA TCC ATG GGG ATG ACC GTC GTA-3', and a specific antisense primer, RevP2, containing a *Sal*I site (5'-GC GTC GAC TCA AGA CTC TTG GAC AAG GC-3'), based on the mRNA sequence of AtSBT1.9 (GenBank accession No. NM_126109). Following RNA extraction, reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the protocol of RNA LA PCRkit (TaKaRa, Japan). Primer RevP2 was used to reverse transcribe the double-stranded cDNA in a 25-µL reaction mixture containing 10 µg total RNA, and the mixture was incubated at 42°C for 60 min. The reaction was terminated by heating to 70°C for 5 min and chilling on ice for 5 min. Primer ForP1 and RevP2 pairs were used for PCR amplification with 1 µL of the double-stranded cDNA reaction mixture as the template. Our PCR program was as follows: pre-denaturation for 4 min at 94°C, then 30 cycles of 30 s at 94°C, 45 s at 62°C, 2 min at 72°C, and finally 10 min at 72°C. The PCR system without the total RNA template was used as a negative control. PCR products were subjected to agar electrophoresis, and the appropriate band removed and purified with a DNA Fragment Recovery Kit (TaKaRa, Japan). The purified fragment was then cloned into a pMD18-T vector and confirmed by DNA sequencing.

Construction of the vector pMAL-*AtSBT1.9*

The presence of a signal peptide was determined with the SignalP 4.1 server (www.cbs.dtu.dk/services/SignalP/). To obtain clones that only contain the mature peptide-coding region of *AtSBT1.9*, another primer (ForMP1), also containing a *Bam*HI site (5'-CGA GGA TCC GAG ACC TCT CCT TAC ATC ATC-3'), was designed, based on the mature peptide coding sequence of the target clone. PCR (with ForMP1 and RevP2 as primer pairs) was then performed following the same PCR program as before. The amplified product of this second round of PCR was cloned into pMD18-T and sequenced. The recombinant vector pMD18-T-*AtSBT1.9* was digested with *Bam*HI and *Sal*I, and the resultant *AtSBT1.9* fragment was cloned into the *Bam*HI and *Sal*I sites of the expression vector pMAL-c2x, and designated as pMAL-*AtSBT1.9*. The vector pMAL-*AtSBT1.9* was introduced into *E. coli* BL21 (DE3) cells by electroporation. The empty vector was also introduced into BL21 (DE3) as a negative control.

Fusion protein production in *E. coli*

E. coli BL21 (DE3) cells containing the plasmid of interest were grown at 37°C for 12 h in 5 mL LB medium containing appropriate antibiotics (100 µg ampicillin/mL). The culture was diluted 1:100 (v/v) to 300 mL of the same medium, followed by incubation at 37°C. When the OD_{600} value reached 0.5, isopropyl β-D-thiogalactopyranoside (IPTG) was added to each culture to a final concentration of 0.2 mM. Then, cells were grown at 18°C for 12 h or at 37°C for 5 h to induce expression.

Purification of fusion protein

Recombinant bacteria induced by IPTG were harvested by centrifugation and re-suspended in column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, and 10 mM β-mercaptoethanol). This suspension was sonicated at 4°C to yield the cell lysate, which was then centrifuged at 20,000 *g* for 20 min. The pellet was removed and the supernatant used for purification. The fusion protein was purified using amylose resin (New England Biolabs, USA) which is an affinity matrix for isolation of proteins fused to maltose-binding protein (MBP). Our

purification protocol was as follows: 1) In a 15-mL conical tube, 2 mL 50 % (v/v) amylose slurry was added and washed three times with cold column buffer, keeping the tubes at 4°C until the samples were ready; 2) The crude extract collected (6 mL) was transferred to the pretreated amylose resin. The system was rocked gently at 4°C for 3 h to allow binding; 3) Unbound material was removed by centrifugation at 4500 *g* for 5 min, at 4°C; 4) The resin was washed three times with cold column buffer; 5) The resin was transferred to a gravity flow column (Sigma-Aldrich, USA); and 6) the protein was eluted with column buffer plus 10 mM maltose. The bound proteins were fractionally eluted outin 250 µL fractions. Quality of the eluted fractions was assessed using sodium dodecyl sulphate poly agarose gel electrophoresis (SDS-PAGE). The fractions containing MBP-AtSBT1.9 were pooled and dialysed against 100 mM Tris-HCl buffer, pH 7, and stored at -80°C until used for the activity assay. SDS-PAGE was performed ona 12% (v/v) gel, which was then stained with Coomassie brilliant blue. Protein concentration was determined by the Bradford method.

Protease activity assay

Protease activity was assessed according to the methods described previously (Cheng et al., 2009) with slight modifications. Briefly, azocasein (Sigma-Aldrich, USA) was used as a substrate for the purified MBP-AtSBT1.9 protein. It was dissolved in 100 mM Tris-HCl buffer, pH 7, to a final concentration of 0.5% (w/v). The azocasein solution was then mixed with 4 µg/mL of the MBP-AtSBT1.9 protein in a 500-µL reaction system. This mixture was incubated at 25°C for 2 h. Finally, 500 µL 40% (w/v) trichloroacetic acid (TCA) was added to the mixture to terminate the reaction. After letting the mixture stand at room temperature for 15 min, the liquid fraction resulting from centrifugation at 13,000 *g* for 10 min was collected, and its absorbency at 335 nm noted. One unit (U) of activity was defined as the amount of enzyme required to increase the absorbency by 0.01 unit per minute. To determine optimal pH and temperature conditions, reactions were carried out in buffers having various pH values (2-12) at 25°C or by incubation at various temperatures (18°-70°C) in buffers, pH, under the conditions described above, respectively. The reaction blank used was 40% TCA. To test effects of MBP tag and AtSBT1.9 signal peptide on protease activity, the fusion protein consisting of MBP tag and full-length AtSBT1.9 (with its signal peptide included) was produced and purified as in the above-mentioned procedures. Cleavage of MBP tags was carried out by Factor Xa Protease using pMAL™ Protein Fusion and Purification System (New England Biolabs, USA). Protease activities of the expressed AtSBT1.9 proteins with different fusion forms (with or without MBP and signal peptide) were tested using standard protocols.

Western blotting

The MBP-AtSBT1.9 fusion protein was expressed in *E.coli* using the conditions described above. Total bacterial protein was prepared and fractionated on 12% (v/v) SDS-PAGE and transferred to nitrocellulose membranes. Immunodetection was performed using an anti-MBP antibody produced in mice (Sigma-Aldrich, USA) against MBP-AtSBT1.9 at a 1:5,000 dilution (v/v) as the primary antibody, and using alkaline phosphatase-conjugated anti-mouse immunoglobulins (1:10,000 dilution, v/v) as the secondary antibody (Sigma-Aldrich). Signals were visualized by employing NBT (nitroblue tetrazolium chloride)/BCIP (5-bromo-4-chloro-3'-indolyl phosphate p-toluidine salt) in the phosphatase reaction.

RESULTS

Cloning of *AtSBT1.9* gene

The cloned *AtSBT1.9* cDNA had a length of 2211 bp. Sequencing results confirm that the cDNA is consistent with the open reading frame of *AtSBT1.9* gene sequence deposited in the NCBI database with GenBank accession No. NM_126109. The *AtSBT1.9* cDNA encodes a deduced protein of 736 amino acids containing a signal peptide of 20 amino acids at the N-terminus domain, based on prediction of signal peptide with the Signal P 4.1 server (Figure 1). Therefore, another set of PCR was carried out with primer pairs that flanked the sequence of AtSBT1.9 mature peptide, resulting in a 2151-bp fragment (Figure 2). Accordingly, AtSBT1.9 mature peptide of 716 amino acids was deduced to possess a calculated molecular mass of 77.5 kDa.

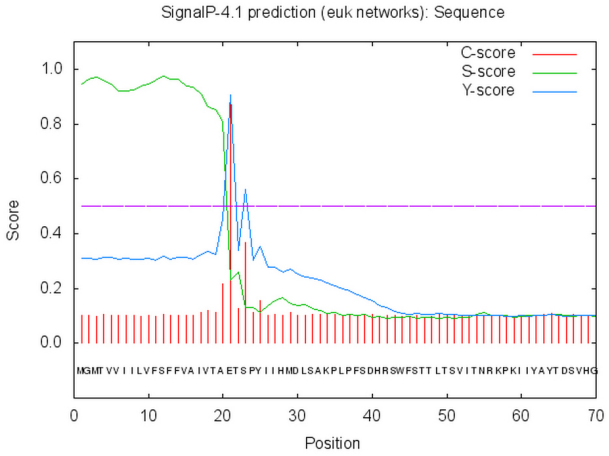

Figure 1. Presence of a signal peptide was determined with the SignalP 4.1 server. The AtSBT1.9 protein has a predicted signal peptide with a cleavage sequence at amino acid 20-21 from its N-terminus. C score for original cleavage site, S score for the signal peptide, Y Score for general cleavage site.

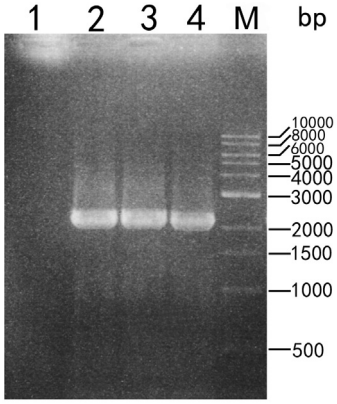

Figure 2. Gel electrophoresis of PCR products encoding the mature peptide-coding region of the AtSBT1.9 protein. *Lane 1* = negative control without template in PCR; *lanes 2-4* = PCR products of different clones; *lane M* = DNA marker.

Bacterial expression of AtSBT1.9 fusion protein

The cDNA corresponding to the AtSBT1.9 mature peptide coding region was cloned into pMD18-T, and incorporated into expression vector pMAL-c2x. Digestion by *Bam*HI and *Sal*I was performed to screen for the recombinant pMAL-*AtSBT1.9* (Figure 3).

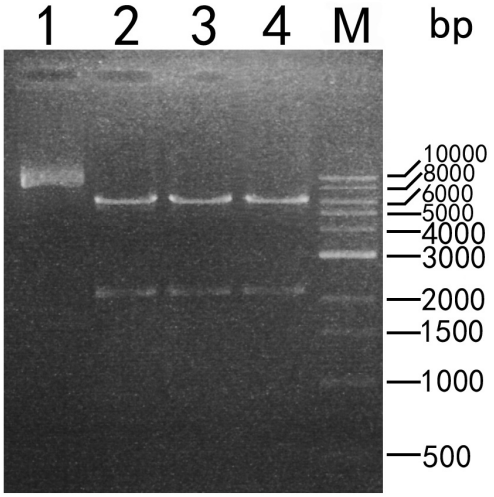

Figure 3. Double digestion with *Bam*HI and *Sal*I to verify presence of pMAL-*AtSBT1.9*. *Lane 1* = negative control without restriction enzymes; *lanes 2-4* = double digestion products; *lane M* = DNA marker.

Upon IPTG induction, the *E. coli* BL21/pMAL-*AtSBT1.9* expressed AtSBT1.9 fusion protein (Figure 4, lane 5). The fusion protein consists of a 40.0-kDa MBP tag (Figure 4, lane 3) and 77.5 kDa of AtSBT1.9 mature peptide, designated as MBP-AtSBT1.9. Thus, the total size of the predicted fusion protein is 117.5 kDa. This roughly corresponds to the molecular size of the detected band of MBP- AtSBT1.9 on SDS-PAGE (Figure 4). The yield of the fusion protein could reach up to 11% (w/w) of total BL21 proteins. Without IPTG induction, there was no detectable AtSBT1.9 fusion protein in the *E. coli* BL21/pMAL-*AtSBT1.9* (Figure 4, lane 1), identical to what was produced by the pMAL-c2x vector (Figure 4, lane 2). Furthermore, the bacterial cell lysate was centrifuged to collect the supernatant and the pellet for SDS-PAGE analysis. The AtSBT1.9 fusion protein, as detected by SDS-PAGE, had been expressed in both the soluble phase and the insoluble form (i.e. as an inclusion body) (Figure 4, lanes 4 and 6).

A pair of parallel experiments with the same bacterial system but with IPTG induction at 18° and 37°C, were performed to test the effect of induction temperature on AtSBT1.9 fusion protein expression in *E. coli*. The difference in the amount of target protein between the liquid and pellet fractions was identified by western blotting analysis using an MBP antiserum (Figure 5). Although inclusion bodies were still observed when induced by IPTG at 18°C, the amount of MBP-AtSBT1.9 protein in this case was much lower than that induced at 37°C (Figure 5). In contrast, induction at 18°C resulted in a significant increase in the amount of AtSBT1.9 fusion protein detected in the soluble phase, as compared with that at 37°C (Figure 5). Thus, the bacterial culture at 18°C was used for the production of the MBP-AtSBT1.9 protein.

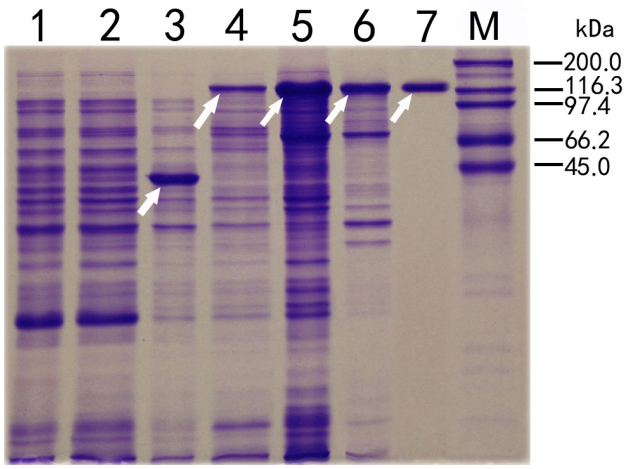

Figure 4. SDS-PAGE analysis of the expressed protein. Mature peptide of full-length AtSBT1.9 was expressed as an N-terminal MBP fusion protein, designated as MBP-AtSBT1.9. *Lane 1* = uninduced BL21/pMAL-*AtSBT1.9*; *lane 2* = uninduced BL21/pMAL-c2x; *lane 3* = induced BL21/pMAL-c2x; *lane 4* = supernatant containing soluble fractions released from the induced BL21/pMAL-*AtSBT1.9* after sonication; *lane 5* = induced BL21/pMAL-*AtSBT1.9*; *lane 6* = pellet containing insoluble fractions after sonication; *lane 7* = purified MBP-AtSBT1.9; *lane M* = molecular mass marker proteins. Arrows indicate MBP-AtSBT1.9 on lanes 4-7 and MBP protein on lane 3.

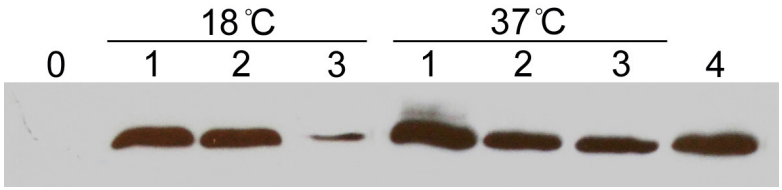

Figure 5. Induction temperature (18° or 37°C) affects the production of the MBP-AtSBT1.9 protein in *E. coli* BL21 (DE3) as detected by anti-MBP Western blotting. *Lane 0* = cell fraction of BL21/pMAL-*AtSBT1.9*, no IPTG induction; *lane 1* = cell fraction of BL21/pMAL-*AtSBT1.9*, by IPTG induction; *lane 2* = soluble lysates of BL21/pMAL-*AtSBT1.9*, by IPTG induction; *lane 3* = insoluble lysates of BL21/pMAL-*AtSBT1.9*, by IPTG induction; *lane 4* = the purified MBP-AtSBT1.9 protein. For 100 µg MBP-AtSBT1.9 induced at 18°C, the amount of protein in the soluble and the insoluble lysates is 92 and 8 µg, respectively. Conversely, when induced at 37°C, the amount of protein in soluble and insoluble lysates is 57 and 43 µg, respectively.

Protease activity of AtSBT1.9 fusion protein

After purification, the MBP-AtSBT1.9 protein (Figure 4, lane 7) showed a specific activity of 9.23 U/mg protein (Table 1). Totally, 87 mg of AtSBT1.9 fusion proteins were obtained from 4 L of the original bacterial culture (Table 1). One of the parameters in our *in vitro* assay, either incubation temperature or pH, was altered at a time, to identify its effects on protease activity. The effect of varying incubation temperature on activity was statistically significant in all assay protocols (Figure 6). As shown in Figure 6, activity increased up to 38% with an incubation temperature of 37°C, whereas it decreased after a 42°C incubation, compared with that at 25°C. A pH value range of 7-8 was detected as the optimal pH for activity (Figure 7). Activity at pH 2 and pH12 decreased by 96 and 83%, respectively, compared with that at pH 7 (Figure 7).

Table 1. Yields and protease activity of the MBP-AtSBT1.9 fusion protein[a].

Purification step	Protein yield (mg)	Recovery (%)	Total activity[b] (U)	Specific activity (U/mg)
Cell lysates	1042 ± 17	100	1229.56 ± 8.12	1.18 ± 0.07
Purified protease	87 ± 2.3	8.35	803.01 ± 3.41	9.23 ± 0.12

[a]The data shown are means (± standard deviation) of three experiments. [b]Protease activity of samples was measured at 25°C, pH 7.

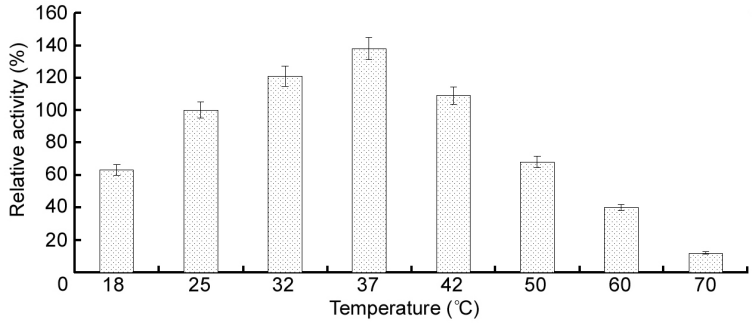

Figure 6. Incubation temperature affects protease activity of MBP-AtSBT1.9 protein *in vitro* assay at pH 7. The activity at 25°C was standardized as 100% (CK sample, corresponding to 9.23 U/mg protein), and other samples were then normalized against the CK for obtaining the relative activities. The data shown are means (± standard deviation) of three experiments.

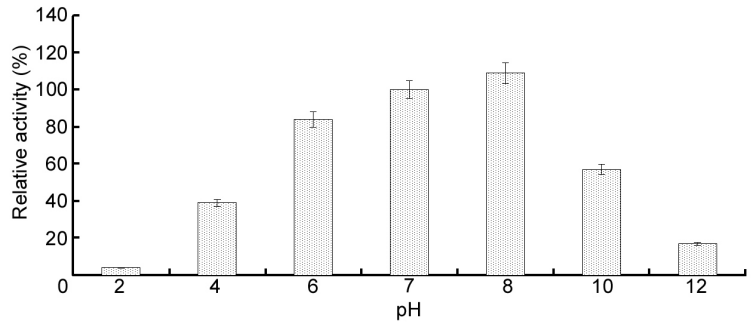

Figure 7. Incubation pH affects the protease activity of MBP-AtSBT1.9 protein *in vitro* assay at 25°C. The activity at pH 7 was standardized as 100% (CK sample, corresponding to 9.23 U/mg protein), and other samples were then normalized against the CK for obtaining the relative activities. The data shown are means (± standard deviation) of three experiments.

To test whether the MBP tag in this fusion protein was responsible for the protease activity, AtSBT1.9 mature peptide after MBP elimination was measured for *in vitro* activity. As shown in Figure 8, a similar activity was presented between the AtSBT1.9 mature peptide and the MBP-tagged one. To test how, if the signal peptide was not cleaved, the activity might be altered compared to properly processed AtSBT1.9 mature peptide, the full-length AtSBT1.9 containing its signal peptide and mature peptide was also fused to the MBP tag. In this case, both the expressed proteins, with and without MBP, showed no protease activity under the same assay conditions (Figure 8).

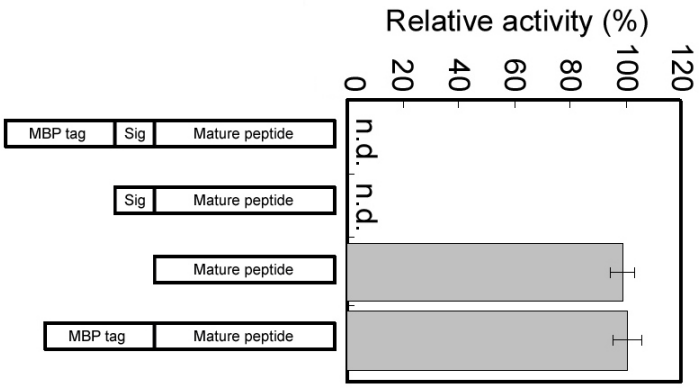

Figure 8. Protease activities of the expressed AtSBT1.9 proteins with different fusion forms. Schematic diagrams of the constructs used for *in vitro* assays are shown at left. Protease activity was monitored at 25°C, pH 7. The activity of MBP-tagged mature peptide of AtSBT1.9 was standardized as 100% (CK sample, corresponding to 9.23 U/mg protein), and other samples were then normalized against the CK for obtaining the relative activities. The data shown are means (± standard deviation) of three experiments. MBP tag, maltose-binding protein; Sig, AtSBT1.9 signal peptide; mature peptide, AtSBT1.9 mature peptide; n.d., not detectable.

DISCUSSION

We cloned an *A. thaliana* subtilase *AtSBT1.9* gene, then demonstrated that this subtilase could effectively be produced as a fusion protein (MBP-AtSBT1.9) in *E. coli*, and that it possessed protease activity *in vitro*. The majority of known subtilases contain a signal peptide domain that targets the protein for extracellular secretion, and a mature peptide domain that exhibits degradative activity (Rautengarten et al., 2005). Processing at N-terminus of the protein appears to be a common feature of *A. thaliana* subtilases (Rautengarten et al., 2005; Sénéchal et al., 2014). The *AtSBT1.9* gene is predicted to encode an N-terminus signal peptide of 20 amino acids. It is interesting that the expressed full-length AtSBT1.9 protein, including its signal peptide in *E. coli* showed no protease activities. These data indicated that the *E. coli* expression system could not recognize the endogenous signal peptide of AtSBT1.9, which resulted in no activation peptides. Therefore, a partial *AtSBT*1.9 cDNA, corresponding to its mature peptide, was expressed in *E. coli*. The AtSBT1.9 protein was expressed using a prokaryotic system for a number of reasons. Compared with other expression systems, *E. coli* serves as an excellent host for recombinant protein production because it provides an economical and fast way to produce the molecules in relatively large amounts, although yields of correctly folded and functional protein can be low because of protein aggregation (Li and Li, 2009). However, the pMAL protein fusion system provides a convenient method for directing fusion proteins to the periplasm where they are allowed to fold. Although expressed MBP-AtSBT1.9 was observed in both the soluble phase and in the form of inclusion bodies, with a nearly equal yield of each at 37°C induction, an optimal induction temperature of 18°C resulted in a significantly increased yield of fusion protein in the soluble phase. Moreover, because of the availability of a commercial anti-MBP antibody, it is convenient to detect MBP-tagged proteins with good sensitivity using western blotting.

Subtilases are characterized by a catalytic triad of the three amino acids: aspartate, histidine, and serine (Dodson and Wlodawer, 1998). Subtilases catalyze many fundamental proteolytic reactions involved in a wide variety of essential processes, including development,

physiology, and adaptation to changing environments (Figueiredo et al., 2014). There are fifty-six subtilase genes (the *AtSBT* genes) in *A. thaliana* genome (Rautengarten et al., 2005). These subtilasescan be subdivided into six distinct subtilase subfamilies based on amino acid sequence similarity (Rautengarten et al., 2005). Nine members (AtSBT1.1 to AtSBT1.9) are included in the subfamily I. Of the nine subfamily I members, only the function of AtSBT1.2 has been investigated. AtSBT1.2 is involved in development of stomatal density and distribution, possibly through generation of peptide signals (von Groll et al., 2002). Although AtSBT1.9 function is currently unknown, we expect that the AtSBT1.9 gene should be functional in *A. thaliana*, because the sequence conservation within the subfamily I, according to the subtilase phylogeny constructed by Rautengarten et al. (2005), reflects some conservation of enzyme function. Moreover, ubiquitous expression of the *AtSBT1.9* gene is indicative of an essential function. However, AtSBT1.9 activity screening is complicated because of the low abundance of the protein in its natural form, and the difficulty involved in purifying it due to its instability. Heterologous expression of the target protein provides an alternative strategy for functional characterization. Here, we expressed an AtSBT1.9 fusion protein in bacteria, which provides a relatively economical method for assessing its activity and biochemical characterization, versus the complexity of purifying the protein from the *A. thaliana* plant. Furthermore, the AtSBT1.9 fusion protein was shown to be a bioactive subtilase. The natural AtSBT1.9, thus, seems likely to contribute to the proteolysis involved in multiple physiological pathways in *A. thaliana*.

In conclusion, protease activity of AtSBT1.9was validated by the expression of its cDNA in *E. coli* for the *in vitro* assay. The recombinant AtSBT1.9 showed maximal activity over broad pH (7-8) and temperature (25°-42°C) optima, which has not been reported to date from *A. thaliana*. These findings suggest that the AtSBT1.9 protein potentially has proteolytic functions involved in *A. thaliana* physiological processes. Moreover, a possible practical application of the AtSBT1.9 fusion proteinneed to be used as a probe to screen putative protein-protein interactions involved in signal pathway networks in *A. thaliana*by co-immunoprecipitation and/or pull-down approaches.

Conflicts of interest

The authors declare no conflict of interest.

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