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Review

Proteases in plant root symbiosis

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Abstract

Proteases catalyze the hydrolysis of peptide bonds in proteins/peptides inside or outside of cells. They play important roles in development and responses to environmental stresses. In arbuscular mycorrhiza (AM), symbiosis-induced protease genes were found by large-scale transcriptome analyses in different plant species, suggesting that proteolytic processes are implicated in AM. In legumes, some of these were also transcriptionally activated during the root nodule symbiosis. However, the precise function of these symbiosis-induced proteases remains unknown. Here we present a compilation of the symbiosis-induced proteases identified so far and discuss their possible roles in symbiosis.

Keywords: *Lotus japonicus*; Protease; Symbiosis; Arbuscular mycorrhiza; Review

Contents

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1. Introduction

The majority of land plants form AM with AM fungi (Read et al., 2000; Brundrett, 2002). In this symbiosis, AM fungi provide inorganic nutrients to host plants, in return, they obtain photosynthetic products (Harrison and van Buuren, 1995). Fossil evidence suggests the existence of AM since plants appeared on land (Remy et al., 1994). Thus, AM fungi are considered important partners of plants for survival on land (Simon et al., 1993; Redecker et al., 2000). Root nodule symbiosis (RNS) between leguminous plants and nitrogen-fixing bacteria, rhizobia evolved more recently (Kistner and Parniske, 2002). In this symbiosis, the host plant develops a symbiotic secondary organ, called nodule, for the accommodation of rhizobia, which deliver fixed nitrogen in exchange for carbon source from the plant (Mylona et al., 1995; Stougaard, 2000). Both symbioses have been studied extensively because of their agricultural and economical importance.

Host development during AM and RNS is triggered by the exchange of different signaling molecules but several signal transduction components are shared (Parniske, 2004; Harrison, 2005; Oldroyd et al., 2005). Genetic screens in several legume species identified mutants, which are defective in RNS, and also exhibit defects in AM (Harrison, 2005). This implies that the evolutionary more recent RNS evolved by recruiting a subset of genes involved in the older AM (LaRue and Weeden, 1994). These genes, required for both AM and RNS, are referred to as common symbiosis (*sym*) genes (Stougaard, 2001; Kistner and Parniske, 2002) and at least seven common *sym* genes exist in legumes (Kistner et al., 2005). Several common *sym* genes have been cloned (Endre et al., 2002; Stracke et al., 2002; Ane et al., 2004; Levy et al., 2004; Imaizumi-Anraku et al., 2005; Kanamori et al., 2006). Among them, receptor kinases, putative channel proteins and nucleoporins turned out to contribute to a common symbiosis pathway.

In parallel with these genetic approaches, large-scale transcriptome analyses of symbiosis-induced genes have been conducted in various plant species; rice, *Medicago truncatula* and *Lotus japonicus* using mutants, EST libraries, cDNA-AFLP and array techniques (Fedorova et al., 2002; Manthey et al., 2004; Asamizu et al., 2005). Among the identified symbiosis-induced genes, genes encoding members of various protease families have been isolated from leguminous and non-leguminous plants (Liu et al., 2003; Guimil et al., 2005; Hohnjec et al., 2005; Kistner et al., 2005). These protease genes display various induction patterns in AM or RNS or both. These results suggest that proteases play a role in symbiotic processes in a broad range of host plants.

Proteases are catalytic enzymes that hydrolyze covalent peptide bonds of proteins or peptides by nucleophilic attack of the carbonyl carbon (Dodson and Wlodawer, 1998). They are classified into serine, cysteine, threonine, metallo, glutamic and aspartic proteases according to their catalytic mechanism (Rawlings and Barrett, 1999). All organisms have a large variety of protease genes and they play essential roles inside or outside of cells. Proteases generally have broad specificity towards substrates and work in degradation of food, storage and damaged proteins into amino acids, which are reused for the production of new proteins. In addition, several recent papers reported interesting regulatory roles of proteases (Seidah et al., 1999; Tanaka et al., 2001; Berger and Altmann, 2000; van der Hoorn and Jones, 2004). A demonstration as to how symbiosis-induced proteases are involved in AM and RNS is still lacking. However, the novel findings about protease functions imply interesting potential roles of symbiosis-induced protease genes. Here we discuss some of these protease genes and their possible functions in symbiosis.

2. Isolation of symbiosis-induced protease genes

In rice and *M. truncatula*, microarray analyzes revealed induction of several classes of protease genes during AM (Liu et al., 2003; Guimil et al., 2005; Hohnjec et al., 2005). Kistner et al. (2005) adopted the cDNA-amplified fragment length polymorphism (cDNA-AFLP) technique for screening of AM-induced genes in *L. japonicus*. In addition to the novel AM-induced protease genes, RNS-induced proteases have been isolated in various nodulating plants.

2.1. Symbiosis-induced subtilase genes

Many of the AM- and/or RNS-induced serine protease genes encode putative members of the subtilisin-like serine protease (subtilase) subfamily (S8A, classified by MEROPS http://merops.sanger.ac.uk/) (Rawlings and Barrett, 1999). Subtilase genes are widely distributed phylogenetically and occur in such diverse groups as archaea, bacteria, fungi and yeast to higher eukaryotes (Siezen and Leunissen, 1997). In plant, subtilases appear to exist in large gene families, as documented in tomato (Meichtry et al., 1999), rice (Yamagata et al., 2000) and *Arabidopsis* (Beers et al., 2004). Subtilase genes are translated as a pre-pro-protein composed of a secretion signal peptide, a pro-region, and a peptidase domain. A catalytic triad consisting of aspartic acid, histidine and serine amino acid residues is present in the peptidase domain and plays a central role in nucleophilic attack (Fig. 1A, B) (Dodson and Wlodawer, 1998). Subtilases contain a protease associated (PA) domain located in the peptidase domain (Mahon and Bateman, 2000). The PA domain is supposed to be involved in protein–protein interactions and definition of substrate specificity. The mature protease is formed after proteolytic cleavage of the signal peptide and the pro-region (Fig. 1B).

2.1.1. SbtS type subtilase genes

The symbiosis-induced subtilase gene *SbtS* was identified as an AM- and RNS-induced gene from *L. japonicus*

(Table 1) (Kistner et al., 2005). The amino acid sequence around the catalytic triad of the putative SbtS protein is conserved in comparison with various plant subtilases, suggesting that SbtS may be an active protease (Fig. 1A, B). Analysis of *SbtS*-promoter-*β*-glucuronidase (GUS) fusion transformants revealed that *SbtS* expression is induced around the infected regions of roots inoculated with AM fungi (Fig. 2A–D). The induction of *SbtS* is strongly attenuated in the common *sym* mutants, *symrk*, *castor*, *pollux*, and *sym15*, but appeared to be less affected by mutations in *NUP133* or *SYM6* (Kistner et al., 2005). The residual expression in *NUP133* and *SYM6* is presumed to be due to a branched or a parallel common symbiosis pathway, however, it is also possible that the temperature sensitive phenotype of *nup133* mutants may be responsible for the *SbtS* expression in this background (Kanamori et al., 2006). While *SbtS* is continuously expressed during AM (Fig. 2B, D), it shows a transient expression during early stages of RNS and no expression in nodules (Kistner et al., 2005). The promoter region of *SbtS* contains two CTCTT motifs (Takeda et al., unpublished data). This sequence motif was identified in the promoter of leghemoglobin, which belongs to late nodulin genes (Sandal et al., 1987). A leghemoglobin containing this motif is also induced during AM in *Vicia faba* (Vieweg et al., 2004). A functional role of the CTCTT motif has not yet been demonstrated, and it is possible that the early and transient expression of *SbtS* is regulated by a different *cis*-element.

The putative SbtS protein is highly homologous to AIR3 in *Arabidopsis thaliana* (Neuteboom et al., 1999). *AIR3* is a subtilase gene that is expressed during the formation of lateral roots. Both genes have similar exon/intron structures (Fig. 1C); the length of individual exons is very similar and the location of the residues crucial for proper enzyme activity is conserved. In both genes, the signal peptide is encoded in exon 1, whereas exon 4 contains the pro-region splicing site. The catalytic triad, Asp, His, and Ser, is located in exon 4, exon 6, and the penultimate exon, respectively. The developmental processes of lateral root formation and nodule formation share some similarities. It has been shown that signaling molecules of AM and RNS stimulate lateral root formation, and common

(A)

		D region		H region		P1 substrate binding region		S region
	pos. V, **D**	*	pos. D, **H**	*	pos. G, **N**	*	pos. F, **S**	*
consensus		VIIGVL**D**T**G**IW**P**		D**D**D**GHGTHT**ASTA**AG**		G**I**LVS**C**SA**GN**S**GP**		FNII **SGTSMSCPH** L**SG**
AtAIR3	133, 139	TIIANL**D**T**G**VW**P**	205, 209	D**L**D**GHGSHT**L**STA**AG**	306, 315	RIVV**V**C**SAGN**S**GP**	540, 547	FNAI **SGTSMSCPH** I**SG**
LjSbtS	133, 139	VIVGVI**D**S**G**VW**P**	211, 215	D**S**D**GHGTHT**ASTI**AG**	310, 319	G**I**LVS**A**SA**GN**S**VF**	538, 545	YNII **SGTSMSCPH** L**SA**
AtARA12	133, 139	VVVGVL**D**T**G**VW**P**	208, 212	D**D**D**GHGTHT**SSTA**AG**	305, 314	G**I**LVS**C**SA**GN**A**GP**	535, 542	FNII **SGTSMSCPH** V**SG**
AtSDD1	140, 146	TIIGVL**D**T**G**VW**P**	218, 222	D**S**T**GHGTHT**ASTV**G**G	315, 324	G**I**S**V**I**C**A**AGN**N**GP**	545, 552	FTVM **SGTSMSCPH** V**SG**
Ag12	139, 145	VIIGVI**D**S**G**VW**P**	212, 216	D**T**L**GHGTHT**ASTA**AG**	309, 318	G**V**VVS**T**SA**GN**A**GP**	530, 537	YNMV **SGTSM**A**CPH**A**SG**
LjSbtM1	144, 150	VIIGVI**D**T**G**IY**P**	205, 209	**E**D**F**F**HGTHT**AA**E**A**AG**	305, 314	G**I**F**V**S**C**SA**A**N**SGP**	528, 535	FDII **SGTSMSCPH** L**SG**
LeP69A	140, 146	VIIGVI**D**T**G**IL**P**	199, 203	D**D**D**GHGTHT**ASTA**AG**	297, 306	G**I**LVS**C**SA**GN**N**GP**	525, 532	FNII **SGTSMSCPH** L**SG**
LeP69B	140, 146	VIIGVI**D**T**G**II**P**	199, 203	D**S**I**GHGTHT**ASTA**AG**	296, 305	G**I**LVS**C**SA**GN**S**GP**	524, 531	FNII **SGTSMSCPH** L**SG**

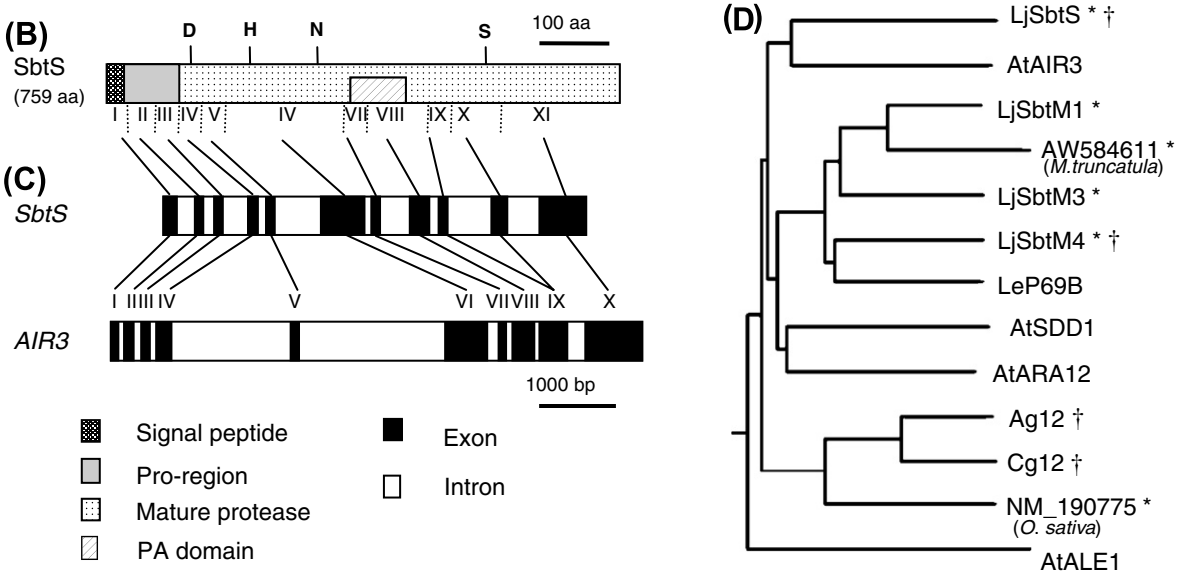

Fig. 1. Alignment and phylogenetic tree of plant subtilases and gene structures of *SbtS* and *AIR3*. (A) Alignment of SbtS, SbtM1, and other plant subtilases around the predicted catalytic triad regions and the substrate binding region. (B) Protein structure of SbtS. The positions of amino acids constituting the catalytic triad (Asp, His and Ser) as well as the stabilizing Asn are given above the schematic protein sequence of SbtS. (C) *SbtS* and *AIR3* have similar exon-intron structures. Exon IX in *AIR3* corresponds to exon IX and X in *SbtS*. (D) Diverse host plants have AM-induced (*) and RNS-induced (†) subtilases (AW584611 is a partial sequence).

Table 1
Protease genes induced in arbuscular mycorrhization

Gene (Accession)	Protease type	Gene induction		Reference
		AM	RNS	
Lotus japonicus				
SbtS	Subtilase	+	+	Kistner et al., 2005
SbtM1	Subtilase	+	–	Kistner et al., 2005
CysS1	Cysteine protease	+	+	Kistner et al., 2005
SbtM3	Subtilase	+	–	Unpublished
SbtM4	Subtilase	+	+	Unpublished
CysS2-6	Cysteine protease	ND	ND	Unpublished
Medicago truncatula				
(AW584611)	Subtilase	+	–	Liu et al., 2003
MtSCP-1	Serine carboxypeptidase	+	–	Liu et al., 2003
(AW586622)	Serine carboxypeptidase	+	–	Liu et al., 2003
(AW585765)	Cysteine protease	+	+	Liu et al., 2003
Oryza sativa				
(NM_190775)	Subtilase	+	/	Guimil et al., 2005
(OSJNBb0019B14.8)	Serine carboxypeptidase	+	/	Guimil et al., 2005
(NM_197584)	Serine carboxypeptidase	+	/	Guimil et al., 2005
(AK193461)	Serine carboxypeptidase	+	/	Guimil et al., 2005
(XM_507511)	Serine carboxypeptidase	+	/	Guimil et al., 2005
(XM_474846)	Cysteine protease	+	/	Guimil et al., 2005
(XM_474836)	Cysteine protease	+	/	Guimil et al., 2005
(XM_450615)	Puromycin-sensitive amino peptidase	+	/	Guimil et al., 2005

ND; not determined.

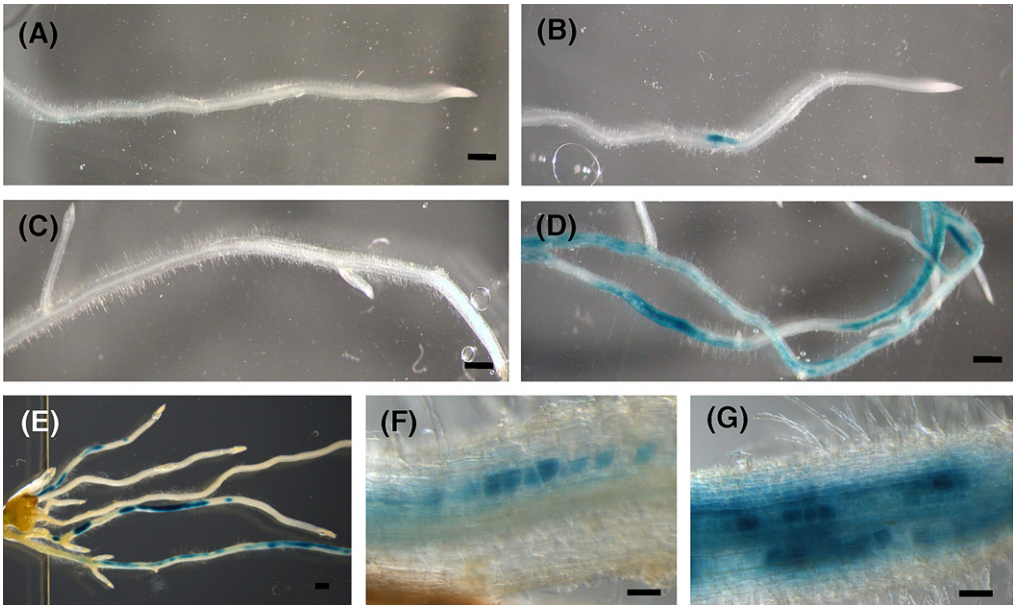

Fig. 2. Histochemical localizations of the *SbtS*-promoter-GUS (A–D) and *SbtM1*-promoter-GUS (E–G) transgene in roots infected with *Glomus intraradices*. (A, C) Roots grown in AM-free substrate for 10 (A), and 30 (C) days. (B, D) Inoculated roots 10 (B) and 30 (D) days after planting to chive nurse pots. Note that *pSbtS*-GUS expression co-localizes with fungal infection sites in the roots. (E) Staining pattern of *pSbtM1*-GUS within hairy roots transformed by *Agrobacterium rhizogenes*. (F) GUS staining is observed along the internal hypha (G). Arbuscule containing cells showed strong staining, indicating that *SbtM1* expression is induced in regions infected by the AM fungus. Bars = 1 mm (A–E) and 0.1 mm (F, G).

sym genes are required for this response (Olah et al., 2005). The similar exon/intron structure of *AIR3* and *SbtS* indicates a common evolutionary origin and suggests an evolutionary relationship between lateral root formation and symbiosis.

2.1.2. *SbtM* type subtilase genes

SbtM1 is a second type of AM-induced subtilase genes (because several homologues were identified, we numbered the genes and *SbtM* was renamed to *SbtM1*) (Kistner et al., 2005; Takeda et al., unpublished data). The basic protein

structure of SbtM1 is similar to SbtS (signal peptide, pro-region, PA domain, and catalytic triad). However, it shares only 34.9% amino acid identity with SbtS (Fig. 1D). While *SbtS* is activated during both root symbioses, *SbtM1* is not activated during RNS but only in AM. Furthermore, while *SbtS* is expressed in *nup133* and *sym6* mutants, there is no induction of *SbtM1* in either of the common *sym* mutants (Kistner et al., 2005). Therefore, *SbtM1* and *SbtS* are likely to be activated by different branches of the symbiotic signaling network.

A *SbtM1*-promoter-GUS fusion construct exhibited a fungal-infection specific expression pattern (Fig. 2E–G). GUS staining was observed in the root cells surrounding internal fungal hyphae and in arbuscule containing cells, but not in the vicinity of external hyphae. The induction of *SbtM1* seems to be triggered by the entry of the fungal hyphae and restricted to the infected region of AM. The observations indicate that close or direct contact of internal hyphae with the host cell is required for *SbtM1* expression. A diffusible factor (or Myc factor) is known to act as a signaling molecule secreted from AM fungi (Kosuta et al., 2003). It is thought that this factor or an unknown fungal factor are secreted from the internal hyphae or displayed on the surface of internal hyphae and activate the signaling pathway.

Plants generally have many subtilase homologues and they often occur in gene clusters in the genome (Rautengarten et al., 2005). Analysis of the genomic sequence of *L. japonicus* revealed the existence of several *SbtM1*-homologous genes induced in AM (Takeda et al., unpublished data). Two of them (*pseudoSbtM2* and *SbtM3*) form a gene cluster with *SbtM1*. One of the homologues, *pseudoSbtM2* is considered to be a pseudo-gene because it has a stop codon in the middle of the protease encoding gene (resulting in a truncated protein of 254 amino acid residues) and the expression of this gene is not induced in mycorrhiza-infected roots. The other homologous gene, named *SbtM3*, has a complete ORF coding a subtilase protein (66.0% identity with SbtM1) and shows AM-induced expression. In contrast to *SbtM1*, *SbtM3* is composed of two exons with the single intron within the ORF.

Two other subtilase homologues, *SbtM4* (57.4% identity) and *pseudoSbtM5* (premature stop codon), which form a gene cluster located in a different genomic region than *SbtM1*, *pseudoSbtM2* and *SbtM3* (Takeda et al., unpublished data). The expression of *SbtM4* is induced during AM, whereas *pseudoSbtM5* is not. *SbtM4* differs from *SbtM1*/*SbtM3* and *SbtS* in that it is induced during RNS and also up regulated in nodules. *SbtM1* and *SbtM3* transcripts cannot be detected in RNS, and *SbtS* expression is limited to an early stage of RNS. The expression patterns of AM-induced subtilase genes therefore indicate different regulation mechanisms for *SbtS*, *SbtM1*/*SbtM3* and *SbtM4* gene activation.

The amino acid sequences of SbtM1, SbtM3 and SbtM4 show high similarity with P69 proteins (e.g. P69B has 54.8% identity with SbtM1) (Tornero et al., 1996; Jorda et al., 1999; Meichtry et al., 1999). *P69* genes are *Pathogenesis Related* (*PR*) genes isolated from tomato (*Lycopersicon esculentum*) which are up regulated during pathogen attack. *P69* genes are organized in a gene cluster that comprises a tandem of four subtilases, *P69A*, *B*, *C* and *D*. Although it is likely that the homologues arose from gene duplications, they are differentially regulated during pathogen infection and development (Jorda et al., 1999). They presumably acquired differential regulation after the gene duplication event. This appears to be a suitable mechanism to redirect an initially AM or lateral root specific expression to the root nodule during RNS evolution.

In *L. japonicus*, array analysis revealed additional subtilase genes induced during RNS (Kouchi et al., 2004). These showed transient expression in early stage of RNS like *SbtS* or high-level expression in nodules like *SbtM4*. As some RNS-induced genes are also induced in AM, it is postulated that there are AM-induced subtilase genes other than *SbtS* and *SbtM* in *L. japonicus*. In addition to the protease genes from *L. japonicus*, large numbers of symbiosis-induced subtilase genes have been identified in other plant species, using differential display, array and proteome analysis. The identification of an AM-induced subtilase (AW584611) was reported in *M. truncatula* (Table 1) (Liu et al., 2003). This subtilase is only induced during AM but not RNS. It has high similarity to *SbtM1* of *L. japonicus* and lower similarity to *SbtM3* (Fig. 1D). This suggests that this AM-induced subtilase in *M. truncatula* may have the same function as SbtM1. In addition, several homologues that have higher homology with AW584611 and *SbtM1* than *SbtM3* are present in EST libraries of *M. truncatula* and one of them (TC103095 in the TIGR *M. truncatula* Gene Index) is induced during AM (Hohnjec et al., 2005). These subtilase genes may form a gene family and a gene cluster like *SbtM* genes in *L. japonicus*. AM-induced subtilase genes were also identified in non-leguminous plants. Microarray analysis of rice roots revealed up regulation of a subtilase gene during AM (Table 1, NM_190775) (Guimil et al., 2005). This subtilase also forms a gene cluster with a homologue. Clustered AM-induced subtilase genes emerge as a common component of angiosperm genomes.

The subtilase gene *ag12* and its orthologue *cg12* were isolated from *Alnus glutinosa* and in *Casuarina glauca*, respectively (Ribeiro et al., 1995; Laplaze et al., 2000). The induction of *ag12* and *cg12* was observed in symbiosis with *Frankia*, but no activation could be detected during endo- or ectomycorrhizal infection (Svistoonoff et al., 2003, 2004). The results suggest that symbiosis-induced subtilases are also involved in the actinorhizal root nodule symbiosis (Benson and Silvester, 1993). Genomic southern analysis showed that there are homologues of *ag12* in *A. glutinosa*. How these homologues relate to symbiosis and whether they form gene clusters is presently unclear.

2.2. Symbiosis-induced serine carboxypeptidase genes

Serine carboxypeptidase (SCP)-like genes (S10, classified by MEROPS) were found to be induced during AM in

M. truncatula (Liu et al., 2003; Hohnjec et al., 2005) and rice (Guimil et al., 2005). SCP genes belong to a large gene family in plants. These genes are also translated as a pre-pro-protein and active mature proteases arise from processing of the pro-protein. The peptidase domain has a catalytic triad in the catalytic center and the amino acid residues of the catalytic triad occur in the order Ser, Asp and His. The order is different from the order of subtilases (Asp, His and Ser).

The *Medicago* homologue *MtSCP1* is specifically activated during AM (Liu et al., 2003). Cortical cells that contain arbuscules or are adjacent to internal hyphae show strong fluorescence of green fluorescent protein (GFP) in the roots transformed with GFP under control of *MtSCP1* promoter. Microarray analysis of rice root transcripts revealed that putative SCP genes are up regulated after infection with AM fungi (Guimil et al., 2005). Moreover, upregulation of a SCP gene was detected during RNS in *L. japonicus*, though induction of this gene has not been observed during AM (Kouchi et al., 2004).

2.3. Symbiosis-induced papain protease genes

AM-induced cysteine protease genes that belong to the papain superfamily (C1A, classified by MEROPS) were identified in *L. japonicus*, *M. truncatula*, and rice (Liu et al., 2003; Guimil et al., 2005; Kistner et al., 2005). The AM-induced cysteine proteases in *L. japonicus* and *M. truncatula* are also induced in RNS (Liu et al., 2003; Kistner et al., 2005). The symbiosis-induced cysteine protease gene in *L. japonicus*, named *CysS*, has five homologues *CysS2* to *CysS6* (Takeda et al., unpublished data). *CysS1* (corresponding to *CysS* in Kistner et al., 2005) and these homologues are located within 35 kb on the same chromosome region and form a gene cluster. Several of them were also found to be induced in macroarray analysis (CysS2: BP070099, CysS4: BP068647 and BP073930) (Kouchi et al., 2004; Suganuma et al., 2004).

Large numbers of RNS-induced cysteine protease genes have been isolated from various nodulating plants and they often have multiple homologues in the genome (Goetting-Minesky and Mullin, 1994; Kardailsky and Brewin, 1996; Naito et al., 2000). Whether these genes are activated in both AM and RNS like the cysteine proteases in *Lotus* and *Medicago* is unclear, as their expression during AM has not been determined.

Papain proteases occur ubiquitously in all kingdoms (Beers et al., 2004). The majority of cysteine proteases is translated as an inactive or less active precursor protein and activated by proteolytic cleavage. CysS1 has a predicted signal peptide and a pro-region at the N terminal end. Because of the signal peptide sequence, CysS1 is predicted to be a secreted protein. The propeptide contains the interspersed ERFNIN motif, a characteristic motif of cathepsin H- and L-like proteases (Karrer et al., 1993). The mature protease forms after cleavage of these regions (Wiederanders, 2003). The peptidase domain of CysS1 has a predicted catalytic dyad, consisting of a Cys (C148) and a His (H285) residue (Groves et al., 1996).

One of the closest homologues of CysS1 from other species is AsNODf32 (74.4% identity) isolated from Chinese milk vetch (*Astragalus sinicus*) (Naito et al., 2000). *AsNODf32* is induced during RNS and the transcripts of AsNODf32 accumulate in the senescence zone of nodules. Thus, AsNODf32 is supposed to act in the senescence process in nodules. It is anticipated that *CysS1* and some of its homologues have a function similar to AsNODf32 in RNS.

In addition to the symbiosis-induced serine and cysteine protease genes, a puromycin-sensitive aminopeptidase (metallo protease) was found to be induced during AM in rice (Guimil et al., 2005). Ongoing large scale analysis of symbiosis-induced genes is likely to produce additional symbiosis-induced protease genes. The expression patterns of symbiosis-induced proteases allow predictions about where and when symbiosis-induced proteases may work. However, there are very few reports on functional analysis of plant proteases, and especially their significance during AM has not been demonstrated. In the next chapter, we discuss possible roles of symbiosis-induced proteases genes based on functional analysis of proteases in various organisms.

3. Predicted roles of proteases in AM and RNS

Successful establishment of symbiosis is accompanied by various biological processes: signal transduction, organogenesis and maintenance of symbiotic structure, metabolite exchange between host and symbiont, senescence and turnover of symbiotic structures. Additional effects of AM have been described, for example increased tolerance against pathogens, drought and other environmental stresses (Gianinazzi-Pearson et al., 1996; Auge, 2001; Porcel and Ruiz-Lozano, 2004). The wide array of functions in which proteases have been implicated in animals and plants suggests that proteases potentially could be involved in any process in symbiosis.

3.1. Formation and senescence of symbiotic structures

A number of proteases exhibit little selectivity for substrates and they are supposed to work in general protein degradation. In plants, such proteolytic processes are observed in seed germination (Cejudo et al., 1992; Cervantes et al., 1994), fruit development (Lin et al., 1993), ovary senescence (Granell et al., 1992), and senescence of nodules. In senescent nodules, proteolytic enzymes degrade proteins of the host plant and the bacteroids. Nodule senescence is not only induced by ageing, but also by other physiological and environmental changes (Gogorcena et al., 1997; Matamoros et al., 1999). Host plants regulate their nitrogen/carbon balance to maintain efficient nitrogen fixation over time. In French bean, the activity of cysteine proteases appeared in senescent nodules, and the cysteine

proteases degraded leghemoglobin and bacteroids (Pladys and Rigaud, 1985; Pladys et al., 1991). In addition, nodules of early senescent nodule mutants showed earlier expression of cysteine protease genes than wild-type nodules (Pladys and Vance, 1993). These observations suggest that cysteine proteases play a role in nodule senescence. Senescence processes are also observed in AM, e.g., in the turn over of arbuscules. Some AM-induced proteases may work in this process.

On the other hand, induction of *SbtS* is observed only in early stages of RNS, not in nodules. This transient expression pattern indicates that SbtS may act in other processes than the senescence of nodule, like nodule formation or maintenance. AIR3, that has high homology with SbtS, is supposed to weaken cell-cell connections by the degradation of structural proteins in the apoplastic space and facilitate the emergence of lateral roots (Neuteboom et al., 1999). Hypothetically, protein degradation by SbtS may facilitate the elongation of internal hyphae of AM fungi and the protrusion of nodule primordia in host roots. Elongation of internal hyphae continues during AM, while protrusion of nodule primordia is limited to the early stage of nodule formation. In this case, the temporary limited requirement of the proteolytic process in nodule formation could explain the transient expression of *SbtS* in RNS.

All putative symbiosis-induced proteases contain predicted secretion signal peptides in their N-terminal regions. The secreted proteases are presumably activated by processing and are believed to digest proteins in the extracellular space. AM fungi develop internal hyphae and vesicles in the apoplastic space of the host root and arbuscules are separated from host cells by the peri-arbuscular space. Promoter-GUS or GFP fusion analyses of *SbtM1* and *MtSCP1* indicated that their expression is induced in the cells in contact with fungal hyphae (Fig. 2) (Liu et al., 2003). This raises the possibility that symbiosis-induced proteases digest not only plant proteins, but also microbial proteins. In nodules, the peri-bacteroid space and bacteroid can be considered as extracelluar compartment. In several plant species infected cells within nodules display a high expression level of symbiosis-induced protease genes (Ribeiro et al., 1995; Vincent and Brewin, 2000). In soybean, a subtilase protein was found by proteome analysis in a peribacteroid membrane fraction that could also contain associated soluble proteins (Panter et al., 2000). If these proteases were located in the peribacteroid space, they could directly interact with rhizobia. The proteases may function in formation or maintenance of nodules by the proteolysis of bacterial proteins or peptidoglycans of the bacterial cell wall.

3.2. Selective processing as a proprotein convertase in a signaling pathway

Proteases can also have very specific functions in signaling and development by selective processing of target proteins (Seidah and Chretien, 1997, 1999). The proteases involved cleave precursor proteins at specific sites thereby converting them into their bioactive forms, which constitute hormones, growth factors, receptors, adhesion proteins, bacterial and viral proteins (Barr et al., 1991; Nakayama, 1997; Berger and Altmann, 2000; Tanaka et al., 2001).

The Toll/Spätzle pathway in *Drosophila* is one of the best-studied examples that revealed an important function of proteases in signaling (Morisato and Anderson, 1994). Toll is a leucine-rich-repeat (LRR) receptor and Spätzle is a peptide ligand of the Toll receptor (Hashimoto et al., 1988). Mutants defective in the corresponding genes show an abnormal embryogenesis. Spätzle is first translated into a proprotein and a serine protease, Easter, which acts in a serine protease cascade, converts the proprotein into the mature ligand (Chasan and Anderson, 1989). The mature Spätzle then interacts with the Toll receptor to trigger downstream signaling (Fig. 3A).

In plants, such relationships between peptide ligands and proteases have also been suggested. Systemin is a plant peptide hormone and plays an important role in systemic defense responses to wounding by insects (Pearce et al., 1991). In this signaling pathway, the prohormone of Systemin is first translated and then processed into the mature hormone (Fig. 3B). It was demonstrated that proteolytic activity is involved in this processing (Schaller and Ryan, 1994). The mature hormone is perceived by the LRR receptor kinase SR160 (Scheer and Ryan, 2002). CLV1/CLV3 is another well-known receptor/ligand model that regulates the development of the shoot meristem (Fig. 3B). CLV3 is hypothesized to act as a ligand of the CLV1 receptor kinase. The mature protein of CLV3 is formed by selective proteolytic cleavage (Ni and Clark, 2006).

Another example is the subtilase Stomatal Density and Distribution 1 (SDD1) which is involved in stomatal development and placed in one signaling pathway with Too Many Mouths (TMM) (Yang and Sack, 1995; Von Groll et al., 2002). TMM is a receptor like kinase and has a LRR domain in the extracellular part (Nadeau and Sack, 2002). Loss-of-function mutants of SDD1 resulted in an increase of stomatal density and clustering of stomata (Berger and Altmann, 2000). Over expression of SDD1 could complement the phenotype of the *sdd1* mutant but not of the *tmm* mutant. Based on these observations, the authors developed the following model about the relationship of SDD1 and TMM according to the examples above: SDD1 processes a hypothetical inactive ligand of TMM and produces an active ligand. This ligand is perceived by TMM to trigger signaling (Fig. 3B).

These examples suggest that maturation of ligands through proteolytic processing could be a widespread phenomenon in plants. Several receptor-like genes have been identified that are involved in symbiosis. NFR1 and NFR5 are thought to be receptors of rhizobial signals in the RNS specific signaling pathway (Madsen et al., 2003; Radutoiu et al., 2003). The LRR receptor-like kinase SYMRK regulates both AM and RNS (Stracke et al.,

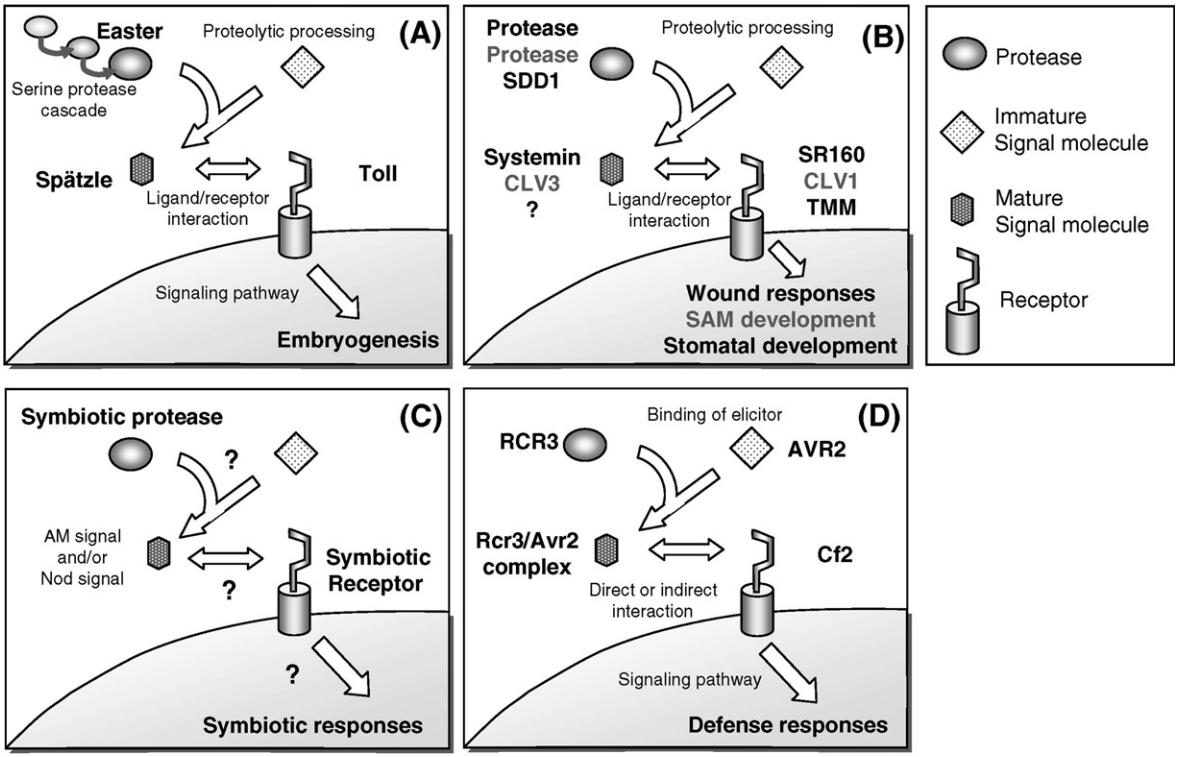

Fig. 3. Models of proteases in signal transduction. (A) Perception of the Toll/Spätzle signal in Drosophila. Pro-ligand (Spätzle) is processed by a protease producing the mature ligand. (B) Predicted plant protease roles in signaling pathway leading to wound response or developmental signaling pathway. (C) One possible but purely hypothetical relationship between symbiosis-induced proteases and symbiotic signal transduction. (D) Other signaling model mediated by interaction between plant protease and fungal protease inhibitor.

2002). *Har1* was isolated from a hypernodulation mutant and encodes a CLV1 like receptor like kinase (Nishimura et al., 2002; Krusell et al., 2002). The ligands of these receptors have not yet been identified. There are several possibilities how symbiosis-induced proteases could be involved in specific signaling or developmental processes. It is possible that some of the symbiosis-induced proteases may mediate the ligand/receptor interaction by processing peptide ligand precursors (Fig. 3C).

An alternative way of involving cysteine proteases in plant microbe recognition processes has been elucidated in the tomato *Cladosporium fulvum* pathosystem. The tomato RCR3 cysteine protease (Krüger et al., 2002) is the target of a fungal protease inhibitor AVR2 (Rooney et al., 2005). The interaction between RCR3 and the fungal inhibitor is recognized by the LRR receptor like protein product of the plant disease resistance gene Cf2 (Fig. 3D) (Krüger et al., 2002). It is conceivable that the cysteine protease RCR3 has some function in defense against fungal invasion, and this function is targeted by the fungal protease inhibitor AVR2. Whether symbiosis-induced protease genes have a role in restricting microbial growth has yet to be determined.

3.3. Potential non-proteolytic functions

It was reported that predicted protease homologues can have other functions than proteolysis. A cysteine protease in tomato has a dual function as a protease and a transcription factor (Matarasso et al., 2005). Some predicted serine carboxypeptidases turned out to have acyl transferease activity, but no peptidase activity (Lehfeldt et al., 2000; Li and Steffens, 2000). In *L. japonicus*, *M. truncatula* and rice, serine carboxypeptidase-like genes were isolated in AM and RNS. These genes were annotated as peptidases based on sequence similarities, however, their activity as peptidases has not been demonstrated. In symbiosis, large amounts of molecules are secreted and exchanged between plants and symbionts. Some of the predicted peptidase-like proteins may catalyze the production or modification of these secondary metabolites or signaling molecules.

Identification of the substrate and determination of enzymatic activity are important to understand the roles of protease genes in symbiosis. Present knowledge about symbiosis-induced protease genes is limited to the transcriptional levels. To our knowledge, studies about their protein activity and function in symbiosis process have not yet been published. However, extensive studies about protease genes are proceeding and recent reverse genetic approaches like TILLING and RNAi promise to clarify the functional significance of these proteases in symbiosis.

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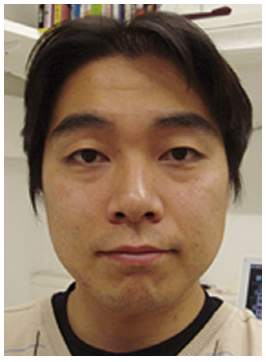

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