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Cysteine proteases in nodulation and nitrogen fixation

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The cysteine proteinases or cysteine endopeptidases (EC 3.4.22) are known to occur widely in plant cells. They are involved in almost all aspects of plant growth and development including germination, circadian rhythms, senescence and programmed cell death. They are also involved in mediating plant cell responses to environmental stress (such as water stress, salinity, low temperature, wounding, ethylene, and oxidative conditions) and plant-microbe interactions (including nodulation). In the development and function of legume root nodules, cysteine proteases could be involved in several important processes:-(i) a defence response to root invasion by microorganisms; (ii) protein turnover required during the formation of new tissue; (iii) cellular homeostasis and metabolism; (iv) adaptation of host cells to physiological stresses; (v) control of nodule senescence. Because of their central importance to plant physiology, cysteine proteases could serve as important targets for the study of nodule development and functioning at the molecular level. Because of their widespread occurrence in nodulating plants they could also serve as candidate genes for targeted plant breeding programmes.

Keywords: Cysteine proteases, Homeostasis, Physiological stresses, Symbiosome, Senescence

Protein degradation through selective proteolysis is an essential process in plant growth, development and environmental responses. Although plants can synthesize all amino acids *de novo*, a substantial proportion of new proteins is normally derived from recycled amino acids[1,2]. Amino acids can be regenerated from the degradation of normal cellular proteins or they can be derived from specialized storage proteins in seeds or vegetative tissues[3]. Under conditions of nutrient stress, protein degradation is often accelerated to maintain the supply of amino acids[4,5]. Thus, proteolysis in plants provides a mechanism for protein turnover and reutilization of nitrogen for maintaining cellular homeostasis and growth.

Plant cysteine proteinases are classified into the papain superfamily[6] on the basis of their general amino acid sequence homology. Characteristically, there is conservation of particular amino acid residues surrounding the active site. These residues have the ability to form disulfide bridges that are involved in the proteolytic activity of this group of enzymes. Cysteine proteinases are widely distributed[7] and have been shown to play important roles in various aspects of germination, plant development and senescence, as summarized in Table 1. Cysteine protease activity in plants is transcriptionally regulated and often occurs when rapid changes in cell metabolism are required. Presumably, protease activity often serves to reutilize intracellular resources stored in the form of hydrolysable proteins.

There have been many reports of the specific expression of cysteine proteases in root nodules[90-95] although in most cases their physiological role is not yet clear. The process of nodulation in legumes induces dramatic changes in metabolic activity like protein turnover and nitrogen balances. These changes are associated firstly with the development of a new plant organ and secondly with the functioning of the mature nodule as a major sink for photosynthate and as a source for the products of nitrogen assimilation. The nodule is also significant because of the possible involvement of cysteine proteases in the controlled process of plant-microbe interaction. This process leads to the colonisation of host cells by rhizobial endosymbionts creating symbiotic organelles termed "symbiosomes". A subsequent stage of plant-microbe interaction involves the controlled senescence of host cells harbouring these endosymbionts in the post-mature tissues of the nodule. Eventually the symbiosome acts as a lytic compartment and triggers the degradation of bacteriods. Thus the host plant apparently retains its capability to treat Rhizobium as a pathogen if the partnership gets out of hand. Cysteine proteases could be involved in several processes in the development and function of legume root nodules: (i) a defence response to root invasion

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Table 1 — Classification of cysteine proteases according to their predicted roles in seed germination plant growth, development and senescence

Predicted roles	Plant	Example	Reference
Catabolism of reserve proteins in seeds	*Vigna mungo*	SH-EP	Akasofo *et al.*, 1989[8], 1990[9]; Yamauchi *et al.* 1992[10] Taneyama *et al.*, 2001[11]; Tsuru-Furuno *et al.*, 2001[12] Okamoto *et al.*, 2001[13]
	Glycine max	P34; GMCP3 C2	Kalinski *et al.*, 1990[14], 1992[15]; Qi *et al.*, 1992[16] Nong *et al.*, 1995[17] Seo *et al* 2001[18]
	Vicia sativa	CPRI, CPR2, Proteinase A CPR4, VsPB2, Proteinase B	Becker *et al.*, 1994[19], 1995[20], 1997[21] Schelereth *et al.*, 2000[22],2001[23] Fisher *et al.*, 2000[24] Teidmann *et al.*, 2001[25] Hara-Nishimura *et al.*, 1998[26]
	Phaseolus vulgaris	EP-CI, Pv CEP-1	Tanaka *et al.*, 1991[27], 1993[28] Sohlberg and Sussex, 1997[29]
	Vicia faba	Vicia faba *CP*	Yu and Greenwood, 1994[30], 1997[31]
	Zea mays	CCP1, CCP2	Domoto *et al.*, 1995[32]
	Hordeum vulgare	EP-A; EP-B, Aleurain	Koehler and Ho, 1988[33], 1990a[34] b[35]; Mikkonen *et al.*, 1996[36] Zhang and Jones, 1996[37]; Davy *et al.*, 2000[38] Holwerda *et al.*, 1990[39]
	Oryza sativa	Oryzains a, b and rL REP-1; CysPcDNApR80 OsEP3A REP-A	Watanbe *et al.*, 1991[40] Kato *et al.*, 1997[41] Shintani *et al.*, 1997[42] Ho *et al.*, 2000[43]
	Pisum sativum	TPE4A	Cercos *et al.*, 1999[44]
	Garden bean (Kidney bean) Castor bean *Medicago truncaluta*	LLP Cyp 15a	Rotari *et al.*, 1997[45] Senyuk *et al.*, 1998[46] Schmid *et al.*, 1998[47]; Sheokand *et al.* unpublished
	Carrot	CSCP	Sakuta *et al.* 2001[48]
	Mung bean Wheat	Cys p Proteinase A	Lee *et al* 1997[49] Jivotovskaya *et al* 1997[50]
Environmental stress responses			
(i) Water stress	*Arabidopsis* *Brassica napus* *Pisum sativum* *Vicia sativa* *Medicago truncatulat* *Lycopersicum esculentum*	RD 19; A 494 bcp-15 Cyp15a CPR2 Cyp15a TD1-65	Koizumi *et al.*, 1993[51] Williams *et al.*, 1994[52] Stroeher *et al.*, 1990[53] Guerrero *et al.*, 1990[54]; Jones and Mullet, 1995[55] Fischer Unpublished Sheokand *et al.*, unpublished Harak *et al.*, 2001[56]
(ii) Salt stress	*Pisum sativum* *Medicago truncatula* *Arabidopsis thaliana*	Cyp15a Cyp15a RD19	Jones and Mullet, 1995[55] Sheokand unpublished Koizumi *et al.*, 1993[51]
(iii) Oxidative stress	*Glycine max*	-	Solomon *et al.* 1999[58]

(Contd)

Table 1 — (*Contd*)

Predicted roles	Plant	Example	Reference
(iv) Low temperature stress	*Brassica napus* *Lycopersicum esculentum*	bcp-15	Stroeher *et al.*, 1997[53] Schaffer and Fisher, 1988[57]
(v) Wound inducible	*Nicotiana tabacum*	Cyp 7 Cyp 8	Linthorst *et al.*, 1993 a[59] b[60]
	Nicotiana rustica		Lidgett *et al.*, 1995[61]
(vi) Hypersensitive response	*Nicotiana tabacum*	Cysteine proteases	Heath 2000[62]
(vii) GLC starvation	*Zea mays*	CCPI	Chevalier *et al.*, 1995[63]
(viii) Nitrogen starvation	*Oryza sativa*	OsEP3A	Ho *et al.*, 2000[43]
(ix) Pathogen attack	*Lycopersicon esculentum*	LCYP-2 Rcr3	Linthorst *et al.*, 1993 a[59];b[60] Kruger *et al.*, 2002 [64]
Senescence			
(i)Leaf senescence	*Zea mays* ***Arabidopsis***	ZMS sel SAG 2 SAG 12 RD21	Griffiths *et al.*, 1997[65]; Hensel *et al.*, 1993[66] Lohman *et al.*, 1994 [67] Drake *et al.*, 1996[68] Yamada *et al.*, 2001[69]
	Lycopersicum esculentum		
	Brassica napus *Vigna unguiculata* Sweet potato *Nicotiana tobacum* *Lolium multiflorum*	LSC 790 SPG31 NTCP-23 See1	Buchanan and Ainsworth, 1997[70] Srivallii *et al.*, 2001[71] Chen *et al.*, 2002[72] Ueda *et al.*, 2000[73] Li *et al.*, 2000[74]
(ii) Flower and ovary senescence	*Pisum sativum*	tpp TPE4A	Granell *et al.*, 1992[75] Cercos *et al.*, 1999 [44]
	Hemerocallis spp. *Phalaenopsis* sp. *Dianthus caryophyllus*	SEN 11 0141 DC-CP1	Guerrero *et al.*, 1998[76] Nadeau *et al.*, 1996[77] Sugawara *et al.* 2002[78]
	Alstroemia	ALS CYP1	Wagstaff *et al.* 2002[79]
Programmed cell death (PCD)	*Zinea elegans*		Minami and Fukuda, 1995[80] Ye and Varner, 1996[81]
	Brinjal		Xu and Chye, 1999[82]
	Brassica napus *Arabidopsis thaliana*	Bn CysP1 XCP1	Wan *et al.* 2002[83]; Funk *et al.* 2002[84];
	Lycopersicum esculentum	TD1-65 Caspases Caspases Caspases	Harrak *et al.* 2001[56] Lam and del Pozo 2000[85] De Jong *et al.* 2000[86] Elbaz *et al.* 2002[87]
	Castor bean		Schmid *et al.* 1999[88]
Circadian rhythms	*Nicotiana tobacum*	NTCP-23	Ueda *et al.* 2000[73] Linthorst *et al.* 1993a[59]

(*Contd*)

Table 1 — (*Contd*)

Predicted roles	Plant	Example	Reference
Leaf development	*Carica papaya*	PLBP cl3	McKee *et al.*, 1986[89]
Metabolism	*Zea mays*	CCP 1	Domoto *et al.*, 1995[32]
Nodulation	*Alnus glutinosa*	AgNOD-CP1	Goetting-Minesky and Mullin, 1994[90]
	Pisum sativum	Ps Cyp1	Kardailsky and Brewin, 1996[91]
	Pisum sativum	CypI5a	Vincent and Brewin, 2000[92]
	Pisum sativum	CyPI5a	Vincent *et al.*, 2000[93]
	Vicia hirsuta	Cyp15a	
	Medicago truncatula	Cyp15a	Sheokand *et al.* unpublished
	Astragalus sinicus	AsNODf32	Yuki Naito *et al.* 2000[94]
	Glycine max	Cyp15a	Panter *et al.* 2000 [95]

by microorganisms; (ii) protein turnover required during the formation of new tissue; (iii) cellular homeostasis and housekeeping; (iv) adaptation of host cells to physiological stresses; (v) control of nodule senescence.

Cysteine proteases in symbiosome senescence

An interesting feature of all nitrogen-fixing symbioses involving angiosperms is that the microbial partner is accommodated within the confines of a living host plant cell. In order to achieve this state, several complex series of events proceed in parallel. Following infection of the plant roots with compatible rhizobia, there is induction of a nodule meristem. Subsequently, the rhizobia are released into the cytoplasmic space of nodule host cells as endosymbiotic bacteriods, enclosed by a plant-derived membrane, the peribacteriod membrane. This functional unit (the bacteroid with its host membrane envelope) is often referred to as a symbiosome to reflect the fact that it resembles an organelle. The physiological status of the symbiosome within the plant cell is a very delicate one that is apparently based on a dynamic metabolic equilibrium. Although the plant develops a mechanism to permit a balanced exchange of metabolites with its endosymbiont, it also retains some of its capability to treat Rhizobium as a pathogen. Nodule senescence could therefore be considered as a delayed reaction of the host plant against *Rhizobium*. It is probably significant that nodule senescence is induced prematurely if the symbiosis is ineffective and if the invading strain of rhizobium fails to fix nitrogen under symbiotic conditions.

Mellor[96] suggested that the symbiosome unit could be considered as a "temporary but independent organelle", in which the presence of various lysosomal proteins such as alpha-mannosidase and acid protease indicates some similarity to the lysosomal compartment. Thus, symbiosomes may represent pre-vacuolar structures accumulating without immediate fusion to the main lytic compartment[97]. It is known that the perisymbiosome membrane possesses tonoplast-like qualities, so it is not surprising to find that a vacuolar cysteine protease is also targeted to this compartment. In an earlier study of root nodule proteolysis[98], it was demonstrated that proteolytic activity was associated with the age-related senescence of alfalfa nodules. In subsequent studies, similar activities have been identified in the age-related senescence of soybean nodules[99] and French bean nodules[100]. The physiological processes of symbiotic nitrogen fixation are probably in a state of dynamic equilibrium: when this equilibrium becomes unbalanced, the relationship moves towards a host-pathogen interaction and the plant rejects the invading microorganism (because the proteases become more active). Moreover, thiol-proteases isolated from senescent French bean nodules[101] have been shown to be capable of digesting the bacteroid peptidoglycans *in vitro*, showing that these enzymes could be involved in mediating a host-pathogen interaction.

Increased Cys protease activity has been shown in early senescing nodules of alfalfa, indicating a specific role for such proteases in the senescent phase of nodule development[102]. A cysteine protease with an acidic *p*H optimum has been described in French bean nodules[101]. Its activity increases markedly with the onset of senescence. Similar observations have been made in soybean[99], black gram[103] and alfalfa[102]. Kardailsky and Brewin[91] have reported an increased expression of Pscyp1 during pea nodule senescence. Another Cys proteinase (AsNODf32) in Chinese milk

vetch has been strongly correlated with cell senescence in nodules[94]. Increased expression of CysP genes in senescent leaves of various plant species have been reported[66,67,82,43]. Thus cysteine proteases appear to play a major role in nitrogen re-mobilisation during senescence.

Molecular analysis of cysteine proteases

The cysteine protease that have been studied at the molecular level in nodules are discussed below:

AgNOD-CPI

AgNOD-CPI has been isolated from the non-legume A*lnus glutinosa*, an actinorhizal plant that establishes a symbiotic nitrogen-fixing symbiosis with the actinomycete Frankia[90]. A characteristic feature of this gene is the presence of a putative vacuole targeting signal (the LQDA motif) at the N terminal region. Four possible roles have been proposed for AgNOD-CPI, although without any supporting experimental evidence (a) a defense response to root invasion by microorganisms; (b) a component of tissue remodeling in root and nodule tissues; (c) a cell cycle component; and (d) an element of a nitrogen-recycling process involving protein turnover.

PsCyp1

Coding sequence for PsCyp1 was amplified from cDNA derived from pea nodule mRNA using PCR (polymerase chain reaction) primers based on conserved regions of DNA sequence in plant cysteine proteases[91]. Expression of this gene, studied both on RNA blots and *in situ*, showed good correlation with the onset of nodule senescence. *In situ* hybridisation studies revealed that PsCyp1 was expressed in senescent infected tissue at the base of the nodule. This signal was just detectable in normal symbiotically wild-type nodules but was much stronger in the early senescing nodules formed by a symbiotically defective mutant of *Rhizobium leguminosarum*. PsCyp1 has an open reading frame that encodes a polypeptide starting with a putative hydrophobic N-terminal signal sequence. The encoded polypeptide consists of 367 amino acid residues and the calculated molecular mass of the processed polypeptide is 38 kDa. The two highest ranking matches for PsCyp1 protein were observed with the putative cysteine protease sequence from *Vicia sativa*[19] and the tpp sequence from *Pisum sativum*[75]. The first of these homologues has been implicated in germination whereas the second one is involved in ovary senescence. Both these developmental stages are associated with a rapid rate of protein turnover.

AsNODf32

A cDNA encoding AsNODf32 was obtained by differential screening of a nodule cDNA library derived from the leguminous plant Chinese milk vetch (*Astragalus sinicus*). This gene product represents a nodule-specific Cys-proteinase similar to AgNOD-CP1 reported for the actinorhizal symbiosis involving *Alnus glutinosa* and Frankia[90]. Like the enzyme from *A. glutinosa*, the ASNODf32 Cys-proteinase carries the putative vacuole targeting signal LQDA at the N-terminus. This motif is missing from Cys-proteinases derived from leguminous plants such as vetch and *Vigna mungo*, which instead have the endoplasmic reticulum targeting signal, KDEL[104] at the C-terminal region. Many other cysteine proteinases of the leguminous plants soybean[29] and pea[91] do not possess either of these motifs.

Northern blot analysis of ASNODf32 revealed specific expression of this gene at the nodulation stage. Although most cysteine proteases of leguminous plants are expressed in germinating seeds[21,24,25], the ASNODf32 transcript showed no discernible hybridization signal in 3, 7 and 10 d imbibed seeds. *In situ* hybridization studies revealed strong expression in the senescence zone (IV). The transcript levels gradually decreased from the senescence zone (IV) to the interzone (II-III) where the expression increased again, forming one to two layers of cells with signals. Cells in regions I and II did not show any discernible signals. These results indicate that the gene for ASNODf32 is expressed differentially in the nodule tissues especially in senescent cells reflecting its possible roles in the development, maintenance and recycling of the nodule tissues.

Cyp15a

This group of proteases has been extensively studied in *Medicago* and pea. PsCyp15a (Accession no x 54358) encodes a Cys protease that is up-regulated in pea (*Pisum sativum* L.) stem tissue in response to water deficit[54,55]. Subsequently, PsCyp15a was also identified as a transcript in the pea root nodule symbiosis with *Rhizobium*[91]. Homologues are also expressed in *Medicago sativa* nodules[93] (accession no AJ 245868) and soybean[95] and the genes from *Pisum* and *Medicago* spp. have been shown to map to syntenic regions of the genome.

Using a proteomics approach, another possible orthologue of Cyp15a (Swiss Prot accession no P25804) has also been identified as a component of isolated symbiosomal membranes from soybean nodules[95]. This provides further evidence that this class of protease is widespread in legume nodules and that it is associated with the symbiosome compartment. Cyp15a is closely related to several stress inducible proteinases such as RD 19 of *Arabidopsis*[51]. It is also related to A 494 from *Arabidopsis*[52] and bcp-15 from *Brassica*[53].

The deduced amino acid sequence for PsCYP15A indicates that it is synthesized as a pre-proprotein that is subsequently targeted to the endomembrane system where it is subsequently activated by peptide cleavage. At the N-terminus there is a propeptide consisting of 110 residues and this is followed by the mature polypeptide sequence comprising 233 residues including the conserved residues Cys-153 and His 299 which form the catalytic dyad. Laser scanning confocal microscopy revealed localization of the antigenicity mainly in large vacuolar bodies and to a smaller extent in cytoplasmic vesicles.

To analyse *Cyp15a* gene functioning and its localization in *Medicago truncatula*, transgenic plants were constructed by transformation of leaf discs (JL Vincent *et al.*, personal communication). These lines harboured the *Cyp15a* promoter in conjunction with the structural gene for glucuronidase (*gus*) construct. Analysis of transgenic plants with the gus construct revealed strong expression in cotyledonary leaves, senescent leaves, root nodules and root tips indicating involvement of this gene in tissues undergoing differentiation as well as in tissues undergoing senescence. A seven-fold increase in *gus* activity was observed with 0.6 *M* mannitol and a five-fold increase with 75 m*M* NaCl (Table 2). These observations further confirm the earlier reports that *Cyp15a* is a stress-inducible gene.

Other transformed lines of *Medicago truncatula* carried the *Cyp15a* construct in antisense orientation positioned downstream of a nodule-specific (nodulin-gene) promoter. Antisense lines showed impaired seed germination indicating a role for Cyp15a in seed germination. Immunocytological analysis of imbibed seeds revealed intracellular localisation of Cyp15a in protein storage vacuoles, indicating a role of this protease in degradation of storage proteins accumulated in the cotyledonary cells adjacent to the embryo axis. Cysteine proteases have been widely reported to be involved in the degradation of storage proteins (Table 1). The phenotypic effects observed in the antisense lines ranged widely. Some lines were extremely slow-growing and some showed conditionally lethal phenotypes, while other lines were abundantly leafy and showed delayed vegetative senescence. Nodulation was also affected adversely in the antisense lines. In some (mildly affected) lines, the nodules contained host cells with enlarged vacuoles and relatively poor colonisation by bacteroids. In other (more severely affected) lines there were abnormal nodules that were very degenerate with poor colonization of host cells. These results suggest a role for Cyp15a in nodule organogenesis. Furthermore, Cyp15a may be involved in cellular homeostasis, stress adaptation, nodule functioning and senescence.

Table 2—Glucuronidase (*gus*) activity observed in leaves of transgenic lines of *Medicago truncatula* following transformation with a reporter gene carrying a promoter for *Cyp15a* (pPsCyp15a::uidA). Seedlings were grown for 7 days with 0.6M mannitol or 75 m*M* NaCl.

Treatment	GUS activity pmol/min/mg protein	+/- S.E.
Control	48.3	4.1
0.6M Mannitol	332.2	12.1
0.075M NaCl	185.4	8.8

Although much work remains to be done on cysteine proteases in nodules, it is apparent from the CPs studied so far that they are frequently involved in almost all aspects of nodulation and nitrogen fixation, starting from nodule initiation through to the stage of nodule senescence. Because of their central importance to plant physiology, cysteine proteases could serve as important targets for the study of nodule development and functioning at the molecular level. Furthermore, because of their widespread occurrence in nodulating plants they could also serve as candidate genes for targeted plant breeding programmes.

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