

Research Note

Characterization of a *Casuarina glauca* Nodule-Specific Subtilisin-like Protease Gene, a Homolog of *Alnus glutinosa ag12*

Laurent Laplaze,¹ Ana Ribeiro,² Claudine Franche,¹ Emile Duhoux,¹ Florence Auguy,¹ Didier Bogusz,¹ and Katharina Pawlowski^{2,3}

¹Physiologie Cellulaire et Moléculaire des Arbres, Laboratoire GeneTrop, IRD, 911 avenue Agropolis, 34032 Montpellier Cedex 01, France; ²Department of Molecular Biology, Dreijenlaan 3, Agricultural University, 6703 HA Wageningen, The Netherlands; ³Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Biochemie der Pflanze, Universität Göttingen, Untere Karspüle 2, D-37073 Göttingen, Germany
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In search of plant genes expressed during early interactions between *Casuarina glauca* and *Frankia*, we have isolated and characterized a *C. glauca* gene that has strong homology to subtilisin-like protease gene families of several plants including the actinorhizal nodulin gene *ag12* of another actinorhizal plant, *Alnus glutinosa*. Based on the expression pattern of *cg12* in the course of nodule development, it represents an early actinorhizal nodulin gene. Our results suggest that subtilisin-like proteases may be a common element in the process of infection of plant cells by *Frankia* in both Betulaceae (*Alnus glutinosa*) and Casuarinaceae (*Casuarina glauca*) symbioses.

Additional keyword: symbiosis.

Actinomycetes of the genus *Frankia* can enter a nitrogen-fixing root nodule symbiosis with several woody plant species belonging to eight different plant families collectively called actinorhizal plants (Benson and Silvester 1993). Actinorhizal root nodules are coralloid structures consisting of multiple nodule lobes, each of which is a modified lateral root without root cap, with a superficial periderm, a central vascular bundle, and both infected and uninfected cortical cells (Pawlowski and Bisseling 1996; Franche et al. 1998).

Several genes expressed specifically or at enhanced levels in actinorhizal nodules, collectively referred to as actinorhizal nodulin genes, have been isolated from *Alnus glutinosa* and *Casuarina glauca* (Guan et al. 1998; Franche et al. 1998). Most of these genes have been classified as late actinorhizal nodulin genes, reflecting their involvement in final stages of nodule formation and functioning. Two families of actinorhi-

zal nodulin genes activated in early stages of nodule development have been identified in *A. glutinosa* (Ribeiro et al. 1995; Pawlowski et al. 1997). One of them, *ag12*, encodes subtilisin-like proteases (Ribeiro et al. 1995). In situ hybridization revealed that *ag12* genes are expressed in *Frankia*-infected cells of *A. glutinosa* nodules. While subtilisin-like proteases of the Kex2-family have been shown to play an important role in the secretory pathway in yeast and mammalia (Vierstra 1996), only a few examples of subtilisin-like proteases from higher plants have been published (Kobayashi et al. 1994; Yamagata et al. 1994; Ribeiro et al. 1995; Tornero et al. 1996; Taylor et al. 1997). The plant subtilases identified seem to be involved in microsporogenesis, fruit ripening, the infection of nodule cells by *Frankia*, and pathogen response, respectively. They contain N-terminal signal peptides, suggesting that they are secreted to the plant extracellular matrix where they are presumably involved in the processing of pericellular substrates (Yamagata et al. 1994; Ribeiro et al. 1995; Tornero et al. 1996; Taylor et al. 1997).

We are working on the *Frankia*-Casuarinaceae symbiosis, a model system that is especially suited to the analysis of plant symbiotic gene expression since *C. glauca* and its close relative *Allocasuarina verticillata*, unlike *A. glutinosa*, can be readily transformed and regenerated (Franche et al. 1997, 1998). To identify an *ag12* homolog in *C. glauca*, degenerate oligonucleotide primers were designed based on the amino

Table 1. Primers used in this study

Primers	Sequences
12 forward	5'-GCIATIG ^G / _C II ^G / _C ITT ^T / _C GCIGCIATGG-3'
12 reverse	5'-GCI ^G / _C ^A / _I IC ^G / _I IGTIGCIGCIGGI ^G / _C ^A / _I CCA-3'
Race1	5'-CGACCGATCTACAGTGCCTCC-3'
Race2	5'-GCTGGGAACAAGGTCCAACC-3'
Race3	5'-CCGGGACATCCATGGCTTGCCTC-3'
Race4	5'-CCCTCATGCTTCTGGCATTGCTGCAC-3'
12RT1	5'-TGCTTCGCCATTGGCCAT-3'
12RT2	5'-CCATTCTGGGCCCTCTTG-3'
Ubi5	5'-ATGCAGAT ^C / _T TTGTGAAGAC-3'
Ubi3	5'-ACCACCACG ^G / _A AGACGGAG-3'

Corresponding author: Emile Duhoux; E-mail: duhoux@mpl.ird.fr

Nucleotide and/or amino acid sequence data can be found at the EMBL data base as accession no. AJ012164.

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acid sequence comparison between *A. glutinosa* Ag12 and *Arabidopsis thaliana* Ara12 (Ribeiro et al. 1995; 12 forward and 12 reverse; Table 1) and used in a reverse transcription-polymerase chain reaction (RT-PCR) to amplify a homologous DNA fragment from RNA isolated from *C. glauca* root nodules. An 0.8-kb PCR product showing sequence similarity to *ag12* was cloned into the pGEM-T vector (Promega, Madison, WI). The insert was excised with *SstII/PstI* and

subcloned in pBluescript KS⁺ (Stratagene, La Jolla, CA), yielding pCg12. Due to its homology to the *ag12* gene, the *C. glauca* gene was designated *cg12*. To obtain the full-length *cg12* cDNA, rapid amplification of 5' and 3' cDNA ends (RACE) PCRs were performed with the Marathon cDNA amplification kit according to the manufacturer's instructions (Clontech, Palo Alto, CA) with primers Race1-Race4 (Table 1). The full products were amplified with the

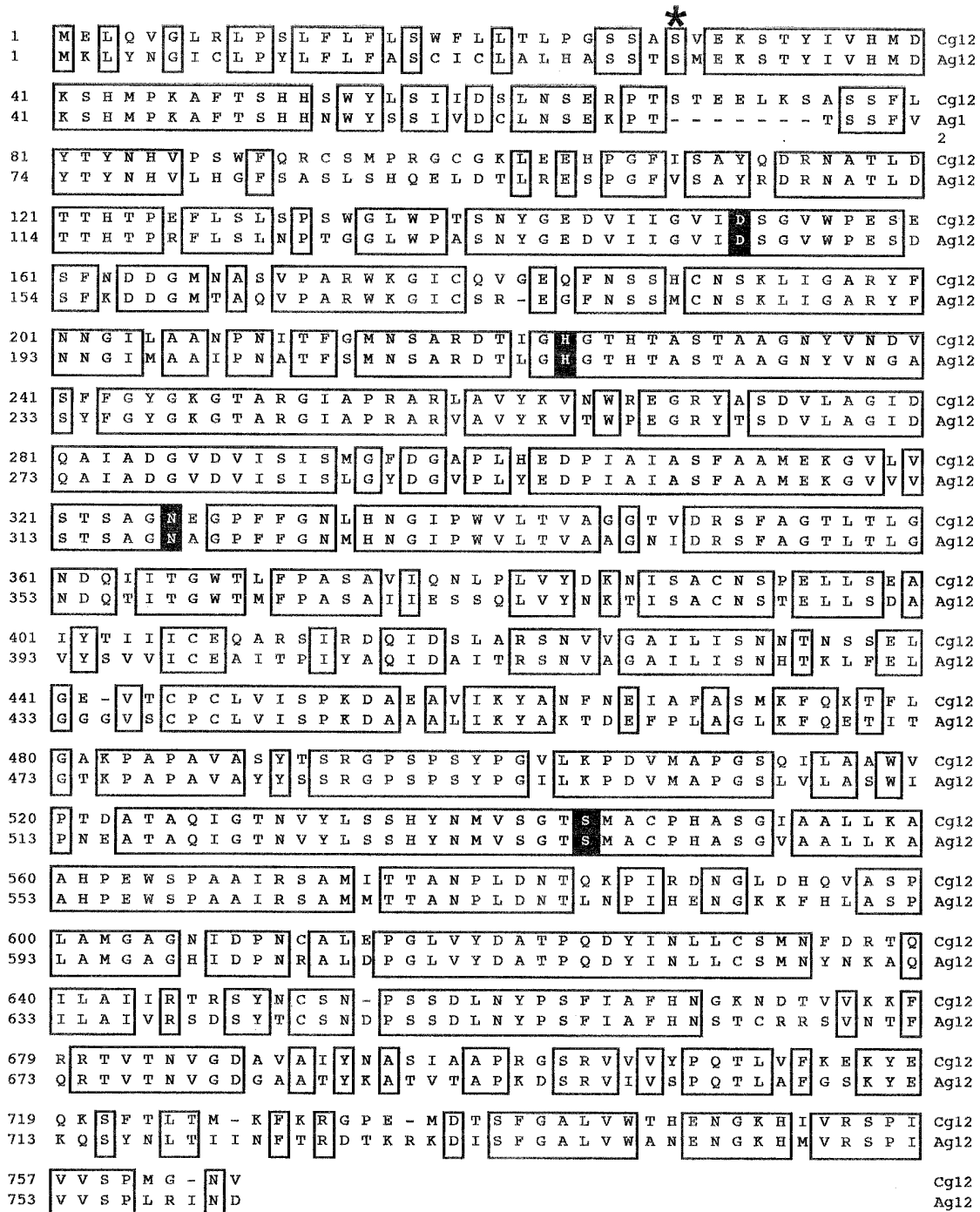


Fig. 1. Amino acid sequence comparison between Ag12 and Cg12. Identical amino acids in conserved positions are boxed. Putative signal peptide cleavage site is indicated by a star (position 29 in Cg12). Amino acids forming the active site of subtilases (D₁₅₂, H₂₂₄, N₃₂₆, and S₅₄₃ of Cg12) are labeled by inverse print.

Advantage cDNA PCR kit (Clontech) and cloned into the pGEM-T vector (Promega). The largest cDNA clone was 2,645 bp long with 5' and 3' untranslated regions (UTRs) of 221 and 108 bp, respectively (EMBL accession number AJ012164; data not shown). The 2,295-bp-long open reading frame (ORF) encoded a protein of 765 amino acids with a calculated molecular mass of 82 kDa and an isoelectric point of 6.5. The deduced amino acid sequence showed 80% similarity with Ag12 and displayed all the features of plant subtilisin-like proteases including the presence of an N terminal signal peptide (Fig. 1). Sequence alignment of Cg12 and Ag12 with tomato subtilases (Meichtry et al. 1999) reveals that these enzymes fall into the *LeSBT3/4* subfamily. Genomic DNA gel blot analysis indicates that a single gene encoding Cg12 is present in *C. glauca* genome (data not shown), while *ag12* and *LeSBT3/4* belong to small gene families (Ribeiro et al. 1995; Meichtry et al. 1999).

cg12 expression in different tissues was studied with both Northern (RNA) blot experiments and RT-PCR. Total RNA was extracted from nodule, uninfected roots, and aerial parts as described (Bugos et al. 1995). Northern blot experiments were conducted as described by Ribeiro et al. (1995) with a *cg12* probe obtained by excision of the insert of pCg12 with *Pst*I and *Sst*II and a plant ribosomal RNA probe (Carnero Diaz et al. 1997) as a loading control. A 2.5-kb transcript could be detected in the nodule RNA but not in RNA isolated from roots or stems/leaves (Fig. 2A). The size of the detected transcript was similar to that of the full size *cg12* clone and to that of *ag12* and *ara12* (Ribeiro et al. 1995). For a more sensitive analysis, RT-PCR was performed based on total RNA isolated from roots, nodules, and stems/leaves as described by Laplaze et al. (1999) with primers 12RT1 and 12RT2 (Table 1). The results showed that *cg12* transcripts are present in nodules and also in aerial parts of *C. glauca* (Fig. 2B). Thus, organ-specific expression of *cg12* was similar to that described for *ag12* in *A. glutinosa* (Ribeiro et al. 1995).

To localize *cg12* gene expression, we performed in situ hybridization on sections of mature *C. glauca* nodules (Gherbi et al. 1997). *cg12* probes were prepared by linearization of pCg12 with *Sst*II and in vitro transcription with T7 RNA polymerase (antisense) or linearization with *Pst*I and in vitro transcription with T3 RNA polymerase (sense), respectively. Parallel sections were hybridized with a probe for the nitrogenase structural gene *nifH* from *Frankia*, prepared as described by Gherbi et al. (1997) to identify infected cells containing nitrogen-fixing bacteria. *cg12* hybridization signals were strong in young, infected cells of the infection zone and weaker and irregular in the infected cells of the fixation zone as denoted by *Frankia nifH* expression (Fig. 3). No hybridization was detected in the meristem, the uninfected cortical cells, or the vascular system. No signal was obtained in control hybridizations with the sense probe (data not shown). A similar expression pattern had been found for *ag12* in mature root nodules of *A. glutinosa* (Ribeiro et al. 1995).

Although molecular phylogeny has revealed that Casuarinaceae and *Alnus* spp. are closely related (Swensen 1996), their nodules differ with respect to oxygen protection mechanisms employed by both plant host and bacterial symbiont (Silvester et al. 1990) and also with respect to nodule carbon metabolism (E. Duhoux, unpublished observation). The conservation of *ag12/cg12* in Casuarinaceae and *Alnus* spp. implies that the

mechanisms of infection of nodule cells by *Frankia* are conserved in both systems.

In summary, we have identified a subtilisin-like protease gene in *C. glauca* and shown that, as already observed in *A. glutinosa*, this protease is probably involved in the process of

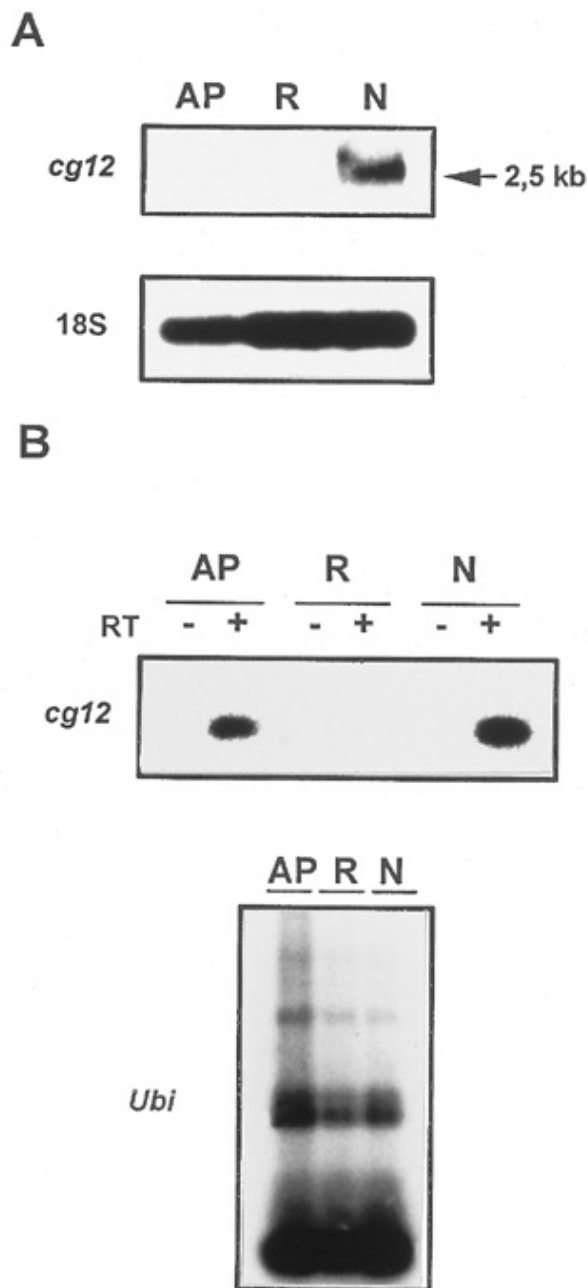


Fig. 2. Transcription analysis of *cg12* in different organs of *Casuarina glauca*. Total RNA was isolated from nodules (N), uninfected roots (R), and aerial parts (AP). **A**, RNA gel blot analysis. RNA samples (20 µg per slot) were separated on an 1.2% agarose gel, transferred to a nylon membrane, and probed with a *cg12* probe and a ribosomal probe (18S; Carnero Diaz et al. 1997). **B**, Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was used for RT-PCR experiments. No amplification was observed when the RT reaction was omitted (RT-), indicating that the RNA preparations did not contain genomic DNA. Ubiquitin (Ubi) was amplified with primers Ubi5 and Ubi3 (Table 1) as an internal control.

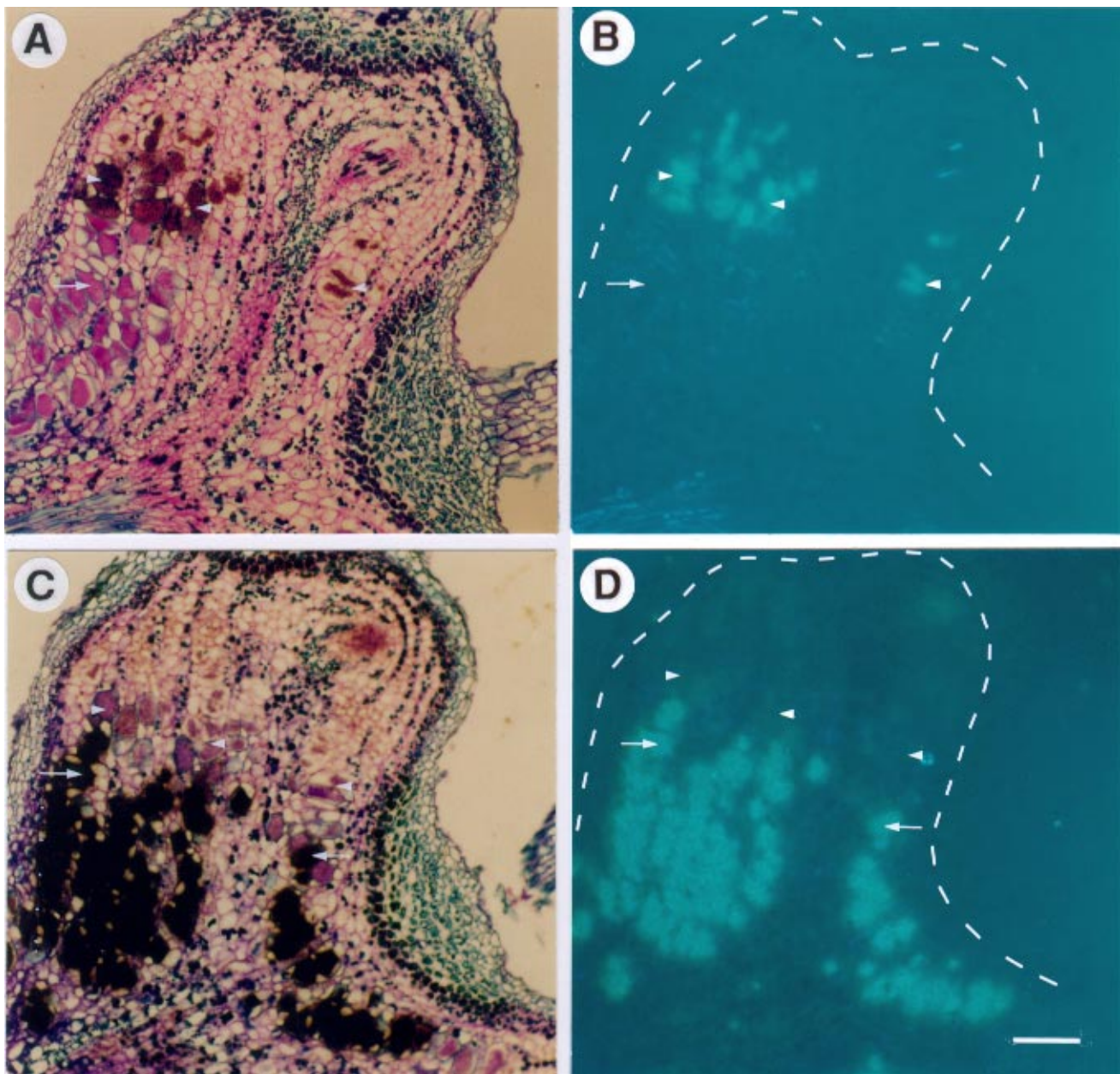


Fig. 3. Localization of *cg12* transcripts in mature, nitrogen-fixing nodules of *Casuarina glauca*. **A** and **C**, Bright-field microscopy; silver grains denoting hybridization appears as black dots. **B** and **D**, Dark-field microscopy and epipolarized light; silver grains are visible as yellow dots. **A** and **B**, Expression of *cg12* in a longitudinal section of a *C. glauca* nodule lobe. **C** and **D**, Expression of *Frankia nifH* in an adjacent longitudinal section of the same nodule lobe. Arrowheads point at infected cortical cells of the infection zone where (**A**, **B**) high levels of *cg12* expression are found but (**C**, **D**) *Frankia* does not yet express *nifH*. Arrows point at infected cortical cells of the fixation zone where *Frankia nifH* is expressed but *cg12* expression has decreased dramatically. No signal was found in control hybridizations with sense RNA as a probe. Bar = 100 μ m.

infection of nodule cells by *Frankia*. The exact function of *cg12* and *ag12* remains to be elucidated. The fact that these proteases are expressed during early stages of the infection of nodule cells suggests that they play a role in protein processing rather than in degradative proteolysis. The isolation of an expressed sequence tag (EST) clone encoding a subtilisin-like protease from nodules of the legume *Lotus japonicus* (Szczygłowski et al. 1997) suggests a widespread function of these proteases in root nodule symbioses. In this context, it is interesting to note that subtilisin-like proteases are also induced in the course of response to pathogens (Tornero et al.

1996, 1997) and have been implicated in the processing of systemin (Schaller and Ryan 1994). Further research will be needed to elucidate the function of subtilases in nodulation in comparison to their role in pathogenic interactions.

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