



Expression pattern of *ara12*^{*}, an *Arabidopsis* homologue of the nodule-specific actinorhizal subtilases *cg12/ag12*

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Abstract

In response to infection by *Frankia*, cells of *Alnus glutinosa* and *Casuarina glauca* nodules strongly express a subtilase-gene (*ag12* and *cg12* respectively). In order to gain a better understanding of the function of these genes in the symbiotic process, we have analysed the gene expression of *ara12*, an *Arabidopsis thaliana* homologue of *cg12* and *ag12*. Transcription fusion of the promoter region of *ara12* to the β -glucuronidase gene was introduced into *A. thaliana*. *Para12-gus* expression was found in young developing tissues. This result suggests that *ara12* might be involved in proteins or polypeptides processing during *A. thaliana* development. We therefore speculate that *cg12* and *ag12*, the actinorhizal homologues of *ara12* might be involved in differentiation of *Frankia* infected cells in nodules.

Introduction

Subtilases are a superfamily of serine proteases that are characterised by a highly similar arrangement of catalytic His, Asp and Ser residues. Based on sequence alignment of the catalytic domain these enzymes have been classified into six families: subtilisin, thermitase, proteinase K, antibiotic peptidase, kexin and pyrolysin (For review see Siezen and Leunissen, 1997). They are widely distributed in many different organisms including bacteria, fungi, plants, cyanobacteria and mammals. In prokaryotes and lower eukaryotes, subtilases display rather low specificity and may act extracellularly and be involved in defense or in development. However, in yeast and in mammals, subtilases have been characterized as having high specificity. For example, the yeast *kex2* endoprotease is involved in the processing of the α -factor pheromone and of killer toxin; mammalian kexins are known to be involved in activation of peptide hormones and growth

factors (for review see Siezen and Leunissen, 1997). In recent years, genomic and cDNA sequences coding for subtilases have been isolated from a variety of plant species including melon (Yamagata et al., 1994), tomato (Meichtry et al., 1999), *Arabidopsis* (Berger et al., 2000; Neuteboom et al., 1999; Yamagata et al., 2000; Zhao et al., 2000), lily (Taylor et al., 1997), and rice (Yamagata et al., 2000). Recent data indicate that some plant subtilases are involved in polypeptide or proprotein processing (Janzik et al., 2000; Kinal et al., 1995; Schaller et al., 1994; Tornero et al., 1996).

Actinorhizal plants are non-leguminous plants capable of forming nitrogen-fixing root nodules as the result of symbiosis with the soil actinomycete *Frankia* (Benson and Silvester, 1993). Actinorhizal nodules consist of several lobes, each of which is similar to a modified lateral root without cap. Nodule lobes have a central vascular bundle, a superficial periderm and both infected and non-infected cortical cells. Starting from the apical pole, 4 zones can be distinguished: (1) a meristematic zone with small dividing cells free from *Frankia*, (2) an infection zone where some

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cells coming from the apical meristem are infected by *Frankia*, (3) a fixation zone where nitrogen fixation takes place and (4) a senescence zone where plant cells and bacteria are degraded (for reviewed see Franche et al., 1998; Mullin and Dobritsa, 1996; Pawlowski and Bisseling, 1996; Wall, 2000). All stages of the symbiotic process can consequently be seen on a transversal section of a nodule lobe, from the infection that take place in the infection zone to the final stages when cells enter senescence.

Our overall aim is to study plant responses to *Frankia* infection. We were particularly interested in understanding the events that take place during the infection process because this step is critical for the establishment of the symbiosis. In actinorhizal plants, most of the nodule-specific genes that have been identified were found to be expressed in the fixation zone and are involved in carbon, nitrogen or oxygen metabolism (Franche et al., 1998). Only few genes that are expressed in the infection zone have been characterized, namely, *Dg93*, a gene from *Datisca glomerata* that is also expressed in the meristematic zone (Okubara et al., 2000), *agNt84/ag164*, two genes from *Alnus glutinosa* which encode two glycine and histidine rich proteins (Pawlowski et al., 1997) and *ag12/cg12*, isolated from *A. glutinosa* and *C. glauca* that encode subtilisin-like serine proteases (Laplaze et al., 2000; Ribeiro et al., 1995). The role of *Dg93* is still unknown, a function of *agNt84/ag164* in metal transport has been suggested (Pawlowski et al., 1997). We previously showed that *cg12* and *ag12* genes are expressed the infection zone 2 of the *C. glauca* and *A. glutinosa* nodule lobes, which suggests that they play a role in the infection process (Laplaze et al., 2000; Ribeiro et al., 1995).

Although the capacity to form nodules is restricted to a few plant families, homologues of genes shown to be induced during nodule formation have been identified in non-nodule forming species (Arredondo-Peters et al., 1998; Kouchi et al., 1999). These results suggest that several functions required for symbioses have been recruited from pre-existing genes that are present in both legume and non-legume (Bladergroen and Spaink, 1998). Ribeiro et al. (1995) previously identified a homologue of *ag12/cg12*, named *ara12*, in *Arabidopsis*. *ara12* is an intronless gene located on the chromosome 5 (Yamagata et al., 2000). Like *ag12*, *ara12* belongs to a small gene family (Ribeiro et al., 1995; Yamagata et al., 2000). In the *Arabidopsis* genome we found 65 genes annotated as subtilases. Two of those subtilases are closely related to *ara12*

(BLASTN e-value $\leq 10^{-9}$). *ara12* codes for a 79.4 kDa protein that shares 61% similarity with the polypeptide encoded by *ag12* (Ribeiro et al., 1995). Like *ag12/cg12*, *ara12* possess a predicted signal peptide that could target the protein to the extracellular compartment (Ribeiro et al., 1995; Yamagata et al., 2000). Northern blot studies showed *ara12* expression in all organs of *Arabidopsis* but the highest levels were detected in developing siliques (Ribeiro et al., 1995; Yamagata et al., 2000).

In order to understand the symbiotic function of *cg12* and *ag12* we studied the *Arabidopsis* homologue gene, *ara12*. We describe the isolation of the *ara12* promoter and its analysis by *gus* fusion in transgenic *A. thaliana*.

Materials and methods

Plant material and bacterial strains

A. thaliana (L.) Heynh., ecotype colombia (Col-0) and Landsberg *erecta* (Ler) were cultivated in growth chambers at 25 °C under 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool fluorescent tubes (Sylvania Gro-Lux) with a 16 h photoperiod at an average of 45% humidity. The *A. tumefaciens* strain C58C1(pGV2260) (Vancanneyt et al., 1990) was used to transform *Arabidopsis*.

Isolation of the *ara12* 5' upstream region and construction of *ara12* promoter *gus* fusion

The *ara12* promoter region was cloned using the Universal Genome Walker kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Primers At12P (5'-CGGCTGAGTCGGAGATAGATCTGAGAG-3') and At12NP (5'-GGAGGTCAAAGACGACGGCGTCTGAG-3') were designed to match the *ara12* cDNA. A 1900 bp product was amplified from the *pVuII* Ler-ecotype *Arabidopsis* DNA library and cloned into pGEM-T (Promega) generating *pPara12*. Two oligonucleotides were synthesised: a 5' primer (5'- CCAAGCTTGGGTAGGACGCGTGGTTCGACGG-3') to introduce a *HindIII* site, and a 3' primer (5'-GCTCTAGAGCGGTCTCTCCCTGGATCGAAGG-3') designed to match the inverse complement of 22 nucleotides starting 17 bp upstream of the initiation codon and to introduce a *XbaI* site. After PCR amplifications a 1837 bp *HindIII/XbaI* fragment was cloned into *HindIII/XbaI*

digested pBI101.3 (Clontech). Cloning was confirmed by DNA sequence analysis.

Plant transformation and analysis of transgenic plants

The *Para12-gus* construct was introduced into Col-0 ecotype *Arabidopsis* using the floral dip method (Clough et al., 1998). Transformants were screened on Murashige and Skoog medium with 50 mg L⁻¹ kanamycin. At the 4-leaf stage, resistant plants were transferred to soil. Plants were grown in standard conditions, allowed to self-pollinate and seeds were harvested. Ten independent *gus*-expressing lines were obtained.

For histochemical analysis whole explants were stained solution containing 1 mM X-gluc (5-bromo-4-chloro-3-indolyl β -D-glucuronide) and incubated for 16 h at 37°C, as recommended by Jefferson et al. (1987). To confine the localization of the blue staining, 2 mM of K₃Fe(CN)₆ and 2 mM K₄Fe(CN)₆ were added as catalysts. Plant samples were fixed for several hours in a solution containing 5% formaldehyde, 5% acetic acid and 50% ethanol, washed several times in 70% ethanol.

Results

The cDNA sequence was used to amplify and clone a 1.834 kb fragment corresponding to the *ara12* 5' upstream region. The sequence of the entire fragment was determined. A transcriptional fusion of *ara12* promoter with the *gus* reporter gene was introduced in *Arabidopsis* by *A. tumefaciens*-mediated transformation. Seedlings derived from 10 independent transformants were tested for GUS activity.

All transgenic plants containing the transcriptional fusion had similar pattern of *gus* expression (Figure 1). No GUS activity could be detected in seeds prior to germination. In germinating seeds, blue staining was detected in the emerging root, near the root meristem, and in the vascular tissue (Figure 1A). No reporter gene activity could be detected at this stage in cotyledons, in root meristem or in the shoot apex (Figure 1A). The same pattern of GUS activity was observed in the primary root of 4-day-old seedlings (data not shown). Blue staining was also detected in cortical cells at the transition between the hypocotyl and root, where secondary roots were about to emerge but no staining was observed in root hairs (Figure 1B). *gus*

expression in the shoot apex remained high throughout the entire course of development (Figure 1C, D). In one-week-old seedlings and in older non-flowering plants, blue color was detected in young developing leaves and in emerging lateral roots near the root tip. Expression was restricted to the vascular system and no expression could be detected in old tissues or in root meristems (Figure 1D, E). In young leaves, GUS activity was seen in the vascular tissue and in the cytoplasm of trichomes (Figure 1D, F). *gus* expression was absent as leaves became older (data not shown). In flowering plants, blue staining was detected in the inflorescence, in the vascular system of pedicels, petals and sepals (Figure 1G). Reporter gene activity was also detected in developing siliques (Figure 1H).

Discussion

In order to determine the function of *ara12*, a homologue of *ag12/cg12*, in *Arabidopsis* we constructed a promoter-*gus* fusion that was analysed in transgenic *Arabidopsis* plants. In this way we explored the developmental process in which the symbiotic homologue *ara12* is involved, and from here we addressed the role of the symbiotic homologues *ag12/cg12* in nodule formation. Promoter activity was detected in young vascular tissues both in roots and shoots, and in developing trichomes and siliques. These results are consistent with previous expression studies (Ribeiro et al., 1995; Yamagata et al., 2000) showing that *ara12* is expressed in all organs and particularly in developing siliques. No activity was detected in the root meristematic zone. Together these data suggest *ara12* function is related to cell elongation and/or differentiation (particularly in the vascular tissue) rather than to cell division.

Two classes of subtilases can be distinguished: degenerative subtilases and processing subtilases. Processing subtilases have high substrate specificity. Well known examples of these processing subtilases belong to the mammalian proprotein convertase family; these subtilases cleave proteins at pairs of basic residues and are involved in activation of peptide hormones, growth factors, and viral proteins. In contrast to processing subtilases, degenerative subtilases have broad substrate specificity and are involved in a wide range of protein degradation that occurs mainly during cell remodeling or cell degradation (Siezen and Leunissen, 1997).

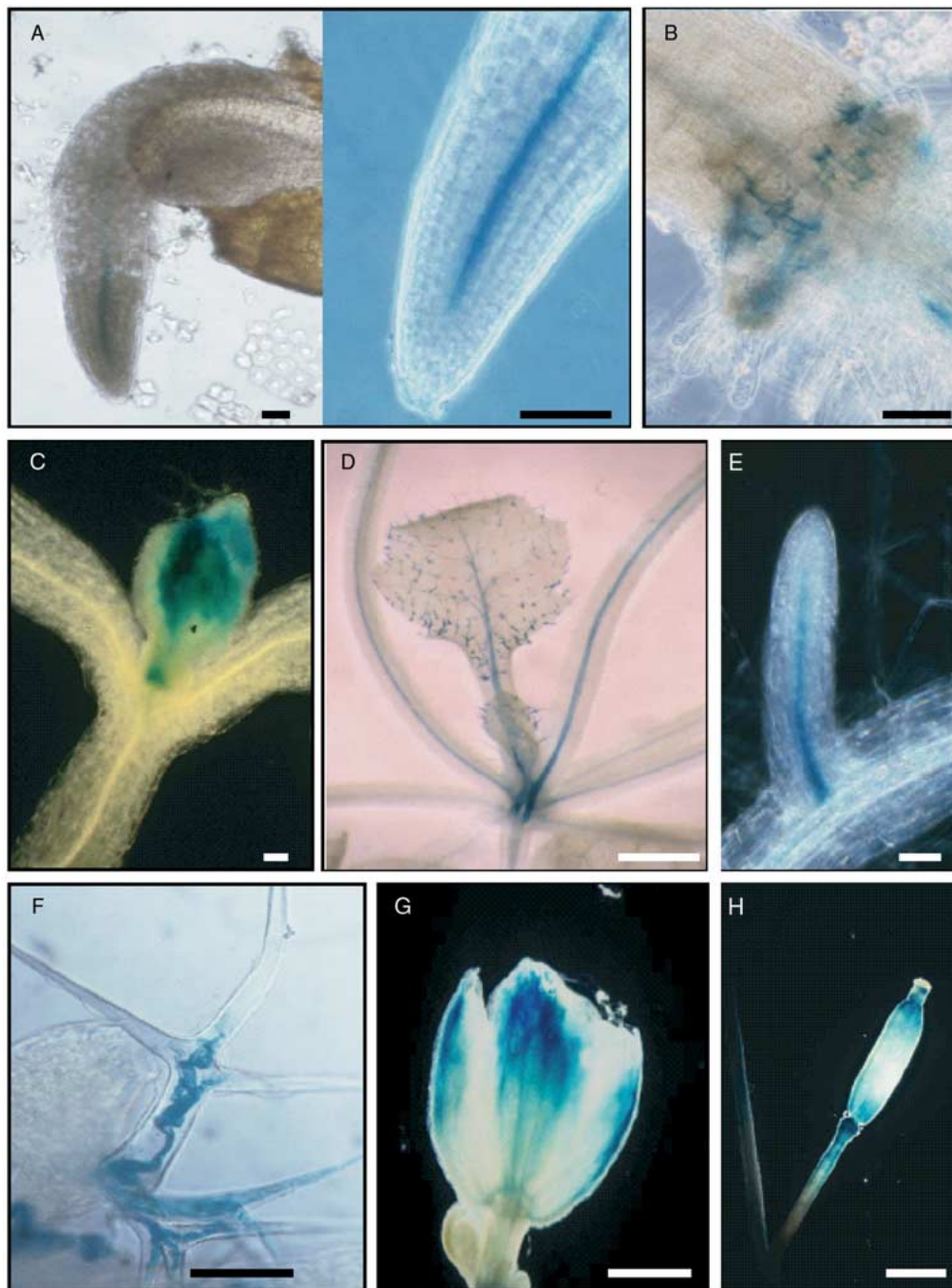


Figure 1. Histochemical localization of *Para12-Gus* activity in transgenic *A. thaliana*. (A) early stage of seedling development; (B, C) 1 – week-old seedlings; (D, E, F) 3 week-old plants; inflorescence; (H) silique of fertilized flower. The scale bar represents 50 μm in A, B, E, F; 200 μm in C, D, G and 500 μm in H.

Based on the similarity of their catalytic domain, all plant subtilases were grouped in the pyrolysins family (Siezen and Leunissen, 1997). Since plant subtilases were found to have a broad range of specificity and not have the preference for sequence motives they were reported to belong to the degenerative class (Arima, 2000; Bogacheva, 1999; Uchikoba, 1995, 1998, 2000). However, the discovery of high levels of specificity for two mammalian pyrolysins (Sakai et al., 1998; Seidah et al., 1999) showed that pyrolysins are not necessarily degenerative and evidence is accumulating that some plant subtilases may also belong to the processing class. In 1994, Schaller et al. found that *kex-2p*-like protease activity in tomato could be involved in the processing of prosystemin and showed that the corresponding protein was immunologically related to subtilisin family. In 1995, Kinal also found specific *kex-2p*-like subtilase activity in tobacco responsible for the processing of a viral antifungal toxin. Processing properties have also been shown for the tomato *P69* subtilase that could be involved in the processing of a leucine rich repeat protein (LRR) during pathogenesis (Tornerio et al., 1996). Recently, Janzik et al. (2000) showed that the tomato subtilase *LeSbt1* preferentially cleaves the peptide bond on the carboxyl side of Gln residues, suggesting that *LeSbt1* may also be a processing subtilase. *ara12* shows strong homology with *LeSBT1* (64% aa identity, 78% similarity) (Yamagata et al., 2000) suggesting a narrow substrate and a processing function. *ara12* might be involved in a young stage-related protein processing/signal transduction associated to cell elongation and/or differentiation. However, a degenerative role cannot be excluded. *ara12* could be involved in cell-wall protein degradation associated with cell elongation, as has been suggested for *LIM9/Tmp* or cucumisin (Meichtry et al., 1999; Taylor et al., 1997; Uchikoba et al., 1995).

In conclusion, the expression pattern of *ara12*, a homologue of the two actinorhizal noduline genes *cg12/ag12*, suggest this subtilase plays a role in cell elongation and/or differentiation during *Arabidopsis* development. Similarly, *ag12* and *cg12* could be involved in differentiation of *Frankia*-infected plant cells in actinorhizal nodules. Biochemical characterisation of *cg12* is underway to try to elucidate its function *in vivo*.

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