

Modified expression of cysteine protease affects seed germination, vegetative growth and nodule development in transgenic lines of *Medicago truncatula*

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Abstract

Transgenic lines of *Medicago truncatula* (R108-1) were constructed to analyse Cyp15a (cysteine protease) gene functioning and localisation. The promoter region of *PsCyp15a* was fused to the coding sequence of *uidA*. GUS-analysis of transgenic plants containing this construct revealed strong expression from the Cyp15 promoter in cotyledonary leaves, senescent leaves, and root nodules. A seven-fold increase in GUS activity was observed following treatment of seedlings with 0.6 M mannitol and a five-fold increase with 75 mM NaCl. This confirmed previous reports that *Cyp15a* is a stress-inducible gene. Immunolabelling of nodule sections showed localization of CYP15A antigen in large vacuolar bodies and to a smaller extent in cytoplasmic vesicles. In seeds, antigenicity was located in the protein storage vacuoles. In a separate series of experiments, part of the coding sequence for *MsCyp15a* was fused in antisense orientation to the promoter region of early nodulin gene MtENOD12 and to the promoter region of the *Medicago* leghaemoglobin gene *Lb1* (a late nodulin gene). Analysis of both sets of antisense lines revealed unexpected developmental effects in vegetative tissues as well as changes in nodule development. Seed germination was significantly impaired compared to untransformed lines, although treatment with gibberellin (1 μ M GA₃) could overcome this inhibition. Transformed plants ranged from slow growing and fatal phenotypes to leafy and late-senescent types. Cytological analysis of nodules revealed effects ranging from mild (infected cells with larger vacuoles and a low number of bacteroids) to severe (degenerate nodules with poor colonization of host cells). These results indicate a role of Cyp15a cysteine protease in germination and stress adaptation and also in nodule organogenesis and function.

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1. Introduction

In plants, cysteine proteases are widely distributed [1]. They are involved in the catabolism of protein reserves in seeds [2–5], leaf and flower senescence [6,7], environmental stress conditions [8,9], oxidative stress [10] and nodulation [11–17].

The present studies were conducted on Cyp15a, a cysteine protease that was first isolated from wilted pea shoots [18]. *PsCyp15a* has also been identified as a transcript in pea root nodule symbiosis with *Rhizobium* [12]. Homologues are also expressed in nodules of *Medicago sativa* (accession no. AJ245868; [14]) and soybean [16]. Genes from *Pisum* and *Medicago* spp. map to syntenic genomic regions [14].

The deduced amino acid sequence for PsCYP15A indicates that it is synthesized as a pre-proprotein consisting of a propeptide (110 residues) and a 233-residue mature peptide. Residues Cys-153 and His-299 form a catalytic dyad that is conserved in this family of proteases. The Merops database for proteolytic enzymes places Cyp15a in clan CA family CI, the glycinain family. Close homologues

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include MCP, a membrane-associated cysteine protease from *Vigna mungo* with possible dual roles in the endoplasmic reticulum and in protein storage vacuoles [19]; CPR2 localized in cotyledons and the embryo axis of *Vicia sativa* [4]; and RD19 a drought-inducible cysteine protease in *Arabidopsis thaliana* [9]. *Medicago truncatula* was chosen as the model system in this study because the small diploid genome size, autogamous genetics and capacity for genetic transformation make it suitable for legume genetics and genomics [20].

In the present study, we analysed gene expression and functionality using transgenic lines of *Medicago truncatula* (R108-1). In order to study the expression pattern of the Cyp15a gene during plant and nodule development, *Medicago truncatula* (Mt) plants were transformed with a construct containing the promoter region of *PsCyp15a* fused to the gene for beta-glucuronidase (*uidA*). In addition, nodule-specific promoters, pMtLb-1 and pMtENOD12 were fused to the partial coding sequence of MsCyp15a in antisense orientation. Our results suggest an important role for Cyp15a in germination, vegetative growth and nodule development.

2. Materials and methods

2.1. Construction of plant transformation vectors

All molecular cloning procedures were carried out using standard methods [21]. A 580 bp fragment of MsCyp15a was ligated in pUC 19. This contained the NOS terminator sequence producing pJV12 with antisense orientation.

Nodulin promoters MtLb1 [22] and MtENOD12 [23] were ligated with the pJV12 plasmids to produce plasmids pJV14.2 and pJV13.2, respectively. Orientation of the promoter fragment with respect to the MsCyp15a-NOS term inserts was confirmed by restriction digestion (antisense). The binary plant transformation vector pE6NOSBAR was used as the recipient of MsCyp15a chimeric constructs. Two binary plasmids were produced containing the MsCyp15a chimeric expression cassettes from pJV13.2 and pJV14.2 and named pE613.2 and pE614.2. A 900 bp promoter region of *PsCyp15a* was cloned upstream of the *uidA* gene to generate pE6P2GUS.

The pE613.2, pE614.2 and pE6P2GUS constructs were introduced into *Agrobacterium tumefaciens* strains AGL1, by triparental mating and used to transform *M. truncatula* R108-1 (C3). The transformation was carried out as described by Trinh et al. [24]. When transformed plantlets developed sufficiently balanced shoot and root systems, they were transplanted to a mixture of coarse sand and grit (1:1) and fed with nutrient solution (Soluplant, N/P/K: 18/6/26; Dulcos International, France) under growth conditions of 16 h light at 24 °C and 8 h dark at 20 °C. Seeds derived by selfing the T₁ transformant lines were germinated as the T₂ generation.

2.2. PCR analysis of regenerants

Regenerated plants were analysed for the presence of the transgene delivered during transformation by AGL1 derivatives. Genomic DNA was sampled from leaf tissue [25] and used as a template for PCR with construct-specific primers. Touchdown parameters [26] were used for PCR with Amplitaq DNA polymerase (Perkin-Elmer) and a Techne Genius thermocycler (Techne Ltd., Cambridge, UK).

2.3. RNA analysis and reverse transcriptase (RT) PCR

Total RNA from imbibed seed was prepared using the RNeasy Kit (Qiagen, Crawley, UK) as outlined by the manufacturer. Poly(A⁺) mRNA was purified from total RNA using oligo (dT)-coated paramagnetic beads (Dynal, Bromborough, UK) following the manufacturer's instructions.

RT-PCR was used to amplify the MtCyp15a from wild type and transgenic lines using a gene-specific primer. A PTC thermocycler was used. Conditions for reverse transcription closely followed the recommendation by Saiki et al. [27].

2.4. Purification of recombinant protein

Recombinant PsCyp15A protein [13] was produced in *E. coli* using the pRSET expression system (Invitrogen, San Diego).

2.5. Development of polyclonal antiserum

Antiserum R79, raised against PsCYP15A [13], was purified by cross-reacting with bacterial antigen [28]. Further purification of PsCYP15A-specific antibodies from the pre-absorbed immune serum was performed by immunoadsorption [29], using immobilized recombinant PsCyp15A on reinforced nitrocellulose membranes (Optitrans, Schleicher and Schuell, Keene, NH). Pre-absorbed pre-immune serum was also processed in this fashion.

2.6. Immunoblotting

Following SDS-PAGE (15 µg per lane) (Mini Protean II system, Bio-Rad Laboratories), western blotting of protein was performed as described previously [30] using nitrocellulose membranes (Schleicher and Schuell) and 3-[cyclohexylamino]-1-propane sulfonic acid (CAPS) transfer buffer. Immunolabelling of western blots was performed as described previously [31] using alkaline phosphatase conjugated secondary antibodies and nitro-blue tetrazolium as substrate.

2.7. Immunolocalization

Immunolocalization by laser scanning confocal microscopy was performed on 100-µm sections of plant material

that had been fixed in 4% (w/v) formaldehyde and stored in pH 6.9 PEM buffer (50 mM PIPES/KOH, 5 mM EGTA and 5 mM MgSO₄), 0.02% (w/v) NaN₃, at 4 °C. Sections were prepared on a vibratome (series 1000, TAAB, Aldermaston, UK) and mounted on microscope slides coated with γ -aminopropyltriethoxysilane (APTES, Sigma–Aldrich, Poole, UK). Sections were treated with 0.05% (w/v) cellulase (Onozuka, Yakult, Honsha, Tokyo) for 10 min before blocking with 3% (w/v) BSA in PEM for 1.5 h. Primary antiserum (purified R79) was applied in a 1% (w/v) BSA solution in PEM and incubated overnight at 4 °C. Sections were then washed in excess PEM for 1 h followed by another blocking step in 1% (w/v) BSA/PEM for 30 min. Secondary antibodies were applied as 1:50 or 1:100 dilution in 1% BSA solution in PEM and inoculated for 2 h at 37 °C. Fluorescent conjugate of secondary antibody was anti-rabbit Alexa Fluor 568 supplied by Molecular Probes Europe (Cambridge, UK). Washing of specimens was then completed over 4 h at room temperature in excess PEM before mounting in fluorescent mounting medium (Vectashield, Vector Laboratories Burlingham, CA) and viewing on a laser scanning confocal microscope (TCS-SP, Leica Microsystems, Wetzlar, Germany). Composite images were generated using processing software (TCS-NT, Leica Microsystems) and arranged for publication using Photoshop 5.0 (Adobe Systems, Mountain View, CA).

2.8. Histochemical GUS assay

Histochemical staining of plant tissue for GUS activity was performed using the method of Jefferson et al. [32].

2.9. Quantitative GUS assay

Quantitative GUS analysis was performed as described by Jefferson et al. [32]. Protein concentrations were measured with the method of Bradford (1976). GUS activity was assayed by enzymatic conversion of 4-methylumbelliferyl- β -D-glucuronide to 4-methylumbelliferone, which was quantified with a fluorimeter.

2.10. Light microscopy

Nodules were fixed in 4% (w/v) formaldehyde, dehydrated in a graded ethanol series and embedded in paraffin. Sections (8–10 μ m thick) were stained with 0.05% (w/v) aqueous toluidine blue and observed under a light microscope.

3. Results

3.1. Transformation with *PsCyp15a* promoter-*uidA* fusion construct

The putative promoter region (P2) of the *cyp15a* gene from pea, *PsCyp15a* [14] was fused with the coding

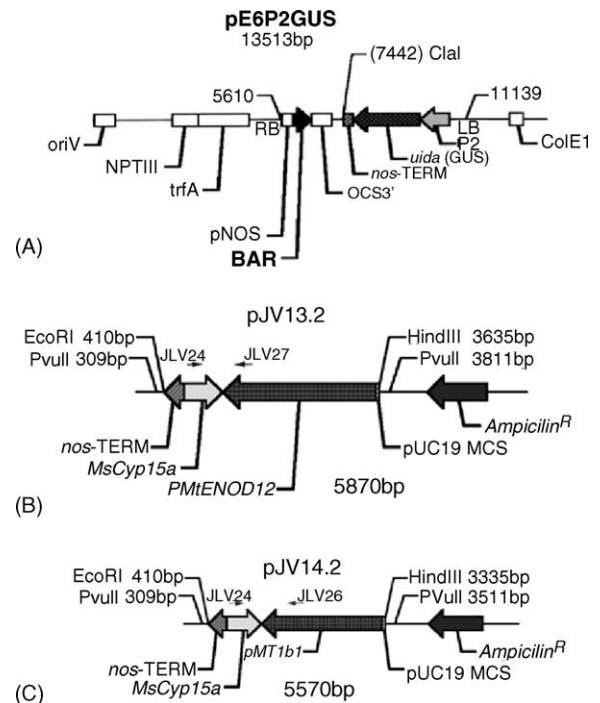


Fig. 1. Vectors for transformation of *Medicago truncatula* R108. (A) Linear map of the final transformation construct pE6P2GUS, a derivative of the vector PE6NOSBAR containing a fusion of the P2 promoter region from *pPsCyp15a* with the *uidA* coding sequence (*pPsCyp15aP2::uidA*). Arrowheads indicate the 5'–3' orientation of the fused sequences. RB and LB mark (respectively) the positions of the right and left borders of the T-DNA. (B) Linear maps of pUC19 derivatives with the completed *Cyp15a* fusion adjacent to *pMtENOD12* (pJV13.2) or (C) *pMtLb1* (pJV14.2). Orientation of the sequences within each fusion is denoted by the direction of the arrowheads, representing a 5'–3' alignment. Arrows indicate primer combinations used for PCR analysis of regenerant plants.

sequence of glucuronidase (*uidA*) as shown in Fig. 1A. Following transformation of Mt R108, nine viable regenerant lines were obtained (T₁ generation). These lines were tested for the presence of transgenic DNA, using PCR-based methods and qualitative GUS analysis. Seven of the T₁ generation lines tested positive for the transgenic DNA, and for GUS activity. Although the intensity of GUS activity was variable among transformed lines, the expression pattern in different tissue types remained similar. Line G9 consistently showed higher level of GUS expression in comparison to the rest of the positive transformants. This line was therefore used to analyze GUS activity in transformed plants.

As shown in Fig. 2A, strong expression of GUS was found in cotyledonary leaves, senescing leaves and nodules. A weaker level of staining was also observed at the root tips. Interestingly, in nodules the highest level of GUS labeling was confined to the nitrogen-fixing region. This was strongest in the mature part of the nodule, nearest to the root, followed by the central region of the nodule cortex. The meristematic region of the nodule apex showed relatively low levels of GUS activity. Seedlings of line G9

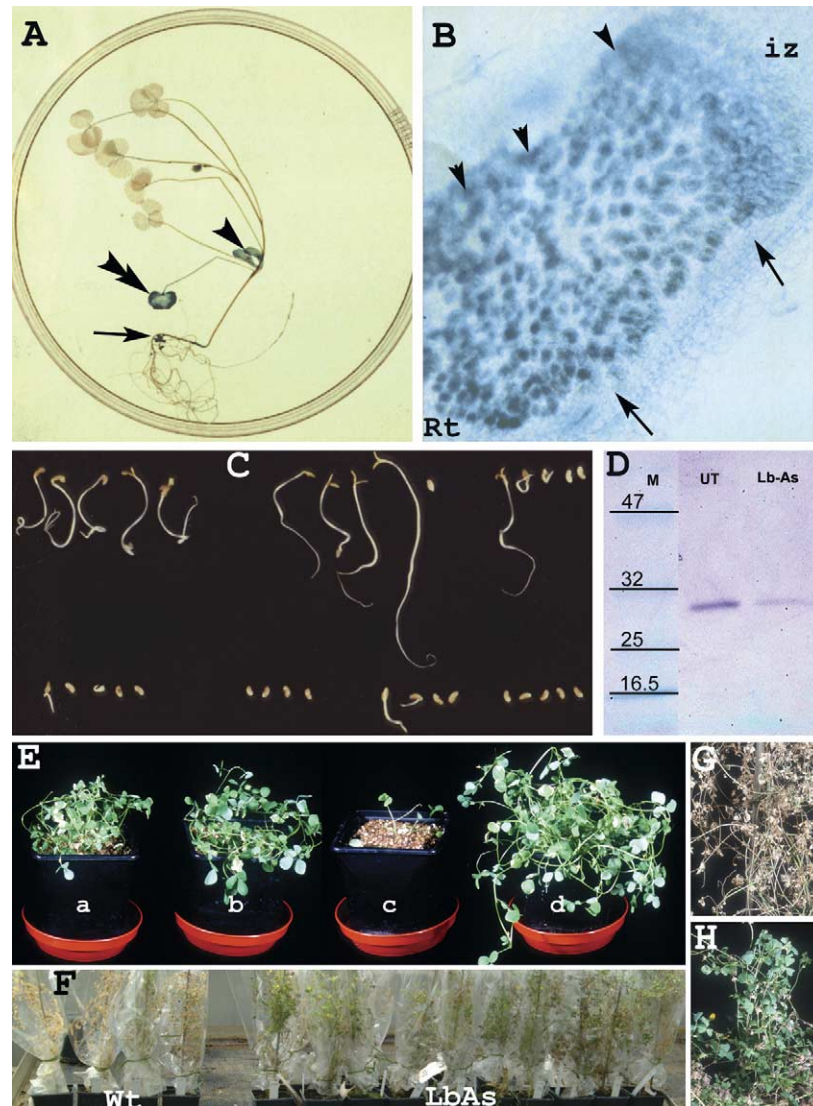


Fig. 2. (A and B) Expression of beta-glucuronidase activity in *Medicago truncatula* R108, line G9, transformed with a *pPsCyp15::uidA* fusion (T_2 generation). (A) Whole seedling, 4 weeks post-germination, showing expression in cotyledonary (arrowhead) and senescing leaves (double arrowhead) and nodules (arrow). Faint GUS labeling was also visible at the root tips. (B) Longitudinal section through a nodule from line G9, showing intense GUS activity in host cells containing bacteroids (arrowheads). Labeling is strongest in the mature infected cells, closest to the root (Rt). Infection zone (iz) and nodule cortical cells (arrow) show faint GUS labeling. (C) Impaired germination of T_3 seed from antisense lines, relative to controls, following imbibition and germination for 5 days. Upper line: wild-type; *pPsCyp15a::uidA* GUS-line (G9); pENOD12-antisense (E12As-2). Lower line: pLb-antisense (LbAs-7, -8, -9, -10). (D) Immunoblot showing reduced levels of CYP15A protein antigen in extracts from leaves of transformed lines (LbAs-7 line, T_2 generation) relative to untransformed (UT) control and molecular weight markers (M) (1000 \times). (E) Abnormal vegetative growth for antisense transformed lines: (a) Untransformed line, (b) GUS transformant (line G9), showing normal growth, (c) Abrupt senescence at the six-leaf stage (LbAs, line 1) (d) Super-leafy plants (LbAs, line 5). (F) Comparison of wild type plant (Wt) with a selection of LbAs lines, comprising lines 1, 2, 3, 5 and 6 (T_2 generation), showing delayed senescence after growth for 20 weeks in the greenhouse. (G and H) Close up views of representative plants from F. (G) Wild-type, (H) LbAs line.

(T_1 generation) were also tested for quantitative expression of GUS activity in leaves following growth for 7 days in the presence of 0.6 M mannitol and 75 mM NaCl. A substantial increase in GUS activity was observed, amounting to seven-fold with 0.6 M mannitol and four-fold with 75 mM NaCl. This indicated that the *PsCyp15a* gene promoter is upregulated in response to osmotic stress (Table 1).

Table 1
GUS activity in the leaves of transgenic *M. truncatula* containing *pPsCyp15a::uidA* fusion construct, following the application of mannitol and NaCl

Treatment	GUS activity pmol/min/mg protein
Control	48.3 \pm 4.1
Mannitol (0.6 M)	332.2 \pm 12.1
NaCl (75 mM)	185.4 \pm 9.8

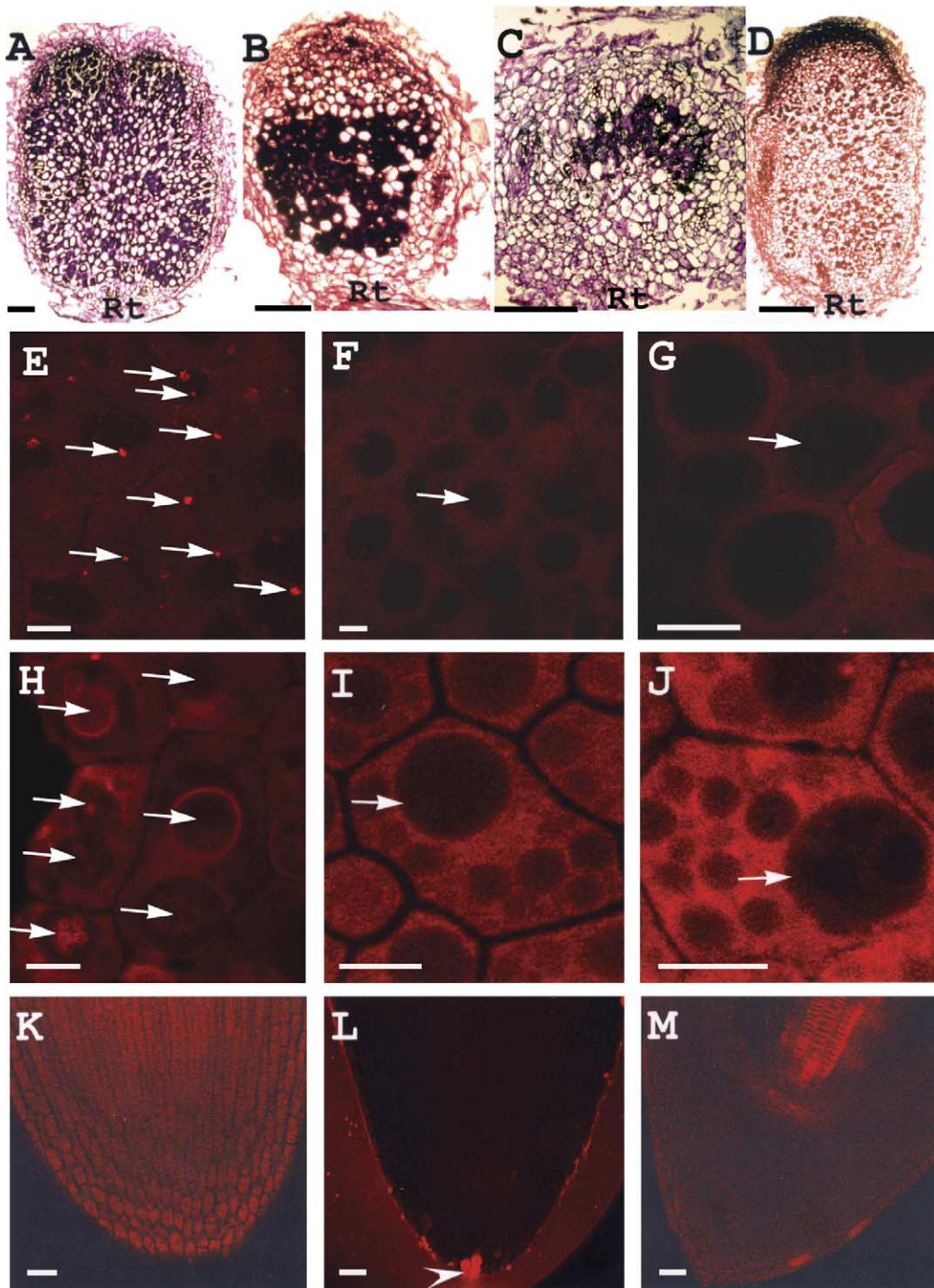


Fig. 3. (A–D) Structure of nodules in wild type and transformed lines, following staining of wax-embedded thick sections with toluidine blue. (A) Wild-type nodule; (B) ENOD12AS-2 (T_2 generation) showing smaller nodules with reduced infection zone; (C) LbAS-1, T_2 generation, a severe example with few infected cells and very small size of the nodule; (D) LbAS-11, T_2 generation showing mild example of the effect of gene suppression. General morphology of the nodule is similar to wild type nodules, although infected cells contain a large vacuole and a reduced number of bacteroids. Rt, root. Bar = 100 μ m. (E–G) Immunolocalisation of CYP15A antigen in the host cells of *Medicago truncatula* nodule tissue from wild-type and antisense lines, visualised by confocal microscopy with AlexaFluor 568 fluorescent secondary antibody. (E) Wild-type (Wt) nodule cells showing vacuolar labeling of infected cells. The label is also present in small vesicles (arrow). Arrowhead shows small size of the central vacuole of the infected cells. (F) ENOD12AS-2 line with very little vacuolar label comparable to wild type, and larger central vacuoles. G. LbAS-11 line showing very large central vacuoles with no labelling. Bar = 20 μ m. (H–J) Immunolocalisation of CYP15A antigen in cotyledonary cells for wild-type and antisense lines. Seeds were examined 4–6 h post-germination (wild-type) or 24 h post-germination (antisense lines). (H) Wild-type cell showing showing labelling of the protein storage vacuoles. Arrow marks the PSVs accumulated with antigen, which is absent from transformed lines; (I) E12AS-2 and (J) LbAS-11. Bar = 20 μ m (K–M). Immunostaining of the embryonic radicals. (K) Wild-type radicle showing antigenic labeling in all of the cells. (L) The radicle of E12AS-2 lines showing confinement of the antigen, exclusively to the three cells at the base of the radicle (arrowheads). Rest of the cells shows complete absence of the label. (M) Antigenic labeling is largely absent from the radicle of LbAS-11 line. Bar = 25 μ m.

3.2. Antisense expression of *MsCyp15a* driven by nodule specific promoters

In order to study the effect of suppression of the *Cyp15a* gene, the coding region of *MsCyp15a* was fused to the tissue-specific promoters in sense and antisense orientation (Fig. 1B and C). The tissue specific promoters of *ENOD12* (*pMtENOD12*) and leghaemoglobin (*pMtlb1*) genes were used with the aim to limiting *Cyp15a* expression in a regulated fashion in the nodule. The expression of *ENOD12* is normally limited to the invasion zone, whereas leghaemoglobin is expressed in the nitrogen-fixing zone.

Sense and antisense constructs of the partial *MsCyp15a* coding sequence, under the control of *ENOD12* and leghaemoglobin promoters, were introduced by transformation from *Agrobacterium tumefaciens* into *M. truncatula*. No transformed regenerants were obtained with either of the sense constructs. Although antisense expression of the partial *MsCyp15a* coding sequence was driven by nodule-specific promoters, the transformed regenerants displayed systemic effects on gene expression, suggesting that *Cyp15a* suppression effects may have occurred outside of the nodule.

3.3. Impaired germination in antisense lines

When compared to untransformed lines or lines carrying the promoter::*uidA* construct, seed germination was significantly impaired in the Lb-AS lines and to a lesser extent also in the *ENOD12*-AS lines (Fig. 2C). This impairment was found to be associated with reduced levels of transcription of the endogenous gene, *MtCyp15a*. A comparative RT-PCR analysis of 24 h imbibed seed gave a detectable PCR product after 20 cycles for untransformed lines, whereas 40 PCR cycles were required for AS lines to produce a detectable transcript (data not shown). Furthermore, reduced levels of CYP15A protein antigen were also detected in the extracts from leaves of AS transformants (Fig. 2D).

Interestingly, application of gibberellic acid (GA_3) significantly improved the germination rate of AS lines (Table 2). However, the transcript levels of *MtCyp15a* remained low in the AS lines, even after application of GA_3 (data not shown). Furthermore, GA_3 treatment of untransformed seed for 48 h did not significantly affect the level of CYP15A protein antigen detectable by western blotting. In

Table 2
Effect of gibberellic acid on seed germination of antisense lines^a

Transgene	% germination	
	H ₂ O	GA ₃
None	>95	>95
<i>pPsCyp15/uidA</i>	>95	>95
<i>pLb/antisense</i>	40	85
<i>pENOD12/antisense</i>	65	100

^a Mean of 30 seeds for Wt and GUS and for pooled LbAS lines 7–11 (T_3 generation); mean of 20 seeds for *ENODAS* line 2 (T_3 generation).

Table 3

GUS activity in imbibed seeds of transgenic *Medicago truncatula* containing the *pPsCyp15a::uidA* construct as affected by treatment with 1 μ M gibberellic acid

Treatment	GUS activity pmol/min/mg protein
Control	35.5 \pm 2.2
Gibberellic acid (1 μ M)	54.5 \pm 4.7

order to see whether *Cyp15a* was induced by GA_3 treatment, the transformants with *uidA* driven by the *Cyp15a* promoter were treated with 1 μ M GA_3 for 48 h. A 50% increase in GUS activity was observed, which is much lower than the induction with mannitol and NaCl (Table 3). These results suggest that GA_3 does not enhance germination by directly affecting the activity of *MtCyp15a*.

3.4. Abnormal vegetative growth in antisense lines

Viable regenerants of LbAS and *ENOD12AS* lines (T_1 generation), showed severely abnormal vegetative growth when grown under low light intensity in the growth cabinets. The abnormalities were more pronounced in the leghaemoglobin AS lines. Some of these LbAS lines collapsed and died after growth to the six-leaf stage while other lines, LbAS-5 for example, developed into super-leafy plants with reduced fertility (Fig. 2E). However, the survival rate of AS lines improved significantly when the plants were grown in greenhouses under better lighting conditions. Moreover, almost all of the antisense lines that survived showed delayed senescence in comparison to the wild type plants (Fig. 2F–H).

3.5. Cytological and antigenic analysis of the nodules

Sections of nodules from transformed lines were examined following staining with toluidine blue (Fig. 3A–D). In *ENOD12AS* lines, mature bacteroids were confined to the base of the nodules, compared to the wild type nodule (Fig. 3A and B). The gene suppressing effect was more pronounced in the LbAS lines. The effects ranged from nodules with larger vacuoles and less bacteroidal region to abnormal nodules, which were degenerate with poor colonization of host cells (Fig. 3C and D).

Tissue localization of CYP15A antigen was examined by using immunopurified R79 (or pre-immune serum) and fluorescent conjugates of secondary antibodies, to immunolabel nodule sections for laser scanning confocal microscopy. In untransformed lines the distribution of the antigen corresponded to the pattern observed with the GUS construct. Intracellular analysis revealed that antigenicity was localized mainly in large vacuolar bodies and to a smaller extent in cytoplasmic vesicles (Fig. 3E). Sections treated with pre-immune serum were not labeled. However, in host cells of nodules from the AS lines, antigen was significantly reduced. (Fig. 3F and G). Host cells of nodules

from AS lines appeared to have fewer bacteroids and a large central vacuole compared to untransformed lines. It seemed probable that the level of nitrogen fixation was severely impaired, but this could not be tested because insufficient plant material was available. However, these plants were able to grow in medium lacking nitrogen and therefore presumably they were capable of nitrogen fixation to some extent.

3.6. Localization of Cyp15a antigen in the imbibed seeds

Because the AS lines were impaired in seed germination (Table 2, Fig. 2C), the localization of CYP15A antigen was examined in imbibed seeds (Fig. 3H–M). Antigen expression was intense in a small number of cells in the embryos and in adjacent cotyledonary cells in the untransformed lines. At the cellular level the antigenicity was localized in the protein storage vacuoles (PSVs) and cytoplasm. (Fig. 3H) with reduced expression in the ENOD-AS and LbAS lines (Fig. 3I,J). The CYP15A protein distribution was also severely reduced in the embryonic radicle tips (Fig. 3K–M). The antigenicity was localized to several cell layers in the tissue at the radicle tips in untransformed lines (Fig. 3K), whereas, in the ENOD-12 AS line the expression was restricted to a few cells at the tip (Fig. 3L). In the LbAS line the expression of the antigen was almost completely suppressed (Fig. 3M).

4. Discussion

To analyse *Cyp15a* gene functioning and its localization, transgenic *Medicago truncatula* plants were generated. Transgenic plants containing the *uidA* reporter gene under the control of the promoter region of *PsCyp15a* revealed expression of GUS activity in cotyledonary leaves, senescent leaves, nodules and root tips (Fig. 2A), indicating involvement of this gene in tissue undergoing differentiation as well as in tissues undergoing senescence. Earlier, Vincent et al. [14] reported that GUS staining from the *PsCyp15a* promoter was observed during the tissue differentiation region in the embryonic callus of *M. truncatula*. To date, a number of sequences homologous to Cyp15a have been reported in the literature or sequence databases, but their proposed physiological functions show a somewhat varied profile including germination, stress response, leaf development and leaf senescence [14]. Promoter-*uidA* activity in the nodules was observed in the whole nitrogen-fixing zone and in the cortex (Fig. 2B). Earlier reports from this lab have indicated a consistent involvement of PsCyp5a during nodule organogenesis and maturation in *Vicia hirsuta* nodules. A strong expression was observed in the meristematic apex [14], however in *Medicago truncatula*, we have observed a relatively weaker expression in the meristematic regions like nodule apex and root tips (Fig. 2A and B). In

nodules, increased cysteine protease activity has been shown in early senescing *Phaseolus* nodules [33]. Another cysteine protease associated with nodule development and senescence has been reported in Chinese milk vetch [15].

A seven- and four-fold increase in GUS activity was observed with 0.6 mM mannitol and 75 mM NaCl (respectively), confirming the hypothesis that Cyp15a activity is required as a part of the osmoregulatory response of plants. Previously, this gene was shown to be strongly expressed in wilted pea shoots [18,8]. Cyp15a is most closely related to several stress-inducible proteinases, such as RD19 a drought inducible protease from *Arabidopsis* [9], CPR2 a germination-induced protease, which has also been shown to be induced by drought in vegetative organs of vetch [3], A1494 from *Arabidopsis* (X74359) [34] and bcp15 a cysteine protease from *Brassica napus* [35].

Antisense lines with nodulin promoter ENOD12 were generated to direct MtCyp15a gene silencing in the nodule apex. However, in vitro hybridization and transgenic promoter fusion analysis with ENOD12 sequences in the pea have indicated expression in stems, floral organs and root tips [36,37] thus indicating areas of ENOD12 activity outside of nodule development. The second promoter MtLb1 was chosen to regulate inhibition of MtCyp15a expression in the central zones of the nodules. Expression of MtLb1 occurs mainly in the central infected tissue as this gene encodes the leghemoglobin protein, although expression analysis of haemoglobin genes has shown that they are expressed in diverse plant tissues, such as leaves, stems and roots [38,39].

In the present investigation, the effects of the antisense constructs were also encountered outside the nodule tissue. The phenotypic effects in the T₁ generation of transformed plants ranged from slow-growing and fatal phenotypes to leafy and late senescing types (Fig. 2E–H). These results indicate a role for Cyp15a initially in plant growth and development and later on in senescence.

Seed germination from antisense lines was significantly impaired compared to untransformed and *uidA* lines (Fig. 2C Table 2). Cysteine proteases (CPRs) have been reported to be responsible for endoproteolytic cleavage during storage protein mobilization in cotyledons of most of the dicotyledonous seeds so far investigated [40,41]. Six cysteine proteases have been reported in legume seed germination [4], of which four are papain-like cysteine proteases.

The closest homologue to PsCyp15a is CPR2 (EMBL 230338), which shows 87% identity at the protein level. This is localized in protein bodies of *Vicia sativa* cotyledons and the embryonic axis. The protease is apparently involved in catalyzing storage globulin mobilization in the embryonic axis [4,5,42,43].

Among the other close homologues reported at the germination stage is MCP. This is localized in ER membrane and vacuoles of *V. mungo* seedlings and functions in post-translational cleavage during the C-

terminal processing of pro-SHEP and in degradation of storage proteins in PSV [19]. Another homologue CCP1 from ripened seeds of corn functions in housekeeping and decomposition of zein during germination [44], and GMCP3 from soybean functions in vacuolar turnover of proteins and enzymes in the diverse metabolic events of the developing cotyledons [45].

Immunocytological analysis of imbibed seeds revealed that antigen expression was intense in the embryo axis and adjacent cotyledonary cells and in a few layers at the apex of the radicle. There was reduced expression in the ENO-D12AS and LbAS lines. Intracellular localization of Cyp15A antigen was in the cytoplasm and protein storage vacuole (PSV) (Fig. 3H) confirming that this protease is involved in degradation of storage proteins accumulated in cotyledonary cells adjacent to the embryo axis. Antisense lines showed significantly less antigenicity of Cyp15A in PSVs (Fig. 3I and J) and could be the cause of decreased germination percentage. This was further confirmed by RT PCR studies where reduced levels of transcription of the endogenous gene were observed in the AS lines as compared to wild-type. One of the most important post-germination events in the majority of dicotyledonous seeds is the mobilization of storage proteins contained within the protein storage vacuole.

Another close homologue MCP has recently been localized in the ER and PSV as an enzymatically active mature form by immunocytochemistry. Subcellular fractionation of cotyledonary cells suggested that the protease was localized in both the ER and protein storage vacuole (PSV) as an active mature form. This is suggested to play a role in the early stage of enzymic degradation of storage proteins [19].

Application of 1 μ M GA₃ could restore the germination efficiency of AS lines. However, RT PCR and western blot analysis showed that GA₃ did not significantly affect the transcript and protein antigen levels. This suggests that GA₃ could be promoting the activity of some other cysteine protease. Although a 50% increase in GUS activity was observed following treatment with 1 μ M GA₃ for 48 h (Table 3), this was a much lower induction than with mannitol [seven-fold] or with NaCl [four-fold]. It may be that the affect of GA₃ could be an indirect effect or a combined induction of a number of cysteine proteases of which Cyp15a may be a part. Although some cysteine proteases have been reported to be induced by GA₃ [46], Jones and Mullet [8] observed that Cyp15a could only be induced to a small extent by GA₃. The antagonism between cytokinins and GA signaling and cysteine protease/senescence in plants has been reported recently [47].

The cellular and intercellular localization of Cyp15A was studied in nodules of transformed and untransformed lines to determine its role in nodule organogenesis and function. Nodulation in the antisense lines was affected adversely. Cytological analysis with toluidine blue staining of sections,

revealed that the effects ranged from nodule infected cells with larger vacuoles and a reduced bacteroidal region to abnormal nodules which were degenerate with poor colonization of host cells (Fig. 3A–D).

The use of laser scanning confocal microscopy further confirmed these observations. The antigenicity was localized mainly in large vacuolar bodies and to a smaller extent in cytoplasmic vesicles. The antisense lines showed reduced levels of the antigen with more pronounced effects in the LbAS lines. The N₂-fixing activity could not be tested due to an insufficient number of nodules in the antisense lines. However, it was observed that the plants could survive in a medium lacking nitrogen, suggesting that the bacteroids present were effective in nitrogen fixation. These results suggest a role for Cyp15a in nodule organogenesis as well as in nodule functioning.

In legume root nodules, there is evidence that cysteine proteases could be involved in nodule function, in the adaptation of host cells to physiological stresses and in the control of nodule senescence [17]. Cysteine proteases induced during nodulation include: PsCyp1 and PsCyp15a from pea (12); Ag NOD-CP1 from *A. glutinosa* [11]; and AsNOD f 32 from Chinese milk vetch [15]. Furthermore, a possible orthologue of Cyp15a (Swiss Prot accession no. P25804) has recently been identified as a component of isolated symbiosomal membranes from soybean nodules [16]. This provides further evidence that this class of protease is widespread in legume nodules and that it is associated with the symbiosome compartment.

5. Conclusion

These data provide preliminary evidence that Cyp15a encodes a gene that has a role in germination and stress adaptation and a further specialized role in nodule organogenesis and function.

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