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The Expression of Genes Encoding Secreted Proteins in *Medicago truncatula* A17 Inoculated Roots

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Subtilisin-like serine protease (MtSBT), serine carboxypeptidase (MtSCP), MtN5, non-specific lipid transfer protein (MtnsLTP), early nodulin2-like protein (MtENOD2-like), FAD-binding domain containing protein (MtFAD-BP1), and rhicadhesin receptor protein (MtRHRE1) were among 34 proteins found in the supernatant of *M. truncatula* 2HA and *sickle* cell suspension cultures. This study investigated the expression of genes encoding those proteins in roots and developing nodules. Two methods were used: quantitative real time RT-PCR and gene expression analysis (with promoter:*GUS* fusion) in roots. Those proteins are predicted as secreted proteins which is indirectly supported by the findings that promoter:*GUS* fusions of six of the seven genes encoding secreted proteins were strongly expressed in the vascular bundle of transgenic hairy roots. All six genes have expressed in 14-day old nodule. The expression levels of the selected seven genes were quantified in *Sinorhizobium*-inoculated and control plants using quantitative real time RT-PCR. In conclusion, among seven genes encoding secreted proteins analyzed, the expression level of only one gene, *MtN5*, was up-regulated significantly in inoculated root segments compared to controls. The expression of *MtSBT1*, *MtSCP1*, *MtnsLTP*, *MtFAD-BP1*, *MtRHRE1* and *MtN5* were higher in root tip than in other tissues examined.

Keywords: secreted proteins, *Medicago truncatula*, root, nodules, RT-PCR, gene expression

INTRODUCTION

To adapt to low nitrogen availability in soil, some plants form a symbiosis with nitrogen fixing bacteria to provide a nitrogen source for the plants. This symbiosis between legumes and rhizobia give rise to a completely new organ called nodule. Nodules (and lateral roots) are initiated from differentiated root cells that become meristematic. In nodules, those meristematic cells will differentiate into different nodule tissues. Nodule formation involves signal exchange and recognition, cell differentiation and cell division that eventually lead to the formation of a completely new organ.

The symbiosis between legume plants and rhizobia involves various physiological processes performed by plants to adapt to their changing environment. This includes signal exchange and recognition, regulation of defence responses and re-initiation of cell divisions that eventually lead to the formation of a completely new organ, a root nodule (Oldroyd & Downie 2008). The symbiotic interaction shows a high degree of host-rhizobia specificity. It is started when roots of host plants secrete flavonoids or isoflavonoids that attract rhizobia to colonize the rhizosphere and induce the rhizobia nodulation (nod) genes (Gulash *et al*. 1984; Peters *et al*. 1986; Redmond *et al*. 1986; Kape *et al*. 1991). Each rhizobial species is activated by a particular set of flavonoids. The induction of nod genes results in the secretion of the nodulation factors (Nod factors), lipo-chitin oligosaccharide molecules with variable structures. Different rhizobia produce different Nod factors that determine the host range of the bacteria (Roche *et al*. 1991; Ehrhardt *et al*. 1995; Lorquin *et al*. 1997). Once the rhizobia attach themselves to the root hairs, the Nod factors induce root hair curling and further distortion into a 'shepherd's crook'. Within the pocket which has been formed by the curled hair, bacterial intrusion takes place and is facilitated by the development of an infection thread (Gage & Margolin 2000). Nod factors also induce cortical cell division of host plant, coinciding with the induction of several specific genes in early nodulation (early nodulin genes) (Bisseling *et al*. 1990).

Nodule development is affected by phytohormones (Scheres *et al*. 1992; Nukui *et al*. 2000; Wasson *et al*. 2006), defence response of the host plant (Niehaus

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et al. 1993; Perotto *et al*. 1994; Martínez-Abarca *et al*. 1998) and the physiological condition of the host plant (Streeter & Wong 1988; Caetano-Anolles & Gresshoff 1991).

Generally, secreted proteins are defined as proteins that are transported intracellularly through the endomembrane system and then delivered to the extracellular environment by means of secretory vesicles that fuse with the cell membrane (Jones & Robinson 1989). In the extracellular environment, secreted proteins can diffuse away and some are freely soluble in the apoplastic space (apoplastic proteins). They also can become attached to the cell wall or become incorporated into the cell membrane where they act as membrane transporters or ion channels, ligand receptors and signalling complexes, or contact points for intracellular cytoskeletal or for the extracellular matrix (Greenbaum *et al*. 2001; Sanderfoot & Raikhel 2003; Lee *et al*. 2004).

Previous studies have revealed that secreted proteins are involved in pathogenic stresses (Rep *et al*. 2002; Grunwald *et al*. 2003; Ndimba *et al*. 2003; Hugot *et al*. 2004), environmental stresses (Fecht-Christoffers *et al*. 2003), cell-cell recognition (Franklin-Tong & Franklin 2003) and development (Pearce *et al*. 2001; von Groll *et al*. 2002; Casamitjana-Martinez *et al*. 2003). In nodule development, physiological processes such as defense response, meristematic cell division and differentiation are taken place.

Examples of proteins involved in the maintenance of shoot apical meristem (SAM) are CLV1 (a receptor like kinase with a leucine rich repeat extracellular domain), CLV2 (a receptor like protein with leucine rich repeat extracellular domain but without the kinase cytoplasmic domain), CLV3 (a secreted protein that acts as a ligand for CLV1) (Clark *et al*. 1997; Jeong *et al*. 1999; Fletcher *et al*. 1999). Secreted protein phytosulfokine-á from *Asparagus officinalis* (Matsubayashi & Sakagami 1996) and *Oryza sativa* (Matsubayashi *et al*. 1997) is involved in promoting cell proliferation and inducing cell differentiation in *Zinnia elegans* (Matsubayashi *et al*. 1999). It is been known that genes encoding receptor-like kinase (RLK) protein, when mutated, cause excessive number of nodules. They are *har1* (from *Lotus japonicus*), *nts1* (from *Glycine max*), *sym29* (from *Pisum sativum*), and *sunn* (from *Medicago truncatula*) (Wopereis *et al*. 2000; Krusell *et al*. 2002; Nishimura *et al*. 2002; Searle *et al*. 2003; Schnabel *et al*. 2005). However, little is known about the potential roles and identities of secreted proteins during the *Rhizobium*-legume symbiosis. Proteome analysis of soybean xylem sap identified a xyloglucan transendoglycosyl transferase was upregulated 8 h after inoculation with *Bradyrhizobium japonicum*. However, silencing of the gene encoding this secreted protein did not result in changes in nodulation (Subramanian *et al*. 2009). Overall, it is likely that secreted proteins play a role in the regulation of meristem initiation and control of defense responses during nodulation.

Here, we used quantitative real time RT-PCR to study the expression level of genes encoding several secreted proteins: subtilisin-like serine protease (*MtSBT*), serine carboxypeptidase (*MtSCP*), *MtN5*, non-specific lipid transfer protein (*MtnsLTP*), early nodulin2-like protein (*MtENOD2-like*), FAD-binding domain containing protein (*MtFAD-BP1*), and rhicadhesin receptor protein (*MtRHRE1*), in *M. truncatula* roots and nodules. Their expression in roots and nodules were also compared to other organs (leaves and cotyledons). Previously, quantitative real time RT-PCR has been used to study the expression level of genes (Prayitno *et al*. 2006a; Holmes *et al*. 2008). Further, this study also studied the expression of those genes in root system and nodules using *GUS* reporter gene assay.

MATERIALS AND METHODS

All chemicals were from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Deionized water 18.2 MÙ/cm ("MQ water") was used throughout the study.

***Medicago truncatula* A17 growth.** *Medicago trucatula* A17 seeds were grown as described in Prayitno *et al*. (2006b). *M. truncatula* seeds were scarified and then surface sterilized with 6.25% (v/v) sodium hypochlorite for 15 minutes. After seven washes with sterile water, seeds were spread on nitrogen-free Fåhraeus agar media (Fåhraeus 1957) containing 0.8% agar (Agar Grade J3, Gelita Pty Ltd., Beaudesert, Queensland, Australia). Seeds were incubated in the dark at 4 ^{o}C for one day to break their dormancy and were then germinated by incubating the plate upside down in the dark at 28 ^{o}C overnight, so that emerging roots grew straight into the air. Seedlings with similar root length were selected and transferred to 15 cm diameter Petri dishes containing nitrogen-free Fåhraeus agar media and sealed ¾ around with parafilm. The seedlings were incubated vertically in the growth chamber with a photon flux density of 100 µmol m^{-2} s^{-1} for 16 h at 20 ^{o}C for four days. To reduce light intensity around the roots, pieces of thick-dark paper were placed in the lower half of the plate between the plates. Under these conditions, seedlings grew on the media

surface, and were easy to inoculate and to observe under sterile conditions. After four days incubation, the seedlings were flood-inoculated at 1 cm above the root tip with 5 µL of diluted *Sinorhizobium meliloti* strain 1021 culture (at OD_{600} of 0.1). Two weeks after inoculation, tissue samples were taken from leaves, cotyledon, root segments, and root tips.

***Agrobacterium rhizogenes*–Mediated Hairy Root Transformation.** *Medicago trucatula* A17 seeds were scarified and surface-sterilised with 6.25% (v/v) sodium hypochlorite for 15 minutes. After seven washes with sterile water, seeds were grown as described in Prayitno *et al*. (2006b). The transformation of *M. truncatula* with *A. rhizogenes* was done as described in Boisson-Dernier *et al*. (2001).

Bacterial Growth. *Sinorhizobium meliloti* strain 1021 was grown in Bergensen's Modified Medium (BMM) agar media (Rolfe *et al*. 1980) for 2 days. A colony of bacteria was then transferred to BMM liquid medium and grown at 28 °C overnight. The culture was then diluted with sterile water to an OD_{600} of 0.1 for inoculation of plants. *Agrobacterium rhizogenes* Arqua1 strain (Boisson-Dernier *et al*. 2001) was maintained on Luria-Bertani (LB) agar plates containing 100 µg mL^{-1} streptomycin.

DNA Isolation. Genomic DNA was isolated using a PUREGENE™ DNA purification kit according to the manufacturer's instructions (Gentra System, Minneapolis, Minn, USA).

Primer Design Promoter Primers. The open reading frame from Softberry was blasted to the University of Oklahoma Medicago truncatula genome blast server (http://www.genome.ou.edu/medicago_blast.html) using blastn to get the contig number (for example mtgsp_008f03.Contig1 for FAD). BAC sequences were downloaded from the University of Oklahoma (ftp://ftp.genome.ou.edu/pub/medicago) and the previously obtained contig number was used to get the complete sequence of the relevant gene. At least two Kb of upstream sequence from the start codon was selected and primers were designed using the online application from Integrated DNA Technologies (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx). Primers were chosen to have a GC content between 40% to 60%, melting temperature above 55 °C and a minimum chance to allow a self dimer of < 8 to form. The oligos were also checked to make sure that they do not have a strong hairpin structure. Primers used for promoter cloning were: SUBT_Promf 5´-CACCCGCTGAGTTAAGACAAGGTTTGTG-3´, SUBT_Promr 5´-TCTCACCCTCTTGATTTTGGAATGAG-3´, FAD_Promf 5´-CACCTCTATAAGTTGGGCGAGGGATA-3´, FAD_Promr 5´-GATTTTAGTTTGTGTTGTGTTTGAGTTTGT-3´, Serine_Promf 5´-CACCAGTTGAAGGAATGGCCGG-3´, Serine_Promr 5´-TGCTGAAGAGGAAGCAGAAAC-3´, nsLTP_Promf 5´-CACCTGCGTATGCGGGAGCTT-3´, nsLTP_Promr 5´-TCACCAATAATTTTGTTGTACTTCAATAAT-3´, MtN5_Promf 5´-CACCGCGAGCCAACATCAACTAATTTTTC-3´, MtN5_Promr 5´-ACTCCTAATTAATTAACCAAATGCTTAGCT-3´, Enod2_Promf 5´-CACCAAGTTCATCAAAAGCATGTCATCAGA-3´, Enod2_Promr 5´-TGTTAATTGCTAAAATATTTAGAGAAACAA-3´, Rhic_Promf 5´-CACCGCCAAAGACGCCCATAATGATC-3´, Rhic_Promr 5´-GTTAAGATCGGTGACAAACAAATTAACC-3´

Quantitative Real Time RT-PCR Primers. Gene-specific primers were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA) and synthesized by GeneWorks (Adelaide, Australia). Those primer were MtSBT1_F 5´-GCTTCAACGATGCTGGGTTT-3´, MtSBT1_R 5´-CTGATTCACACGCGCCTTT-3´, MtSCP1_F 5´-CCAACTTGCACAACTCATTGTTC-3´, MtSCP1_R 5´-TCCCTATTGCAATTCCCTTGA-3´, MtN5_F 5´-AGGGCAGATGAGGCTTGCT-3´, MtN5_R 5´-CGCAGAGGCAAGGAAGATTG-3´, MtnsLTP_F 5´-TTTGCTGCTCGGCTATTGC-3´, MtnsLTP_R 5´-GCAATCCTGAATCCTTATAACGACAT-3´, MtENOD2-like_F 5´-CAAACCCAATCAAGAAGTTCGAA-3´, MtENOD2-like_R 5´-GGCCCTGCTCTGTCCAAAG-3´, MtFAD-BP1_F 5´-GGCATCGGTGGCCTCAT-3´, MtFAD-BP1_R 5´-CCAAGGCCATACTTTCTCATCAT-3´, MtRHRE1_F 5´-TCATTGGCACGTATTGACTATGC-3´, MtRHRE1_R 5´-TGCACGTGGGTGAGTGTGA-3´

Polymerase Chain Reactions. Polymerase Chain Reactions (PCR) were performed in Thermo Hybaid PCR Express Thermal Cycler (Fisher Scientific, Pittsburgh, PA, USA) using Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes, Finland). The initial denaturations were carried out at 98 °C for 30 seconds followed by a total of 35 cycles as follows: denaturation at 98 °C for 10 seconds, annealing between 55 °C and 65 °C for 30 seconds, and extension at 72 °C for 1 min/kb. The final extension was carried out at 72 °C for 5 minutes.

Gateway Cloning. The GATEWAY™ Cloning Technology was used to generate expression vectors incorporating entry vectors. PCR products were cloned into an entry vectors using pENTR™ Directional TOPO Cloning Kit (Invitrogen, Carlsbad, CA, USA) to create a Gateway® entry clones. TOPO

Cloning reactions were transformed into Premade Z-Competent™ *E. coli* cells (Zymo Research, CA, USA) following the manufacturer's instructions. Plasmid DNA (entry clones) were isolated using QIAprep Spin Miniprep kit (QIAGEN, Melbourne, Australia) and entry clones were confirmed by PCR and gel electrophoresis.

The sequences of the entry constructs were confirmed by DNA sequencing at the Biomolecular Resource Facility, The John Curtin School of Medical Research, the Australian National University. Subsequently, correct sequences of interest were transferred from the entry clones into Gateway® destination vectors to generate expression clones by performing the LR recombination reaction using Gateway® LR Clonase™ II enzyme mix (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

LR reaction mixtures were transformed into Premade Z-Competent™ *E. coli* cells (Zymo Research, CA, USA) as described in the manufacturer's protocol and the cells were grown overnight on LB plates containing 50 μg mL^{-1} spectinomycin and 100 μg mL^{-1} streptomycin. Putative positive clones (transformed *E. coli* containing expression clones) were cultured overnight in liquid LB containing the same antibiotics. Plasmid DNA (expression clones) were isolated using QIAprep Spin Miniprep kit (QIAGEN, Melbourne, Australia) and expression clones were confirmed by PCR and gel electrophoresis. One μg of expression clones DNA was transformed into 100 μL *Agrobacterium rhizogenes* ARqua1 strain (Boisson-Dernier *et al*. 2001) using the freeze-thaw transformation method (Höfgen & Willmitzer 1988). Transformants were selected by resistance to spectinomycin and streptomycin.

β-Glucuronidase (GUS) Staining. GUS activity was localized in whole transgenic root of *M. truncatula* following the protocol described previously (Vitha *et al.* 1995).

Statistical Analysis. Student's *t* test was performed using Microsoft Excel 2003.

Gene Expression Analysis. The expression levels of the seven selected genes were examined by using relative transcript expression which was obtained from quantitative real-time RT-PCR. The examined tissues were root segments (approximately 2 cm segments where nodules usually formed), root tips (0.5 cm from the tip), leaves and cotyledons. Each tissue was divided into two groups, i.e. non-inoculated (control) and inoculated with *Sinorhizobium liloti*. Samples were harvested 14-day post inoculation with consideration that it is quite difficult to differentiate nodules and lateral root primordial before 14 day. Quantitative real-time RT-PCR data was analyzed using Student's *t* test.

RESULTS

Gene Expression Analysis. As shown in Figure 1, the expression level of subtilisin-like serine protease (*MtSBT1*), serine carboxypeptidase (*MtSCP1*), *MtN5*, non-specific lipid transfer protein (*MtnsLTP*), FAD-binding domain containing protein (*MtFAD-BP1*) and rhicadhesin receptor (*MtRHRE1*) were higher in root tips compared to its expression in other tissues (leaves, cotyledons and root segments). Only early nodulin 2-like (*MtENOD2-like*) was expressed higher in leaves than in roots. The high expression level of *MtN5* in root tips (Figure 1C) was also reported previously by Holmes *et al*. (2008). Interestingly, as shown by Figure 1C, *MtN5* expression was up-regulated 15-fold ($P < 0.05$) in inoculated root segments compared to control root segments (non-inoculated). There was no significant difference in *MtN5* expression between inoculated leaves, cotyledons, root tips and their control (non-inoculated) counterparts (Figure 1C). There were also no significant difference in the expression level of *MtSBT1*, *MtSCP1*, *MtnsLTP*, *MtFAD-BP1*, and *MtRHRE1* between control (non-inoculated) and inoculated group for any tissue examined.

The location of the expression of genes encoding six secreted proteins in roots was also examined using *A. rhizogenes*-mediated hairy root transformation. Six promoters were successfully isolated and cloned while the promoter of *MtN5* could not be isolated successfully after several attempts. Therefore, the location of *MtN5* expression in roots and nodules could not be reported here.

In transformed roots, MtSBT1:*GUS* transgenic roots showed strong expression in the vascular bundle and root apex (Figure 2A). At different stages of lateral root development, MtSBT1:*GUS* was expressed stronger in the zone where lateral roots are initiated (Figure 2A). In nodules, it was expressed in all areas of the nodule but stronger in the vascular bundle, apex and basal nodule region (Figure 3A). Figure 2B demonstrates the expression of MtSCP1:*GUS* in transgenic roots, which showed expression in the vascular bundle, in both roots and nodules. It was also expressed strongly in the apex zone of root tips and where lateral roots were initiated, confirming the RT-PCR data that *MtSCP1* was expressed higher in root tips than other tissues.

In transformed roots, MtnsLTP:*GUS* did not seem to be expressed in the main root, except in the pex

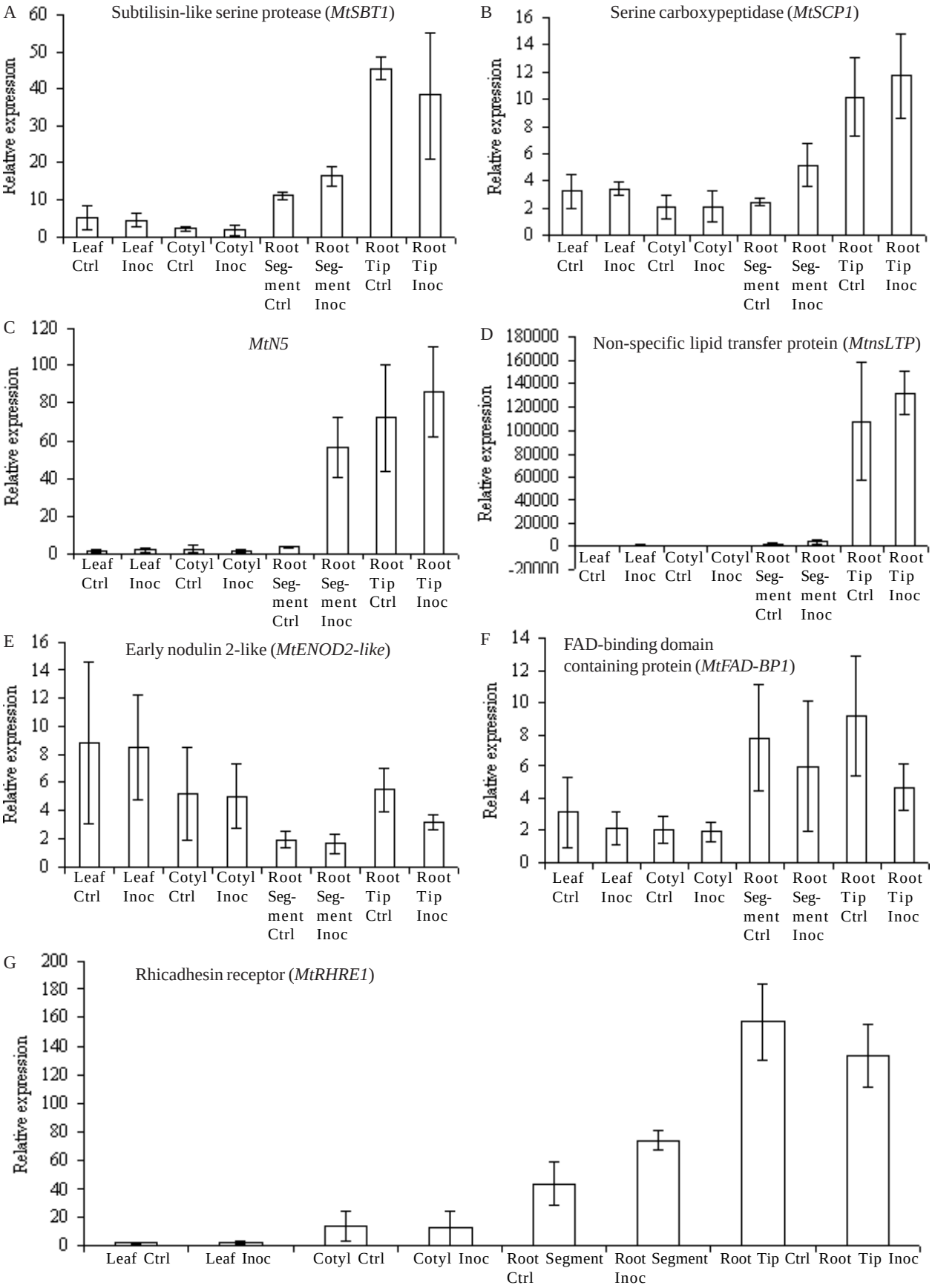

Figure 1. The expression level of genes encoding seven predicted secreted proteins. Gene expression level of (A) *MtSBT1*, (B) *MtSCP1*, (C) *MtN5*, (D) *MtnsLTP*, (E) *MtENOD2-like*, (F) *MtFAD-BP1*, (G) *MtRHRE1*. Relative transcript expression is obtained by mean of real-time RT-PCR. Sample used was *Medicago truncatula* A17 which was divided into two sample groups, i.e. control – non-inoculated plants (Ctrl) and 14 day post inoculated plants (Inoc). Each sample group presents leaf, cotyledon (Cotyl), root segment and root tip. Error bars show standard deviations.

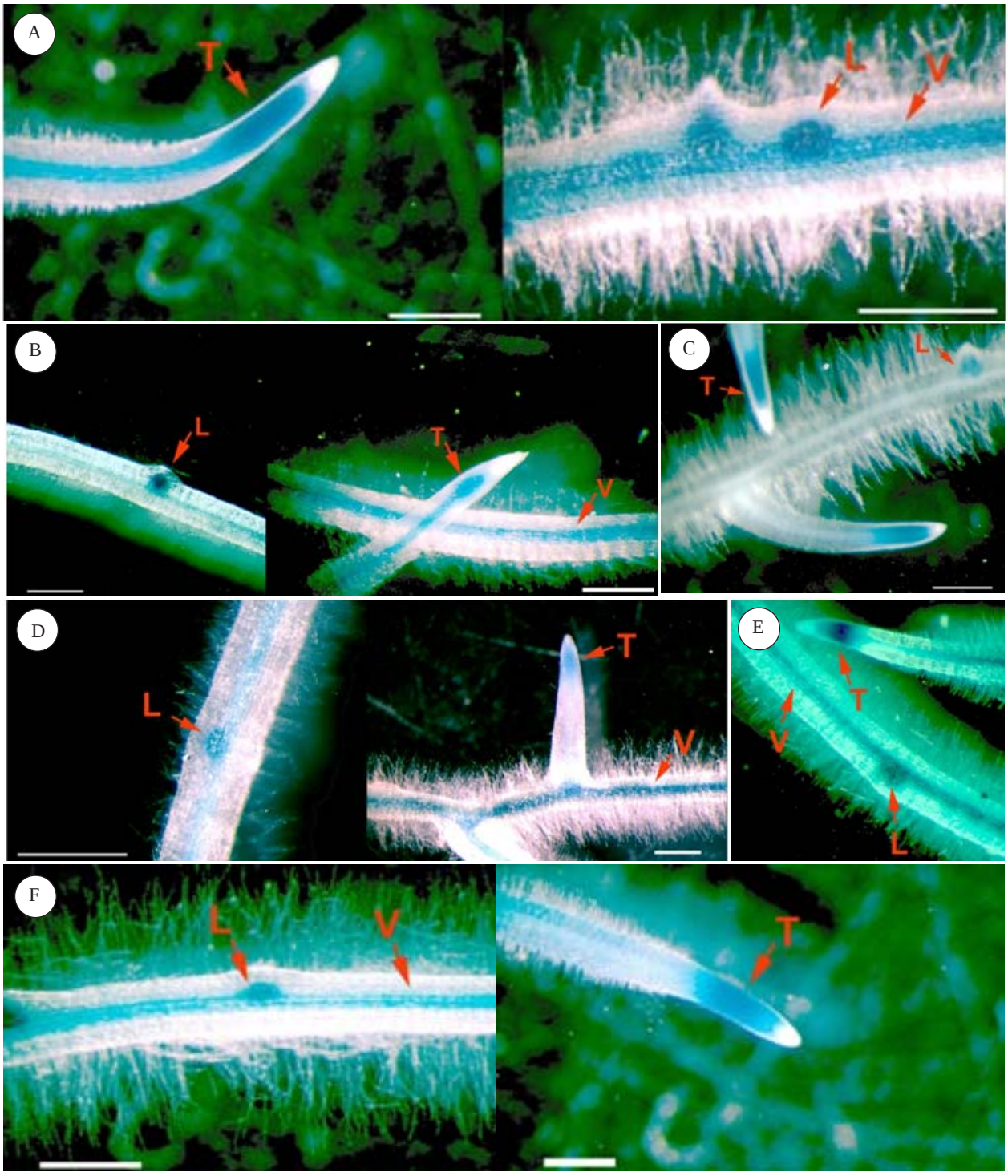

Figure 2. *GUS* reporter gene assay for transgenic root of *M. truncatula* A17. The expression of GUS reporter gene in transgenic root driven by promoter of (A) *MtSBT1*, (B) *MtSCP1*, (C) *MtnsLTP*, (D) *MtENOD2-like*, (E) *MtFAD-BP1*, (F) *MtRHRE1*. *GUS* expression was observed in different stages of lateral root development (L), root tip (T), and vascular bundle (V). White bars represent 0.5 mm.

zone and the first centimeter of root tip (Figure 2C). Nonetheless, its expression in some of the lateral roots was detected, not limited to the vascular bundle. During different developmental stages of the lateral root, MtnsLTP:*GUS* expression was localized in the apex of root tip and where lateral roots were initiated (Figure 2C). In inoculated roots, MtnsLTP:GUS was expressed in the vascular bundle and in the entire nodule (Figure 3C). Its expression was stronger in apex. In general, there was a parallel between RT-PCR data and MtnsLTP:*GUS* expression localization. RT-PCR data showed that *MtnsLTP* expressed the highest in root tips (Figure 1D) and MtnsLTP:*GUS* showed a high expression in root apex and the first centimetre from the tip (Figure 2C).

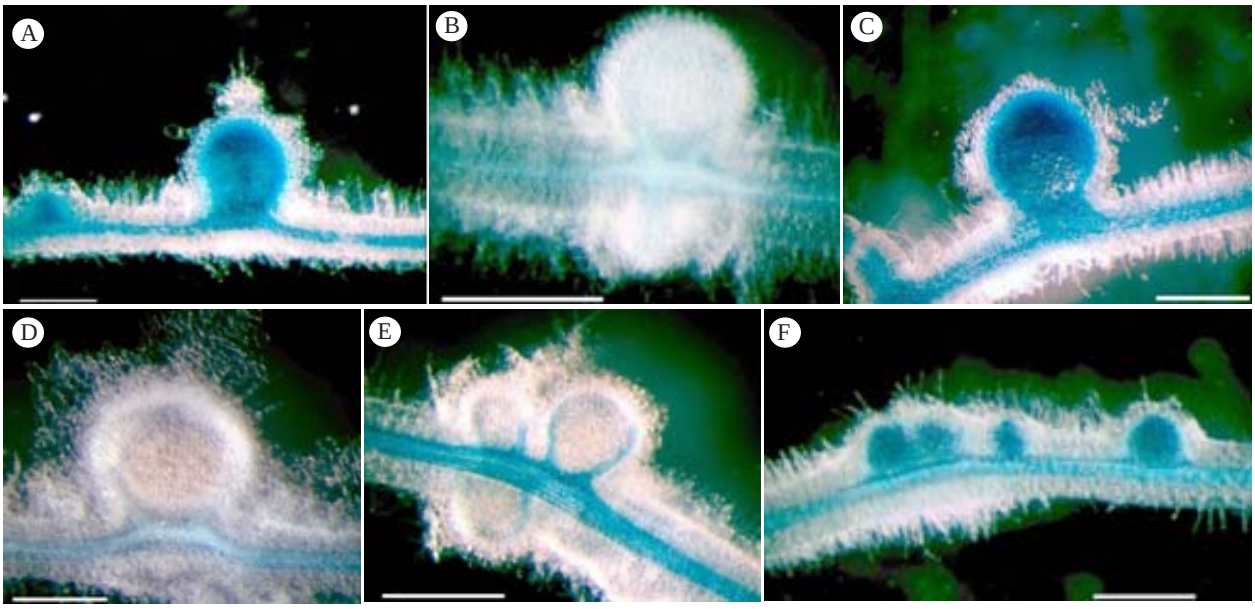

Figure 3. GUS staining of nodules formed in transgenic roots expressing promoter:*GUS* construct. The expression of GUS reporter gene in transgenic root driven by promoter of (A) *MtSBT1*, (B) *MtSCP1*, (C) *MtnsLTP*, (D) *MtENOD2-like*, (E) *MtFAD-BP1*, (F) *MtRHRE1*. White bars represent 0.5 mm.

In transformed roots, MtENOD2-like:*GUS* expression was mainly in the vascular tissue (Figure 2D). It was also expressed in the apex, either in the root tip or in the location where lateral root primordial is initiated. In 14-day old nodules, it was expressed in apex and vascular tissue (Figure 3D). RT-PCR data showed that *MtENOD2-like* was expressed higher in aerial organs than in root segments and inoculated root segments (Figure 1E). MtENOD2-like:*GUS* expression in transformed roots also showed a relatively low expression in 14-day old nodules (Figure 3D).

As can be seen in Figure 2E, *GUS* reporter gene (under MtFAD-BP1 promoter) was expressed strongly in vascular tissues and apex. During lateral root development, it was expressed in dividing cells where the growth was initiated and in vascular tissue. In nodules, it was expressed in vascular tissue, in the apex and base of the nodule (Figure 3E).

In Figure 2F, *GUS* reporter gene (under MtRHRE1 promoter) was expressed in vascular tissue and the root apex, either in root tips or in lateral root primordia. In 14-day old nodules, it was expressed in the entire nodule but stronger in nodule apex (Figure 3F).

DISCUSSION

The expression of selected genes encoding secreted proteins was studied using two different dataset, i.e. *GUS* expression in transformant roots and quantitative real time RT-PCR data.

GUS Expression in Transformant Roots. In all transgenic roots, *GUS* expression was detected in nodules (Figure 3). It was also detected in the dividing and enlarging cells where the lateral roots were initiated in root apex and in vascular bundle (Figure 2). The expression of *GUS* in actively proliferating cells suggests that these secreted proteins have role in cell division and growth. Further, the use of promoter:*GUS* constructs showed that all six promoters examined were strongly expressed in vascular bundles (Figure 2 & 3). They were *MtSBT1*, *MtSCP1*, *MtnsLTP*, *MtFAD-BP1*, *MtRHRE1*, and *MtENOD-like2*. Their strong expression in the vascular bundle suggests that they might be involved in vascular bundle development or function, and that the gene products would be released into apoplast on a continuous basis along the entire length of the vasculature.

It is interesting to note that transgenic roots carrying MtSBT1:*GUS*, MtnsLTP:*GUS*, and MtRHRE1:*GUS* were expressed strongly the whole nodule. Therefore, it is possible that those secreted proteins have a role in nodule development.

Expression Level of *MtSBT1, MtSCP1, MtN5, tnsLTP, MtFAD-BP1* and *MtRHRE1*. Using quantitative real time RT-PCR, six genes encoding secreted proteins examined showed higher expression in root tips than in leaves, cotyledons or root segments. These six genes were *MtSBT1*, *MtSCP1*, *MtN5*, *MtnsLTP*, *MtFAD-BP1* and *MtRHRE1*. Only *MtENOD2-like* showed lower expression in roots (either inoculated or non-inoculated) than in leaves and cotyledons (Figure 1).

Subtilisin-Like Serine Protease (*MtSBT1*) and Serine Carboxypeptidase (*MtSCP1*). In *Alnus glutinosa*, a subtilase, *Ag12*, was detected in nodules but not in roots, cotyledons, flowers and developing fruits (Ribeiro *et al*. 1995). In *Casuarina glauca*, *Cg12* (a *Ag12* homolog) was also expressed in nodules but not in roots, stems and leaves (Laplaze *et al*. 2000). Both were expressed stronger in the young infected cells of the infection zone, weaker in the fixation zone and no expression of *Ag12* and *Cg12* was detected in senescent infected cells (Ribeiro *et al*. 1995; Laplaze *et al*. 2000). Since young infected cells are active in synthesizing proteins, therefore, a stronger expression of *Cg12* and *Ag12* in the young infected cells suggests that *Cg12* and *Ag12* are involved in protein processing rather than proteolysis (Ribeiro *et al*. 1995; Laplaze *et al*. 2000). In this study, *MtSBT1* was expressed strongly in nodule apex, root apex and dividing cells. Therefore, it is possible that MtSBT1 may have a role in cell division and growth in *M. truncatula*.

In *A. thaliana*, brassinosteroid insensitive 1 (*BRI1*) encodes a leucine-rich repeat receptor serine/threonine kinase (Li & Chory 1997; Friedrichsen *et al*. 2000) cell surface receptor for brassinosteroids, a plant growth and development regulator (He *et al*. 2000). A suppressor of weak bri1 allele, *BRS1* (brassinosteroid insensitive 1 suppressor) was found by a gain-of-function genetic screen (Li *et al*. 2001). Using *35S:BRS1-GFP* transgenic plants, it was shown that *BRS1* (brassinosteroid insensitive 1) encodes a secreted serine carboxypeptidase (Zhou & Li 2005). Overexpression of *BRS1* in wild-type plants showed no obvious phenotype alteration while overexpression of *BRS1* in *bri1-5* and *bri1-9* mutants resulted in the partially restored phenotype caused by the point mutation in extracellular domain of *BRI1* by 52 and 75%, respectively (Noguchi *et al*. 1999; Friedrichsen *et al*. 2000; Li *et al*. 2001). In addition, overexpressing *BRS1* in a cytoplasmic kinase domain mutant, *bri1-1*, failed to restore the phenotype (Li *et al*. 2001). These observations suggest that BRS1, a secreted serine carboxypeptidase, involves in early events of BRI1 signaling in the apoplast.

In this study, transgenic roots expressing *GUS* under MtSCP1 promoter showed that, in mature nodules, *GUS* was expressed only in vascular bundle (Figure 3B), suggesting that MtSCP1 involved in vascular bundle development or function.

Early Nodulin 2-Like Protein *(MtENOD2-like*), FAD-Binding Domain Containing Protein (*MtFAD-BP1*) and Rhicadhesin Receptor (*MtRHRE1*). Previously, *ENOD2* is known as a nodulin gene which is expressed specifically in nodules (Gloudemans & Bisseling 1989), and is used as a molecular marker for the early stage of nodule formation. Previous studies on nodulins compared their expression in nodules to their expression in roots but not to their expression in other organs (Fuller *et al*. 1983; Govers *et al*. 1985; Lang-Unnasch & Ausubel 1985), or compared nodulin encoded proteins to *Rhizobium* encoded proteins (Fuller *et al*. 1983; Lang-Unnasch & Ausubel 1985). However, the expression of *ENOD2* has been detected in uninfected roots of *Lotus japonicus* (Szczyglowski *et al*. 1997), *Glomus intraradices* colonised *M. sativa* roots (van Rhijn *et al*. 1997) and in cytokinin-treated *M. sativa* roots (van Rhijn *et al*. 1997). Further, *ENOD2* expression was also detected in the parenchyma cells of alfalfa bacteria free-nodules (van de Wiel *et al*. 1990). Additionally, *ENOD2-like* expression was detected in the stems, flowers and roots of non-nodulating tree legumes, *Styphnolobium japonicum* and *Cladrastis kentukea* (Foster *et al*. 2000). Due to the growing evidence that what was first thought to be nodule specific genes are also expressed in other parts of different plants, it has been proposed that genes involved in nodulation, such as ENOD2, are evolved from genes involved in general physiological processes which are common to most plants (Gualtieri & Bisseling 2000).

In this study, it was shown by quantitative real time RT-PCR that *MtENOD2-like* was expressed higher in leaves and cotyledons than in roots. Therefore, it is possible that MtENOD2-like may not have a specific role in root growth.

In transgenic roots that express reporter gene under MtENOD2-like promoter, *GUS* was expressed in the vascular tissue, in the apex of root tips and in the place where lateral roots are initiated. In 14-day old nodule, *GUS* expression was detected in vascular bundle at the base of the nodule and a very weak expression was detected at the apex of the nodule. A similar pattern was shown by transgenic roots that express *GUS* reporter gene under *MtFAD-BP1* promoter. In those roots, *GUS* was expressed very strongly in vascular bundle, in the apex of root tips and in the lateral root primordium. In 14-day old nodule, *GUS* was expressed in the vascular bundle at the base of the nodule and also the apex of the nodule.

Three germin-like proteins in *A. thaliana* have been characterized to be associated with extracellular matrix (Membré *et al*. 2000). Similarly, rhicadhesin receptor examined in this study may also be associated with extracellular matrix. It was predicted as a secreted protein by bioinformatics tools and is

expressed in vascular bundle as shown by *GUS* reporter gene.

Rhicadhesin receptor from *Pisum sativum*, *PsGER1*, the first known plant germin-like protein to be associated with nodules, is expressed strongly in leaves, stem, roots and nodules, while its expression in flower is weak (Gucciardo *et al*. 2007). The proposed role of rhicadhesin receptor is to mediate the attachment of the bacteria to the plant surface (Swart *et al*. 1994). However, if rhicadhesin receptor (MtRHRE1) examined in this study functions to mediate the attachement of rhizobia to the plant surface, then it should be expressed in the root hairs and epidermis of the whole root, which was not the case. As seen in the Figure 3F, *GUS* expression was detected in the whole nodule and vascular bundle but not in the epidermis of the root, suggesting that MtRHRE1 might not function as attachment protein for rhizobia.

Lipid Transfer Proteins (MtN5 and MtnsLTP). In *P.sativum*, a lipid transfer protein was expressed in the region of expanding cells just proximal to the apical meristem and was expressed weakly in the epidermis (Gucciardo *et al*. 2007). A non-specific lipid transfer protein in *M. truncatula*, MtN5, is proposed to be involved more in nodule organogenesis than in nitrogen fixation for two reasons. First, it is expressed in uninfected Nar nodules (nodulation in the absence of *Rhizobium*) (Truchet *et al*. 1989). Second, its expression in early nodulation before nitrogen fixation takes place is stronger than its expression in mature nodules. In this study, promoter of *MtN5* could not be cloned and only transformant roots expressing *GUS* under *MtnsLTP* promoter were generated. Figure 3C showed that *GUS* was expressed in root vascular bundle and the whole body of 14-day old nodule with stronger expression in the apex zone. It is possible that MtnsLTP might have general role in nodule development.

Quantitative real time RT-PCR showed that *MtN5* and *MtnsLTP* (non-specific lipid transfer protein) were both expressed higher in roots and root tips than in aerial organs (Figure 1C & D). Moreover, *MtN5* expression was significantly (P value < 0.05) up-regulated (15-fold) in inoculated root segments relative to control root segment indicating that MtN5 may be involved in nodule development or host defense during nodulation.

Secreted Proteins are Expressed Stronger in Vascular Bundles. Previous studies identified soluble secreted proteins by isolating them from xylem sap and confirmed that they are secreted proteins by identifying their N-terminal sequences of the mature proteins that do not contain N-terminal signal sequence (Kehr *et al*. 2005; Alvarez *et al*. 2006; Djordjevic *et al*. 2007). In the previous study (Kusumawati *et al*. 2008), proteins were isolated from the medium of cell suspension culture and were identified using MALDI TOF-TOF and searching MtGI database. Using bioinformatic programs, the identified proteins were predicted to be secreted.

The use of promoter:*GUS* constructs, showed that all six promoters examined were strongly expressed in vascular bundles and root tips. They were *MtSBT1*, *MtSCP1*, *MtnsLTP*, *MtFAD-BP1*, *MtRHRE1*, and *MtENOD-like2*. Their strong expression in the vascular bundle suggest that they might be involved in vascular bundle development or function and that the gene products would be released into apoplast on a continuous basis along the entire length of the vasculature. Their strong expression in the vascular bundle might also suggest that these six secreted proteins are secreted to the cells in the vasculature and therefore it is likely that they might become part of the xylem sap. This conclusion is supported by the literature. Subtilisin-like serine protease was found in the xylem sap of *Glycine max* (Subramanian *et al*. 2009), *Zea mays* (Alvarez *et al*. 2006), *Brassica napus*, *Brassica oleracea*, *Cucurbita maxima*, and *Cucumis sativus* (Buhtz *et al*. 2004). Serine carboxypeptidase was among the xylem sap proteins isolated from *Brassica napus* (Kehr *et al*. 2005). A lipid transfer protein was detected in xylem sap of *Glycine max* (Djordjevic *et al*. 2007).

In conclusion, the expression localization study carried out using the native promoter:*GUS* fusions could give an indication concerning which tissues expressed the genes encoding secreted proteins. A reasonable assumption would be that these proteins were then secreted directly into the apoplast surrounding these cells. This could be verified either by generating a secreted-protein-*GFP* fusion or by generating secreted protein specific antibodies and determining the location of the secreted proteins using immunolocalization. Using *GUS* reporter gene assay, this study showed that *MtSBT1*, *MtSCP1*, *MtnsLTP*, *MtFAD-BP1*, *MtRHRE1*, and *MtENOD-like2* strongly expressed in vascular bundles.

Using quantitative real time RT-PCR, six genes encoding secreted proteins examined here showed higher expression in root tips than in leaves, cotyledons or root segments. These six genes were *MtSBT1*, *MtSCP1*, *MtN5*, *MtnsLTP*, *MtFAD-BP1*, and *MtRHRE1*. Only *MtENOD2-like* showed lower expression in roots (either inoculated or non-inoculated) than in leaves and cotyledons (Figure 1).

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