Molecular Cytological Analysis of Cysteine Proteinases from Nodules of *Lotus japonicus*

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Summary  Some cysteine proteases are expressed in nodules of leguminous plants. AsNodf32, one of the nodulins of *Astragalus sinicus*, is a cysteine protease of the papain super family and is expressed strongly in mature nodules. To date, functions of proteinases in the nodules have not been clarified. Homologues of AsNodf32 were identified in the EST library of the model legume *Lotus japonicus*. The expression of homologous proteinase genes was strongly enhanced in mature nodules. Subcellular localization of one of the *L. japonicus* proteinases, LjCyp2, was studied using tobacco BY-2 cells. The localization of the LjCyp2-GFP-fusion protein changed depending on the cell growth phase. GFP fluorescence was observed in the cytoplasm of rapidly growing cells while the fluorescence was observed solely in the vacuoles of cells at the stationary phase. Cyclical changes of the localization observed in BY-2 cells may reflect control mechanism of LjCyp2 functions in the nodule.

Key words  Nodule, Cysteine protease, *Lotus japonicus*, Symbiosis, BY-2.

Leguminous plants establish symbioses with soil bacteria. The process to establish endosymbiosis and symbiotic nitrogen fixation are important topics in the plant sciences. Many nodule-specific genes known as nodulins have been isolated from various legumes to study their functions in the root nodule developmental process. We isolated more than 100 clones from Chinese milk vetch, *Astragalus sinicus* (Fujie et al. 1998, Kasai et al. 2000, Naito et al. 2000). One of the clones, AsNodf32, encodes a cysteine protease of the papain super family and its expression was nodule specific (Naito et al. 2000). AsNodf32 was expressed in mature indeterminate-type nodules and especially in the senescent zone. It is interesting that expression of AsNodf32 was also observed transiently in the interzone before differentiation (Naito et al. 2000). Plant cysteine proteinases have various functions in germination, plant development and senescence (Sheokand and Brewin 2003). The AsNodf32-protein is similar to a protein encoded by the AgNOD-CP1 gene of *Alnus glutinosa*, including a putative vacuolar-targeting signal, LQDA (Goetting-Minesky and Mullin 1994). *A. glutinosa* is not a leguminous plant, but it forms actinorrhizal nodules with Frankia. Like AsNodf32, the AgNOD-CP1 gene is expressed in the nodule specifically. Other cysteine proteinases that are expressed in the nodule are also reported, such as PsCyp1 and Cyp15a (Kardailsky and Brewin 1996). Expression of the homologues of Cyp15a was also reported from *Medicago* (Vincent et al. 2000) and soybean (Panter et al. 2000). The expression of PsCyp1 and Cyp15a is associated with senescence. The functions of PsCyp1 and Cyp15a may also be related to degradation of storage proteins (Sheokand and Brewin 2003). It is noteworthy that antisense lines of Cyp15a formed altered nodules (Sheokand and Brewin 2003). From these observations, some possible roles
of the cysteine protease in root nodules are thought to be as follows: (i) a defense response to invasion of microorganisms, including rhizobia, (ii) protein turnover required during the formation of new tissue, and (iii) control of nodule senescence (Sheokand and Brewin 2003).

Recently, *Lotus japonicus* has been widely used as a model leguminous plant. Expression sequence tags (EST) have been generated for *L. japonicus* (Asamizu et al. 2004, Colebatch et al. 2002). We have used *L. japonicus* to investigate the functions of AsNodf32 using molecular genetic methods.

The purpose of this study was to characterize the cysteine proteases precisely. Homologues of AsNodf32 were identified in the EST library of the *L. japonicus* and their expressions in the course of the nodule development were investigated. The subcellular localization was studied using cultured cells tobacco BY-2, since BY-2 cells are much suitable for cytological observation than using leguminous plants. BY-2 cells are easy to transform and their monocellular structure eliminates promoter selective regulation and/or variation of translation efficiency that is possible in the nodule. In BY-2 cells, the localization of the proteinase changed cyclically depending on the cell growth condition.

Materials and methods

*Plant material, bacterial strains, and growth conditions*

Seeds of *Lotus japonicus* Miyakojima MG-20 were gifted from Dr. Kawaguchi. *L. japonicus* were grown in a pot and infected with *Mesorhizobium loti* (MAFF303099) as described by Kawaguchi et al. (2001) at 28°C. Suspension cultured cells of tobacco BY-2 (*Nicotiana tabacum* cv. Bright Yellow 2) were cultured in modified LSD medium by the method of Nagata et al. (1981) in an orbital shaker at 120 rpm and at 26°C in the dark. The cells were transferred to new medium at weekly intervals. *Agrobacterium tumefaciens* LBA4404 was used for transformation of BY-2 cultured cells.

*Microscopic observation*

BY-2 cells were washed with fresh LSD medium and images were recorded with an epifluorescence microscope (Olympus BH-2, Tokyo, Japan) and a CCD camera (Keyence VB-6010, Tokyo, Japan), or with a confocal laser scanning microscope (Olympus FLUOVIEW FV300) for the fluorescence of GFP. BY-2 cells were also observed without washing to evaluate the effect of the washing. Images were electronically recorded and processed with Photoshop (Adobe, San Jose, California, USA).

*DNA and RNA analysis*

Genomic DNA of *L. japonicus* was prepared using the DNeasy plant mini kit (Qiagen Sciences, Maryland, USA) according to the manufacturer’s instructions. Purification of plasmid DNA, cloning, and endonuclease analyses were performed as described by Sambrook and Russell (2001). Total RNA was prepared from each organ of *L. japonicus* using the RNeasy plant mini kit (Qiagen Sciences) according to the manufacturer’s instructions.

*Site-directed mutagenesis*

The nucleotide sequence of the gene encoding LjCyp2 was changed to remove BamHI sites in the ORF for the manipulation of the gene using the primer 5’-CAAGATCCAGTGAAGAGAC-CACTAGAATAAATTGAAATCTCTG-3’ without altering coding amino acids. Mutagenesis was carried out using the LA PCR in vitro Mutagenesis Primer Set for pBluescript II (Takara, Ohtsu, Japan) according to the manufacturer’s instructions. Mutations of the objective nucleotides were confirmed by DNA sequencing.
qRT-PCR analysis

qRT-PCR was performed as described previously (Alemzadeh et al. 2006). Total RNA was treated with RNase free DNaseI (Roche, Basel, Switzerland) before cDNA synthesis. First-strand cDNAs were synthesized from 1 μg of total RNA with the ReverTraAce reverse transcriptase kit (Toyobo, Osaka, Japan) and oligo(dT)20, according to the manufacturer’s instructions. Quantitative PCR was performed with the SYBR Premix Ex Taq kit (Takara). The 10 μl reaction mixture contained 0.5 μl RT reaction solution, 5 pmol each primer, and 5 μl SYBR Premix Ex Taq. Primer pairs used in this experiment are shown in Table 1. The specificity of each primer pair for the four clones was confirmed by melting point analysis (data not shown). Reactions were run and analyzed on a LineGene thermal cycler (Bioflux, Tokyo, Japan), according to the manufacturer’s instructions. Relative expression levels were calculated as the ratio of normalized gene expression against the actin gene of L. japonicus (AU089544; Endo et al. 2000). Reactions were performed in triplicate and average values were calculated.

Preparation of LjCyp2 antibody

A part of LjCyp2 cDNA was amplified by PCR (primer A: 5’-TCAGTCGACGCTTCAATTGGAGAGACAGC-3’, and primer B: 5’-ACTGCGGCCGGCGGACTCATCCTAAAACCCCCTT-3’). Products were cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA), then cut by SalI and NotI and separated on an agarose gel. A 0.4 kbp fragment was recovered from the gel and cloned into pGEX-4T-3 (GE Healthcare UK Ltd., Buckinghamshire, England) to express the protein fused with GST in E. coli. The GST-LjCyp2 fusion protein was expressed in E. coli strain XJa Autolysis (Zymo Research, Orange, CA, USA), and purified according to the manufacturer’s instructions. The purified fusion protein was cleaved with thrombin (4 μg/ml, 2.5 mM CaCl2) and separated on a GST column. The eluted peptide was injected into rabbits for the preparation of antisera. GST-binding antibodies were removed from the antisera and LjCyp2-specific antibodies were affinity purified as described by Onimatsu et al. (2006).

Immunoblot analysis

Frozen samples (20 mg) were ground in liquid nitrogen, suspended in 60 μl of 2×sample buffer (50 mM Tris·HCl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.01% (w/v) bromophenol blue) and boiled at 100°C for 5 min. After a brief centrifugation, the proteins in the supernatant were size-separated by SDS-PAGE on a 15% resolving gel, and blotted onto a PVDF membrane (Immobilon, Millipore Corp., MA). For detection of LjCyp2, the membrane was reacted with the primary antibody (anti-LjCyp2) at a dilution of 1 : 2500 for 2 h, and then incubated with alkaline phosphatase-conjugated anti-rabbit IgG (H+L) antibody (AP132A,

<table>
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<tr>
<th>Clone name</th>
<th>Nucleotide sequences</th>
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<tr>
<td>LjCyp1 (GNf032f12)</td>
<td>Forward: 5’-TAGCAACAATGAGCAGGCAC-3’&lt;br&gt;Reverse: 5’-TAACCCACAACAGCACAACCC-3’</td>
</tr>
<tr>
<td>LjCyp2 (GNf037h07)</td>
<td>Forward: 5’-GGAGAACAATGGGGTGAGA-3’&lt;br&gt;Reverse: 5’-GCCACAAAACCCCACTGCTG-3’</td>
</tr>
<tr>
<td>LjCyp3 (GNf071h01)</td>
<td>Forward: 5’-TACTCCCAATCAAGGACCAAGG-3’&lt;br&gt;Reverse: 5’-GAACCTTTAAGCCTCCTGCTC-3’</td>
</tr>
<tr>
<td>LjCyp4 (GNf089d01)</td>
<td>Forward: 5’-GTGGGATGAGAATGATGATG-3’&lt;br&gt;Reverse: 5’-GCTTACAAACCATTACTATTACAC-3’</td>
</tr>
<tr>
<td>Actin</td>
<td>Forward: 5’-CTTTTATACCCCCCTGATGATG-3’&lt;br&gt;Reverse: 5’-GGTGGTAAAGAATAACACCTTC-3’</td>
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Chemicon, Temecula, CA, USA) at a dilution of 1:2500 according to the manufacturer’s instructions. Nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate toluidinium (NBT-BCIP) was used to detect the antibody. For detection of GFP, the blotted membrane was reacted with the primary antibody (anti-GFP (Mouse IgG1-k) monoclonal (GF200), Nacalai Tesque, Kyoto, Japan) at a dilution of 1:2500 for 2 h, and then incubated with alkaline phosphatase-conjugated anti-mouse IgG (H+L) antibody at a dilution of 1:2500. For detection of LjCyp2, peroxidase-conjugated secondary antibody was also applied and detected with the ECL system (GE Healthcare).

Plasmid construction for plant transformation

A GFP cassette (CaMV35S promoter::GFP-NosT) was excised from psmGFP (U70495) by HindIII and EcoRI, then was cloned into HindIII and EcoRI sites of pCAMBIA1301 (CAMBIA; 346 Cytologia 74(3) M. Fujie et al.).
Canberra, Australia), and the plasmid was named pHSGFP. To make a fusion gene, the entire LjCyp2 sequence was amplified by PCR using primer pairs (Forward: 5'-ATTCTAG-
TACTTGGGA
TCC
ATCACTG-3' /H11032, Reverse: 5'-/H11032-GTTTAACAATTGGA
TCC
TGGGGTAAGAAGC-
3'/H11002) containing BamHI sites. The PCR products were cut by BamHI and cloned into the BamHI site (/H11002 17 bp of smGFP gene) of pHSGFP in frame to express the fusion protein.

Transformation of BY-2 cells

Plasmids were transformed into A. tumefaciens (strain LBA4404) by electroporation using the Gene Pulser Xcell (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. BY-2 cells were infected with the A. tumefaciens LBA4404 which contains the plasmid, and transformed cells were selected as described by An (1987). Transformed BY-2 cells were maintained on the solid LSD medium until experiments were carried out.

Results

Site-directed mutagenesis

Two BamHI sites at nucleotide positions +791 and 830 of LjCyp2 ORF were removed by substituting C at 795 to A, and C at 834 to G. This mutated sequence was used for further manipulation of the LjCyp2 ORF.

Comparison of amino acid sequences of nodule-specific cysteine proteases

We searched the EST database of L. japonicus (constructed by Kazusa DNA Research Institute) and found a homologue, GNf037h07 (LjCyp2, AB300460), of AsNodf32. The nucleotide sequence of its entire region was determined, which encoded a cysteine protease of the papain superfamily (Barett 1986). Some other ESTs encoding cysteine proteases were also found as senescence-associated genes (Hata et al., personal communication). Homologues of GNf037h07 included GNf032f12 (LjCyp1, AB300459), GNf071h01 (LjCyp3, AB300461), and GNf089d01 (LjCyp4, AB300462). Fig. 1 shows an alignment of the deduced amino acid sequences of AsNodf32, LjCyp1, LjCyp2, LjCyp3 and LjCyp4. Amino acid residues (in AsNodf32 numbering)
involved in the catalysis (C-149 and H-286), active-site formation (Q-143, N-307, S-308 and W-309), and disulfide bridges (C-146/C-189, C-180/C-222, and C-280/C332) are conserved among the LjCyp1-4 (Kamphius et al. 1985). A characteristic feature common to LjCyp1, LjCyp2 and LjCyp4 is a putative vacuolar targeting signal, the LQDA motif, at the N-terminal region (Naito et al. 2002, Goetting-Minesky and Mullin 1994). There are 2 putative cleavage sites predicted by Wolf PSORT program (Horton 2007) and by comparison with AgNOD-CP1 (Goetting-Minesky and Mullin 1994), so-called ERFNIN motifs that inhibit protease activity were found in the propeptide regions of LjCyp1, LjCyp2, LjCyp3, and LjCyp4 (Karrer et al. 1993). Extracellular localization of LjCyp1, LjCyp2, LjCyp3, and LjCyp4 was predicted by the PSORT program (Nakai and Horton 1999) and by the WOLF PSORT program (Horton et al. 2007).

**RT-PCR analysis of cysteine protease expression**

The expression levels of the mRNA of LjCyp1, LjCyp2, LjCyp3, and LjCyp4 were measured by real time RT-PCR as relative values to the amount of actin mRNA (Fig. 2). Changes of the expression level of the 4 clones were similar to each other in the course of development. One day after infection, the levels were lower than those preceding infection. Expression levels decreased at 2 weeks post infection. The levels increased again in the nodules older than 4 weeks post infection. The expression levels were highest in the mature nodules. Relative expression levels of LjCyp2 and LjCyp1 were higher than those of LjCyp3 and LjCyp4.

**Immunoblot analysis of LjCyp2**

A part of the LjCyp2-protein (a.a. 125–239; 12 kD) was expressed in *E. coli* as a GST fusion protein. GST was removed from the fusion protein by digestion with thrombin and the remaining peptide was used to prepare antiserum against LjCyp2. GST-binding antibody was removed from the antiserum, and the LjCyp2-binding antibody was then purified using affinity columns. The expression of the protease during nodule development was analyzed by immunoblot. In mature nodules (older than 4 weeks), the signals were observed at 26 kD (Fig. 3B). This molecular mass corresponds to the estimated size of mature LjCyp2 processed at a second cleavage site (23.3 kD). This indicates that the processed LjCyp2 protein accumulates and may work specifically in the
mature nodule. No significant signal was detected when pre-immune serum was used as a primary antibody (data not shown).

**Expression of LjCyp2 in BY-2 cells**

We further intended to study subcellular localization of the LjCyp2-GFP-fusion protein using pHSGFP (Fig. 4). We made transgenic nodules of *L. japonicus* that express LjCyp2-GFP-fusion protein but we could not observe any GFP signals (data not shown). Therefore, we tried to express the fusion protein using cultured cells of tobacco BY-2. BY-2 cells were transformed with chimeric genes, encoding LjCyp2 and smGFP (26.8 kD). Stable transformants were selected on a solid medium containing hygromycin B (25 μg/ml). Selected transformants were maintained in liquid LSD medium. Transformed cells were transferred to new liquid medium at weekly intervals for at least 4 weeks before microscopic analysis. The expressions of chimeric genes were confirmed by immunoblot analysis using the anti-GFP monoclonal antibody (Fig. 5A). In rapidly growing cells (1 to 5 d after transfer into fresh medium), GFP fusion protein was detected at 66 kD. This size corresponds to the sum of the estimated molecular mass of LjCyp2 (37.4 kD) and smGFP (26.8 kD). In stationary phase (7 d after transfer), signals were detected at 27 kD and 29 kD. The signal at 27 kD represents smGFP itself and the signal at 29 kD may represent a fusion protein of a broken piece of C-terminal peptide of LjCyp2 and GFP. This suggests the fusion protein was cleaved at the junction between LjCyp2 and GFP in aged cells. When the anti-LjCyp2 antibody was used as the primary antibody (Fig. 5B), GFP fusion protein was detected at 66 kD in rapidly growing cells (1 to 5 d after transfer to the fresh medium), as was detected using the anti-GFP antibody. In stationary phase (7 d after transfer), a signal was detected at 38 kD. This size corresponds to the LjCyp2. This also suggests the fusion protein was cleaved at the junction between LjCyp2 and GFP in stationary phase BY-2 cells.

**Localization LjCyp2 in BY-2 cells**

BY-2 cells transformed with pHSGFP, which expresses GFP in plant cells, showed green fluorescence localized in the cytoplasm and nuclei throughout the culture period. Figs. 6A, B show BY-2 cells transformed with pHSGFP at 3 d and 8 d after transfer to fresh medium, respectively.
The BY-2 cells were examined with a fluorescent microscope and/or with a laser-scanning microscope. Localization of GFP did not change during the cultivation cycle of the cells. When BY-2 cells were transformed with the chimeric gene encoding the LjCyp2-GFP fusion protein, localization of the fluorescence changed cyclically during the culture cycle depending on the time after transfer to fresh medium. At 3 d after transfer to fresh LSD medium, green fluorescence was

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**Fig. 6.** Confocal images of BY-2 cells transformed with GFP (A, B) and LjCyp2-GFP fusion proteins (C–F). Rapidly growing cells (3 d after transfer to fresh medium; A, C) and stationary phase cells, (8 d after transfer to fresh medium; B, D, E, F) are shown. In the cells transformed with GFP, signals were observed on the nuclei and cytoplasm at 3 d after transfer, and no significant signals were observed on the vacuoles at 8 d after transfer. When transformed with LjCyp2-smGFP fusion proteins, localization changed from cytoplasm (3 d after transfer, C) to vacuoles (8 d after transfer, D–F). Signals in the vacuole were weaker in some cells, and a number of spots of GFP fluorescence were observed along the cell wall and the surface of nuclei (E). Fluorescence was observed as a network-like structure on the cell surface (F). Bar=50 μm.
observed in the cytoplasm, as networks passing through the vacuole, around the nuclei, and along the plasma membrane. Significant signals were not observed in the nuclei (Fig. 6C). At 8 d after transfer to fresh medium, green fluorescence was observed in the vacuole but there was no significant signal observed in the nuclei (Fig. 6D). In some cells, the signals were weaker, and a number of spots of GFP fluorescence were observed along the cell wall and the surface of the nucleolus (Fig. 6E). The cells that were observed immediately after sampling with no washing procedure also gave consistent images (data not shown). Fluorescence was observed as a network-like structure on the cell surface (Fig. 6F).

Discussion

Plant cysteine proteases are classified into the papain super family on the basis of their amino acid sequence homology (Barrett 1986). Some cysteine proteases are expressed in the course of senescence and are thought to have roles in nitrogen recycling. As shown in this study, LjCyp1, 2, 3 and 4 were strongly expressed in mature nodules as previously demonstrated for AsNodf32 in *A. sinicus* and AgNOD-CP1 in *A. glutinosa*. Therefore, the major functions of LjCyp1, 2, 3 and 4 are considered to be senescence related.

The *L. japonicus* *sen1* mutant forms ineffective nodules in which nodule development is arrested at the stage of bacterial differentiation into nitrogen-fixing bacteroids (Suganuma *et al.* 2003). The expression of many hydrolases including LjCyp1, LjCyp2, LjCyp3 and LjCyp4 was enhanced in the nodules of the *sen1* mutant (Suganuma *et al.* 2004). It was suggested that such hydrolases might function in the early senescence of the *sen1* mutant. The expression of LjCyp1, 2, 3 and 4, however, showed a temporal increase in 1-week-old nodules. In the nodule of *A. sinicus*, temporal expression of AsNodf32 mRNA was also observed in the developing cells in the interzone (Naito *et al.* 2000). Therefore, it is possible that LjCyp1, LjCyp2, LjCyp3 and LjCyp4 have also some functions in relatively early stages of nodule development.

To determine the functions of the cysteine proteinases, it is valuable to study the subcellular localization within the nodules. In previous studies, it was suggested that AsNodf32 and AgNOD-CP1 are localized in the vacuole (Naito *et al.* 2000, Goetting-Minesky and Mullin 1994). More than 10 plant vacuolar targeting signals have been reported to date and most of them are signals for protein storage vacuoles (Vitale and Hinz 2005). Limited information is available for targeting signals to lytic vacuoles. LjCyp2 contained the LEDV sequence motif in the amino terminal. This sequence may correspond to the LQDA motif of AsNodf32 in *A. sinicus* (Naito *et al.* 2000) or AgNOD-CP1 in *A. glutinosa* (Goetting-Minesky and Mullin 1994), which is thought to be a vacuolar targeting sequence. The 43-amino-acid amino-terminal peptide of phytohemagglutinin containing a 20-amino-acid signal sequence directs the linked invertase to the vacuole in yeast cells. This targeting appeared to be dependent on the LQRD sequence at amino acid positions 38–41. This LQRD sequence is sometimes found in legume lectins (Tague *et al.* 1990, Chrispeels 1991). To study subcellular localization of LjCyp2, we made transgenic nodules that express LjCyp2-GFP-fusion protein by a hairy root method, but no significant GFP signals were observed (data not shown). Therefore, we used BY-2 cultured cells to study the localization of LjCyp2.

The size of the fusion protein in the cytoplasm was 66 kD in BY-2 cells transformed with the LjCyp2-GFP fusion protein, which corresponds to the sum of the masses of the individual LjCyp2 and GFP proteins. In the stationary phase BY-2 cells, signals were observed at 27 and 29 kD when detected with the anti-GFP monoclonal antibody. These sizes almost correspond to the molecular mass of GFP (26,795 D). When the fusion protein was detected with the anti-LjCyp2 antibody, the signal was detected at 38 kD in stationary phase BY-2 cells. It was suggested that GFP has a stable structure and the additional peptide at the N- or C-terminus is susceptible to attack by proteinases (Tamura *et al.* 2003). LjCyp2-GFP fusion protein transferred to the vacuole may have been
truncated by proteinases in the course of the cultivation cycle. The band at 27 kD may correspond to the GFP itself. The band at 29 kD may represent a fusion protein of GFP and a C-terminal peptide of LjCyp2.

In this study, the localization of LjCyp2-GFP fusion protein changed cyclically in BY-2 cells depending on the time after transfer to fresh medium. It is reported that GFP in the vacuole is degraded in the light (Tamura et al. 2003). Those authors suggested that blue light induced a conformational change of the GFP, and GFP was degraded by papain-type vacuolar cysteine protease(s) under acidic pH. In this study, BY-2 cells were cultured in the dark and observed in a dark room to avoid light-induced degradation before observation. It is possible that some GFP had degraded in rapidly growing cells before observation during the washing procedure, where cells were washed to remove culture medium in the light. GFP signals were observed in the vacuole in 8-day-old cells after 4 h exposure to normal light conditions within the laboratory (data not shown). Furthermore, the localization of the GFP was consistent even when the cells were observed without washing (data not shown). These observations indicate that the cyclic change of the GFP localization observed in this study was not caused by light-dependent degradation.

Stably transformed BY-2 cells expressing SP-GFP (composed of the signal peptide of 2S albumin and GFP) driven by the CaMV35S promoter localized the protein in the cytoplasm and did not show any cyclical change of protein localization throughout the growth of the transgenic cells (Mitsuhashi et al. 2000). BY-2 cells that expressed SP-GFP-2SC (composed of signal peptide, GFP and C-terminal 18-amino-acid sequence of pumpkin 2S albumin containing a putative vacuolar targeting signal, NLPS) showed almost the same cyclical change of GFP localization (Mitsuhashi et al. 2000) as the LjCyp2-GFP fusion protein observed in this study. Mitsuhashi and coworkers used the CaMV 35S promoter in their study, and an almost consistent expression was expected throughout the culture cycle of BY-2 cells. They concluded such cyclical change might be due to a cyclical change in proteolytic activity in the vacuoles. They suggested that in the stationary phase cells, the proteolytic activity was highest and that this may cause degradation of GFP in the vacuole.

The reason for the cyclical change in localization of LjCyp2 is not certain. The expression level of the protein is considered to be constant since the promoter used in this study is CaMV35S. It is possible that proteolytic activity in the vacuoles may change as described previously (Mitsuhashi et al. 2000). There remains a possibility that the sorting mechanism changes in the cell culture cycle. Western blot analysis showed the existence of a 66 kD fusion protein in rapidly growing BY-2 cells (3-d-old) without GFP fluorescence in the vacuoles. In such cells, GFP signals were observed in the cytoplasm. In the 8-d-old cells, the fusion protein appeared to be cleaved in the vacuole and the fluorescence signal in the cytoplasm was weak. These observations suggest that the changes in subcellular localization are related to changes in protein sorting mechanisms during the cell culture cycle, and not to GFP degradation activity. Immunocytological observation will provide further information on the localization of LjCyp2 in the nodule. Unfortunately, the antibody prepared against LjCyp2 did not work for the immunocytological purpose (data not shown).

Very strong expressions of the proteinases were observed in the mature nodules of L. japonicus and in the senescent zone of A. sinicus (Naito et al. 2000). The functions of the proteinases in the mature cells may be degradation of old cells to recycle nitrogen compounds. Cyclical change of the protease localization in BY-2 cells may have some correlation to the functions of the proteinases in the developing young nodules. RD19 is a cysteine proteinase of Arabidopsis thaliana and is related to the resistance for the plant pathogenic bacteria, Ralstonia solanacearum. Beronoux et al. (2008) reported that subcellular localization of RD19 changes interacting with PopP2, an R. solanacearum type III effector. They proposed that RD19 works for activation of resistance response in the nucleus. It is possible that cyclic change of LjCyp2 localization may reflect the control mechanism of LjCyp2 functions.
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