Nuclear DNA Endoreduplication and Expression of the Mitotic Inhibitor Ccs52 Associated to Determinate and Lupinoid Nodule Organogenesis

Alfonso González-Sama,^{1,2} Teodoro Coba de la Peña,² Zoltan Kevei,¹ Peter Mergaert,¹ M. Mercedes Lucas,² María R. de Felipe,² Eva Kondorosi,¹ and José J. Pueyo²

¹Institut des Sciences du Végétal, Centre National de la Recherche Scientifique, Unité Propre de Recherche 2355, Avenue de la Terrasse, 91198 Gif-sur-Yvette, France; ²Department of Plant Physiology and Ecology, Instituto de Recursos Naturales, Centro de Ciencias Medioambientales, CSIC, Serrano 115-bis, E-28006 Madrid, Spain

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Lotus japonicus determinate nodules differ greatly from indeterminate nodules in their organogenesis and morphological characteristics, whereas *Lupinus albus* lupinoid nodules share features of determinate and indeterminate nodules. The mitotic inhibitor Ccs52A is essential for endoreduplication and ploidy-dependent cell enlargement during symbiotic cell differentiation in *Medicago truncatula* indeterminate nodules. *ccs52A* homolog genes were isolated from lupin and lotus nodules; the deduced Ccs52A proteins showed high sequence similarity with other Cdh-1-type activators of the anaphase-promoting complex and were grouped with A-type Ccs52 proteins from different plants. In lupin, *ccs52A* expression was restricted to the earlier stages of nodule development, whereas *ccs52A* transcripts accumulated in lotus nodule primordia and, to a lesser extent, in mature nodules. Nodule development in *Lupinus albus* involved a progressive increase in nuclear and cellular size and ploidy level; similarly, *Lotus japonicus* nodules contained polyploid nuclei and enlarged cells in the infected zone. Nevertheless, in situ hybridization experiments showed the highest *ccs52A* expression in the inner cortex cells of the lupin nodule primordium, probably associated to the increased size of these cells in mature nodules. In view of our results, Ccs52A-mediated endoreduplication appears to be a universal mechanism required for nodule cell differentiation during the establishment of nitrogen-fixing symbioses.

Additional keyword: polyploidy.

The formation of nitrogen-fixing root nodules requires a precise exchange of recognition signals (Hirsch 1992) that trigger a series of plant morphogenetic changes, including the repeated division of root cortical cells. Nodule organogenesis involves a complex plant cell differentiation program. Rhizobia penetrate the root cortex, invade nodule primordium cells, and eventually evolve inside infected cells to their nitrogen-fixing form, the bacteroid (Brewin 1991). Two main types of nodules can be distinguished according to the persistence of meristematic activity and the location of the first cell divisions in the

root cortex. Typical determinate nodules (lotus, soybean, common bean) are spherical, they develop from the outer cortex, and their meristematic activity ceases after a defined period of time. Characteristic indeterminate nodules (pea, alfalfa) are initiated in the inner cortex and have a cylindrical shape as a result of a persistent uninfected apical meristem. Nevertheless, certain nodule subtypes, such as aescynomenoid nodules or lupinoid nodules, share features of determinate and indeterminate nodules (Sprent 2001).

A longitudinal section of a typical indeterminate nodule reveals cells at different stages of development, exhibiting a differentiation gradient from the apical part to the basal tissues attached to the root (Newcomb et al. 1979). The apical meristem is composed of small proliferating uninfected cells. In the pre-fixation zone, the symbiotic differentiation program involves cell division arrest, followed by several rounds of endoreduplication (endocycles) and cell enlargement. Enlarged polyploid cells are invaded and host the bacteroids, whereas small diploid cells remain uninfected. In the fixation zone, both the bacteria and the plant cells are differentiated for symbiotic nitrogen-fixing activity (Truchet 1978).

Endoreduplication is a common phenomenon in developmental programs of plant polyploid organs and tissues (Kondorosi et al. 2000; Larkins et al. 2001) and is characterized by repeated S and Gap phases. In animals, endoreduplication is less common but is well-documented in salivary glands and Malpighian tubules of dipters and in the trochoblast, deciduas, and myocardial cells of mammals (Conlon and Raff 1999). During endocycles, nuclear DNA is replicated without mitosis or cytokinesis, resulting in cells with a DNA content greater than 2C (C stands for the amount of DNA corresponding to the haploid complement). One way to induce endoreduplication is the inactivation of mitotic CDK/cyclin complexes before the M-phase transition point (Fang et al. 1998; King et al. 1995). This inhibition of mitosis, required for cell division arrest prior to differentiation, can be achieved by early activation of the anaphase promoting complex (APC) pathway, which is responsible for ubiquitin-dependent proteolysis of mitotic cyclins.

Two different genes encoding APC activators, *ccs52A* and *ccs52B*, have been identified in *Medicago truncatula* and *Arabidopsis thaliana* (Cebolla et al. 1999; Tarayre et al. 2004). In *Medicago truncatula*, *ccs52A* is clearly responsible for mitotic cyclin degradation and for the regulation of endoreduplication during symbiotic cell differentiation in the indeterminate nodule. *ccs52B* is expressed in shoot apices but not in the symbiotic organ, indicating divergence in the expression patterns or

Corresponding author: J. J. Pueyo; Telephone: +34 917452500; Fax: +34 915640800; E-mail: pueyo@ccma.csic.es

Nucleotide sequence data is available in the GenBank database under accession numbers AY350619 for *Llccs52A*, AY350620 for *Laccs52A*, and DQ059035 for *Ljccs52A*.

functions, or both, of both homologs (Vinardell et al. 2003). *ccs52A* is not restricted to the nodulation process (Cebolla et al. 1999; Favery et al. 2002; Foucher and Kondorosi 2000; Koltai et al. 2001). In *Medicago* indeterminate nodules, *ccs52A* expression is localized in meristematic proliferating cells and in the prefixation zone differentiating cells, and its involvement has been established in nuclear DNA endoreduplication associated to nodule development (Cebolla et al. 1999; Vinardell et al. 2003).

Lotus japonicus was selected as a model legume to study determinate nodules. It is nodulated by *Mesorhizobium loti* and produces spherical nodules of finite development that originate from the outer cortex. The genus *Lupinus* establishes effective symbiosis with *Bradyrhizobium* sp. (*Lupinus*). Lupin nodules are recognized as a unique subtype of indeterminate nodules (Bergersen 1982). They are considered indeterminate because of their basi-lateral meristem, which can cause the nodule to grow until it surrounds the root. Growth often ceases earlier, however, and the nodule remains spherical, as for determinate nodules. Several other features distinguish lupinoid nodules from other nodule types: their initiation takes place in the outer cortex, the meristematic region contains bacteroids, the nodule does not show the typical zonation, and the infected central tissue does not have uninfected interstitial cells (Lotocka et al. 2000; Tang et al. 1992, 1993). Moreover, the infected zone originates from the division of a single infected cortical cell (González-Sama et al. 2004).

To acquire more insight into the development of determinate and lupinoid nodules and to verify the occurrence of *Ccs52*-mediated endoreduplication, which may differ from that described in *Medicago truncatula*, we isolated and characterized *ccs52A* homologs from lotus and lupin and studied their role in the endoreduplication process triggered by rhizobial infection during establishment of nitrogen-fixing symbioses.

Fig. 1. Ploidy levels in **A**, *Lupinus albus*, and **B**, *Lotus japonicus* plants. Distribution (mean percentage) of nuclei with different DNA content in several organs. Standard error bars are displayed. NP = nodule primordium (10 days postinoculation [dpi]), YN = young nodule (17 dpi), MN = mature nodule (28 dpi, lupin; 21 dpi, lotus).

RESULTS

Nodule development in *Lupinus albus* and *Lotus japonicus* involves nuclear DNA endoreduplication.

The nuclear DNA content was measured in several organs at different developmental stages in lupin and lotus plants. The results showed a high diversity of ploidy levels in white lupin (Fig. 1A). All plant organs examined, except young leaves, contained polyploid nuclei with different levels of endopolyploidy. The highest degree of endoreduplication was detected in nodules (2C, 18%; 4C, 38%; 8C, 30%; 16C, 13%; 32C, 1%) and petioles (2C, 16%; 4C, 42%; 8C, 38%; 16C, 4%). When comparing both legumes, higher levels of ploidy were observed in lupin for several organs (root tip, stem, petiole, and leaf). In lotus (Fig. 1B), roots (2C, 23%; 4C, 68.8%; 8C, 8%; 16C, 0.2%) and, specially, nodules (2C, 6%; 4C, 78%; 8C, 9%; 16C, 1%; 32C, 6%) were the only polyploid organs, and the polyploid nuclei distribution pattern differed from that found in lupin nodules.

Figure 2 shows the cellular and nuclear size in lotus nodules and in different stages of lupin nodule development, corresponding to the ploidy values (Fig. 1). Lupin nodule primordia (10 days postinoculation [dpi]) consisted of cells containing mainly 2C and 4C nuclei and a low proportion (approximately

Fig. 2. Fluorescence micrographs showing nuclear and cell sizes. **A** and **B**, *Lupinus albus* nodule primordia (10 days postinoculation [dpi]). **C** and **D**, *L. albus* young nodules (17 dpi). **E** and **F**, *L. albus* mature nodules (28 dpi). **G** and **H**, *Lotus japonicus* mature nodules (21 dpi). Nuclei were stained with 4',6-diamidino-2-phenylindol. C = cortex; IZ = infected zone. Scale bars = 100 μm (A, C, E, and G) and 50 μm (B, D, F, and H).

10%) of polyploid cells with DNA contents greater than 4C. 2C and 4C nuclei may correspond to cells in the G1 and G2 cell cycle phases, respectively, and cells with 8C nuclei may be the result of a single endoreduplication event. The infected zone of the nodule primordium was composed of compacted cells with a nuclear size similar to that of the nodule cortex cells (Fig. 2A and B). Later in nodule development, young nodules (17 dpi) increased their ploidy level and 16C nuclei were detected, indicating that a new round of endoreduplication had taken place. Contents of polyploid cells (>4C) reached 36% of the total cell number. DAPI (4',6-diamidino-2-phenylindol) staining showed a dramatic increase in nuclear and cell size in the infected zone relative to the previous developmental stage (Fig. 2C and D). Mature nodules (28 dpi) contained the highest proportion of cells with polyploid nuclei, accounting for 44% of total nuclei. An additional round of endoreduplication led to the appearance of a small group of cells with 32C nuclei (approximately 1%). At this stage, nuclei and cells of the infected zone had reached maximum size (Fig. 2E and F).

A correlation could also be observed among nuclear size, cell size, and rhizobial infection in lotus nodules. DAPI staining of the nodule sections showed a clear difference between large infected cells with large nuclei and small noninfected cells with considerably smaller nuclear sizes (Fig. 2G and H).

Lupin and lotus have a *ccs52A* homolog.

Since *ccs52A* is involved in the occurrence of endoreduplication cycles (endocycles) during the *Medicago* nodule differentiation program, we isolated the lupin and lotus *ccs52A* homologs.

Screening of a yellow lupin (*Lupinus luteus*) cDNA library, generated from developing root nodules, with a *Medicago sativa* *ccs52A* probe yielded a positive hybridization signal corresponding to a full-length cDNA clone (2,076 bp), which coded for a 469-amino acid polypeptide. A full-length lotus cDNA clone was obtained by reverse transcription-polymerase chain reaction (RT-PCR) from nodule RNA. The clone contained an open reading frame encoding a polypeptide of 487 amino acids. Blastx searches to sequence databases showed that the isolated cDNA corresponded to *ccs52A* homologs, which we termed *Llccs52A* (GenBank accession number AY350619) and *Ljccs52A* (GenBank accession number DQ059035).

Alignment of *LlCcs52A* and *LjCcs52A* with other known or putative plant *Ccs52A* and *Ccs52B* proteins revealed conservation among the different protein regions (Fig. 3A). The greatest divergence was found at the N-terminal region, whereas the C-terminal region proved to be highly conserved. *LlCcs52A* structure was very similar to that described for *MscCs52A* and *MtCcs52A* (Cebolla et al. 1999; Tarayre et al. 2004). *LlCcs52A* and *LjCcs52A* bore a putative PEST motif at the N-terminal region and several putative CDK phosphorylation sites that might be involved in posttranscriptional regulation of the protein. The C-terminus had seven WD40 repeats. Moreover, the lupin and lotus homologs contained the C-box and CSM (Cdh1-specific motif) conserved motifs and the IR tail required for *Ccs52A* protein binding to APC.

We generated a phylogenetic analysis based on a multiple alignment with plant homologous proteins (Fig. 3B). The tree separates two major clusters corresponding to the A- and B-type *Ccs52* proteins. *LlCcs52A* and *LjCcs52A* were grouped with the A-type *Ccs52* proteins and, within them, with legume *Ccs52A* proteins. The phylogeny of the *Ccs52A* proteins follows the general phylogeny of the plant kingdom, indicating that these proteins are, indeed, Cdh1-type of APC activators and orthologs of the *Medicago* *Ccs52* protein.

ccs52 is expressed during *Lupinus albus* and *Lotus japonicus* nodule development.

To obtain a homologous probe for gene expression studies in white lupin nodule organogenesis, we isolated a partial cDNA clone from developing *Lupinus albus* (white lupin) nodules using RT-PCR. We cloned and sequenced a 1,368-bp cDNA product corresponding to the N-terminal region and part of the WD40 repeats (GenBank accession number AY350620). This *Lupinus albus* *LacCs52A* fragment shared 98.5% amino acid identity with the corresponding region of *Lupinus luteus* *Llccs52A*, differing in only seven residues.

Northern blot and RT-PCR were used to elucidate the *ccs52* temporal expression pattern in *Lupinus albus* and *Lotus japonicus*, respectively. For lupin (Fig. 4A), total RNA was isolated from the nodulation-competent zone of inoculated roots (7 dpi), nodule primordia (10 dpi), young nodules (17 dpi), mature nodules (28 dpi), and other plant organs (root tip, nodulation-competent zone of uninoculated roots, young leaf, mature leaf, and cotyledon). *ccs52* mRNA was detected at very early stages of nodule development when nodule primordia are not yet visible on the root surface (7 dpi); the highest expression levels were found in nodule primordia (10 dpi). Much lower expression was observed in young nodules (17 dpi), root tips, and uninoculated roots. In lotus, gene expression along nodule development was analyzed by RT-PCR (Fig. 4B) in inoculated roots, young nodules (7 dpi), and mature nodules (21 dpi). Ubiquitin was used as a constitutive control and leghemoglobin as an indicator of nodule development. The highest expression was also detected at the initial steps of development, in nodule primordia (7 dpi).

In situ hybridization experiments were performed on lupin nodule primordia (10 dpi) (Fig. 5). Sections were hybridized with *ccs52a* sense and antisense RNA probes. The sense probe yielded no hybridization signal above background level in negative controls. Transcripts were detected preferentially in the nodule inner cortex surrounding the infected zone, while we observed lower, uniform labeling in the infected zone.

DISCUSSION

As early as 1908, Fred noted that the mitotic figures in cells of the nodules of some fifty leguminous plants were "larger, very irregular and not well marked, with uneven number of chromosomes" as compared with root-tip cells (Fred 1908). Although polyploidy has been reported in determinate, indeterminate, and lupinoid nodules (Kodama 1970), studies of the endoreduplication process associated with nodule organogenesis have been carried out exclusively on *Pisum*, *Medicago*, and other indeterminate nodules. One important finding in these studies was the identification of the *Ccs52A* protein, a Cdh1-type of APC activator, as a central regulator of endoreduplication and ploidy levels (Cebolla et al. 1999; Vinardell et al. 2003). *Ccs52A* promotes the exit of meristematic cells from the cell division cycle and their entrance into endoreduplication cycles via activation of ubiquitination-dependent proteolysis of mitotic cyclins (Cebolla et al. 1999). The importance of endoreduplication for normal symbiotic cell differentiation in nodules was demonstrated by manipulating *ccs52A* expression in transgenic plants (Vinardell et al. 2003). Here, we describe the isolation and molecular characterization of *Ccs52A* homologs from lupin and lotus and their participation in the increase in ploidy levels observed during nodule development. Lotus and lupin are models for determinate and lupinoid nodules, respectively, whose developmental processes exhibit distinct features.

Endoreduplication is a frequent phenomenon in plant organ development. The organ-specific but reproducible ploidy pattern in plants suggests that endoreduplication cycles are critical in

A

B

Fig. 3. Sequence analysis of *Lupinus luteus* and *Lotus japonicus* Ccs52A homologs. **A**, Multiple alignment (Clustal W) of the amino acid sequence of plant Ccs52A proteins. Conserved sequences (C-box and CSM) are indicated on black-shaded background and WD40 repeats with double arrows. Putative PEST motifs are underlined, and S and T residues of putative CDK phosphorylation sites are gray-shaded. IR tail is indicated in bold type. **B**, Phylogenetic analysis of plant Ccs52A proteins. The unrooted tree was constructed with the PHYLIP program (Felsenstein 1989) from multiple alignment Clustal W. At = *Arabidopsis thaliana*, Gm = *Glycine max*, Lj = *Lotus japonicus*, Ll = *Lupinus luteus*, Ms = *Medicago sativa*, Mt = *Medicago truncatula*, Ps = *Pisum sativum*, Os = *Oryza sativa*. Sequence accession numbers are (in order of alignment) AY350619, DQ059035, AF079404, AF134835, DQ059036, AY128834, AY091235, AP003298, AY357299, A1736659, and AY099581.

differentiation programs (Grafi 1998). In *Lupinus albus* and *Lotus japonicus*, different levels of DNA content were detected, depending on the tissue and organ tested, but the highest endopolyploidy was found in mature nodules, as reported in *Medicago* plants (Cebolla et al. 1999). Comparison of ploidy levels in different organs nonetheless evidences variations in the distribution patterns and in the DNA content of polyploid nuclei, suggesting different requirements for endopolyploidy in each legume. The percentage of polyploid nuclei (greater than 4C) in lupin petioles (42%) is double that of *Medicago* petioles (20%), although 32C nuclei, absent in lupin petioles, are present in *Medicago* petioles. In contrast, no polyploid nuclei are detected in lotus petioles. Nodules were characterized by a high level of endopolyploidy in the three legumes, although there were differences among them. *Medicago* (Cebolla et al. 1999) and lotus nodules have a large proportion of 32C nuclei (11% and 6%, respectively) compared with lupin nodules (approximately 1%), although the total percentage of polyploid nuclei (>4C) in the latter is much higher (44%) than in *Medicago* (24%) or *Lotus* (approximately 16%). These differences in distribution and content of polyploid nuclei might be the result of distinct nodule developmental programs. During *Medicago* and *Lotus* nodule development, the diploid meristematic cells cease to divide, enter the differentiation program, are infected by rhizobia, and undergo endoreduplication. During lupin nodule development, however, plant cells are infected first and the processes of cell division and symbiotic differentiation take place afterwards (González-Sama et al. 2004).

Comparison of the LjCcs52A and LjCcs52A sequences with the protein databases indicated that the lupin and lotus homologs share high similarity with Ccs52-type APC activators. Phylogenetic analysis showed that LjCcs52A and LjCcs52A are grouped in the same branch with other Ccs52A proteins and are clearly distinct from the Ccs52B proteins. Phylogeny of the Ccs52A proteins follows that of the plant kingdom, suggesting that the LjCcs52A and LjCcs52A proteins are orthologs of the *Medicago* Ccs52 protein and that they are involved in

control of endoreduplication during nodule development, as described for *Medicago truncatula* MtCcs52A (Vinardell et al. 2003). LjCcs52A and LjCcs52A contain motifs and amino acid residues involved in the protein function, such as a putative PEST motif for protein degradation and several CDK-phosphorylation sites, as well as the C-box and CSM motifs involved in interaction with other proteins, the seven WD40 repeats, and the IR tail (Tarayre et al. 2004).

Southern blot analysis (not shown) suggested that the lupin genome has at least two *csc52* homologs, as described in other plants such as *Medicago truncatula* and *Arabidopsis* (Tarayre et al. 2004). The sequence divergence between the *csc52A* and *csc52B* homologs and their distinct expression patterns indicating that they might participate in different developmental processes (Tarayre et al. 2004). The nature and function of putative *csc52B* homologs in lupin and lotus remain to be studied.

During organogenesis of nitrogen-fixing nodules in *Medicago truncatula*, *Mtccs52A* expression is not detected during nodule initiation. It is switched on in the central region of the nodule primordium just before cell differentiation starts, and is maintained in the meristematic and preinfection zones of mature nodules (Cebolla et al. 1999; Vinardell et al. 2003). Conversely, in *Lupinus albus*, *Llccs52A* expression can be detected during the earliest steps of nodule organogenesis, when the nodule primordium is essentially constituted by a few small proliferating cells inside the root cortex; maximum expression is reached in freshly emerging nodule primordia. In both types of nodules, no expression is detected in the fully-differentiated nitrogen-fixing symbiotic cells. In lotus, highest expression occurs in very young nodules, but transcript accumulation is also detected in mature nitrogen-fixing nodules.

In situ hybridization experiments showed the highest expression levels in the inner cortex of the lupin nodule primordium, in a cell layer that will give rise to a cortex zone in the mature nodule. This cortex layer is composed of enlarged cells with thickened walls and broad intercellular spaces, a special feature of the lupin nodule cortex that has been related to the oxygen diffusion barrier (Iannetta et al. 1993). The very early *Llccs52A* expression in inoculated roots could be indicative of polyploid cell formation prior to infection, such that only polyploid cells would be infected by rhizobia, which also appears to be the case in *Medicago truncatula* nodules (Cebolla et al. 1999). In lupin nodules, the infected zone originates from the repeated division of a single infected cell (González-Sama et al., 2004).

Our results show that the *csc52A* gene has a role in endoreduplication associated to nitrogen-fixing symbiosis that is widespread and independent of the nature of the nodule morphogenesis and differentiation program.

Fig. 4. Analysis of *csc52A* expression in *Lupinus albus* and *Lotus japonicus*. **A**, *csc52A* expression in *Lupinus albus* organs as analyzed by Northern blot. Lane 1, root tip; lane 2, nodulation-competent zone of uninoculated root; lane 3, nodulation-competent zone of inoculated root (7 days postinoculation [dpi]); lane 4, nodule primordium (10 dpi); lane 5, young nodule (17 dpi); lane 6, mature nodule (28 dpi); lane 7, young leaf; lane 8, mature leaf; and lane 9, cotyledon. Ethidium bromide-staining of rRNA was used to ensure equal RNA loading. **B**, Analysis of *csc52A* expression in *Lotus japonicus* as analyzed by reverse transcription-polymerase chain reaction. Lane 1, nodulation-competent zone of uninoculated root; lane 2, young nodule (7 dpi); lane 3, mature nodule (21 dpi); and lane 4, water control. Ubiquitin was used as a constitutive control and leghemoglobin as an indicator of nodule development.

Fig. 5. In situ localization of *Laccs52A* transcripts in lupin nodule primordia. **A**, Nodule primordium section (10 days postinoculation) hybridized with an antisense probe. Arrowheads indicate the accumulation of transcripts in the inner cortex. **B**, Control hybridization with a sense probe. C = cortex, VB = vascular bundles, and IZ = infected zone. Scale bars = 100 µm.

MATERIALS AND METHODS

Bacterial strain, plant material, and growth conditions.

Bradyrhizobium sp. (*Lupinus*) ISLU16 and *Mesorhizobium loti* MAFF303099 were grown at 28°C with gentle shaking in a yeast extract-mannitol liquid medium (Vincent 1970).

White lupin (*Lupinus albus* L. cv. Multolupa) seeds were surface-sterilized in 1% HgCl₂ with gentle shaking for 5 min, were rinsed several times in distilled water, and were allowed to imbibe for 2 h. Seeds were sown in pots containing sterilized vermiculite and were inoculated with 1 ml of bacterial inoculum. Plants were grown in a growth chamber under controlled conditions at 60% relative humidity, 25°C day and 15°C night temperatures, and 16-h light photoperiod (250 μ mol photon m⁻² s⁻¹). Plants were watered with distilled water for one week, with nitrogen-free nutrient solution (Hoagland and Arnon 1938) at one-fourth and one-half strength during the second and third weeks, respectively, and with undiluted Hoagland solution thereafter.

Seeds of *Lotus japonicus* Miyakojima MG20 were surface-sterilized with sulphuric acid for 15 min, followed by thorough washing with water. Seeds were germinated on humidified Whatman paper, and seedlings were planted in pots containing perlite imbibed in nitrogen-poor growth medium (Solution I; EMBO Practical Course on the new plant model system *Medicago truncatula* website). Plants were grown in a growth chamber with a 16-h photoperiod at 24°C day and 17°C night temperatures, 50% humidity, and regular watering with Solution I. One week after planting, plantlets were inoculated with approximately 1 ml of a *Mesorhizobium loti* suspension at OD₆₀₀ = 0.1 per plant.

Flow cytometry analysis.

DNA content of nuclei from different organs was determined by flow cytometry as described (Coba de la Peña and Brown 2001). Freshly collected plant material was finely cut with a razor blade in Galbraith buffer containing 0.1% Triton X-100 and 0.1% β -mercaptoethanol to release intact nuclei, as described previously (Galbraith et al. 1983). Isolated nuclei were filtered through 30- μ m nylon mesh and were stained with 5 μ g ml⁻¹ 4',6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co., St. Louis). DNA histograms were generated by analyzing at least 5,000 nuclei per measurement in a minimum of three independent experiments, using an EPICS V flow cytometer (Beckman-Coulter, Roissy, France).

Fluorescence microscopy.

Nodule primordia and nodules of lupin and lotus at different stages of development were collected, fixed immediately at room temperature in FAA solution (3.7% formaldehyde, 50% ethanol, and 10% acetic acid), and were stored overnight, dehydrated in an ethanol dilution series and embedded in Paraplast (Sigma) as described by McKhann and Hirsch (1993). Sections (10 μ m) were obtained from sample tissues. Paraffin was removed by xylene treatment, and tissue was rehydrated. Nuclei were stained with 1 μ g of DAPI containing 0.1% Triton X-100 per milliliter, and cell walls were stained with 0.3 mg of calcofluor per milliliter (Sigma). After washing with phosphate buffered saline, pH 7.2, sections were observed and photographed using a Zeiss Axioplan photomicroscope and UV light excitation.

Isolation of *ccs52A* cDNA clones.

A cDNA library of yellow lupin developing nodules constructed into the λ UniZAP XR cloning vector (Stratagene, La Jolla, CA, U.S.A.) and containing approximately 4.4×10^{10} CFU ml⁻¹ was screened onto nylon filters (Biotrans, ICN Bio-

medicals, Costa Mesa, CA, U.S.A.) by standard procedures (Sambrook et al. 1989). A random-primed ³²P-labeled probe was generated using the *Medicago sativa ccs52A* cDNA as a template with the Megaprime DNA labeling kit (Amersham Pharmacia, Buckinghamshire, U.K.), according to manufacturer's instructions. Two rounds of screening were performed to obtain single positive clones. Phagemids were excised from λ UniZAP to the plasmid form following manufacturer's instructions. Plasmid DNA was isolated according to standard procedures (Sambrook et al. 1989), and the insert was sequenced.

cDNA was prepared from 1 μ g of total RNA from white lupin young nodules using the First strand cDNA synthesis kit (Roche, Branchburg, NJ, U.S.A.) for RT-PCR, according to manufacturer's protocols. One-tenth of the RT reaction volume was used for PCR. Oligonucleotide design was based on the *Llccs52A* cDNA sequence (forward primer, 5'-CGATTATTCCTTCTCCTTCTCC-3' and reverse primer, 5'-ATGCTCCGATTTCAGTGTCC-3'). The PCR reaction consisted of 35 cycles (30 s at 95°C, 45 s at 55°C, and 1 min at 72°C) and an extension time of 10 min at 72°C. The PCR product (1,368 bp) was cloned into a pDrive cloning vector (Qiagen, Hilden, Germany) according to manufacturer's instructions. After cloning, the *Lacccs52A* cDNA partial clone was confirmed by sequencing.

cDNA was prepared from 3 μ g of *Lotus japonicus* and *P. sativum* nodule total RNA with the PowerScript reverse transcriptase (BD Biosciences, Palo Alto, CA, U.S.A.), according to manufacturer's guidelines. *Pscs52A* cDNA was PCR-amplified with oligonucleotides based on the *Mtccs52A* sequence (forward primer, 5'-ATGGACGGAACCGGTAACCG and reverse primer, 5'-TCACCTAATAGTAGTTCTTCC). *Ljccs52A* cDNA was obtained by PCR, using primers designed on the basis of available EST sequences (accessions BI420244, AU252139, CB828401, BP061405, and BP034390). Primer sequences were 5'-ATGGACGATTCTTCCGGCC, 5'-GACACCTCCAGCGCCTACACCACT, 5'-GAATCCG AGCCCCGTCAAGAT, 5'-AGTCTTGACGGGGCTCGGATTC, 5'-TGGTGGCCGGGAT AAGAATA, 5'-TATTCTTATCCCCGGCCACCA, 5'-CCACCCCCAGATGCAAGAAG TCCA, and 5'-TCACTTATAATAGTTCTTCC.

Gene expression analysis.

ccs52A expression in lupin was studied by Northern blot analysis. RNA was isolated by phenol extraction and LiCl precipitation as described (Goormachtig et al. 1995). Total RNA samples (20 μ g) were denatured in formamide/formaldehyde by heating (65°C, 5 min) and then were separated electrophoretically on a 1.2% agarose gel containing formaldehyde. The presence of equal amounts of different RNA samples was confirmed by ethidium bromide staining. RNA was transferred to Hybond N membrane (Amersham Pharmacia Biotech) as described previously (Sambrook et al., 1989). RNA was fixed to the membrane by UV irradiation. Probes were ³²P-labeled by random priming (Megaprime DNA labeling kit), using the 1,368-bp fragment of *Lacccs52A* cDNA as template. Hybridization was performed overnight at 65°C in Rapid Hyb buffer (Amersham Pharmacia Biotech). Three 15-min washes were performed consecutively in 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5 $\times$ SSC, and 0.1 $\times$ SSC, containing 0.1% SDS, at 65°C. Hybridization signals were visualized with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Expression analysis in lotus was performed by semiquantitative RT-PCR. cDNA was prepared from 3 μ g of root, young (7 dpi) nodule, or mature (21 dpi) nodule total RNA with the PowerScript reverse transcriptase (BD Biosciences) according to manufacturer's instructions. For each semiquantitative PCR

reaction, one two hundredth of the cDNA samples was used. Standard PCR conditions were used with 28 cycles for amplification of the ubiquitin and leghemoglobin fragments and 35 cycles for the *ccs52A* fragment. PCR products were analyzed on standard agarose gels. The primers were: for ubiquitin, forward primer, 5'-CTACAACATTTCAGAAGGAGTCCA and reverse primer, 5'-CACAGGCCAG AAGAGGCCACAACA; for leghemoglobin, forward primer, 5'-GTTCTACACCGTTATAT TGGAGATAG and reverse primer, 5'-GCAGGGCTTCTTTAA CCACCACG; and for *ccs52A*, forward primer, 5'-TGGTGGC CGGGATAAGAATA and reverse primer, 5'-CCACCCCCAG ATGCAAGAAGTCCA.

In situ hybridization.

³⁵S-labeled sense and antisense probes were prepared by in vitro transcription with T7 polymerase (SP6/T7 DIG-RNA labeling kit; Roche) from linearized plasmid containing the 1,368-bp *Lupinus albus ccs52a* cDNA insert cloned in both orientations. The probes were hydrolyzed by alkaline treatment (carbonate buffer, pH 10.2, at 60°C for 38 min) to generate approximately 200-bp fragments.

Nodule primordia and nodules at different stages of development were fixed immediately at room temperature in FAA solution and were stored overnight, dehydrated in an ethanol dilution series and embedded in Paraplast. Sections (8 µm) of each tissue were mounted on Superfrost®/Plus slides (Menzel-Glaser, Braunschweig, Germany). Immediately before hybridization, paraffin was removed by xylene treatment and tissue was rehydrated. Probe penetration was increased by the treatment of sections with proteinase K (1 µg/ml) for 30 min at 37°C.

Hybridization was performed overnight at 42°C in hybridization buffer (50% deionized formamide, 0.3 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1× Denhardt's solution, 10% dextran sulphate, 70 mM dithiothreitol (DTT), 40 units of RNase inhibitor per milliliter, and 150 µg tRNA/ml). The corresponding sense probe was used as negative control. After hybridization, sections were washed for 1 h in 2× SSC containing 10 mM DTT at room temperature and for 1 h in 0.1× SSC containing deionized formamide and 10 mM DTT at 47°C. Sections were stained with 0.01% toluidine blue. Observations were made and photographs obtained with an epifluorescence Reichert Polyvar microscope. Radioactive in situ hybridization images were obtained with a combination of dark-field and epifluorescence illumination.

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