

**Oxylipin-specific cytochrome P450s (CYP74s) in *Lotus japonicus*:
Their implications in response to mechanical wounding and nodule
formation**

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Oxylipin-specific cytochrome P450s (CYP74s) in *Lotus japonicus*: Their implications in response to mechanical wounding and nodule formation

Plant oxylipins are involved in defense responses against pathogens or herbivores. Each oxylipin compound exerts its distinctive physiological function. The ability of many legumes to fix nitrogen with rhizobia gives them special importance in natural environments and agriculture. It has been postulated that oxylipins are somehow related to symbiosis; however, this is still a controversial issue. In this study, we isolated five genes at the branching point of the oxylipin pathway in *Lotus japonicus*, and their biochemical functions were identified as allene oxide synthases (AOSs), 13-hydroperoxide lyase (13HPL), and 9/13-HPLs. When the leaves were mechanically wounded, *AOS* and *9/13HPL* were upregulated in leaves and roots, respectively, from which their implications in wound response were suggested. When the plants were inoculated with rhizobia, no big change in the expression levels of genes was found. When high N was supplied to the nodulated plants, the number of nodules decreased, and simultaneously, *AOS* in the leaves was downregulated. Significance of *AOS* in response to the N status in the plants was suggested.

Keywords: oxylipin, *Lotus japonicus*, allene oxide synthase, hydroperoxide lyase, symbiosis, jasmonate

Abbreviations: AOS, allene oxide synthase; HPL, hydroperoxide lyase; 13HPOT, 13-hydroperoxy-(*Z,E,Z*)-9,11,15-octadecatrienoic acid; 13HPOD, 13-hydroperoxy-(*Z,E*)-9,11-octadecadienoic acid; 9HPOT, 9-hydroperoxy-(*E,Z,Z*)-10,12,15-octadecatrienoic acid; 9HPOD, 9-hydroperoxy-(*E,Z*)-10,12-octadecadienoic acid.

Introduction

Fatty acid hydroperoxides produced by lipoxygenases, either 9- or 13-hydroperoxides of C18 fatty acids (linoleic and linolenic acid), are intermediates in the oxylipin pathway in plants (Stumpe and Feussner 2006). 13-Hydroperoxides of fatty acids are the substrates for the oxylipin-specific P450s, such as allene oxide synthase (13AOS) or hydroperoxide lyase (13HPL). 13AOS and 13HPL comprise an unusual class of P450s and demonstrate low affinity for carbon monoxide and do not require molecular oxygen or NADPH-dependent P450 reductase, therefore, classified as subfamily CYP74A and CYP74B, respectively. 13AOS converts the hydroperoxide into allene oxide that is further converted into oxophytodienoic acid (OPDA) or jasmonates (JAs). They are signaling molecules regulating defense and development in plants (Koo and Howe 2009). 13HPL cleaves the hydroperoxides to yield C6 volatile aldehydes and the corresponding C12 oxo acids (Matsui 2006).

The other types of CYP74s act on 9-hydroperoxides of C18 fatty acids (Stumpe and Feussner 2006, Matsui 2006). CYP74C cleaves both 9- and 13-hydroperoxides and named 9/13HPL. AOS specific to 9-hydroperoxide (9AOS) is also known. Divinyl ether synthase (DES; CYP74D) catalyzes conversion of either 9- or 13-hydroperoxide into the corresponding divinyl ether. Most plants have several genes for CYP74s. The end product of each branch of oxylipin pathway takes a distinctive role in defense responses against biotic and abiotic attack, and structural diversity of each oxylipin compound permits functional specificity. The function of JAs has been extensively studied, and their roles as plant hormones related to stress responses and development have been established (Koo and Howe 2009). The products of HPL branch function as molecules exerting direct and indirect defense against pathogens or herbivores (Matsui 2006). In this context, it should be plausible that the metabolic flow of each branch of the oxylipin pathway is tightly controlled, most probably through regulation at the branching point catalyzed by each CYP74.

The ability of many legumes to fix nitrogen in association with rhizobia gives them special importance in natural environments and agriculture (Doyle and Luckow 2003). The symbiosis between legumes and rhizobium needs mutual exchange of signal molecules, such as flavonoids and Nodulation (Nod) factors (Ferguson et al. 2010). Several CYP74 genes in leguminous plants have been isolated so far, and their

biochemical nature has been studied (Noordermeer et al. 2000, Stumpe et al. 2005, Hughes et al. 2006, Domenico et al. 2007, Kongrit et al. 2007). However, their expression has been seldom reported. Leguminous plants accept rhizobia and arbuscular mycorrhizal fungi, and establish symbiotic interactions with them, while they start defense responses against the pathogenic microorganisms, insects, or parasitic plants in order to deter them. Thus, there should be a device that can distinguish the foes and friends. Oxylipins are generally involved in defense responses against biotic invaders (Koo and Howe 2009, Matsui 2006), hence, the oxylipin pathway should be coordinately regulated with the distinguishing device.

In order to get insight into the coordination, we report here the isolation of genes encoding CYP74 enzymes in *Lotus japonicus*. *L. japonicus* is one of the model legumes, and its genome is almost completely sequenced (Sato et al. 2008). The aim of the present work was to gain an understanding of the molecular basis of CYP74-dependent metabolism of fatty acid hydroperoxides in *L. japonicus*. Owing to the wealth of knowledge of plant-symbiont interactions in *L. japonicus*, this system is likely to provide a good model for assessing the role of oxylipins in plant symbiosis with rhizobia.

Materials and methods

Plant materials

L. japonicus (Miyakojima, MG-20) seeds were damaged using a sheet of sandpaper in a mortar and sown on Kimwipe moistened with water. The seeds were grown at 22°C under a 14/10 h light/dark cycle. After three days, the seedlings were placed between ‘pillows’ (11 x 11 x 4 cm) made of a nylon mesh bag filled with vermiculite and perlite (6/1, v/v). The plants were grown with B & D medium containing 0.05 or 5 mM KNO₃ under the same condition. Rhizobium (*Mesorhizobium loti*, MAFF 303099) was cultured in fresh TY medium for 72 hr at 28°C. The culture (1 µl) containing 1 x 10⁶⁻⁷ cells was diluted with 200 ml of fresh B & D medium. Inoculation was carried out by drenching the diluted suspension of the bacteria to the pillows.

Cloning and expression of CYP74s

EST clones were obtained from Frontier Science Research Center, University of Miyazaki, Japan. The list of the clones is shown in Supplemental Table. S1. For AOS2, PCR cloning from the genomic DNA was carried out. The primers used in this study are listed in Supplemental Table S2. For expression of each gene in *E. coli*, restriction enzyme sites were generated using PCR with the primers listed in Table S2, then, the open reading frame was inserted into the pQE vector (QIAGEN, Valencia, CA). *E. coli* M15 (QIAGEN) was used for expression. The recombinant enzymes were prepared as described previously (Matsui et al. 2000).

Substrate and product specificities of recombinant CYP74s

Fatty acid hydroperoxides were prepared by using soybean lipoxygenase-1 or tomato fruit lipoxygenase as described previously (Matsui et al. 2000). Hydroperoxide decomposing activity was determined by recording the absorption at 234 nm for conversion of the hydroperoxides at 25°C. AOS activity was determined by reacting the enzyme with 10 mM 13-hydroperoxy-(*Z,E,Z*)-9,11,15-octadecatrienoic acid (13HPOT) suspended with 50 mM Tris-HCl, pH 7.5 for 10 min at 25 °C. The AOS products were analyzed with HPLC by comparing those formed from 13HPOT by *E. coli*-expressed soybean AOS (Kongrit et al. 2007). HPL activity was determined by reacting the enzyme with 10 mM 13HPOT or 9-hydroperoxy-(*E,Z*)-10,12-octadecadienoic acid (9HPOD) suspended with 50 mM MES-KOH, pH 5.5 for 5 min at 25°C. The products were analyzed as their hydrozoane derivatives with HPLC (Matsui et al. 2000).

Determination of heme b and volatiles

For heme *b* determination, the roots were frozen in liquid N₂ and homogenized in 50 mM phosphate buffer (pH 7.4) by using a homogenizer (MicroSmash, TOMY, Tokyo, Japan). After centrifugation, the supernatant was filled up to 1.5 ml with cold acetone. The mixture was incubated for 10 min on ice, then, for 10 min at 25°C, and centrifuged. The supernatant was dried up and solubilized with 0.5 ml each of pyridine and 1N NaOH, and 1.5 ml of H₂O. The mixture (pyridine ferro-hemochrome) was divided into

two parts, and the one part was reduced with sodium hydrosulfite. The amount of heme *b* was determined from reduced minus oxidized difference spectra, through determining the absorbance at 555 and 535 nm.

For volatile analysis, the leaves or roots (ca. 40 mg fr wt) of *L. japonicus* (21 day old, without inoculation) was collected and homogenized with 1.5 ml of 50 mM Na-phosphate (pH 6.3) containing 0.1 mM DETAPAC on ice. The homogenate was put into a glass vial and incubated at 26°C for 10 min. After addition of 1.5 ml of saturated CaCl₂ solution in order to kill the enzymes, an internal standard (nonyl acetate, 100 ng) was added. SPME fiber (50/30 μm DVB/Carboxen/PDMS, Supelco, Bellefonte, PA, USA) was exposed to the headspace of the vial for 30 min at 80°C. The volatiles were analyzed with GC-MS (QP-2010Plus, Shimadzu) equipped with 0.25 μm x 30 m DB-WAX column (Agilent Technologies, Santa Clara, CA). The column temperature was programmed as 40°C for 5 min, to 200°C for 10 min at 5°C min⁻¹ with a carrier gas (He) at 5 ml min⁻¹. The temperature of the injector and interface was 200 and 230°C, respectively. The mass detector was operated in an electron impact mode (70 eV). In order to assign each compound, retention indices and MS profiles of corresponding authentic specimens were used.

RNA extraction and real time RT-PCR

RNA was extracted with RNeasy Plant Mini Kit (QIAGEN). cDNA was synthesized with ThermoScript RT-PCR System (Invitrogen). RT-PCR was performed with ABI PRISM 7300 (Life Technologies Co., Carlsbad, CA, USA)) using an Express SYBR GreenER qPCR Super mix (Invitrogen, Life Technologies Co.). The ubiquitin gene (acc. no. DQ249171) was used as an internal standard. Primers used in this study are listed in Supplemental Table S2.

Results

Assigning five CYP74s in L. japonicus

We performed BLAST search with the EST database of *L. japonicus* (*Lotus japonicus*

EST index, <http://est.kazusa.or.jp/en/plant/lotus/EST/>) (Asamizu et al. 2004). We found 41 entries derived from four genes that showed high similarity to CYP74 genes (see Supplementary Table S1). The longest EST clone of each was sequenced, and from the sequences we validated that they were mostly full-length. Based on their sequences, they were tentatively identified as *LjAOS1* (*LjCYP74A1*, DDBJ acc. no., AB600747), *Lj13HPL* (*LjCYP74B*, AB600748), *Lj9/13HPL1* (*LjCYP74C1*, AB600749), or *Lj9/13HPL2* (*LjCYP74C2*, AB600750). We also performed BLAST search with the genome database of *L. japonicus* ([miyakogusa.jp, http://www.kazusa.or.jp/lotus/index.html](http://www.kazusa.or.jp/lotus/index.html)) (Sato et al. 2008). We found another CYP74-related sequence within a BAC clone (CM0314) other than the four genes. The sequence showed high similarity to *LjAOS1*, and it was tentatively named *LjAOS2* (*LjCYP74A2*). The genes for DES (CYP74D) and 9AOS were not found. When the phylogenetic tree was constructed, the CYP74s in *L. japonicus* were located in the clade as expected from their tentative assignments (Figure 1). In the clade, *LjCYP74s* were located nearby the counterparts of the other leguminous plant, *Medicago truncatula*, except for *LjAOS2*, whose counterpart was absent from *M. truncatula*. Among the five *LjCYP74s*, two (*Lj9/13HPL2* in chromosome III and *LjAOS1* in chromosome V) occur in the synteny blocks found between chromosomes of *L. japonicus* and *M. truncatula* with their counterparts in *M. truncatula* (Cannon et al. 2006) (Supplemental Figure S1). Prediction of subcellular localization with TargetP analysis (<http://www.cbs.dtu.dk/services/TargetP/>) suggested that *LjAOS1*, *LjAOS2* and *Lj13HPL* were localized in the chloroplasts with 37, 57, and 85 amino acid stretch of transit peptide, respectively. The TargetP analysis showed that *Lj9/13HPL1* and 2 did not have any sequences directing them to a specific organelle.

Functional expression of recombinant proteins

Active recombinant enzymes of *LjAOS1*, *LjAOS2*, *Lj13HPL*, and *Lj9/13HPL2* were expressed with *E. coli*. *Lj9/13HPL1* could not be functionally expressed in *E. coli* with unknown reasons. 13HPOT was the best substrate for the recombinant *LjAOS1*, and 13-hydroperoxy-(*Z,E*)-9,11-octadecadienoic acid (13HPOD) followed (Figure 2A). Recombinant *LjAOS1* showed only 20% activity with 9HPOD, and 9-hydroperoxy-(*E,Z,Z*)-10,12,15-octadecatrienoic acid (9HPOT) was not essentially the

substrate. When products from 13HPOT formed by recombinant LjAOS1 was analyzed, α - and γ -ketols, and oxophytodienoic acid (OPDA) were detected (Figure 2B), but HPL products could not be detected. Collectively, it was concluded that *LjAOS1* encoded 13-hydroperoxide-specific AOS. Unlike most AOSs examined so far (Stumpe et al. 2006, Kongrit et al. 2007, Lee et al. 2008), the activity of recombinant LjAOS2 with 13HPOD was significantly higher than that with 13HPOT. Products formed by recombinant LjAOS2 from 13HPOT were also α - and γ -ketols, and OPDA. These analyses confirmed that LjAOS2 also encoded 13-hydroperoxide-specific AOS but with a preference to the hydroperoxides of dienoic acids rather than those of trienoic acids as substrates.

Recombinant Lj13HPL showed highest activity against 13HPOT, and 13HPOD followed. 9HPOs were hardly catalyzed by Lj13HPL as found with most 13HPLs of the other plant sources (Stumpe and Feussner 2006, Matsui 2006). (Z)-3-Hexenal and 12-oxo-(Z)-9-dodecenoic acid were the major products, and the AOS products could not be detected (Figure 2C). Thus, it was clarified that Lj13HPL encoded 13-hydroperoxide-specific HPL. Recombinant Lj9/13HPL2 showed high activity with 13HPOs, but also showed a substantial activity against 9HPOD, which is a distinguishing feature of most 9/13HPLs. The recombinant Lj9/13HPL2 yielded a typical HPL product, (Z)-3-nonenal from 9HPOD (Figure 2C); thus, it is reasonable to assign the gene as the one encoding 9/13HPL.

Expression of CYP74 genes

Expression levels of *LjAOS1* and 2, and *Lj9/13HPL2* were largely equivalent among leaves, stems, and roots of 21 days old seedlings grown without inoculation of rhizobia but with B & D medium containing 5 mM KNO₃ (Figure 3A). The levels of the other two *LjCYP74* genes in these organs varied. *Lj13HPL* transcript level was higher in leaves, and lower in stems and roots. *Lj9/13HPL1* transcript level was higher in roots than in leaves or stems. The same tendency was observed with the rhizobia-inoculated plants (cf. Figure 5); therefore, organ-specificity of expression of each *CYP74* in *L. japonicus* was little affected by symbiosis. The organ-specific expression of *Lj13HPL* and *Lj9/13HPL* gene was further confirmed by the analysis of volatile products of these two types of HPLs (Figure 3B). In leaves the amounts of C6 volatiles, such as

(*E*)-2-hexenal, (*Z*)-3-hexenal, and the alcohols and acetates derived from them, were significantly higher than those in roots. On the contrary, C₉ volatiles, such as (*E,Z*)-2,6-nonadienal and (*E*)-2-nonenal were more abundant in roots than in leaves.

Oxylipin metabolism is known to be involved in wound-inducible defense gene expression in various plant species (Koo and Howe 2009, Matsui 2006). When the leaves of 21 days old non-inoculated seedlings of *L. japonicus* were mechanically wounded, *LjAOS2* was upregulated in leaves (Figure 3A). *LjAOS1* was also slightly upregulated. The transcript levels of *Lj13HPL* showed no change. *Lj9/13HPL2* was upregulated in roots when the leaves were wounded even though upregulation of the gene in the leaves was not observed. *Lj9/13HPL1* was also slightly upregulated in roots after mechanical wounding on leaves.

Effect of nodulation and autoregulation

In order to examine the effects of nodulation and autoregulation of nodulation (AON) caused by high supply of N (Ferguson et al. 2010, Oka-Kira and Kawaguchi 2006) to the nodulated *L. japonicus* plants on expression of *LjCYP* genes, the seedlings (7 days old) were inoculated with *Mesorhizobium loti*, then, grown with low (0.05 mM) or high (5.0 mM) N supply (Figure 4A). At 14 days after inoculation, formation of nodules was evident with the plants grown with low N supply while few nodules were observed with the ones with high N supply (Figure 4B). At 14 days after inoculation (i.e., at 21 days after germination), the nodulated plants grown with low N were divided into two groups; then, one group was further grown with low N supply while the other group was grown with high N supply in order to onset autoregulation of nodulation (AON) (Ferguson et al. 2010, Oka-Kira and Kawaguchi 2006). Seven days after the supply of high N (i.e., 21 days after inoculation and 28 days after germination), the number of nodules was reduced to less than half of that with low N because of AON (Figure 4B, C). The AON was also evident when the amount of heme *b*, which was an indicator of active nodules containing leghemoglobin, was quantified (Figure 4D).

The transcript level of leghemoglobin extensively increased after inoculation when the plants were grown with low N supply (Figure 5). When the inoculated *L. japonicus* plants were grown with high N supply, the transcript level of leghemoglobin was effectively reduced. Such effect of high N supply on the transcript level of

leghemoglobin was evident even with the nodulated *L. japonicus* plants, and a shift of N supply from 0.05 to 5 mM from 14 days after inoculation extensively reduced the expression of leghemoglobin, from which it was confirmed that AON proceeded by high N supply under the experimental condition employed here. When the transcript levels of *CYP74s* were examined, they were mostly constant irrespective of the presence or absence of rhizobium, or to the N availability. The only exception being *LjAOS2* in leaves. The amount of *LjAOS2* transcript in the leaves of inoculated *L. japonicus* plants (14 days post inoculation) grown with high N was lower than that found with the inoculated plants grown with low N (Figure 5). This is also the case with the plants at 21 days post inoculation. The amount of *LjAOS2* transcript was extensively suppressed at 7 days after changing the N supply from 0.05 mM to 5 mM when suppression of nodulation caused by AON was obvious.

Discussion

Comprehensive analysis showed that *L. japonicus* had two *AOSs*, one *13HPL*, and two *9/13HPLs*. The phylogenetic analysis based on their amino acid sequences indicated that each *CYP74* in *L. japonicus* was placed in the appropriate clade, such as *CYP74A*, *B* or *C*, which was consistent with its enzymatic activity identified in this study. When BLASTP search was performed with genome sequences of *M. truncatula*, *Glycine max*, *Cucumis sativus*, and *Prunus persica* with phytozome (<http://www.phytozome.net/>) by using either of *CYP74s* in *L. japonicus* as a query, five hypothetical gene families, namely, #26459994, #26457455, #26477720, #26478987, and #26455604, were identified. They were corresponding to *LjAOS1*, *LjAOS2*, *Lj9/13HPL2*, *Lj9/13HPL1*, and *13HPL*, respectively. Among them, the gene corresponding to *Lj9/13HPL1* was found only with leguminous plants but not found with the Cucurbitales and Fagales. Thus, it was assumed that the five genes except for that corresponding to *Lj9/13HPL1* were acquired before segregation of the clade consisting of Fabales, Rosales, Cucurbitales, and Fagales in the eudicots, but duplication of *9/13HPL* genes occurred after establishing leguminous plants (Stevens 2008). Every gene for *13HPL*, including that in monocots and eudicots, was found at the same clade in the phylogenetic tree, which suggested that the gene was acquired

before segregation of monocots and dicots (Lee et al. 2008). Among dicots, the genes corresponding to *9/13HPL* have been found only with rosids. Some monocots also have *9/13HPLs*; however, they were placed apart from those of rosids on the phylogenetic tree. Therefore, it is assumed that *9/13HPL* genes in rosids and monocots acquired their catalytic function independently after divergence of eudicots and monocots.

With *L. japonicus* seedlings, 13HPL highly expressed in leaves, while both the *9/13HPLs* expressed profoundly in roots. Volatile analysis indicated that C6 volatiles were more abundant in leaves but C9 volatiles were more abundant in roots, which coincided with the expression profiles of *Lj13HPL*, and *Lj9/13HPL1* and 2. C6 volatiles thus formed in leaves are thought to be involved in direct or indirect defense against pests as reported with *Arabidopsis* (Shiojiri et al. 2006); however, the physiological significance of C9 volatiles formed in roots is scarcely known. In cucumber, it was expected that one of the products of *9/13HPL*, C9 aldehyde, functioned as toxic agents against pathogens (Matsui et al. 2006). Azelaic acid, which might be formed from the counterpart of *9/13HPL* products, 9-oxononanoic acid, is a mobile metabolite accountable for plant systemic immunity involved in priming defenses (Jung et al. 2009). Taken together, root expression of *9/13HPL* might be involved in plant defense against pathogens in the rhizosphere. We found that mechanical wounding on leaves resulted in upregulation of *Lj9/13HPLs*. It was reported that wounding of pepper leaves by infestation of whitefly (*Bemisia tabaci*) resulted in induced resistance at the below-ground organs against a soil-borne pathogen, *Ralstonia solanacearum*, and also in modifications of the rhizosphere microflora (Yang et al. 2011). The systemic induction of *Lj9/13HPL* expression in roots observed in this study was in line with this orchestrated defense responses between the above-ground and below-ground organs, which supported the hypothesis for the role of *Lj9/13HPLs* in defense responses against pathogens in rhizosphere. Because *LjAOS2* was upregulated in leaves after mechanical wounding, the products of the corresponding enzymes, for examples, OPDA or JAs, might be involved in the systemic signaling from the above-ground to the below-ground. JA as a long-distance signaling molecule that is transported in the phloem system has been reported in various plant species (Schilmiller and Howe 2005).

Oxylipin pathway is induced under biotic stresses in leguminous plants. When *M. truncatula* was infected by pathogenic fungi or by a parasitic plant, the genes for JA

synthesis were upregulated (Uppalapati et al. 2009, Anderson et al. 2010, Hiraoka et al. 2009). Aphid infestation resulted in induction of oxylipin pathway through upregulation of *LOX* and several *CYP74* genes in *M. truncatula* (Gao et al. 2007). Damage by herbivores enhanced emission of C6 compounds (Shimoda 2010). On the contrary, our study showed that inoculation of rhizobia did not result in upregulation or downregulation of the *LjCYP74* genes. Apparently, legume plants have a mechanism to distinguish the foes and friends. In fact, β -glucan and chitin tetramer, which are pathogen-related microbial elicitors for plants, induced accumulation of JA while no accumulation of JA could be observed after treating *M. truncatula* with Nod-factors, which are also oligosaccharides (Leitner et al. 2008). Thus, leguminous plants have a machinery to distinguish the foes and friends, and because of it, leguminous plants induce expression of *CYP74s* only when they were attacked by destructive invaders but do not modulate expressions of *CYP74s* when they recognize their symbionts.

Leguminous plants control the number of nodules through a mechanism known as the autoregulation of nodulation (AON) pathway involving long-distance root-shoot signaling (Ferguson et al. 2010, Oka-Kira and Kawaguchi 2006). Several reports pointed out a possible involvement of JAs in AON. Spraying methyl jasmonate on shoots strongly suppressed nodulation in *L. japonicus* (Nakagawa and Kawaguchi 2006). Involvement of JA and AOS in controlling the degree of nodulation has also been reported with soybean (Kinkema and Gresshoff 2008). However, it was recently reported that JA might not be involved in a typical AON that needed a perception of shoot-derived signal by a product of gene named *Too Much Love (TML)* because the suppression of nodulation by JA was also apparent with a mutant *tml* that showed a hypernodulating phenotype (Magori and Kawaguchi 2010). The result shown in this study indicated that expression of *CYP74* genes, except for *LjAOS2*, in *L. japonicus* showed no big change when the plants were forced to launch AON by supplying high N. In contrast, *LjAOS2* was highly suppressed in the leaves when the nodulated plants were grown with high N supply. From this, it was suggested that any *CYP74* genes including the two *AOSs* were not involved in launching or maintaining AON. However, at this moment, it is unknown why *LjAOS2* in leaves was downregulated after onset of AON. Nitrogen status affects nodulation because the symbiosis would be no longer necessary when N availability is high. JA-treatment on leaves of tomato enhanced allocation of N to roots (Gómez et al. 2010). It was also reported that surplus N supply induced

expression of JA-responsive genes in soybean (Staswick et al. 1991). JA was also involved in partitioning of nitrogen among organs of alfalfa (Meuriot et al. 2004). Thus, it was assumed that the downregulation of *LjAOS2* under high N status might be related to the nitrogen dynamics in *L. japonicus* that was coordinated with AON.

Appendix A. Supplemental data

Supplemental Figure S1.

Supplemental Table S1.

Supplemental Table S2.

Acknowledgments

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Legends for Figures

Figure 1. Phylogenetic tree analysis of the CYP74 family. Amino acid sequences were aligned using ClustalW, then, the tree was constructed with Njplot. LjCYP74s are highlighted, and the entries whose enzymatic activities were confirmed are underlined. At13AOS; *Arabidopsis thaliana* AOS (CAA63266), At13HPL; *A. thaliana* 13HPL (AAC69871), AsDES; *Allium sativum* DES (CAI30435), Ca13HPL; *Capsicum annuum* 13HPL (AAK27266), CasAOS; *Camelia sinensis* AOS (ACU30142), CiAOS; *Citrus cinensis* AOS (AAO72741), Cj13HPL; *Citrus jambhiri* 13HPL (BAC55161), Ci13HPL; *Citrullus lanatus* 13HPL (AAU12570), CmAOS; *Cucumis melo* AOS (AAM66138), Cm9/13HPL; *C. melo* 9/13HPL (AAK54282), Cs9/13HPL; *Cucumis sativum* 9/13HPL (AAF64041), Gm13AOS1; *Glycine max* 13AOS1 (ABB91776), Gm9/13HPL; *G. max* 9/13HPL (ABC68416), HvAOS1; *Hordeum vulgare* AOS1 (CAB86384), Hv13HPL; *H. vulgare* 13HPL (CAC82980), LoAOS; *Lonicera japonica* AOS (ABC17856), Lu13AOS; *Linum usitatissimum* 13AOS (P48417), Ma13HPL; *Musa acuminata* 13HPL (CAB39331), MtAOS; *Medicago truncatula* 13AOS (CAC86897), Mt9/13HPL1; *M. truncatula* 9/13HPL1 (CAC86899), Mt9/13HPL2; *M. truncatula* 9/13HPL2 (CAC86898), Ms13HPL1; *Medicago sativa* 13HPL1 (CAB54847), NtDES; *Nicotiana tabacum* DES (AAL40900), Os9/13HPL1; *Oryza sativa* 9/13HPL1 (AK105964), Os9/13HPL2; *O. sativa* 9/13HPL2 (AK107161), PaAOS; *Parthenium argentatum* 13AOS (CAA55025), PdHPL; *Prunus dulcis* HPL (CAE18065), Pg13HPL; *Psidium guajava* 13HPL (AAK15070), PtAOS; *Populus trichocarpa* AOS (B9GP05), PpAOS2; *Physcomitrella patens* AOS (CAC86919), PpHPL; *P. patens* HPL (CAC86920), PtHPL; *Populus trichocarpa* HPL (XP_002305404), RcHPL; *Ricinus communis* (XP_002529334), SbAOS; *Sorghum bicolor* AOS (XP_002463829), S113AOS; *Solanum lycopersicon* 13AOS (CAB88032), S19AOS3; *S. lycopersicon* 9AOS (AAN7687), SIDES; *S. lycopersicon* DES (AAG42261), S113HPL; *S. lycopersicon* 13HPL (CAB43022), St9AOS; *S. tuberosum* 9AOS (CAI30876), StDES; *S. tuberosum* DES (CAC28152), TaAOS; *Triticum aestivum* 13AOS (CAC28152), VvAOS; *Vitis vinifera* AOS (XP_002283780), VvHPL1; *V. vinifera* HPL1 (ACZ17394), VvHPL2; *V. vinifera* HPL2 (XP_002281213), ZmHPL; *Zea maize* HPL (AAS47027).

Figure 2. Substrate specificities (A) and product specificities (B, C) of recombinant CYP74s derived from *L. japonicus*. The hydroperoxide-decomposing activity was determined spectrophotometrically with 13-hydroperoxy-(*Z,E,Z*)-9,11,15-octadecatrienoic acid (13HPOT), 13-hydroperoxy-(*Z,E*)-9,11-octadecadienoic acid (13HPOD), 9-hydroperoxy-(*E,Z,Z*)-10,12,15-octadecatrienoic acid (9HPOT), or 9-hydroperoxy-(*E,Z*)-10,12-octadecadienoic acid (9HPOD). The highest activity for each enzymatic activity, 1.66 (LjAOS1), 3.62 (LjAOS2), 2.16 (Lj13HPL), or 11.8 (Lj9/13HPL2) $\mu\text{kat ml}^{-1}$, is set as 100%, respectively. For analysis of products, HPLC for detection of AOS products (B) or of HPL products (C) was carried out as in Method section, and the representative chromatograms are shown. The profiles of products formed from Lj13HPL and Lj9/13HPL2 from 13HPOT were essentially same, then, only one chromatogram is shown. Peaks 1; γ -ketol, 2; α -ketol, 3; OPDA, 4; 12-oxo-(*Z*)-9-dodecenoic acid, 5; (*Z*)-3-hexenal, 6; (*Z*)-3-nonenal.

Figure 3. Expression of each *LjCYP74* in leaves, stems, and roots of *L. japonicus* (21 days old, uninoculated) and the effect of mechanical wounding (A). The transcript level was first normalized to the level of ubiquitin in each sample, and the expression level in the roots before wounding was then set as 1. The leaves were mechanically wounded, and the expression levels after 6 h was determined. The means \pm SE (n=3) are shown. The values followed by the same letter indicate means that are not significantly different ($P>0.05$, ANOVA). Volatiles formed after disruption of leaves or roots of the seedlings (B). The relative area ratio normalized with the area of internal standard calculated with total ion chromatogram is shown (means \pm SE, n=3). The values followed with asterisks indicate means significantly different between leaves and roots ($P>0.05$, t-test). Compounds 1; *n*-hexanal, 2; (*Z*)-3-hexenal, 3; (*E*)-2-hexenal, 4; 2-octanone, 5; (*Z*)-3-hexenyl acetate, 6; (*Z*)-2-penten-1-ol, 7; *n*-hexan-1-ol, 8; (*Z*)-3-hexen-1-ol, 9; *n*-nonanal, 10; (*E*)-2-hexen-1-ol, 11; 1-octen-3-ol, 12; (*E*)-2-nonenal, 13; 2,6-(*E,Z*)-nonadienal.

Figure 4. The growth schedule of *L. japonicus* seedlings (A), photos of plants used in this study (B), number of nodules (C) and the amount of heme *b* derived from leghemoglobin in roots (D). The seeds were germinated and grown with 0.05 mM

KNO₃ for 7 days. Then, the seedlings were inoculated with *M. loti*, and grown with 0.05 or 5.0 mM KNO₃. At 14 days after inoculation, the ones grown with 0.05 mM KNO₃ were divided into two halves, and one of them were grown with 0.05 mM KNO₃ while the others were grown with 5.0 mM KNO₃ for additional 7 days. The plants used for the analyses were named as *a* to *f*. On the photos (B), the nodules are shown with white arrows. For nodule number (C) and the amount of heme *b* (D), the means \pm SE (n=3) are shown. The values followed by the different letter indicate means that are significantly different ($P>0.05$, ANOVA). In panel D, the shape and color of nodules isolated from plant *d* and *f* is shown in the inset.

Figure 5. Expression of each CYP74 in nodulated *L. japonicus* grown with different N availability. The plants were grown as shown in **Results** section, then, the expression level of each gene with the plant *a* to *f* was determined. For the details of the plants, refer Figure 4A. The transcript level was first normalized to the level of ubiquitin in each sample, and the expression level in the roots of 7 days old seedling was then set as 1. The means \pm SE (n=3) are shown.

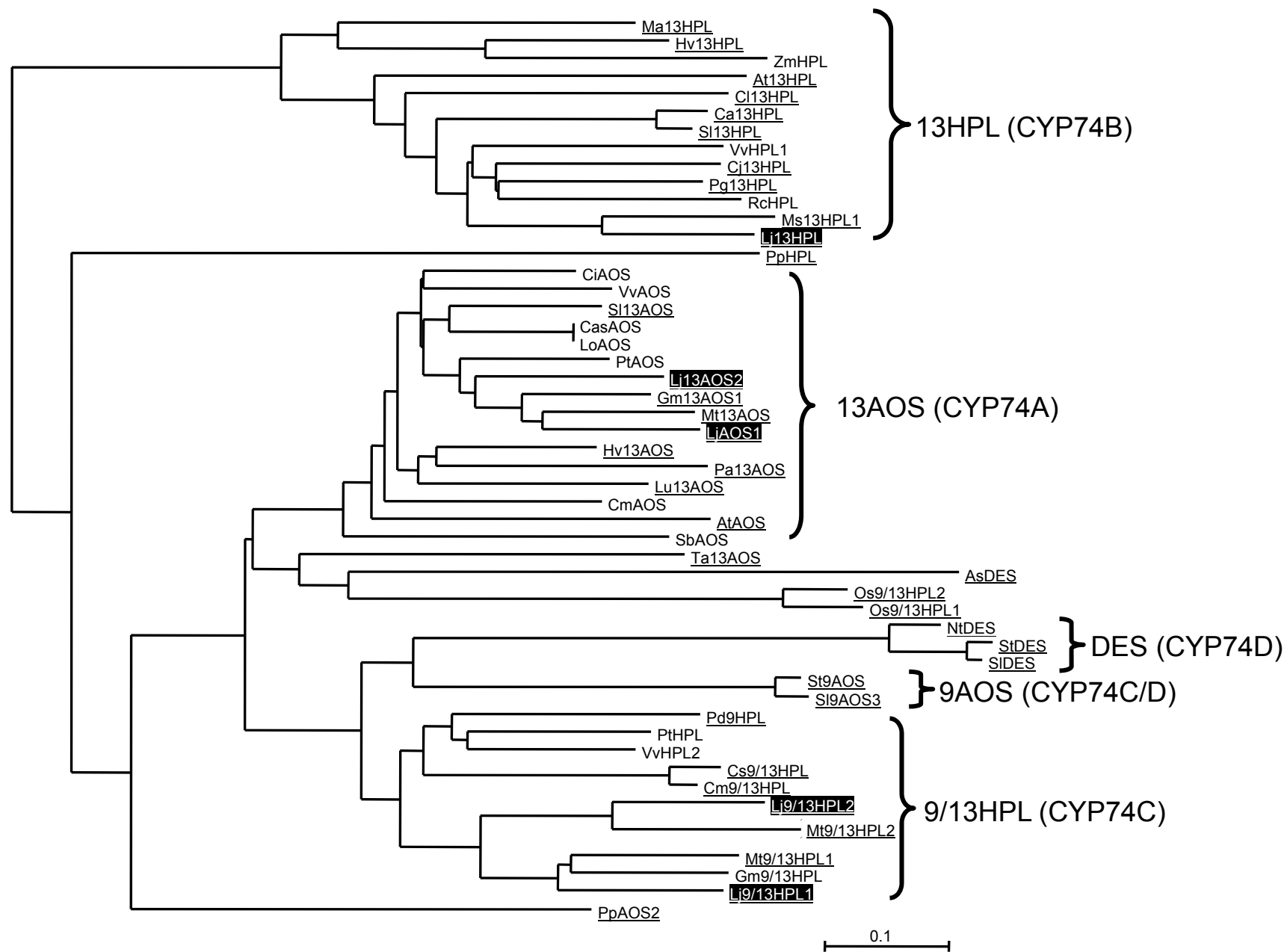


Fig. 1

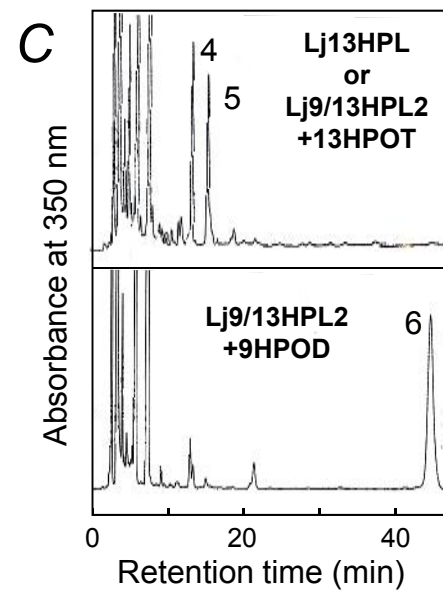
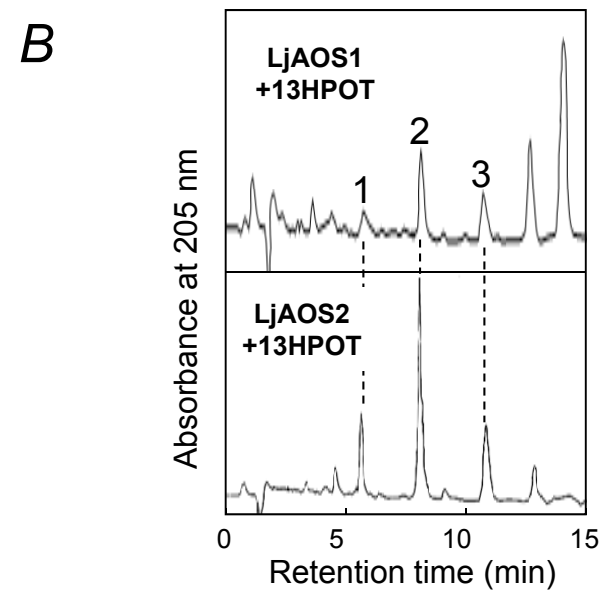
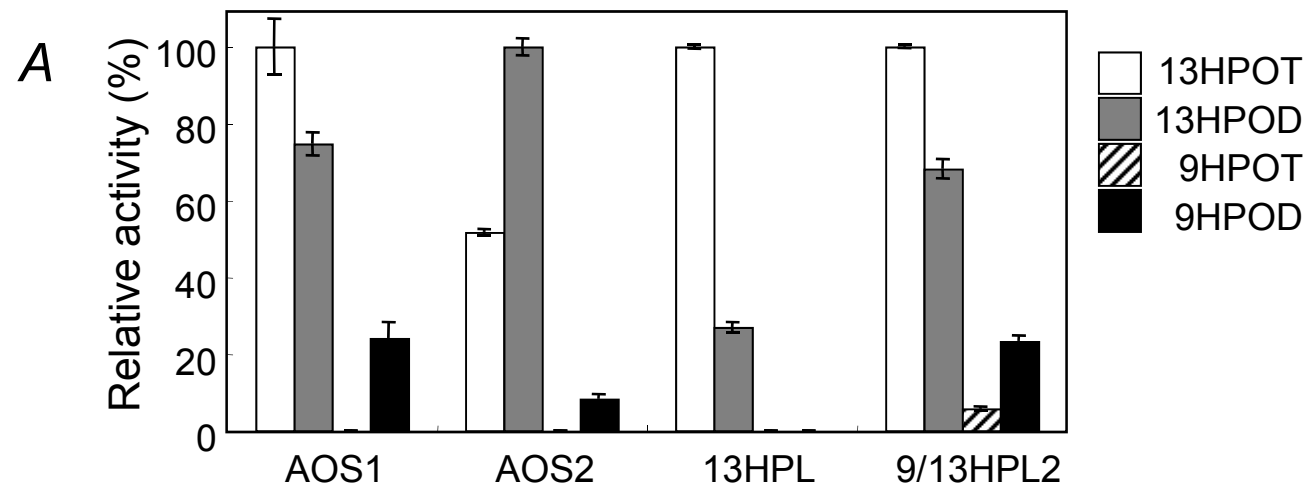
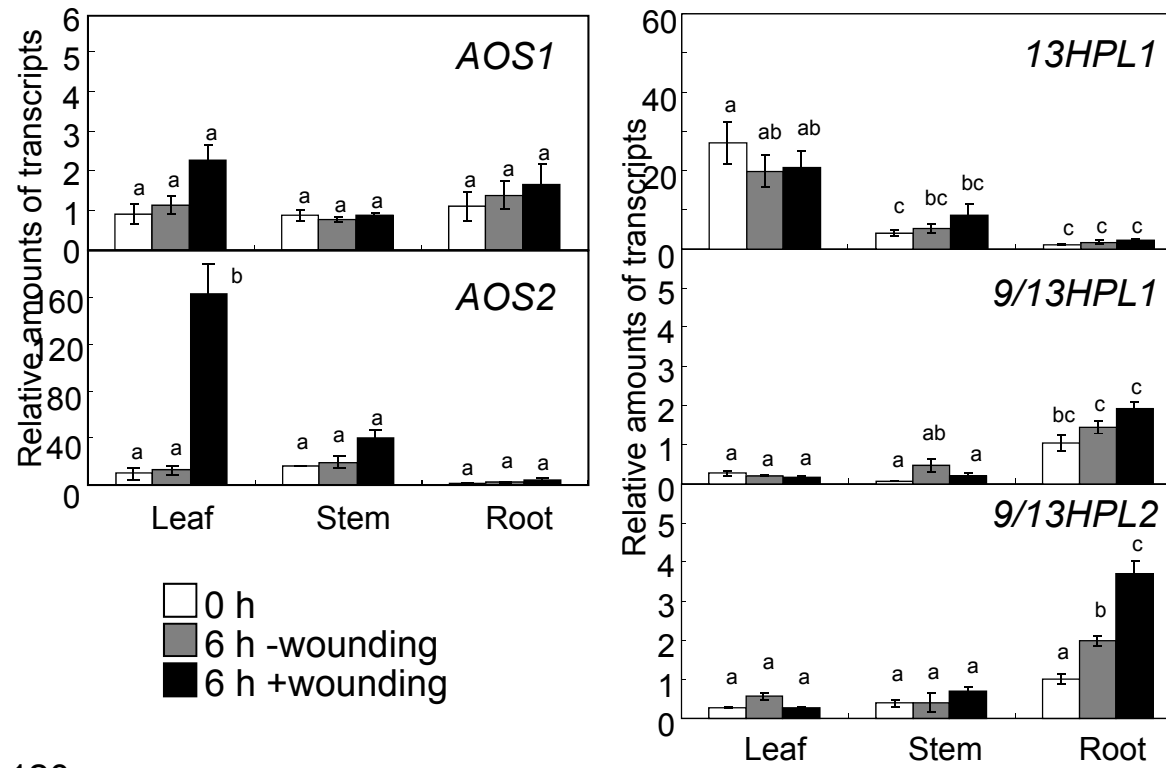


Fig. 2

A



B

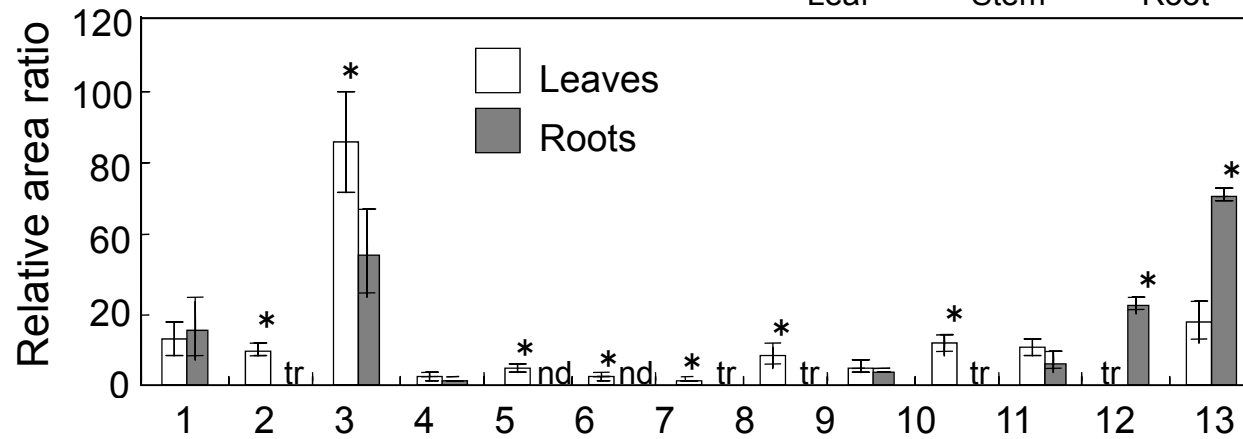


Fig. 3

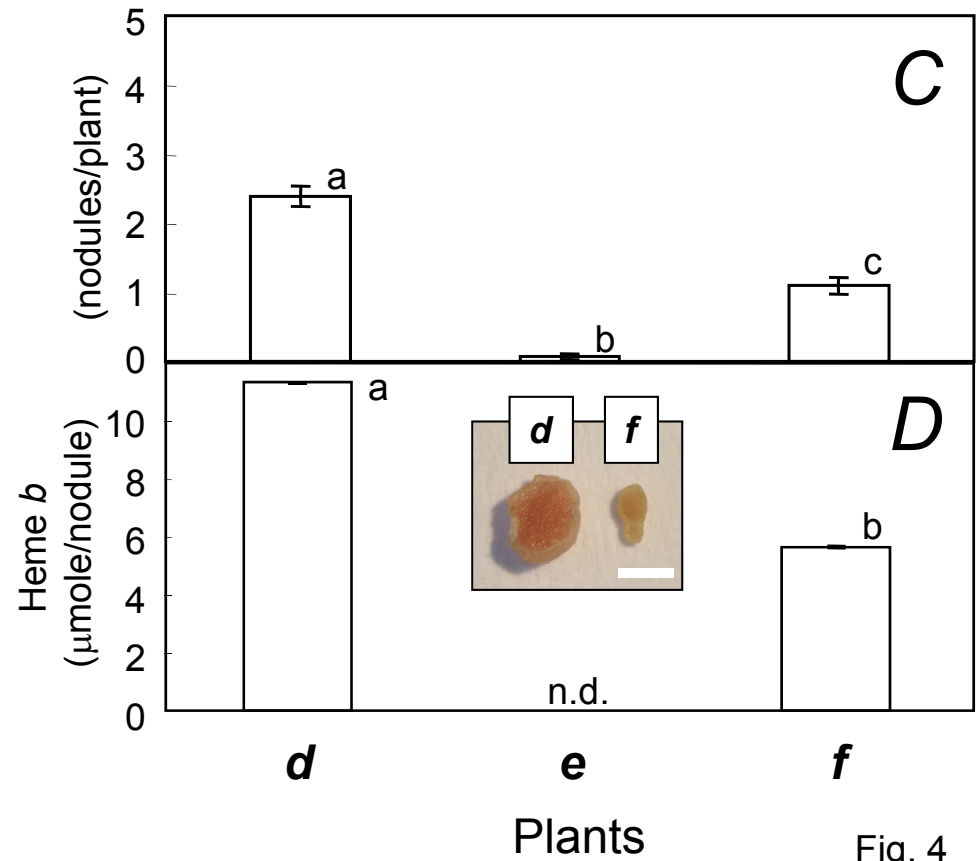
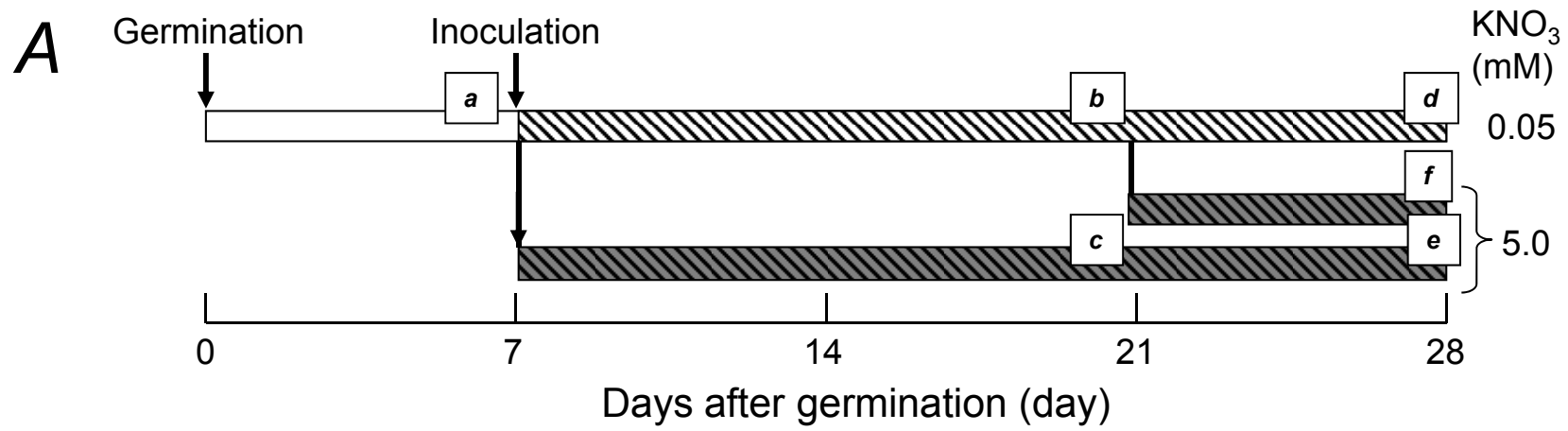


Fig. 4

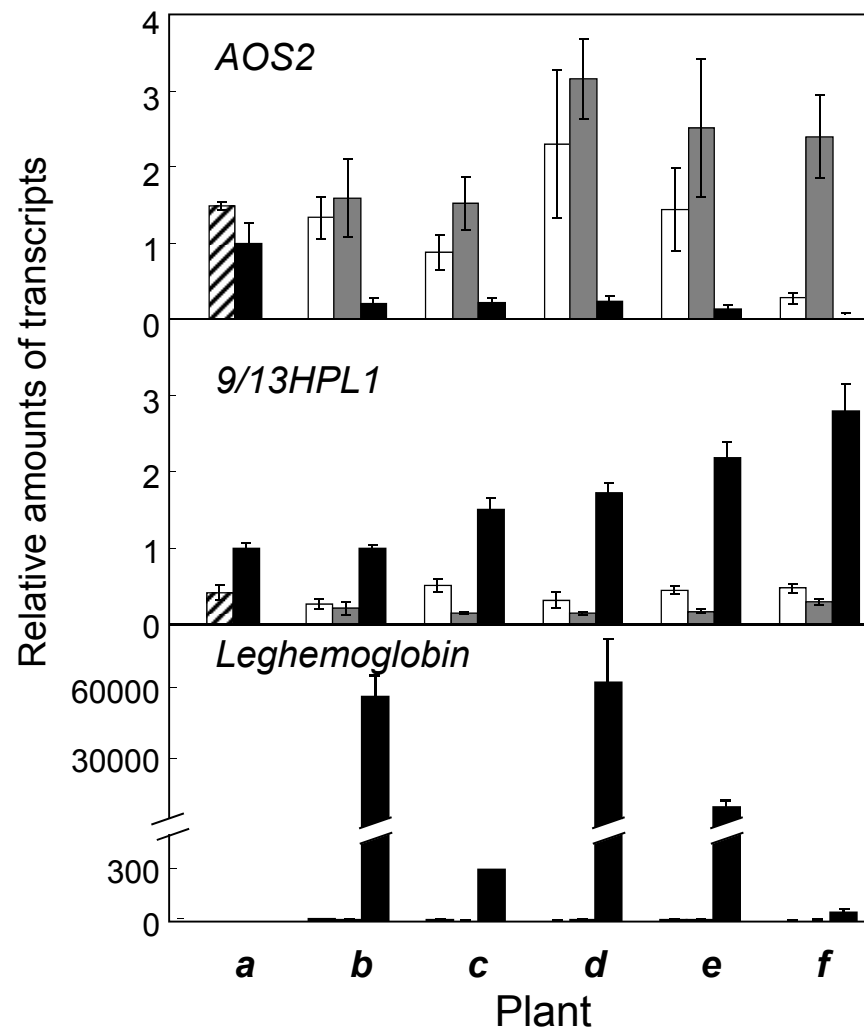
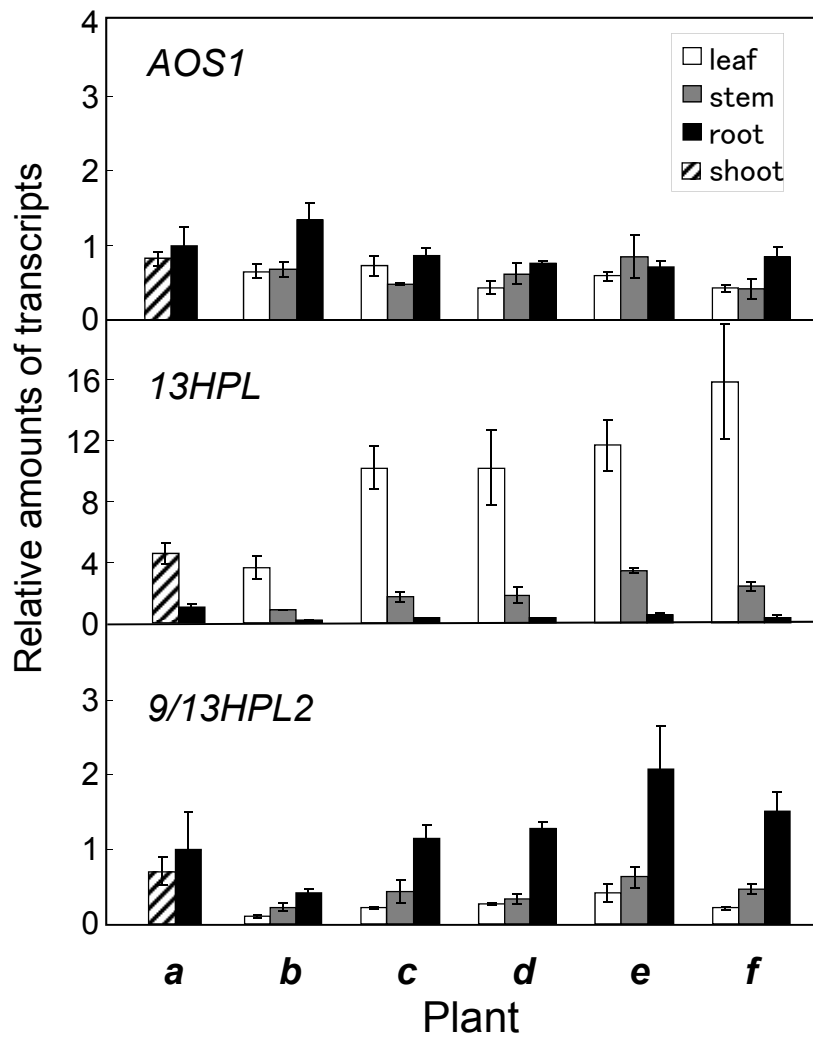
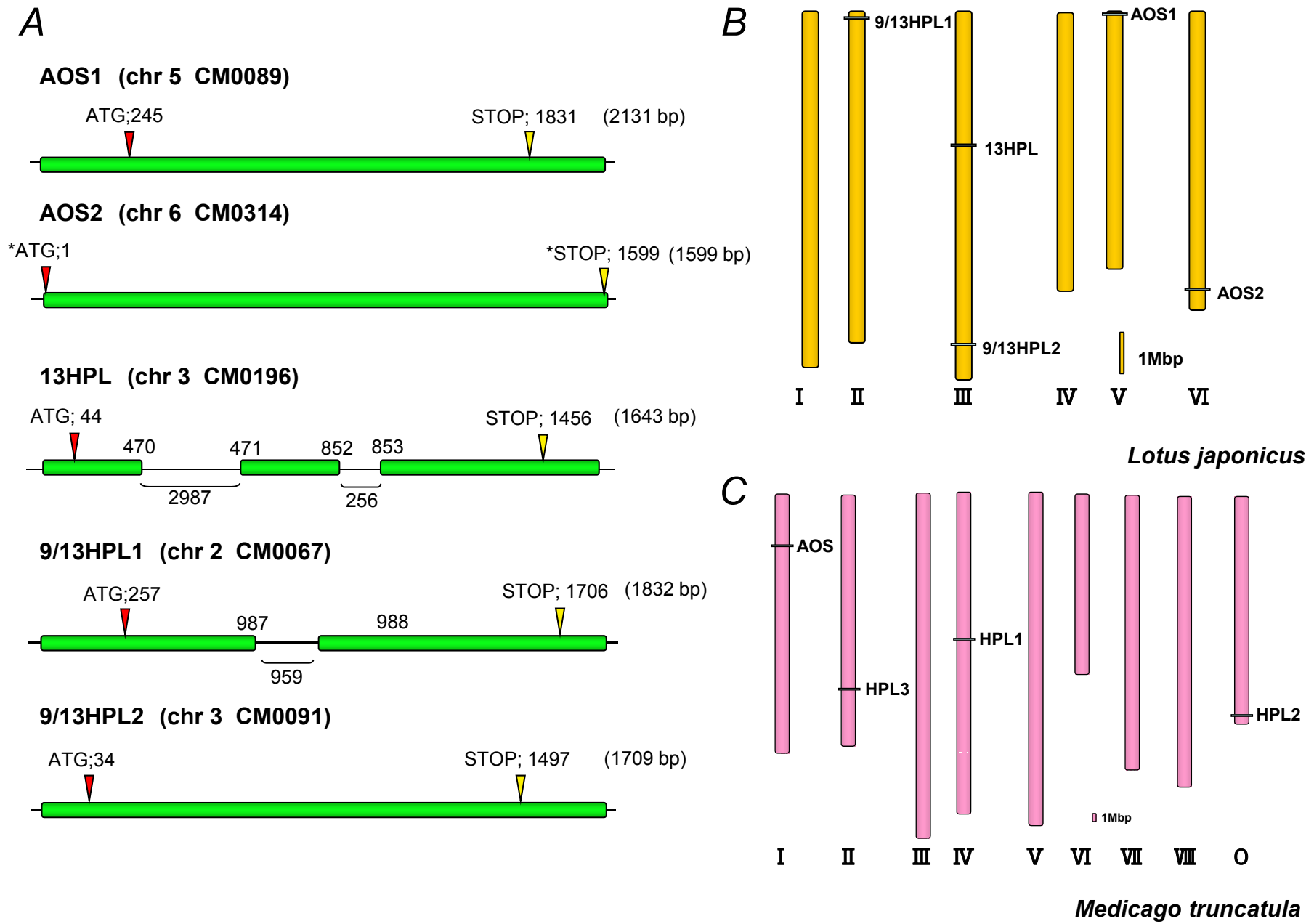


Fig. 5



Supplemental Figure S1. A: Gene organization of each clone in *L. japonicus*. B: Location of the gene in chromosome of *L. japonicus*. C: Location of the counterpart of *M. truncatula* in its chromosome.

Supplemental Table S1. A list of EST clones analyzed in this study.

Gene id	Clone number	Origin
<u>LjAOS1</u>		
BP049277	SPD066e09_f, 3' end	pod (less than 20 mm in length)
BP032674	clone:MF083c10_f, 3' end	flower
DC599445	MR055b07_r, 5' end sequence	root
BP047127	SPD039g04_f, 3' end	Pods
BP080857	MR063g01_f, 3' end	root
BP070314	GNf040e02, 3' end	nodulating root
DC599546	MR063g01_r, 5' end sequence	root
BP080186	MR054f12_f, 3' end	root
BP080217	MR055b07_f, 3' end	root
BP076764	MR011b09_f, 3' end	root
<u>Lj13HPL</u>		
BP050909	SPD087a01_f, 3' end	Pods
AV764698	MWM003f01_f, 3' end	whole plant
BP031737	MF065b05_f, 3' end	flower
BP034383	MFB007a05_f, 3' end	flower bud
BP049607	SPD070e06_f, 3' end	Pods
BP038899	MFB067h11_f, 3' end	flower bud
BP041292	MFB100h06_f, 3' end	flower bud
BP038668	MFB064h03_f, 3' end	flower bud
BP050435	SPD081a10_f, 3' end	Pods
BP038798	MFB066f01_f, 3' end	flower bud
BP047200	SPD040f08_f, 3' end	Pods
BP048442	SPD056b10_f, 3' end	Pods
AV780334	MPDL074h05_f, 3' end	pod
AV422018	MWM003f01_r, 5' end	whole plant
BP032825	MF086e05_f, 3' end	flower
AV429010	MWM107h02_r, 5' end	whole plant
<u>Lj9/13HPL1</u>		
AV767736	MWM196e02_f, 3' end	whole plant
BP035363	MFB019g01_f, 3' end	flower bud
BP030430	MF041c06_f, 3' end	flower
AV422876	MWM019f01_r, 5' end	whole plant
AV410864	MWL079f10_r, 5' end	whole plant
BP082233	MR081e01_f, 3' end	root
AV421537	MWM196e02_r, 5' end	whole plant
<u>Lj9/13HPL2</u>		
AV408399	MWL040f01_r, 5' end	whole plant
AV409979	MWL065c04_r, 5' end	whole plant
AV422965	MWM020c07_r, 5' end	whole plant
AV426352	MWM066d03_r, 5' end	whole plant
AV410421	MWL072a07_r, 5' end	whole plant
AV421222	MWM192e02_r, 5' end	whole plant
AV407553	MWL025d02_r, 5' end	whole plant
AV428633	MWL025a08_r, 5' end	whole plant

Supplemental Table S2. The primers used for cloning and RT-PCR.

Target gene	Forward primer	Reverse primer
(RT-PCR primers)		
Lj Ubiquitin	5'-TGGGTCTCTGTTGACCCTTT-3'	5'-AGAAGAGGCCACAACAAACG-3'
Lj AOS1	5'-GGGTTATGAATTATGATGCATGCTA-3'	5'-TCCAGAACTGCATGATGACAGAGT-3'
Lj AOS2	5'-ACGGAGGGATTAGGGTTTTCTT-3'	5'-CACCCCTGCTCGACCAAT-3'
Lj 13HPL	5'-CAGCTTCTGGTACCCACACA-3'	5'-CAGCCTTCCAGGTGAAGAAG-3'
Lj 9/13HPL1	5'-ATCACAAGGTGGAGGCTTTG-3'	5'-CATGTGCAGGTTGTTCTTGG-3'
Lj 9/13HPL2	5'-CTTCGCCGGACACTGTTTAT-3'	5'-GGATCCGAAGGTGTTTGAGA-3'
Lj Leghemoglobin	5'-CTCCAAGCCCATGCTGAAAA-3'	5'-TGGCATCTGCAAGTGTCCTTC-3'
(Cloning primers)		
AOS1	5'- CTGCAGCATCTTCTACTATCCCCTCTTCTT -3'	5'- CTGCAGTTAAAAGCTTGCTCTCTTCAATGA -3'
AOS2	5'- AACGAGGGTACCCATGTACGCATCAACCATAATGGC -3'	5'- AACGAGGGTACCCTAAAACTTGCCCTCTTAAGAGA -3'
13HPL	5'- GGATCCTCACTACCACCTGTCCCGCCACCG -3'	5'- GGATCCTCATTTGGCTTTTTGAAGGGCAGT -3'
9/13HPL1	5'- AACGAGGCATGCGCTTCTTCTTCAGACACCAAGCAG -3'	5'- AACGAGGCATGCTCACAAGGTGGAGGCTTTGGTGAG -3'
9/13HPL2	5'- AACGAGGCATGCGCAGCAGCATCTTCAGACGACACC -3'	5'- AACGAGGCATGCTCAGACTGTGGTGGTGGCCTTGGT -3'