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ABSTRACT OF THESIS

Evolutionary and functional characterization of *Os-POLLUX*, a rice gene orthologous to a common symbiosis gene in legume

Root symbioses with arbuscular mycorrhizal fungi and rhizobial bacteria share a common signaling pathway in legumes. Among the common symbiosis genes are *CASTOR* and *POLLUX*, the twin homologous genes in *Lotus japonicus* that encode putative ion channel proteins. Orthologs of *CASTOR* and *POLLUX* are ubiquitously present in both legumes and non-legumes, but their function in non-legumes remains to be elucidated. Here, we use reverse genetic approaches to demonstrate that the rice (*Oryza sativa*) ortholog of *POLLUX*, namely *Os-POLLUX*, is indispensable for mycorrhizal symbiosis in rice. Furthermore, we show that *Os-POLLUX* can restore nodulation, but not rhizobial infection, to a *M. truncatula dmi1* mutant.

Key word: Reverse genetics, rice, root symbiosis, mycorrhization, nodulation

Cui Fan

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Evolutionary and functional characterization of Os-*POLLUX*, a rice gene orthologous to a
common symbiosis gene in legume

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THESIS

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The Graduate School
University of Kentucky

2008

Evolutionary and functional characterization of Os-*POLLUX*, a rice gene orthologous to a common symbiosis gene in legume

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture
at the University of Kentucky

By

Cui Fan

Lexington, Kentucky

Director: Dr. Hongyan Zhu, Assistant Professor of Crop Science

Lexington, Kentucky

2008

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Dedicated to my parents and grandparents who give me the love, support and help.

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SECTION 1

INTRODUCTION

Many terrestrial plants can grow under nutrient-limiting conditions by forming mutually beneficial root symbioses with soil microbes. These underground symbiotic networks contribute significantly to the functionality and sustainability of agricultural and natural ecosystems. Arbuscular mycorrhizal (AM) and rhizobial symbioses represent two important symbioses formed between plants and microbes. Through the AM symbiosis, AM fungi assist the plant in assimilating mineral nutrients, particularly inorganic phosphate, from the soil, whereas the legume-rhizobia symbiosis results in the formation of the root nodule in which the bacteria fix atmospheric nitrogen for use by the plant. Since phosphorous and nitrogen are two limiting nutrients for plant growth, the two symbioses are of crucial importance in sustainable agriculture.

Arbuscular mycorrhizal symbiosis

The most widespread symbiosis is arbuscular mycorrhiza (AM), the ‘fungus root’ formed between the vast majority of vascular flowering plants and biotrophic fungi belonging to the phylum Glomeromycota (Smith et al., 1997). The AM symbiosis originated more than 400 million years ago and possibly had played a key role in helping the first plants to colonize land (Heckman et al., 2001; Redecker et al., 2000; Remy et al., 1994). In addition to the supply of mineral nutrients to the plant, AM symbioses also improve plant health through enhanced tolerance to biotic and abiotic stresses (Ruiz-Lozano, 2003; Pozo et al., 2007; Liu et al., 2007).

Initiation of the AM symbiosis is mediated by signal exchanges between the two symbiotic partners. The host plant releases strigolactones, known as ‘branching factors’ (Figure 1-A), into the rhizosphere that induce hyphal branching, thereby increasing the chance of AM fungi to contact the root (Buee et al., 2000; Akiyama et al., 2006; Akiyama et al., 2005; Besserer et al., 2006). The fungal partner subsequently produces yet unknown diffusible signals, termed ‘Myc factors’ (Figure 1-B), to trigger the host responses (Kosuta et al., 2003; Navazio et al., 2007). During the process, AM fungi enter the plant root and form within the inner cortical cells highly ramified, tree-like fungal structures, called arbuscules (Smith et al., 1997; Harrison, 1997). AM fungi also develop extensively branched hyphae outside the plant root. The extraradical hyphae function to expand the rhizosphere, thereby enhancing access to mineral nutrients from the soil, whereas the intraradical hyphae (arbuscules) serve as an intracellular microbe-plant interface where soil nutrients move to the plant and plant photosynthates flow to the fungus (Harrison, 1997; Harrison, 2005).

The ability of fungal spores to germinate in the soil depends on the presence of the host plant root. In other words, the spores germinate in response to plant-derived signals. Previous studies have shown that some factors from the host plants are the early recognition signals that the germinated spores respond to. These factors initiate new direction and different branching pattern of the hyphal growth, which lead to the formation of an fungal infection structure called appressorium on the root surface. In contrast, roots of non-host plants were unable to induce such fungal responses

(Schreiner RP et al., 1993; Giovannetti M et al., 1993; Vierheilig H et al., 1996). Once the presymbiotic branching was induced by the so called ‘branching factors’, it was accompanied by changes in RNA accumulation patterns in the fungi. Cloning and sequencing the accumulated cDNA fragments shows that root exudates could stimulate the fungal respiratory activity (Tamasloukht et al., 2003).

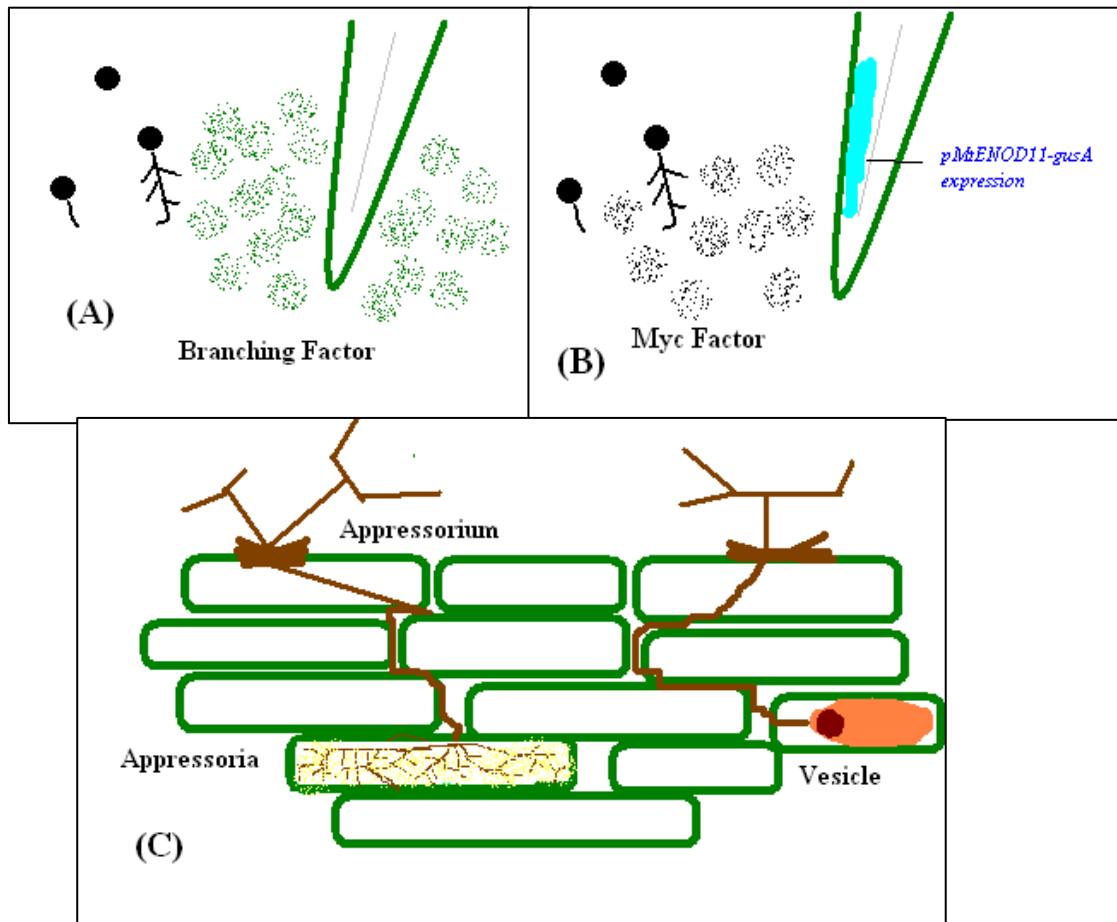


Figure 1. The developmental processes of AM symbiosis. (A) AM fungi respond to the plant-derived signals-strigolactone; (B) Plants sense the AM fungi-derived signals-Myc factors. Myc factors induce the p*ENOD11*::GUS expression in plants; (C) Appressorium formation, intercellular hyphae spreading; arbuscule and vesicle development in the inner cortex cells.

Akiyama et al. (2005) successfully isolated and identified a branching factor from root exudates of *Lotus japonicus* as 5-deoxy-strigol, a strigolactone (Akiyama et al., 2005). Previous studies have shown that strigolactones could prompt the seed germination in *Striga* and *Orobancha*, parasitic weeds of many monocotyledonous and dicotyledonous plants (Bouwmeester et al., 2003). A very low concentration of the natural strigolactones could induce the extensive hyphal branching of the germinating fungal spores (Akiyama et al., 2005).

Kosuta et al. (2003) demonstrated that fungal hyphae germinated from spores release a diffusible signal, which could be perceived by *Medicago truncatula* roots without physical contact. The experiments were based on the separately cultured AM fungi and *M. truncatula*. *pMtENOD11-gusA*, which works as a reporter for pre-infection and infection stages of nodulation and AM symbioses, was transformed into *M. truncatula* roots, since the expression of *MtENOD11* is induced by both Nod factors and Myc factors (Journet et al. 2001). In wide-type plants which were exposed to membrane-separated AM fungi, Gus expression was detected in the root cortex (Figure 1-B). The expression of *pMtENOD11-gusA* is exclusively induced by AM fungi but not by many other pathogenic fungi tested (Paszkowski, 2006). A series of experiments further confirmed the existence of diffusible Myc factors. Interestingly, the putative Myc factors are able to activate the *MtENOD11* expression within the *Nod⁻/Myc⁻ dmi* mutants before physical contact, implying that pre-symbiotic recognition occurs independently from the *DMI* symbiotic pathway (Paszkowski, 2006). It is also possible that different Myc factors

induce different mechanisms for pre-symbiotic recognition and symbiotic process.

When the extensively branched hyphae reach the plant roots, the hyphae differentiate into appressorium. The formation of appressorium represents the successful presymbiotic recognition process of the AM symbiosis (Paszkowski, 2006). The appressorium is a flattened and elliptical structure that attaches to the surface of the host cells (Garriock et al., 1989) (Figure 1-C), which facilitates the penetration of the fungal hyphae into the root epidermal cells. It was shown that the switch of the hyphae into appressorium was accompanied by transcriptional changes of the fungal genes (Breuninger et al., 2004). Appressorium could not be formed on the non-host cell walls or the non-epidermal cell walls (Harrison, 2005; Nagahashi G, 1997). In an *in vitro* study using isolated cell walls with required features, the appressoria were formed but no hyphal penetration occurred (Nagahashi G, 1997). Therefore, while required cell wall features are sufficient for generating appressoria, the further penetration of the root requires other factors.

Following the intercellular hyphal growth, hyphal branches reach the inner cortex and penetrate the cortical cell walls. Further differentiation within the cortical cells leads to the formation of tree-like structures called the arbuscules that could fill the whole cortical cell (Harrison, 1999). Arbuscules represent the typical character of AM symbiosis, which constitute an extensive intracellular interface that brings the two symbionts together. Parallel to the changes of AM fungi, the host cells also response with changes, such as the fragmentation of vacuoles, the migration of the nucleus to a

central position, and the increase of the organelle numbers (Harrison, 1999). Although the two symbionts make intensive efforts to form an arbuscule, the life length of an arbuscule is only a few days. Once an arbuscule collapses, the cortical cell remains intact and ready to host another arbuscule. In addition to the formation of arbuscules, the lipid-filled vesicles could also be formed for some species of AM fungi (Figure 1-C).

Nitrogen-fixing root nodule symbiosis

In addition to the AM symbiosis, certain members of the Eurosid I angiosperms can enter into a root symbiosis with soil bacteria (Soltis et al., 1995). The symbiosis culminates in the formation of the root nodule, an optimal micro-environment for bacteria to fix atmospheric nitrogen and for nutrient exchange between the symbiotic partners. The root nodule symbiosis takes place in two major forms, involving either the Gram-negative bacteria of the family Rhizobiaceae collectively called rhizobia or the Gram-positive Frankia bacteria that are filamentous actinomycetes (Pawlowski, 1996). The majority of leguminous plants as well as a non-leguminous plant, *Parasponia*, can interact with rhizobia, whereas a diverse group of non-leguminous plants, so called actinorhizal plants, are able to enter into an interaction with Frankia bacteria. The legume-rhizobia symbiosis is the best characterized at cellular and molecular levels. The interaction is set in motion by an intimate communication between the host and rhizobia (Long, 1996; Spaink, 2000). The legume roots secrete into the rhizosphere (iso)flavonoids that attract the rhizobia to the root and trigger the production of a

bacterial signal known as Nod factor. Nod factors are chitin-like lipo-chito-oligosaccharides that are essential for activating the host symbiotic responses, including an early ion influx, calcium spiking, root hair deformation and curling, transcriptional reprogramming of the host symbiotic genes, and cortical cell divisions, that ultimately result in the formation of the rhizobium-infected root nodules (Oldroyd et al., 2004; Oldroyd et al., 2006; Oldroyd et al., 2008; Jones et al., 2007; Stacey et al., 2006).

The nodulation process starts from root hairs curling, which is caused by a growth direction reorientation of the root hair induced by bacterial infection (Limpens et al., 2003; Emons et al., 2000). The bacteria gather in the pocket of the curl, where both plant and bacteria are making efforts for a change of structure: the plant cell wall gets degraded, the cell membrane tends to draw back and a new delivery material forms. The infection thread is initiated at this time at the center of curled root hairs. As the infection proceeds, a dense microtubule network will surround the growing infection thread and a connection will be made from the nucleus to the infection thread tip, finally the infection thread will reach the cortex (Timmers et al., 1999). At the same time, cortical cells reenter into the cell cycle and form into a primordium, where a nodule could develop. Once the infection thread reaches the primordium, the bacteria could flow into the primordial cells through the thread. Then, the bacteria begin to differentiate into bacteroids (a nitrogen-fixing form) within the plant cytoplasm (Oke et al., 1999; Limpens et al., 2003).

Nod factors are produced by the rhizobia after flavonoids secreted by which the legume root activates the bacterial transcriptional regulator NodD, which induces the expression of other *nod* genes (Limpens et al., 2003). The basic structure of Nod factors synthesized by different rhizobial species is very similar. It consists of a β -1, 4-linked *N*-acetyl-D-glucosamine (GlcNAc) backbone accompanied with 4 or 5 residues, of which the non-reducing terminal residue is substituted at the C2 position with an acyl chain. Based on the rhizobial species and its presence of *nod* genes, the structure of the acyl chain can vary, and specific changes at the reducing and non-reducing terminal glucosamine residues could be found. These differences lead to the diverse biological activity of the Nod factors and determine the host specificity (Spaink, 2000). For example, the Nod factor structure of *Sinorhizobium meliloti* (Figure1-2), which is the microsymbiont of *Medicago*, is tetrameric and consists of a 16-carbon acyl chain with two unsaturated bonds (C16:2). The two specific terminal residues are an *O*-acetyl group at the non-reducing terminal sugar residue and an *O*-sulfated group at the reducing end (Spaink, 2000). The sulfate substitution of this Nod factor is required for the induction of most *medicago* symbiotic responses (Gressent et al., 1999).

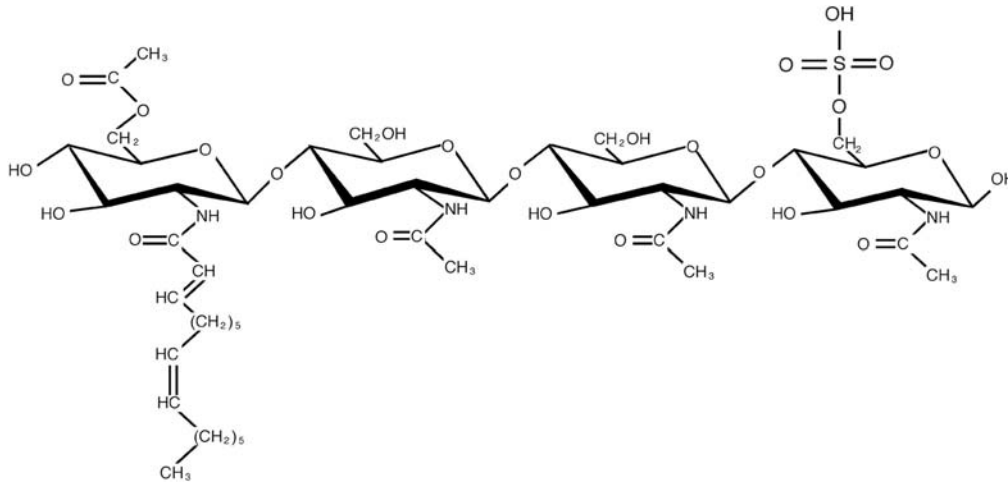


Figure 2. The Nod factor structure of *Sinorhizobium meliloti* (Lerouge et al., 1990). The major Nod factor produced by *Sinorhizobium meliloti*, is tetrameric (four glucosamine units) and consists of a 16-carbon acyl chain with two unsaturated bonds (C16:2). The two specific terminal residues are an *O*-acetyl group at the non-reducing terminal sugar residue and an *O*-sulfated group at the reducing end.

Symbiosis signaling and cross-talk between AM and nodulation symbioses

Although the AM and rhizobial symbioses are morphologically distinct (arbuscules vs. nodules), the two are mechanistically related in legumes. A number of legume genes that are required for nodulation also are essential for the AM interaction (Kistner et al., 2005). Moreover, a subset of the host genes that is induced during legume-rhizobia symbiosis is also up-regulated during AM symbioses (Albrecht et al., 1999; Kistner et al., 2002). The overlap of the two symbiotic pathways has led to the hypothesis that the evolutionarily younger legume root nodule symbiosis may have evolved from the more ancient AM symbiosis (LaRue et al., 1994; Gianinazzi-Pearson, 1996; Zhu et al., 2006).

In recent years, the development of genetic and genomic tools for the two model legumes *Medicago truncatula* and *Lotus japonicus* has greatly facilitated the cloning of

genes required for root symbioses (Stacey et al., 2006). Analysis of these genes has begun to reveal the Nod factor and mycorrhizal signaling pathways (Figure 3). The Nod factors are likely perceived directly by the receptor-like kinases, such as Lj-NFR1/Mt-LYK3 and Lj-NFR5/Mt-NFP, that contain peptidoglycan-binding LysM domains in the extracellular region (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006; Smit et al., 2007). Downstream of the Nod factor receptors are a set of proteins that play a dual role in AM and nodulation symbioses. These proteins include the leucine-rich repeat receptor kinase Mt-DMI2/Ms-NORK/Lj-SYMRK/Ps-SYM19 (Endre et al., 2002; Stracke et al., 2002), the ion channel proteins Lj-CASTOR and Lj-POLLUX/Mt-DMI1/Ps-SYM8 (Ane et al., 2004; Imaizumi-Anraku et al., 2005; Edwards et al., 2007), the Ca²⁺/calmodulin-dependent protein kinase Mt-DMI3/Lj-CCaMK/Ps-SYM9 (Levy et al., 2004; Mitra et al., 2004; Tirichine et al., 2006), and two nucleoporins Lj-NUP85 and Lj-NUP133 (Kanamori et al., 2006; Saito et al., 2007). Except for Mt-DMI3/Lj-CCaMK/Ps-SYM9, all these common symbiosis components act upstream of the Nod factor-induced calcium spiking, a periodic, transient increases in cytosolic calcium levels (Ehrhardt et al., 1996; Oldroyd et al., 2004; Oldroyd et al., 2006). Most recently, a DMI3-interacting protein, called IPD3, has been identified (Messinese et al., 2007). IPD3 likely represents a novel protein required for both nodulation and mycorrhizal symbioses (Chen et al., 2008).

Besides the common symbiosis pathway, a number of other Nod factor signaling

transduction components were identified (Figure 3). Within the nuclear inner membrane, the calcium-spiking signal is perceived by a calcium and calmodulin-dependent protein kinase (CCaMK) MtDMI3/LjCCaMK/PsSYM9 (Levy et al., 2004; Mitra et al., 2004), which subsequently activates the nodulation-specific transcription factors: two GRAS (GAI, RGA, SCR) domain proteins MtNSP1 and MtNSP2 (Kalo et al., 2005; Smit et al., 2005; Heckmann et al., 2006; Murakami et al., 2006); a ERF (Ets2 repressor factor) transcription factor MtERN (Middleton et al., 2007). In contrast to the Nod factor signaling, the mycorrhiza-specific signaling components beyond the common symbiosis pathway are largely unknown.

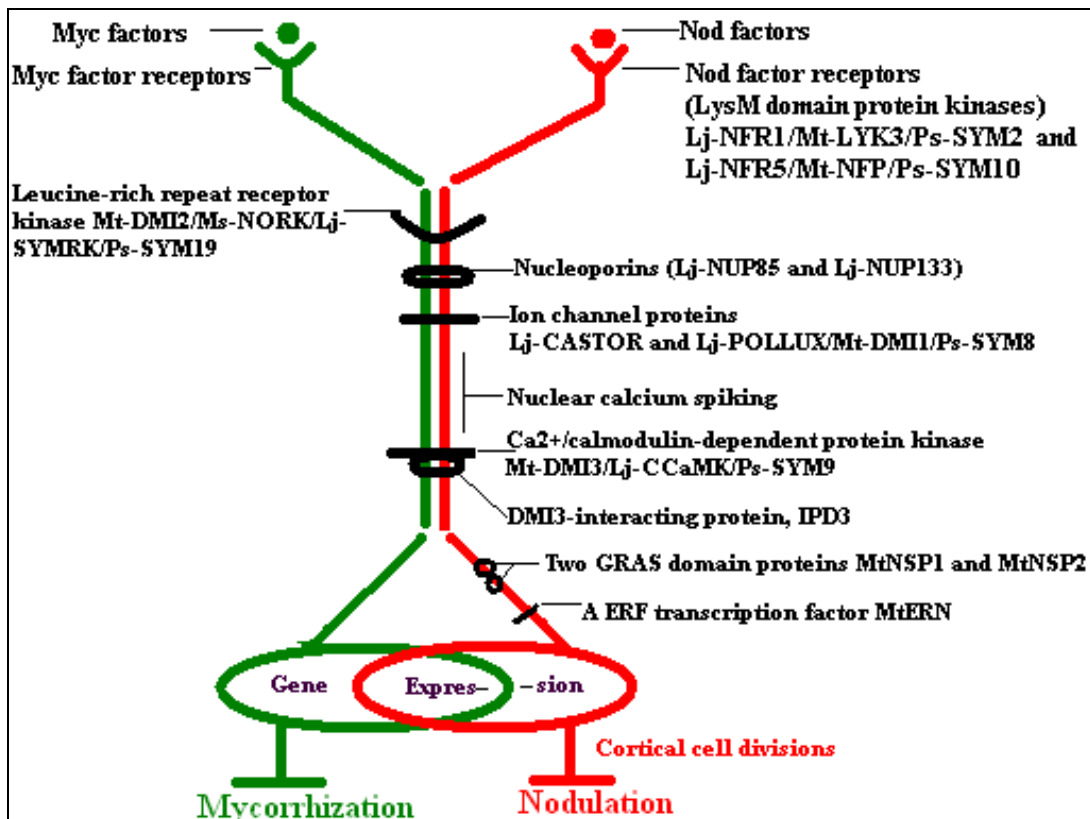


Figure 3. Cross-talk between nodulation and AM signaling pathways. Nod factors and Myc factors are perceived by LysM receptor kinases and other unknown specific receptors, respectively. The two signaling pathways share several components: DMI proteins, nucleoporins, nuclear calcium spiking as well as DMI3 interacting protein. Nodulation signals further transduced through GRAS and ERF transcription factors, which activate the expression of early nodulation genes and initiate the cortical cell divisions. In contrast, the mycorrhiza-specific signaling components beyond the common symbiosis pathway are largely unknown. For the mycorrhization transduction pathway. However, the downstream gene expression for the two pathways overlaps.

Symbiosis-related genes are highly conserved in non-legumes

Intriguingly, all cloned legume symbiosis genes, including both the common symbiosis genes and genes only required for rhizobial symbiosis, have their orthologs in non-legumes (Zhu et al., 2006). This finding offers an opportunity to address the evolution of root symbioses in plants by characterizing ortholog functionality across the legume and non-legume boundary. For example, the Lj-*NFR1* ortholog in Arabidopsis (At1g21630) has been shown to be essential for chitin signaling, indicating an evolutionary relationship between the chitin and Nod factor perception (Zhu et al., 2006; Miya et al., 2007; Wan et al., 2008). As part of our effort to address the function of non-legume orthologs of legume genes required for root symbioses, we seek to determine whether the common symbiosis genes in legumes also are required for mycorrhizal symbioses in non-legumes using rice as a study model. We, as well as others, have demonstrated that Os-*DMI3*, the rice ortholog of Mt-*DMI3*/Lj-*CCaMK*/Ps-*SYM9*, is required for AM symbiosis in rice and able to complement a *M. truncatula dmi3* mutant (Godfroy et al., 2006; Chen et al., 2007). Two recent studies revealed that the Mt-*DMI2*/Ms-*NORK*/Lj-*SYMRK*/Ps-*SYM19* orthologs from the two actinorhizal plants,

Casuarina glauca and *Datisca glomerata*, are essential for root symbioses with both AM fungi and *Frankia* bacteria (Gherbi et al., 2008; Markmann et al., 2008).

Here we extend these studies to include Os-*CASTOR* and Os-*POLLUX*, the rice orthologs of the twin common symbiosis genes Lj-*CASTOR* and Lj-*POLLUX*. Rice is a mycorrhizal plant with a completely sequenced genome and available resources for high throughput reverse genetic analysis, so rice is chosen as our study model (Miyao et al., 2007; Jeong et al., 2006; Chen et al., 2007). The objective of this research is to use rice T-DNA/Tos17 insertion lines to characterize the function of Os-*POLLUX* in AM symbiosis and further to use full-length cDNAs of Os-*POLLUX* to complement Mt-*dmi1* mutants. This research aims to answer the questions that whether Os-*POLLUX* is required for AM symbiosis in rice and whether Os-*POLLUX* could fulfill equivalent roles in AM and nodulation symbioses to Mt-*DMII*.

SECTION 2

MATERIALS AND METHODS

Rice and *M. truncatula* mutants

The rice Tos17 insertion line (NC6423) in the ‘Nipponbare’ background was provided by the Rice Genome Resource Center of the National Institute of Agrobiological Sciences (RGRC-NIAS), Japan. The rice T-DNA insertion line B02432 in the genetic background of Dongjin was provided by the Pohang University of Science and Technology (POSTECH), Korea. The *M. truncatula dmi1-1* mutant (C71) was obtained from Dr. Douglas Cook's lab at the University of California, Davis.

Isolation of homozygous *Os-pollux* mutant lines

Seeds of the Tos17/T-DNA insertion lines from the providers were from the progeny of a primary transgenic or tissue-culture derived plant. To isolate homozygous mutants, we carried out two rounds of PCR analyses. The first-round PCR was to identify plants with Tos17/T-DNA insertion using the Tos17/T-DNA-specific and the Os-POLLUX-specific primer pairs. The second-round PCR was conducted to identify homozygous mutant plants using the primer pairs flanking the putative Tos17/T-DNA insertion sites. The positions of these primers are indicated in Figure 5A. Primer sequences are as follow: F: 5'-CGATTTGATCTCTCCCCGTA-3'; R: 5'-GCTGACAACATAAAGCGCAA-3'; T1: 5'-ATTGTTAGGTTGCAAGTTAGTTAAGA-3'; T2: 5'-CCACAGTTTTTCGCGATCCAGACTG-3'.

Binary vector construction

Two full length cDNA clones of Os-*POLLUX*, AK067564 and AK073102, were cut with *EcoRI* and *KpnI* to generate the full length cDNAs of Os-*POLLUX*. The Os-*POLLUX* full length cDNAs were then ligated into the corresponding sites of a modified binary pHellsgate8 vector driven by the CaMV-35S promoter (Helliwell et al., 2002). To correct the point mutation on each of the full length cDNAs, two rounds of restriction and ligation were carried out. *EcoRI* and *NruI* were first used to produce a small (2400bp) and a large fragment. The small fragment of AK067564 containing an insertion of 'T' at the position 2136 was replaced by the corresponding fragment from AK073012. A similar strategy was used to correct an 'A-to-T' transversion at the position 593 for AK073012.

Hairy root transformation of *M. truncatula*

The *dmi1-1* mutant of *M. truncatula* was transformed with Os-*POLLUX* using *A. rhizogenes*-mediated hairy root transformation (Boisson-Dernier et al., 2001). Two full-length cDNA clones of Os-*POLLUX* (AK067564 and AK073162) were cloned into a binary vector modified from pHellsgate8 driven by the 35S promoter (Helliwell et al., 2002). The binary vector was introduced into the *A. rhizogenes* strain ARqual and transformed into the roots of the *dmi1-1* mutant. Transformed roots were selected on Färhaeus medium (Färhaeus et al., 1957) containing 20 mg/L kanamycin for 2 weeks at 20°C.

Inoculation of rice roots with *Glomus intraradices*

The AM fungus *G. intraradices* was ordered from Premier Tech Biotechnologies (Canada). The inoculation method was as described by Chen et al. (2007). Briefly, the rice plants were grown in 11-cm pots with sterilized Turface covered with 3-cm depth of sand in a growth chamber with a 13-h-light, 28°C/11-h-dark, 24°C regime. The plants were fertilized twice weekly with half-strength Hoagland solution (Arnon and Hoagland, 1940) supplemented with 100 μ M KH₂PO₄. Roots of 2-week-old rice plants were inoculated by adding 1,000 spores to the sand at 1.5-cm depth. Roots were harvested at 5 weeks after inoculation. A random sample of the root tissues was used for phenotyping analysis, and the remaining tissues were used for RNA isolation.

Mycorrhizal colonization was phenotyped by means of Trypan Blue staining according to the protocol described by Koske and Gemma (1989). The cleaned roots were first fixed in 50% (v/v) ethanol. The fixed roots were then incubated at 90°C in 10% KOH for ~20 min. After rinsing with water, the roots were soaked in 1% HCl at room temperature for overnight. The roots were then stained at 90°C for 30 min in an acidic glycerol solution containing 0.1% Trypan Blue. After de-staining in acidic glycerol, the roots were examined using light microscope (Olympus BX40F-3) and images were captured by a microscope digital camera system (Olympus DP71).

Inoculation of *A. rhizogenes*-transformed *M. truncatula* roots with *Sinorhizobium meliloti*

Nodulation assay was conducted as described by (Limpens et al., 2003). Three weeks after transformation, composite plants were transferred to sterile Turface saturated with Färhaeus medium [without Ca (NO₃)₂] for three days at 21°C, 16/8 h light/dark. Each plant was then inoculated with 1 mL of culture (OD600 0.1) of *S. meliloti* strain 2011 carrying the lacZ reporter gene in plasmid pXLGD4 (Catoira et al., 2000). The nodulation was scored 2 weeks after inoculation.

Nodule sectioning, staining, and microscopy

Nodules were stained with X-gal to detect the presence of bacteria. For nodule sectioning, nodules were cut in half longitudinally, placed in FAA solution [100ml: 45ml 95% EtOH, 40ml D-H₂O, 5ml glacial acetic acid, 10ml 37%(w/w) formaldehyde] and vacuum infiltrated until they sank. The FAA fixation was followed by several steps of ethanol dehydration (50, 60, 70, 80, 95 and 2 changes of 100% EtOH each for 30 min). The samples were then gradually infiltrated with Hemo-De (20, 50 and 75% Hemo-De solutions, each for 30min, then 2 changes of 100% Hemo-De each for 1h). Once hydrated with Hemo-De, the samples were infiltrated with Paraplast Plus by successive adding chips of paraplast to Hemo-De at 42°C. After removing the paraplast/Hemo-De solution, melted paraplast was added and incubated at 60 °C for at least 8 hours (This step was repeated for at least 6 changes of Paraplast). The samples were then embedded and

sectioned. The samples were sectioned with a Leica RM2135 Microtome.

For light microscopy, sections of 5µm thick were dried onto glass slides. The slides went through stepwise de-Paraplast and hydration and were stained with 1% (wt/vol) Toluidine blue in 95% EtOH. Photographs were taken on an Olympus BX40F-3 light microscope and images were captured by an Olympus DP71 microscope digital camera system.

Analysis of gene expression

Total RNA was isolated by the Qiagen Plant RNeasy kit. Two micrograms of RNA was used to perform RT reactions using M-MLV reverse transcriptase (Invitrogen) in a 20-µL reaction mixture. Two microliters of the RT reaction was used as a template in a 20-µL PCR reaction solution. The PCR primers were as follows: *Os-Actin*, 5'-GCGATAATGGAAGTGGTATG-3' and 5'-CTCCATTTCTGGTCATAGTC-3'; *Os-POLLUX*, 5'-CGATTTGATCTCTCCCCGTA-3' and 5'-GCTGACAACATAAAGCGCAA-3'; *Os-PTII*, 5'-ATGGCTCGACGGACAGTAAG-3' and 5'-GATCAGCTGGATCATGTACCT-3'. Quantitative RT-PCR was performed on an Applied Biosystems StepOne Real-time PCR System using the SYBR Green I detection kit (BioRad). The *Os-ubiquitin* gene was selected as a constitutive internal control. PCR primers used for the real-time PCR experiments were: *Os-ubiquitin*, 5'-TGCACCCTAGGGCTGTCAAC-3' and 5'-TGACGCTCTAGTTCTTGATCTTCTTC-3'; *Os-CASTOR*,

5'-CAAGAGGGTGATGAGGTGCTAGTA-3' and

5'-GGTAACCTCTCATAACCTTGGGTAAT-3' and Os-POLLUX,

5'-CCTCGGATGGAGCGACAA-3' and 5'-ACGACACCACCACCAATACTCTT-3'.

SECTION 3

RESULTS AND DISCUSSION

Antiquity and evolution of the *CASTOR* and *POLLUX* homologs in plants

CASTOR and *POLLUX* are two homologous genes encoding putative ion channels that are components of the common symbiosis pathway in *L. japonicus* (Imaizumi-Anraku et al., 2005). The twin genes likely evolved from an ancient gene duplication event that dated before the monocot-dicot divergence (Zhu et al., 2006) (Figure 4). Under this evolutionary scenario, the duplicated gene copies have degenerated to perform complementary rather than redundant functions through a process called subfunctionalization (Lynch et al., 2000). It was speculated that *CASTOR* and *POLLUX*, analogous to many other ion channels, may act as hetero-multimeric complexes (Jiang et al., 2002; Imaizumi-Anraku et al., 2005). The fact that *CASTOR* and *POLLUX* homologs also are present in *Physcomitrella patens* (moss), a basal lineage of land plants that can establish root symbioses with AM fungi (Ane et al., 2004), suggests that the progenitor of *CASTOR* and *POLLUX* is ancestral to land plants.

The *CASTOR* and *POLLUX* orthologs are ubiquitously present in nearly all examined plant taxa for which sequence information is currently available, including *M. truncatula*, soybean (*Glycine max*), poplar (*Populus trichocarpa*), grapevine (*Vitis vinifera*), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), and maize (*Zea mays*). The between-species orthologous relationship of the *CASTOR-POLLUX* homologs can be

readily inferred based on their microsyntenic genomic position and/or phylogenetic analysis (Zhu et al., 2006) (Figure 4). The only exception is *Arabidopsis thaliana* which contains the ortholog of *POLLUX* (At5g49960) but lacks the ortholog of *CASTOR* (Zhu et al., 2006; Ane et al., 2004). Notably, *Arabidopsis* also lacks the orthologs of Mt-*DMI2*, Mt-*DMI3*, and Mt-*IPD3*, a gene encoding an interacting protein of Mt-*DMI3* in *M. truncatula* (Zhu et al., 2005; Zhu et al., 2006; Messinese et al., 2007). Such gene deletions in *Arabidopsis* (and likely the lineage leading to the Brassica family) offer an explanation of the inability of the *Brassica* plants to form root symbioses with mycorrhizal fungi (Ocampo et al., 1980; Glenn et al., 1985). As shown in Figure 4, the phylogenetic position of At-*POLLUX* (At5g49960) is not congruent with species tree, suggesting that purifying selection on At5g49960 may have been relaxed due to a lack of ability of *Arabidopsis* to establish AM symbiosis.

The *CASTOR* and *POLLUX* homologs are highly conserved over the C-termini of ~650 amino acids, with a sequence identity ranging from 69% to 98% depending on the phylogenetic distances between species (Table 1). As shown in Table 1, the level of sequence identity between orthologs (80-98%) is always higher than that between within-species paralogs (72-76%). Moreover, multiple sequence alignments of the conserved regions revealed numerous amino acid residues that could discriminate between *CASTOR* and *POLLUX* orthologs (Figure A.1), further supporting the bifurcation of the two orthologous groups in phylogenetic analysis (Figure 4). In contrast to the C-terminal region, the N-terminus of these proteins appears to evolve more

rapidly. Within each of the CASTOR and POLLUX orthologous groups, the N-terminal sequences are conserved only between closely-related species but highly diverged between distantly-related species. Visual and *in silico* analysis using the SIMPLE algorithm revealed that the N-terminal regions of CASTOR and POLLUX proteins are rich in simple sequence repeats (SSRs) encoded by SSRs at DNA level (data not shown), which may have contributed to the fast-evolution feature of this region (Hancock et al., 2005).

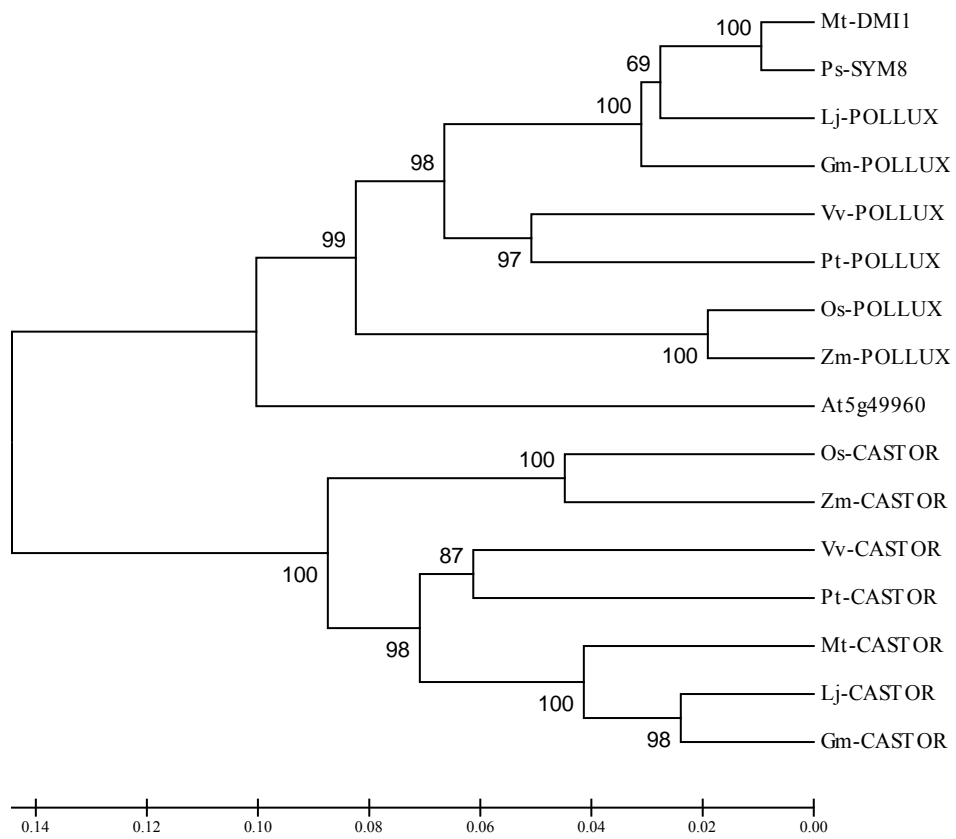


Figure 4. Phylogenetic tree (unrooted) of CASTOR and POLLUX homologs in *M. truncatula* (Mt), *L. japonicas* (Lj), soybean (*Glycine max*, Gm), poplar (*Populus trichocarpa*, Pt), grapevine (*Vitis vinifera*, Vv), *Arabidopsis thaliana* (At), rice (*Oryza sativa*, Os), sorghum (*Sorghum bicolor*, Sb), and maize (*Zea mays*, Zm). The tree was based on C-terminal ~650 amino acids of the proteins. Sequence alignments were performed using ClustalX (Thompson et al., 1997) and manually curated. The tree was constructed by MEGA3.1 (Kumar et al., 2004), using the UPGMA method. Numbers below the branches represent the percentages of 1,000 bootstrap replications supporting the particular nodes.

Table 1. Percentages of amino acid sequence identity between CASTOR and POLLUX homologs*.

	P _s -SYM8	L _j -POLLUX	Gm-POLLUX	Pt-POLLUX	Vv-POLLUX	Os-POLLUX	Zm-Pollux	Sb-POLLUX	At5g49960	Mt-CASTOR	L _j -CASTOR	Gm-CASTOR	Pt-CASTOR	Vv-CASTOR	Os-CASTOR	Zm-CASTOR	Sb-CASTOR
Mt-DMI1	98	94	94	87	86	83	84	84	80	72	75	75	74	74	74	73	74
P _s -SYM8		94	93	87	87	83	84	84	80	72	75	75	74	75	74	73	74
L _j -POLLUX			93	87	86	83	84	84	81	74	75	76	74	74	74	72	73
Gm-POLLUX				89	87	84	84	84	81	73	75	76	74	75	74	73	74
Pt-POLLUX					90	84	84	85	83	74	76	77	75	75	75	74	75
Vv-POLLUX						84	85	85	83	74	76	77	76	74	76	75	75
Os-POLLUX							96	96	80	73	75	75	74	74	74	75	75
Zm-POLLUX								98	80	73	75	76	75	74	75	75	76
Sb-POLLUX									81	73	75	76	74	75	75	76	75
At5g49960										70	71	72	71	70	70	69	70
Mt-CASTOR											90	92	84	85	83	80	82
L _j -CASTOR												95	87	86	85	82	84
Gm-CASTOR													88	87	85	82	85
Pt-CASTOR														88	85	83	84
Vv-CASTOR															84	82	85
Os-CASTOR																91	94
Zm-CASTOR																	98

*Percentages of identity were calculated based on C-terminal 650 amino acids of the proteins via the NCBI BLAST2 program.

Characterization of *Os-CASTOR* and *Os-POLLUX* in rice

We have selected rice as a model system to assess the function of non-legume orthologs of the legume symbiosis genes, because rice is a mycorrhizal plant with a completely sequenced genome and available resources for high throughput reverse genetic analysis (Miyao et al., 2007; Jeong et al., 2006; Chen et al., 2007). *Os-CASTOR* and *Os-POLLUX* were identified as Os03g62650 and Os01g64980, respectively, in the rice genome (Nipponbare) based on the TIGR Rice Genome Annotation (Zhu et al., 2006). Alignment of full-length cDNAs with the genomic sequences revealed a gene structure of 12 exons for both genes (Figure 5A), which is conserved with their legume and

non-legume counterparts listed in Table 1 (Ané et al., 2004; Imaizumi-Anraku et al., 2005). Both Os-*CASTOR* and Os-*POLLUX* also produce transcript variants that result from alternative splicing (data not shown). Quantitative reverse transcription-PCR (qRT-PCR) analysis indicated that Os-*CASTOR* and Os-*POLLUX* are expressed in all tissues tested, including roots, leaves, stems, and panicles; and the expression levels in the root were not enhanced by mycorrhizal colonization (Figure 5B). This observation was further supported by the analysis of the Rice Gene Index database, from which the cognate expressed sequences of Os-*CASTOR* (TC285740) and Os-*POLLUX* (TC285508 and TC334749) were derived from cDNA libraries of various plant tissues. The expression pattern of Os-*CASTOR* and Os-*POLLUX* appears to be similar to that observed for Lj-*CASTOR* and Lj-*POLLUX* in *L. japonicus* (Imaizumi-Anraku et al., 2005), but in contrast to that of Mt-*DMII* (orthologous to Lj-*POLLUX*) which is predominantly expressed in *M. truncatula* roots (Ané et al., 2004).

The regular transcripts of Os-*CASTOR* and Os-*POLLUX* encode predicted proteins of 893 and 943 amino acids, respectively, with a domain structure identical to their legume orthologs (Ané et al., 2004; Imaizumi-Anraku et al., 2005). Both proteins possess four transmembrane (TM) helices, a central region homologous to the RCK (the regulator of conductance of K^+) domain of bacterial calcium-gated potassium channels, and several motifs, such as the filter, the pore helix and the hinge, that are characteristic of the structurally characterized MthK channel from *Methanobacterium thermoautotrophicum* (Jiang et al., 2002) (Figure 3). Over the C-terminal 650 amino

acids, starting from a site between the second and third transmembrane helices, Os-CASTOR and Os-POLLUX are >91% identical to their maize and sorghum orthologs, and 83-85% identical to their legume counterparts (Table 1).

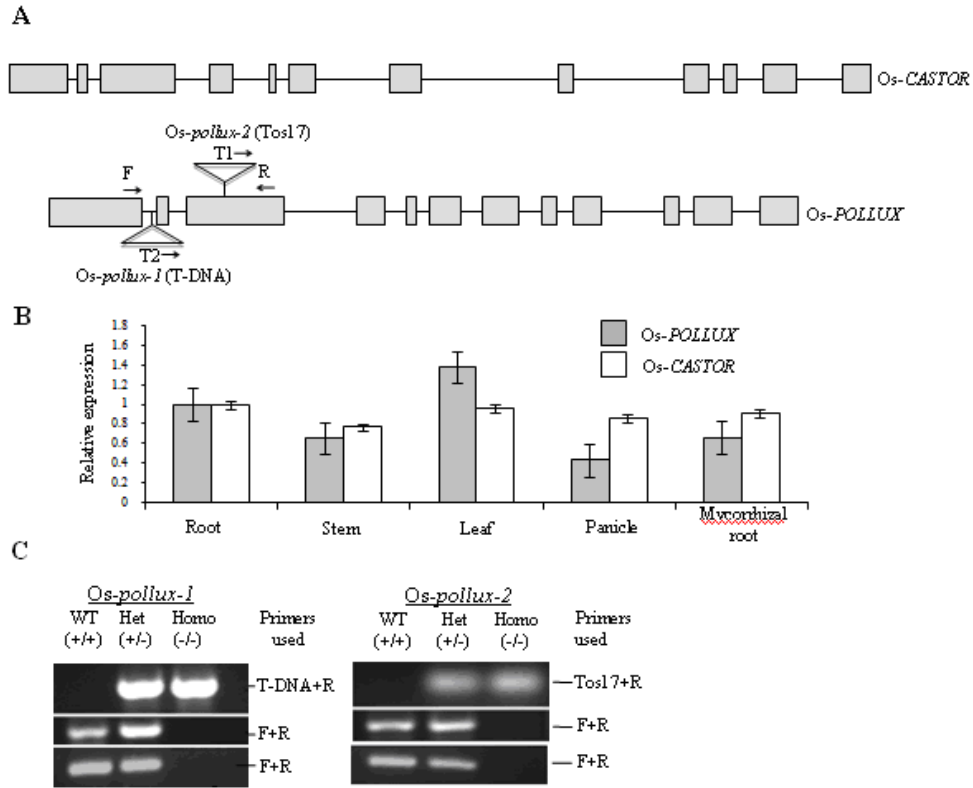


Figure 5. Isolation and characterization of Tos17/T-DNA insertion mutants of *Os-POLLUX*. (A) Gene structure of *Os-CASTOR* and *Os-POLLUX*, and the Tos17/T-DNA insertion sites. The exons and introns are indicated by boxes and lines, respectively. Insertion sites of Tos17/T-DNA are indicated. (B) *Os-CASTOR* and *Os-POLLUX* expression levels in roots, stems, leaves, panicles, and mycorrhizal roots. Relative transcript abundance was determined by quantitative RT-PCR and normalized against *Os-Ubiquitin1*. Error bars represent standard deviations from three independent biological replications. (C) Identification of homozygous ($-/-$) insertion mutants by PCR. Top, Identification of positive insertion plants ($+/-$ or $-/-$) by PCR using a pair of Tos17/T-DNA- and gene-specific primers. Middle, PCR analysis to distinguish between homozygous ($-/-$) and heterozygous ($+/-$) mutant plants using a primer pair flanking the Tos17/T-DNA insertion site that allowed the amplification of only the wild-type allele under given PCR conditions. Bottom, RT-PCR analysis of *Os-POLLUX* expression in the wild-type and mutant plants. The primer positions are indicated in panel A.

A. Os-CASTOR

MPLDPDS SPAPP HRDWF FPAPP FLPS SRAR TPRAPP PST
SRSSNPYSFPDRRPPPTFRSRSR SPLPPPEQKQQQPPT
TPPPAPRRRDPYAGVRRGDVRTLTAEKAAAAAVPTAAQ
VHGSKSAASATTLRWSGMVSVAATVLCFSSLVRSNSSLHD
TM-1
QVHHLKAQLAEATTKLQSCITFESSMOMSSILSYQSNNSTS
QNRGLKNFSLLLSLSTLYAPLLILKYMDLFLKLRSSQDSE
TM-2
EEVPINKRLAYRVDIFLSLQPYAKPLVLLVATLLIGLGG
TM-3
LALYGVNDDSLDCLWLSWTFVADSGNHANAEGFGPKLVS
Pore helix Filter
VSI SIGGMLVFAMMLGLVTDSEKFD SLRKGKGRSEVIBQS
TM-4 Hinge
HTLVLWGSQKLGSLLNQIAIANESLGGGTIVMMAEKDKEE
MEADIAKMEFDLKGTAICRSGSPLILADLKKVSVSKARA
RCK domain
IWLVAEEGNADQSDARALR TVLSLTGVKEGLRGHIWVLS
DLDNEVLVCLVGGDLVETVVAHDVIGRLM IQCARQPLAQ
IWEDILGFENCEFYIKRWPQLDGMQFEDVLI SF PDAIPCG
IKVASYGKGIILNPDDFYVLQEGDEVLVIAEDDDTYAPAP
LPKVMRGYL PKDFWPKSPERILFCGWRROMEDMIMVLDA
FLAPGSELWMFNDVPBMDRERKLIDGGLDFSRLENITLVH
REGNAVIRRHLESPLLESFDSILILADESVEDSA IQADSR
SLATLLLRDIQAKRLPFR EAMVSHVTRG SFCEGSWIGEM
QQASDKSVIISEILDPRTKNLLSVSKISDYVLSNELVSMA
LAMVAEDRQINDVLEELFAEQGNEMQIRPADLYLREDEEL
NFFEVMLRGRQRKEIVIGYRLVDAERA IINPDKVSRRRW
SAKDVFVWITEKE

B. Os-POLLUX

MAESDGGEASPSGGGGGEGSPDPRRPPARPQLTKSRTISG
SAASAFDRWGTSNSSSSILVRRSSTAPLPPGAAPRGLLTV
AVDEP SYAAPNGGAAMLDRDWCYPSFLGPHASRFRPPRSQ
QQTPTTTAAAAADSR SPTP AAPPQTASVSQR EEEKSLASV
VKRPMILLDERRSLSPPPQQRAPRFDLSPYLVLMLVWTVI
TM-1
SFLAIWQMMKATVLQEKIRSCCVSTVDCKTTTEAFKIN
GQHGSDFINSADWNLASC SRMLVFAIPVFLVKYIDQLRRR
TM-2
NTDSIRLRSTE EEPV LKKRIAYKVDVVFSGHPYAKLLALL
LATIILIASGGIALYVWSGSGFLEALWLSWTFVADSGNHA
TM-3 Pore helix Filter
DQVGLGPRIVSVSISGGMLVFA TMLGLVSDAISEKVDSW
TM-4 Hinge
RKGKSEVIEVNHILILGWSDKLGSLLKQLAIANKSIGGGV
VWVLAER DKEEMEMDIGKLEDFDMGTSVICRSGSPLILAD
RCK domain
LKKVSVSKARAIIVLASDENADQSDARALRVMLSLTGVE
GLRGHWVEMSDL DNEPLVCLVGGELIETVVAHDVIGRLM
IQCALQPLAQIWEDILGFENAEFYIKRWPQLDGMRFQDV
LISFPDAVPCGVK IASKAGKILMNPNDYVLQEGDEVLVI
AEDDDTYVPASLPQVRKGF LPNIPTPPKYPEKILFCGWR
DIHDMIMVLEAFLAPGSELWMFNEVPEKERERKLTGGMD
IYGLTNIKLVHKEGNAVIRRHLESPLLETDFDSILILADES
VEDSIVHSDSRSLATLLLRDIQSKRLPSKELK SPLRYNG
FCHSSWIREMQHASDKSIIISEILDSTRNLVSVSKISDY
VLSNELVSMALAMVAEDKQINRVLEELFAEEGNEMCIRSA
EFYLYBQEELSFFDIMWRARERDEWVIGYRLANDDQA IIN
PEQKSEIRKWSLDDVFVVISKGD

Figure 6. Sequence and domain structure of Os-CASTOR and Os-POLLUX. The characteristic motifs and domains are underlined, including the four transmembrane (TM) helices, the filter, the pore helix, the hinge, and the regulation of conductance of K⁺ (RCK) domain.

Isolation of *Os-pollux* mutants in rice

We searched the rice mutant databases for putative Tos17 and T-DNA insertion lines to be used for functional analysis of *Os-POLLUX* in root symbioses (Miyao et al., 2007; Jeong et al., 2006). We identified two insertion alleles for *Os-POLLUX*, named *Os-pollux-1*

and *Os-pollux-2*. The *Os-pollux-1* mutant was a T-DNA insertion line in the genetic background of Dongjin (line No. B02432), in which the T-DNA was inserted into the first intron. The *Os-pollux-2* was a Tos17 insertion line in the genotype Nipponbare derived from tissue culture (line No. NC6423). In *Os-pollux-2*, the retrotransposon Tos17 was inserted into the third exon. For the two mutants, the expression of *Os-POLLUX* was disrupted based on RT-PCR analyses (Figure 5C, bottom).

From progeny of each primary mutant line, positive T-DNA/Tos17 insertion plants were identified by PCR analysis using a pair of T-DNA/Tos17 and gene-specific primers (Figure 5C, top). A second-round PCR analysis was followed to discriminate between homozygous mutant ($-/-$) and heterozygous ($+/-$) plants using a primer pair flanking the T-DNA/Tos17 insertion sites that enabled the amplification of only the wild-type alleles under given PCR conditions (Figure 5C, middle). Since T-DNA and Tos17 mutant lines may comprise multiple insertion sites, the wild-type plants segregated from the progeny of the heterozygous mutant lines were used as additional controls for experiments described below.

***Os-pollux* mutant plants are defective in mycorrhizal symbiosis**

To test whether *Os-POLLUX* is required for AM symbiosis in rice, we inoculated the mutant and wild-type rice roots with the fungus *Glomus intraradices*. At 35 days after inoculation, wild-type plants were densely colonized by *G. intraradices*, exhibiting all typical symbiotic structures such as intercellular and intracellular hyphae, vesicles, and

arbuscules. In each of the 60 wild-type plants comprising of the genotypes Nipponbare and Dongjin, ~60-85% of the total root length was colonized. Similar levels of colonization also were observed for wild-type plants segregated from heterozygous mutant plants (Figure 7). In contrast, intracellular fungal structures, including vesicles and arbuscules, were never observed on roots of a total of 72 *Os-pollux-1*, and 60 *Os-pollux-2* homozygous mutant plants. For homozygous mutant plants, extraradical hyphae and appressoria were frequently observed on the root surface (Figure 7), but the fungus was unable to penetrate the roots beyond the epidermis. These observations indicate that the knockout of *Os-POLLUX* has completely abolished the ability of the AM fungus to enter the plant root. The defective phenotypes were similar to those reported for the *pollux* (i.e., *Lj-sym23* and *Lj-sym86*) (Kistner et al., 2005) mutants in *L. japonicas*, the *dmi1* mutants in *M. truncatula* (Catoira et al., 2000), and the *sym8* mutant in *Pisum sativum* (Balaji et al., 1994). For the weak alleles of the *L. japonicas pollux* mutants, the fungus was occasionally able to penetrate the cortical cells and form arbuscules (Senoo et al., 2000; Kistner et al., 2005), but this leaky phenotype was not observed for the knockout lines of rice.

Transcriptional profiling has revealed a number of host genes that were expressed exclusively in the root colonized by AM fungi (Harrison, 2002; Liu et al., 2003; Guimil et al., 2005; Kistner et al., 2005). Thus, the expression of these AM-specific genes can serve as a molecular marker for the occurrence of successful symbiotic interaction between the two symbionts. In addition to the phenotypic analysis at the cytological

level, we analyzed the expression of *Os-PT11*, a rice mycorrhiza-specific phosphate transporter (Paszkowski et al., 2002), in roots of the wild type and mutants under inoculated and non-inoculated conditions. The results showed that *Os-PT11* was expressed only in the wild-type roots inoculated with *G. intraradices* but not in the mutant roots (Figure 8). The cytological and molecular evidence strongly indicate that *Os-POLLUX* is required for the establishment of AM symbiosis in rice.

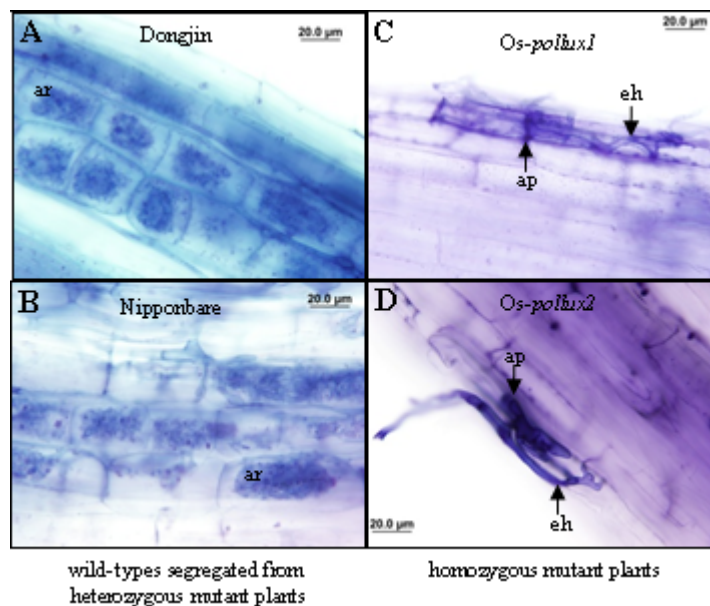


Figure 7. *Os-pollux* is defective in AM symbiosis. (A-B) Roots of wildtype plants segregated from heterozygous mutants formed arbuscules upon inoculation with *G. intraradices*. (C-D) Roots of homozygous mutants failed to form AM symbiosis, despite the presence of fungal hyphae on the root surface. Photographs were taken from roots at 5 weeks postinoculation with *G. intraradices*. Mycorrhizal colonization was assessed by Trypan Blue staining according to the procedures described by Koske and Gemma (1989). Stained roots were examined using a light microscope (Olympus BX40F-3) and images were captured by a microscope digital camera system (Olympus DP71). eh, extraradical hypha; ih, intraradical hypha; ar, arbuscule; ap, appressorium.

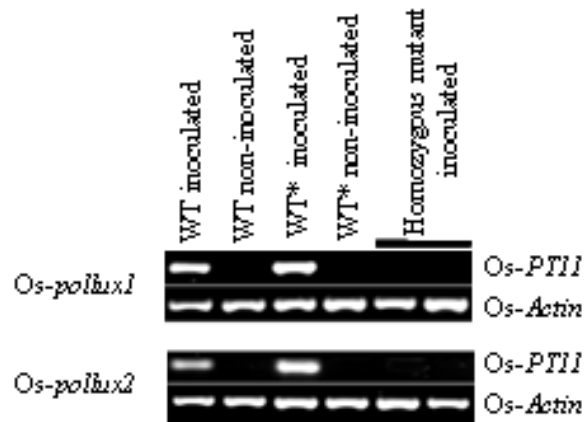


Figure 8. Expression of the rice AM-specific phosphate transporter *Os-PTII* in the mutant lines of *Os-POLLUX* under inoculated and non-inoculated conditions. WT* indicates wild-type plants segregated from a corresponding heterozygous mutant plant.

***Os-POLLUX* can restore nodulation, but not rhizobial infection, to a *M. truncatula dmi1* mutant**

To determine whether the non-legume *POLLUX* orthologs possess an equivalent function to their legume counterparts, we introduce two *Os-POLLUX* full-length cDNAs (AK067564 and AK072312), under the control of the 35S promoter, into the *M. truncatula dmi1-1* mutant (allele C71) (Catoira et al., 2000; Ane et al., 2004) using *Agrobacterium rhizogenes*-mediated hairy root transformation (Boisson-Dernier et al., 2001). AK067564 and AK072312 encode predicted protein of 943 and 965 amino acids (Figure 11), which is due to alternative splicing that leads to the differences at their 3' end. Based on sequence alignments with other *POLLUX* orthologs, the 943-aa version is

found to be canonical. Nevertheless, the two isoforms share the first 941 amino acids. It is worthy mentioning that both cDNA clones provided by the Rice Genome Resource Center (Japan) contain a point mutation likely resulting from the reverse transcription process (Figure 9): for the AK067564, there was an extra insertion of ‘T’ at the position 2136; for the AK072312, an ‘A-to-T’ transversion was found at the position 593. Both errors are corrected in the transformation experiments described below (Figure 11). We do not observe any obvious differences resulting from using the two constructs, suggesting that the two protein isoforms (Figure 11) are equally functional.

```

AK067564      GAGTGGAGCTTTGAGCTTTGCTAATCTTGGGGCGTTGAGGGAGCTTGAGCTCGCTGCTAA
AK072312      --TGGGAGCTTTGAGCTTTGCTAATCTTGGGGCGTTGAGGGAGCTTGAGCTCGCTGCTAA
                *****

AK067564      TGGCGGAGAGCGATGGCGGCGAGGCTAGCCCAGCGGTGGCGGGCGGAGAGGGGAGCC
AK072312      TGGCGGAGAGCGATGGCGGCGAGGCTAGCCCAGCGGTGGCGGGCGGAGAGGGGAGCC
                *****

AK067564      CCGATCCCCGGCGGCCACCGGCTCGGCCGAGCTCACCAAGTCGCGCACCATCTCCGGCT
AK072312      CCGATCCCCGGCGGCCACCGGCTCGGCCGAGCTCACCAAGTCGCGCACCATCTCCGGCT
                *****

AK067564      CCGCCGCGTCCGCGTTTCGACAGATGGGGCACCAGCAACAGCAGCAGCAGCATCCTAGTTC
AK072312      CCGCCGCGTCCGCGTTTCGACAGATGGGGCACCAGCAACAGCAGCAGCAGCATCCTAGTTC
                *****

AK067564      GCCGCTCCTCCACAGCGCCGCTCCCGCCGGGCGCGCGCCGCGGGGGTGTCTACCGTGG
AK072312      GCCGCTCCTCCACAGCGCCGCTCCCGCCGGGCGCGCGCCGCGGGGGTGTCTACCGTGG
                *****

AK067564      CCGTGGACGAGCCCTCCTACGCGGCGCCCAACGGCGGGGCGGCCATGCTCGACCCGCGACT
AK072312      CCGTGGACGAGCCCTCCTACGCGGCGCCCAACGGCGGGGCGGCCATGCTCGACCCGCGACT
                *****

AK067564      GGTGCTACCCGTCGTTCTCGGCCCGCACGCCTCGCGGCCGCGGCCCGCGGTTCGCGAGC
AK072312      GGTGCTACCCGTCGTTCTCGGCCCGCACGCCTCGCGGCCGCGGCCCGCGGTTCGCGAGC
                *****

AK067564      AGCAGACGCCGACGACGACGGCTGCTGCTGCTGCCGACAGCCGAGCCCTACCCCGCCG
AK072312      AGCAGACGCCGACGACGACGGCTGCTGCTGCTGCCGACAGCCGAGCCCTACCCCGCCG
                *****

AK067564      CGCCCGCGCAGACGGCTCGGTCTCGCAGCGGGAGGAGAGAAGAGCCTCGCTTCCGTGG

```

AK072312 CGCCGCCGACGGCGTTCGGTCTCGCAGCGGGAGGAGAGAAGAGCCTCGCTTCCGTGG

AK067564 TGAAGCGGCCGATGCTGCTGGATGAGAGGAGATCGCTCTCGCCGCCGCCGCCGCAAGCAGC
AK072312 TGAAGCGGCCGATGCTGCTGGATGAGAGGAGATCGCTCTCGCCGCCGCCGCCGCAAGCAGC

AK067564 GCGCCCCGCGATTTGATCTCTCCCGTATCTTGTACTAATGCTGGTTGTAAGTGTGATAA
AK072312 GCGCCCCGCGATTTGATCTCTCCCGTATCTTGTACTAATGCTGGTTGTAAGTGTGATAA

AK067564 GTTTTTCCTTGGCTATATGGCAGTGGATGAAAGCTACCGTACTCCAGGAAAAGATCAGAT
AK072312 GTTTTTCCTTGGCTATATGGCAGTGGATGAAAGCTACCGTACTCCAGGAAAAGATCAGAT

AK067564 CATGCTGTAGTGTAGTACTGTGGACTGTAAGACAACACTGAGGCGTTCAAGATTAACG
AK072312 CATGCTGTAGTGTAGTACTGTGGACTGTAAGACAACACTGAGGCGTTCAAGATTAACG

AK067564 GACAGCATGGTTCTGATTTTATCAACTCCGCGGACTGGAATTTAGCTTCCTGCAGCAGAA
AK072312 GACAGCATGGTTCTGATTTTATCAACTCCGCGGACTGGAATTTAGCTTCCTGCAGCAGAA

AK067564 TGCTTGTTTTTCGATACCTGTATTCTTGGTTAAGTACATTGATCAGCTTCGACGAAGGA
AK072312 TGCTTGTTTTTCGATACCTGTATTCTTGGTTAAGTACATTGATCAGCTTCGACGAAGGA

AK067564 ACACAGATTCCATTAGGCTAAGAAGCACCCGAGGAAGAAGTGCCTCTGAAGAAGAGGATTG
AK072312 ACACAGATTCCATTAGGCTAAGAAGCACCCGAGGAAGAAGTGCCTCTGAAGAAGAGGATTG

AK067564 CTTATAAGGTTGATGTGTTCTTCTCAGGACACCCGTACGCAAAGCTTCTTGCCTCTCTGT
AK072312 CTTATAAGGTTGATGTGTTCTTCTCAGGACACCCGTACGCAAAGCTTCTTGCCTCTCTGT

AK067564 TAGCCACAATAATCCTCATTGCTTCTGGCGGATTGCGCTTTATGTTGTCAGCGGCAGTG
AK072312 TAGCCACAATAATCCTCATTGCTTCTGGCGGATTGCGCTTTATGTTGTCAGCGGCAGTG

AK067564 GATTCTGGAGGCTCTTTGGCTTTCATGGACTTTTGTGGCAGATTGAGGAAACCATGCTG
AK072312 GATTCTGGAGGCTCTTTGGCTTTCATGGACTTTTGTGGCAGATTGAGGAAACCATGCTG

AK067564 ATCAGGTTGGCTGGGCCAAGGATTGTGTCGATGCTAGCTCTGGGGGTATGCTGG
AK072312 ATCAGGTTGGCTGGGCCAAGGATTGTGTCGATGCTAGCTCTGGGGGTATGCTGG

AK067564 TGTTTGGCACAATGCTTGGGCTTGTGTCAGATGCAATATCAGAGAAGGTAGATTCTGGC
AK072312 TGTTTGGCACAATGCTTGGGCTTGTGTCAGATGCAATATCAGAGAAGGTAGATTCTGGC

AK067564 GCAAGGAAAAAGTGAAGGTGATAGAGGTCAACCATATACTAATCCTCGGATGGAGCGACA
AK072312 GCAAGGAAAAAGTGAAGGTGATAGAGGTCAACCATATACTAATCCTCGGATGGAGCGACA

AK067564 AGTTGGGCTCTCTCCTGAAGCAGCTAGCTATAGCAAACAAGATATTGGTGGTGGTGTG
AK072312 AGTTGGGCTCTCTCCTGAAGCAGCTAGCTATAGCAAACAAGATATTGGTGGTGGTGTG

AK067564 TTGTTGTTCTGGCAGAAAGAGACAAGGAAGAAATGGAGATGGACATTGGGAAGCTAGAGT
AK072312 TTGTTGTTCTGGCAGAAAGAGACAAGGAAGAAATGGAGATGGACATTGGGAAGCTAGAGT

AK067564 TTGACTTTATGGGAACATCTGTAATATGTAGAAGTGGCAGTCCTCTAATTTAGCTGATT
AK072312 TTGACTTTATGGGAACATCTGTAATATGTAGAAGTGGCAGTCCTCTAATTTAGCTGATT

AK067564 TGAAGAAGGTTTCTGTTTCTAAAGCACGTGCTATTATGTTTGTAGCATCCGATGAAAATG
AK072312 TGAAGAAGGTTTCTGTTTCTAAAGCACGTGCTATTATGTTTGTAGCATCCGATGAAAATG

AK067564 CAGACCAAAGTGATGCACGGGCTTTACGTGTGGTCTGAGCCTGACTGGAGTTAAAGAGG
AK072312 CAGACCAAAGTGATGCACGGGCTTTACGTGTGGTCTGAGCCTGACTGGAGTTAAAGAGG

AK067564 GTTTAAGGGGACATGTTGTTGTAGAGATGAGTGACCTTGACAACGAGCCTTTGGTGAAAC
AK072312 GTTTAAGGGGACATGTTGTTGTAGAGATGAGTGACCTTGACAACGAGCCTTTGGTGAAAC

AK067564 TGGTTGGAGGTGAAC TAATTGAAACAGTTGTTGCCATGATGTGATTGGACGTTTGATGA
AK072312 TGGTTGGAGGTGAAC TAATTGAAACAGTTGTTGCCATGATGTGATTGGACGTTTGATGA

AK067564 TACAATGTGCACTCCAACCAGGCCGTCACAGATATGGGAGGATATTTGGGGTTTGAAA
AK072312 TACAATGTGCACTCCAACCAGGCCGTCACAGATATGGGAGGATATTTGGGGTTTGAAA

AK067564 ACGCAGAGTTCTACATTAAGATGGCCAGAATTAGATGGCATGCGATTGGGGATGTCT
AK072312 ACGCAGAGTTCTACATTAAGATGGCCAGAATTAGATGGCATGCGATTGGGGATGTCT

AK067564 TAATCTCATTTCCTGACGCTGTTCCATGTGGAGTAAAGATTGCTTCAAAGCTGGAAAA
AK072312 TAATCTCATTTCCTGACGCTGTTCCATGTGGAGTAAAGATTGCTTCAAAGCTGGAAAA

AK067564 TCTTAATGAATCCTGATAATGATTATGTTTTGCAAGAAGGTGATGAGGTCTTGTATAG
AK072312 TCTTAATGAATCCTGATAATGATTATGTTTTGCAAGAAGGTGATGAGGTCTTGTATAG

AK067564 CAGAAGATGACGATACTTATGTTCTCCTCCTCTACCACAGGTGCGAAAGGTTTCTAC
AK072312 CAGAAGATGACGATACTTATGTTCTCCTCCTCTACCACAGGTGCGAAAGGTTTCTAC

AK067564 CTAATATCCCAACCCCTCCTAAATACCCAGAGAAAATTTGTTCTGCGGTTGGCGACGTG
AK072312 CTAATATCCCAACCCCTCCTAAATACCCAGAGAAAATTTGTTCTGCGGTTGGCGACGTG

AK067564 ACATTATGACATGATAATGGTCTTAGAAGCA~~TTT~~CTCGCTCCAGGTTCTGAATTATGG
AK072312 ACATTATGACATGATAATGGTCTTAGAAGCA~~TTT~~CTCGCTCCAGGTTCTGAATTATGG

AK067564 ATGTTTAAACGAGGTGCCAGAGAAGGAGAGGAGAGAAAAC TACTGACGGTGGTATGGAT
AK072312 ATGTTTAAACGAGGTGCCAGAGAAGGAGAGGAGAGAAAAC TACTGACGGTGGTATGGAT

AK067564 ATTTATGGACTAACAAACATTAACCTTGTACATAAAGAAGGGAATGCCGTATCAGCGG
AK072312 ATTTATGGACTAACAAACATTAACCTTGTACATAAAGAAGGGAATGCCGTATCAGCGG

AK067564 CACTTAGAAAGTTTGCCTCTGGAGACATTCGATTCTATTTAATTCTTGCAGATGAGTCA
AK072312 CACTTAGAAAGTTTGCCTCTGGAGACATTCGATTCTATTTAATTCTTGCAGATGAGTCA

AK067564 GTGGAGGATTCATCGTACATTCTGATTACGTTCTTTAGCTACACTTCTTCTAATTTCG
AK072312 GTGGAGGATTCATCGTACATTCTGATTACGTTCTTTAGCTACACTTCTTCTAATTTCG

AK067564 GACATTCAGTCTAAACGTCTTCCATCAAAGGAACTAAAATCACCTCTGCGTTACAATGGC
AK072312 GACATTCAGTCTAAACGTCTTCCATCAAAGGAACTAAAATCACCTCTGCGTTACAATGGC

AK067564 TTCTGTACAGCTCCTGGATTCCGGAGATGCAGCACGCATCAGATAAATCAATAATTATC
AK072312 TTCTGTACAGCTCCTGGATTCCGGAGATGCAGCACGCATCAGATAAATCAATAATTATC

AK067564 AGTGAATTCTGGATTCCAGAACCAGAAACCTTGTATCTGTCTCCAAAATCAGCGACTAT
AK072312 AGTGAATTCTGGATTCCAGAACCAGAAACCTTGTATCTGTCTCCAAAATCAGCGACTAT

AK067564 GTTCTATCAAATGAAC TTGTCAGCATGGCATTAGCAATGGTAGCAGAGGACAAAACAAATC

AK072312 GTTCTATCAAATGAACTTGTGTCAGCATGGCATTAGCAATGGTAGCAGAGGACAAACAAATC

AK067564 AACAGAGTTCCTTGAAGAACTTTTGGCCGAGGAGGGCAACGAGATGTGCATACGTTACAGCT
AK072312 AACAGAGTTCCTTGAAGAACTTTTGGCCGAGGAGGGCAACGAGATGTGCATACGTTACAGCT

AK067564 GAGTTTTACCTCTATGAGCAAGAGGAATTGAGTTTCTTGTATATAATGGTCAGGGCTCGT
AK072312 GAGTTTTACCTCTATGAGCAAGAGGAATTGAGTTTCTTGTATATAATGGTCAGGGCTCGT

AK067564 GAAAGAGACGAGGTTGTCATTGGCTACCGCCTTGCCAACGATGACCAGGCAATCATTAAAT
AK072312 GAAAGAGACGAGGTTGTCATTGGCTACCGCCTTGCCAACGATGACCAGGCAATCATTAAAT

AK067564 CCAGAACAGAAGTCTGAGATTAGGAAGTGGTCGCTAGACGATGTTTTTGTGTGATCTCA
AK072312 CCAGAACAGAAGTCTGAGATTAGGAAGTGGTCGCTAGACGATGTTTTTGTGTGATCTCA

AK067564 AAAGCGGGCAATGCAACATATTTTGTGAAGACCACGGTGTGCGGTCTAACCCAGTTGTG
AK072312 AAAG-----GTGACTAATTTT-----ACAATAGCACCTCACTTTTTTCGAGTTA--
**** * ** ***** ** * * * * *
AK067564 AAAGCGGGCAATGCAACATATTTTGTGAAGACCACGGTGTGCGGTCTAACCCAGTTGTG
AK072312 AAAG-----GTGACTAATTTT-----ACAATAGCACCTCACTTTTTTCGAGTTA--
**** * ** ***** ** * * * * *
AK067564 TATTCCTCTACATTTTAATTTGACTAATTCCTGCT--CCTCCGTTTGCTGTTATTTTTCGA
AK072312 -GTAAGTATATATTCTAAC--ACTAGTGTGCTAACCAATGCTAACTGAAATGCTTTAA
*
AK067564 TTT--AAATTAGTGGGTCCAGGCTCCAG--CAACTTAATAT-AGATGGCTAAATTTG
AK072312 GTTGCTAACCCAGGCAACACACGTCACAGTACAGTTCTGATCTCATATGTTGCTAGCTCT
*
AK067564 CCATTGGCCTGCTGTTGGCTGGATCTCTCATCCAAGGTTTCATATGAATGCTCACCCC
AK072312 GTACATGTTGATTTGCCATCTCCATAGCATTAGTTGATCTTAACATAAATAATACACA
*
AK067564 CAGGCTTGATGAGGTCATCTGGATAGAGCTGGCATGGCTAAAGGAATGGTTGCCTGGA
AK072312 TCAGC--ATTGAAACTGTTGTTACTAA--TTGACATTACAAAATTTTCAGT-GCGTCGA
*
AK067564 TACTCTCATCCAAGGTTTCATATGAATGCTCTACCCCAGGCTTGATGAGGGTCTCTACC
AK072312 T----TCAGC-AGAGATAATTTGTAATTTGTAACCTACTGTTTCATTTGAGAGCATGCT
*
AK067564 CCCAGGCTTGATGAGGGCTAAAGGAATGGTTGCCTGGATACTCTCATCCAAGGTTTCATA
AK072312 A--ATCATTG-----
* * * * *
AK067564 TGAATGCTCTACCCCAGGCTTGATGAGGTCATCCTGATAGAGCTGGCCCCAGGCTTA
AK072312 -----
AK067564 TATGAATGCTCTACCCCAGGCTTGATGAGGTCATCCTGATAGAGCTGGCCCCAGGCT
AK072312 -----
AK067564 TGTATGAGGTCATCCTGGATAGAGCTGGCATGGCTAAAGGAATGCAAAATTTGCAAAATG
AK072312 -----
AK067564 TATGTTTGGCTGTGCTTGTGGAATTCAGTTTGTGGACAGATGTATAGGGCCAGTTTT
AK072312 -----
AK067564 GGAGGCAATGGCAAAGCTGCGGTATTCCAATAATATACTGTACAATTGCTG
AK072312 -----

Figure 9: Sequence alignments of two *Os-POLLUX* full-length cDNAs (AK067564 and AK072312). Both sequences were obtained from the Rice Genome Resource Center (Japan). The point mutants of the two cDNA clones have been highlighted: for the AK067564, there was an extra insertion of 'T' at the position 2136; for the AK072312, an 'A-to-T' transversion was found at the position 593.

```

AK067564      MAESDGGEASPSGGGGGEGSPDRRPPARPQLTKSRTISGSAASAFDRWGTSN
AK072312      MAESDGGEASPSGGGGGEGSPDRRPPARPQLTKSRTISGSAASAFDRWGTSN
                *****

AK067564      SSSSILVRRSSTAPLPPGAAPRGLLTVAVDEPSYAAPNGGAAMLDLDRWCYP SFLGPHASR
AK072312      SSSSILVRRSSTAPLPPGAAPRGLLTVAVDEPSYAAPNGGAAMLDLDRWCYP SFLGPHASR
                *****

AK067564      PRPPRSQQQTPTTTAAAAADSRSPTPAAPPTASVSQREEEKSLASVVKRPMLLDERRSL
AK072312      PRPPRSQQQTPTTTAAAAADSRSPTPAAPPTASVSQREEEKSLASVVKRPMLLDERRSL
                *****

AK067564      SPPPPQQRAPRFDLSPYLVLMLVVTVISFSLAIWQWMKATVLQEKIRSCCSVSTVDCKTT
AK072312      SPPPPQQRAPRFDLSPYLVLMLVVTVISFSLAIWQWMKATVLQEKIRSCCSVSTVDCKTT
                *****

AK067564      TEAFKINGQHGSDFINSADWNLASC SRMLVFAIPVFLVKYIDQLRRRNTDSIRLRSTEEE
AK072312      TEAFKINGQHGSDFINSADWNLASC SRMLVFAIPVFLVKYIDQLRRRNTDSIRLRSTEEE
                *****

AK067564      VPLKKRIAYKVDVFFSGHPYAKLLALLLATIILIASGGIALYVVS GSGFLEALWLSWTFV
AK072312      VPLKKRIAYKVDVFFSGHPYAKLLALLLATIILIASGGIALYVVS GSGFLEALWLSWTFV
                *****

AK067564      ADSGNHADQVGLGPRIVSVSIS SGGMLVFATMLGLVSDAISEKVDSWRKKGSEVIEVNI
AK072312      ADSGNHADQVGLGPRIVSVSIS SGGMLVFATMLGLVSDAISEKVDSWRKKGSEVIEVNI
                *****

AK067564      LILGWSDKLGSLLKQLAIANKSIGGGVVVLAERDK EEMEMDIGKLEFDFMGTSVICRS
AK072312      LILGWSDKLGSLLKQLAIANKSIGGGVVVLAERDK EEMEMDIGKLEFDFMGTSVICRS
                *****

AK067564      SPLILADLKKVSVSKARAIIVLASDENADQSDARALRVVLSLTGVKEGLRGHV VVEMSDL
AK072312      SPLILADLKKVSVSKARAIIVLASDENADQSDARALRVVLSLTGVKEGLRGHV VVEMSDL
                *****

AK067564      DNEPLVKLVGGELIETVVAHDVIGRLMIQCALQPGLAQI WEDILGFENA EFYIKRWPELD
AK072312      DNEPLVKLVGGELIETVVAHDVIGRLMIQCALQPGLAQI WEDILGFENA EFYIKRWPELD
                *****

AK067564      GMRFGDVLISFPDAVPCGVKIASKAGKILMNPNDNDYVLQEGDEV LVIAEDDDTYVPASLP
AK072312      GMRFGDVLISFPDAVPCGVKIASKAGKILMNPNDNDYVLQEGDEV LVIAEDDDTYVPASLP
                *****

AK067564      QVRKGF L PNIPTPKYPEKILFCGWRRDIHDMIMVLEAF SRSRF -----
AK072312      QVRKGF L PNIPTPKYPEKILFCGWRRDIHDMIMVLEAF LAPGSELWMFN E VPEKERERK
                *****

AK067564      -----
AK072312      LTDGGMDIYGLTNIKLVHKEGNAVIRRHLES LPLETFDSILILADESVEDSIVHSDSRSL

AK067564      -----
AK072312      ATLLLIRD IQSKRLPSKELKSPLRYNGFCHSSWIREMQHASDKSII ISEILDSRTRNLVS

AK067564      -----
AK072312      VSKISDYVLSNELVSMALAMVAEDKQINRVLEELFAEEGNEMCIRSAEFYLYEQEELSFF

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AK067564 -----
 AK072312 DIMVRRERDEVVIGYRLANDDQAIINPEQKSEIRKWSLDDVFFVVISKGD

Figure 10: Protein sequence alignments of two uncorrected Os-*POLLUX* isoforms. Protein sequences are translated from the uncorrected AK067564 and AK072312 full-length cDNAs using ExPASy translate tool. For the isoform translated from uncorrected AK067564, the extra insertion of ‘T’ within the cDNA leads to a stop codon at the position 697 of isoform sequence; for the isoform translated from uncorrected AK072312, an ‘Q-to-L’ transversion was found at the position 179 of isoform sequence.

```

AK067564_corrected MAESDGGEASPSGGGGEGSPDRRPPARPQLTKSRTISGSAASAFDRWGTSN
AK072312_corrected MAESDGGEASPSGGGGEGSPDRRPPARPQLTKSRTISGSAASAFDRWGTSN
*****

AK067564_corrected SSSSILVRRSSTAPLPPGAAPRGLLTVAVDEPSYAAPNGGAAMLDRDWCYPSFLGPHASR
AK072312_corrected SSSSILVRRSSTAPLPPGAAPRGLLTVAVDEPSYAAPNGGAAMLDRDWCYPSFLGPHASR
*****

AK067564_corrected PRPPRSQQQTPTTTAAAAADSRSPTPAAPQTASVSQREEEKS LASVVKRPMLLDERRSL
AK072312_corrected PRPPRSQQQTPTTTAAAAADSRSPTPAAPQTASVSQREEEKS LASVVKRPMLLDERRSL
*****

AK067564_corrected SPPPPQQRAPRFDLSPYLVMLVVTVISFSLAIWQWMKATVLQEKIRSCCSVSTVDCKTT
AK072312_corrected SPPPPQQRAPRFDLSPYLVMLVVTVISFSLAIWQWMKATVLQEKIRSCCSVSTVDCKTT
*****

AK067564_corrected TEAFKINGQHGSDFINSADWNLASCSRMLVFAIPVFLVKYIDQLRRRNTDSIRLRSTEE
AK072312_corrected TEAFKINGQHGSDFINSADWNLASCSRMLVFAIPVFLVKYIDQLRRRNTDSIRLRSTEE
*****

AK067564_corrected VPLKKRIAYKVDVFFSGHPYAKLLALLLATIILIASGGIALYVVS GSGFLEALWLSWTFV
AK072312_corrected VPLKKRIAYKVDVFFSGHPYAKLLALLLATIILIASGGIALYVVS GSGFLEALWLSWTFV
*****

AK067564_corrected ADSGNHADQVGLGPRIVSVSISGGMLVFATMLGLVSDAISEKVD SWRK GKSEVIEVNHI
AK072312_corrected ADSGNHADQVGLGPRIVSVSISGGMLVFATMLGLVSDAISEKVD SWRK GKSEVIEVNHI
*****

AK067564_corrected LILGWSDKLGSLLKQLAIANKSIGGGVVVLAERDKEEMEMDIGLE FDFMGTSVICRS
AK072312_corrected LILGWSDKLGSLLKQLAIANKSIGGGVVVLAERDKEEMEMDIGLE FDFMGTSVICRS
*****

AK067564_corrected SPLILADLKKVSVSKARAIIVLASDENADQSDARALRVVLSLTGVKEGLRGHV VVEMSDL
AK072312_corrected SPLILADLKKVSVSKARAIIVLASDENADQSDARALRVVLSLTGVKEGLRGHV VVEMSDL
*****

AK067564_corrected DNEPLVKLVGGELIETVVAHDVIGRLMIQCALQPGLAQIWEDILGFENAEFYIKR WPELD
AK072312_corrected DNEPLVKLVGGELIETVVAHDVIGRLMIQCALQPGLAQIWEDILGFENAEFYIKR WPELD
*****

AK067564_corrected GMRFGDVLISFPDAVPCGVKIASKAGKILMNPNDNDYVLQEGDEV LVIAEDDDTYVPASLP
AK072312_corrected GMRFGDVLISFPDAVPCGVKIASKAGKILMNPNDNDYVLQEGDEV LVIAEDDDTYVPASLP
*****

AK067564_corrected QVRKGFLPNIPTPKYPEKILFCGWRRDIHDMIMVLEAF LAPGSELWMFNEVPEKERERK
AK072312_corrected QVRKGFLPNIPTPKYPEKILFCGWRRDIHDMIMVLEAF LAPGSELWMFNEVPEKERERK
*****

AK067564_corrected LTDGMDIYGLTNIKLVHKEGNAVIRRHLES LPLETFDSLILILADESVEDSIVHS DSRSL
AK072312_corrected LTDGMDIYGLTNIKLVHKEGNAVIRRHLES LPLETFDSLILILADESVEDSIVHS DSRSL
*****

AK067564_corrected ATLLLRDIQSKRLPSKELKSPLRYNGFCHSSWIREMQHASDKSIIISEILDSRTRNLVS

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AK072312_corrected    ATLLLRDIQSKRLPSKELKSPLRYNGFCHSSWIREMQHASDKSIIISEILDSRTRNLVS
*****
AK067564_corrected   VSKISDYVLSNELVSMALAMVAEDKQINRVLEELFAEEGNEMCIRSAEFYLYEQEELSFF
AK072312_corrected   VSKISDYVLSNELVSMALAMVAEDKQINRVLEELFAEEGNEMCIRSAEFYLYEQEELSFF
*****
AK067564_corrected   DIMVRARERDEVVIGYRLANDDQAIINPEQKSEIRKWSLDDVVFVVISKAGNATYFVKTTV
AK072312_corrected   DIMVRARERDEVVIGYRLANDDQAIINPEQKSEIRKWSLDDVVFVVISKGD-----
*****
AK067564_corrected   MRSNPVYVSSTF
AK072312_corrected   -----

```

Figure 11: Protein sequence alignments of two corrected Os-*POLLUX* isoforms. Protein sequences are translated from the corrected AK067564 and AK072312 full-length cDNAs using ExPASy translate tool. The two corrected isoforms from AK067564 and AK072312 encode predicted protein of 943 and 965 amino acids and share the first 941 amino acids.

Upon inoculation with *Sinorhizobium meliloti*, the *M. truncatula dmi1-1* mutant fails to exhibit root hair curling, infection thread formation, cortical cell division, and nodule development (Catoira et al., 2000). To determine whether Os-*POLLUX* is able to complement the non-nodulation phenotype of the *dmi1-1* mutant, we inoculate the composite transgenic plants with *S. meliloti* strain 2011 carrying the *lacZ* reporter gene in plasmid pXLGD4 (Catoira et al., 2000). Two weeks post inoculation, nodule-like organs are observed on roots of 36 (out of 59) *dmi1-1* plants transformed with Os-*POLLUX*. However, unlike wild-type plants that form long, pink, cylindrical-shaped nodules, the transgenic mutant roots produce small, white, round-shaped nodules that are similar to the phenotypes of the nitrogen fixation mutants of *M. truncatula* (Starker et al., 2006). GUS staining and microscopy of sectioned nodules reveal that the small white nodules were devoid of bacteria and no infection threads are observed, thus these nodules are non-functional (Figure 12). The lack of nitrogen-fixing ability of these white

nodules also is reflected by the poor growth and chlorotic leaves of the composite transgenic plants under nitrogen-starving conditions (Figure 13).

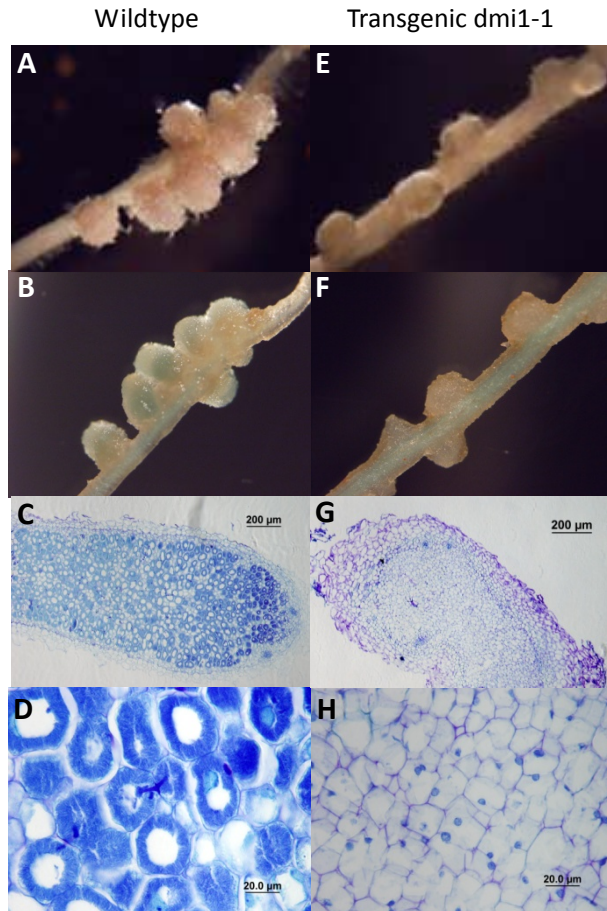


Figure 12. Complementation of the *M. truncatula dmi1-1* mutant (C71) with *Os-POLLUX* using *Agrobacterium. rhizogenes*-mediated hairy root transformation (Boisson-Dernier et al., 2001). A full-length cDNA clone of *Os-POLLUX* (AK067564) was cloned into a binary vector modified from pHellsgate8 driven by the 35S promoter (Helliwell et al., 2002). The binary vector was introduced into the *A. rhizogenes* strain, *ARqual*, and transformed into the roots of the *dmi1-1* mutant. (A-D), wild-type; (E-H), the *dmi1-1* mutant transformed with 35S:*Os-POLLUX*. (A) and (E), nodule phenotypes of wildtype and transgenic *dmi1-1* roots, respectively. (B) and (F), GUS staining of nodules shown in A and E, respectively. (C) and (G), toluidine blue staining of sectioned nodules from wildtype and transgenic roots, respectively. (D) and (H) are a close-up of the sectioned nodules.



Figure 13: *dmi1* mutant and wide-type plants transformed with 35S:Os-*POLLUX* grown under nitrogen-starving conditions. Left: the *dmi1-1* mutant transformed with 35S:Os-*POLLUX*; right: wide-type plant transformed with 35S:Os-*POLLUX*.

The observation that Os-*POLLUX* can complement the nodule organogenesis but not the infection process is similar to that reported for the *dmi3* mutants complemented by Os-*DMI3* (Godfroy et al., 2006; Chen et al., 2007). It was also reported that Os-*DMI2* (or Os-*SYMRK*), a ‘reduced-length’ version of the Mt-*DMI2*/Lj-*SYMRK* ortholog, restored nodule organogenesis of an Lj-*sym10* mutant but failed to support the formation of infection threads, despite that the ‘full-length’ versions from several other non-legume orthologs can complement both processes (Markmann et al., 2008). These observations are consistent with the finding that the nodule organogenesis and bacterial infection can be uncoupled (Gleason et al., 2006; Tirichine et al., 2006; Tirichine et al., 2007; Murray et al., 2007). Taken together, these data suggest that 1) in addition to early steps of nodule initiation, the common symbiosis genes also are involved in the

control of the infection process and 2) the infection process in nodule development appears to be under more stringent genetic control than the nodule organogenesis. It has been shown that knockdown mutants of the *DMI2* orthologs maintained the ability to form nodules but failed to form symbiosomes in the legume nodules (Limpens et al., 2005; Capoen et al., 2005). This taxonomic-specific functionality could be due to adaptation through changes in gene regulation and expression or sequence diversification between leguminous and non-leguminous plants.

SECTION 4

CONCLUSIONS

Root symbioses with AM fungi and nitrogen-fixing bacteria share common signaling components, suggesting that the nitrogen-fixing root nodule symbioses have evolved from the ancient AM symbiosis (Kistner et al., 2002). This hypothesis is further supported by the fact that all the legume common symbiosis genes are present in non-legumes that have the ability to establish the AM (Zhu et al., 2006). However, the function of these non-legume orthologs needs to be addressed in order to gain an insight into the evolution of the root symbioses in plants. Thus far, four of the seven known common symbiosis genes have been shown to be required for AM symbiosis in non-legumes (Chen et al., 2007; Gherbi et al., 2008; Markmann et al., 2008). We speculate that all common symbiosis genes in legumes would be required for the AM symbiosis in non-legumes. Interestingly, those genes required only for rhizobial symbiosis but not essential for the AM symbiosis in legumes are also present in non-legumes. Elucidating the function of non-legume orthologs of nodulation-specific genes will provide further insights into the evolution of root symbioses in land plants.

In our research, we use reverse genetic approaches to demonstrate that the rice *Os-POLLUX* is indispensable for mycorrhizal symbiosis in rice. Furthermore, we show that *Os-POLLUX* can restore nodulation, but not rhizobial infection, to a *M. truncatula dmi1* mutant.

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APPENDIX

Os-POLLUX **WPLKKRIAMNDVDFSSGHPTAKLLALALATILLIAGGIALVVSQSGLEALWLSWTFVADSGNHADQVGLGPRIVSVS** 80
 Zm-POLLUX **WPLKKRIAMNDVDFSSGHPTAKLLALALATILLIAGGIALVVSQSGLEALWLSWTFVADSGNHADQVGLGPRIVSVS** 80
 Vv-POLLUX **WPLKKRIAMNDVDFSSGHPTAKLLALALATILLIAGGIALVVSQSGLEALWLSWTFVADSGNHADRVGTGQRIVSVS** 80
 Pt-POLLUX **APLKKRVAMNDVDFSSGHPTAKLLALALATILLIAGGIALVVSQSGLEALWLSWTFVADSGNHADRVGTGPRIVSVS** 80
 Mt-DM11 **WPLKKRVAMNDVDFSSGHPTAKLLALALATILLIAGGIALVVSQSGLEALWLSWTFVADAGNHAETEGTQQRIVSVS** 80
 Ps-SYM8 **WPLKKRVAMNDVDFSSGHPTAKLLALALATILLIAGGIALVVSQSGLEALWLSWTFVADAGNHAETEGMQRIVSVS** 80
 Lj-POLLUX **WPLKKRIAMNDVDFSSGHPTAKLLALALATILLIAGGIALVVSQSGLEALWLSWTFVADSGNHAETCGTQQRIVSVS** 80
 Gm-POLLUX **WPLKKRVAMNDVDFSSGHPTAKLLALALATILLIAGGIALVVSQSGLEALWLSWTFVADSGNHAETEGTQQRIVSVS** 80
 At5g49960 **WPLKKRLAMNDVDFSSGHPTAKLLALALATVVLIVVGGIALVVSQSGLEALWLSWTFVADSGSHADRVGVGARIVSVA** 80
 Os-CASTOR **VPINKRLAMNDVDFSSGHPTAKPLVLLVATLLIAGGIALVVSQSGLEALWLSWTFVADSGNHANAEVGLGPKLVSVS** 80
 Zm-CASTOR **VPINKRLAMNDVDFSSGHPTAKPLVLLVATLLIAGGIALVVSQSGLEALWLSWTFVADSGNHANAEVGLGPKLVSVS** 80
 Lj-CASTOR **VSLNRCIAMNDVDFSSGHPTAKPLVLLVATLLIAGGIALVVSQSGLEALWLSWTFVADSGNHASREGIGPRLVSVS** 80
 Gm-CASTOR **VSLNRCIAMNDVDFSSGHPTAKPLVLLVATLLIAGGIALVVSQSGLEALWLSWTFVADSGNHASREGIGPRLVSVS** 80
 Mt-CASTOR **VSLNRCIAMNDVDFSSGHPTAKPLVLLVATLLIAGGIALVVSQSGLEALWLSWTFVADSGNHASREGIGPRLVSVS** 80
 Vv-CASTOR **VSLNRCIAMNDVDFSSGHPTAKPLVLLVATLLIAGGIALVVSQSGLEALWLSWTFVADSGNHANAEVGLGPKLVSVS** 80
 Pt-CASTOR **ALNRCIAMNDVDFSSGHPTAKPLVLLVATLLIAGGIALVVSQSGLEALWLSWTFVADSGNHASREGIGPRLVSVS** 80
 ruler 1.....10.....20.....30.....40.....50.....60.....70.....80



Os-POLLUX **TSGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 Zm-POLLUX **TSGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 Vv-POLLUX **TSGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 Pt-POLLUX **TSGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 Mt-DM11 **TSAGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 Ps-SYM8 **TSAGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 Lj-POLLUX **TSGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 Gm-POLLUX **TSGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 At5g49960 **TSAGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 Os-CASTOR **TSIGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 Zm-CASTOR **TSIGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 Lj-CASTOR **TSFGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 Gm-CASTOR **TSFGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 Mt-CASTOR **TSFGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 Vv-CASTOR **TSFGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 Pt-CASTOR **TSFGGMLVFAMMLGLVSDAISEKVDLSRKGSEVVEKNHILILGSDILGSLNQIAIANKSIQGGVIVVLAERKEENE** 160
 ruler90.....100.....110.....120.....130.....140.....150.....160



Os-POLLUX **MDIARKLEDFMGTSVICRSGSPLILADLKKVSVSKARAIIIVASDENADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 Zm-POLLUX **MDIARKLEDFMGTSVICRSGSPLILADLKKVSVSKARAIIIVASDENADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 Vv-POLLUX **MDIARKLEDFMGTSVICRSGSPLILADLKKVSVSKARAIIIVASDENADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 Pt-POLLUX **MDIARKLEDFMGTSVICRSGSPLILADLKKVSVSKARAIIIVASDENADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 Mt-DM11 **MDIARKLEDFMGTSVICRSGSPLILADLKKVSVSKARAIIIVASDENADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 Ps-SYM8 **MDIARKLEDFMGTSVICRSGSPLILADLKKVSVSKARAIIIVASDENADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 Lj-POLLUX **MDITKLEDFMGTSVICRSGSPLILADLKKVSVSKARAIIIVASDENADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 Gm-POLLUX **MDIARKLEDFMGTSVICRSGSPLILADLKKVSVSKARAIIIVASDENADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 At5g49960 **TDIARKLEDFMGTSVICRSGSPLILADLKKVSVSNARAIIIVASDENADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 Os-CASTOR **ADIAKMEFDLGTAVICRSGSPLILADLKKVSVSKARAIIIVAAEENADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 Zm-CASTOR **ADIAKMEFDLGTAVICRSGSPLILADLKKVSVSKARAIIIVAAEENADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 Lj-CASTOR **LDIARKLEDFMGTSVICRSGSPLILADLKKVSVSKARAIIIVAAEDGNADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 Gm-CASTOR **LDIARKLEDFMGTSVICRSGSPLILADLKKVSVSKARAIIIVAAEDGNADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 Mt-CASTOR **LDIARKLEDFMGTSVICRSGSPLILADLKKVSVSKARAIIIVAAEDGNADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 Vv-CASTOR **LDIARKLEDFMGTSVICRSGSPLILADLKKVSVSKARAIIIVAAEDGNADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 Pt-CASTOR **MDIARKLEDFMGTSVICRSGSPLILADLKKVSVSKARAIIIVAAEDGNADQSDAFALRVVLSLTGVVEGLRGRHVVVENSDDL** 240
 ruler170.....180.....190.....200.....210.....220.....230.....240




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***          *          *          *          *          *          *          *          *          *          *          *
Os-POLLUX DNEPLVKLVGGELIETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGMRTGDVLIISFPDAVPCGVX 320
Zm-POLLUX DNEPLVKLVGGELIETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGMRTGDVLIISFPDAVPCGVX 320
Vv-POLLUX DNEPLVKLVGGELIETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGMRTGDVLIISFPDAVPCGVX 320
Pt-POLLUX DNEPLVKLVGGELIETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGMRTGDVLIISFPDAVPCGVX 320
Mt-DMI1 DNEPLVKLVGGELIETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGLLFDLILISFPDAIPCGVX 320
Ps-SYM8 DNEPLVKLVGGELIETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGLLFDLILISFPDAIPCGVX 320
Lj-POLLUX DNEPLVKLVGGELIETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGLSFDLILISFPDAIPCGVX 320
Gm-POLLUX DNEPLVKLVGGELIETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGLSFDLILISFPDAIPGVX 320
At5g49960 DNEPLVKLVGGELIETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGMRTGDVLIISFPDAIPCGVX 320
Os-CASTOR DNEPLVKLVGGDLVETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGMRTGDVLIISFPDAIPCGIX 320
Zm-CASTOR DNEPLVKLVGGDLVETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGMRTGDVLIISFPDAIPCGIX 320
Lj-CASTOR DNEPLVKLVGGDLVETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGMRTGDVLIISFPDAIPCGIX 320
Gm-CASTOR DNEPLVKLVGGDLVETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGMRTGDVLIISFPDAIPCGIX 320
Mt-CASTOR DNEPLVKLVGGDLVETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGMRTGDVLIISFPDAIPCGIX 320
Vv-CASTOR DNEPLVKLVGGDLVETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGMRTGDVLIISFPDAIPCGIX 320
Pt-CASTOR DNEPLVKLVGGDLVETVYADVIGRLMIQCALQPGGLACIWEIDILGSENAEFTIKRPFELDGMRTGDVLIISFPDAIPCGIX 320
ruler .....250.....260.....270.....280.....290.....300.....310.....320

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**          *          *          *          *          *          *          *          *          *          *          *
Os-POLLUX VASVAGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 399
Zm-POLLUX VASVAGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 399
Vv-POLLUX VASVAGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 399
Pt-POLLUX VASVAGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 399
Mt-DMI1 VASVAGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 399
Ps-SYM8 VASVAGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 399
Lj-POLLUX VASVAGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 399
Gm-POLLUX VASVAGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 399
At5g49960 VASVAGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 399
Os-CASTOR VASVGGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 399
Zm-CASTOR VASVGGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 399
Lj-CASTOR VASVGGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 399
Gm-CASTOR VASVGGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 399
Mt-CASTOR VASVGGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 400
Vv-CASTOR VASVGGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 399
Pt-CASTOR VASVGGKILINPDNDIVLQEGDEVLVIAEDDDITAPASLP-QVREGVLPNIPTPPKYPKRILCGWRREDIDDMIMVLEAF 399
ruler .....330.....340.....350.....360.....370.....380.....390.....400

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**          *          *          *          *          *          *          *          *          *          *          *
Os-POLLUX LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 479
Zm-POLLUX LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 479
Vv-POLLUX LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 479
Pt-POLLUX LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 479
Mt-DMI1 LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 479
Ps-SYM8 LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 479
Lj-POLLUX LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 479
Gm-POLLUX LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 479
At5g49960 LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 478
Os-CASTOR LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 479
Zm-CASTOR LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 479
Lj-CASTOR LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 479
Gm-CASTOR LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 479
Mt-CASTOR LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 480
Vv-CASTOR LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 479
Pt-CASTOR LAPGSELQMNVEVPEREERKILTDGGIDVGLTNIKLVRREGNAVIRRHLESLPLETFLSILLIADESVEDSIVHSDSRS 479
ruler .....410.....420.....430.....440.....450.....460.....470.....480

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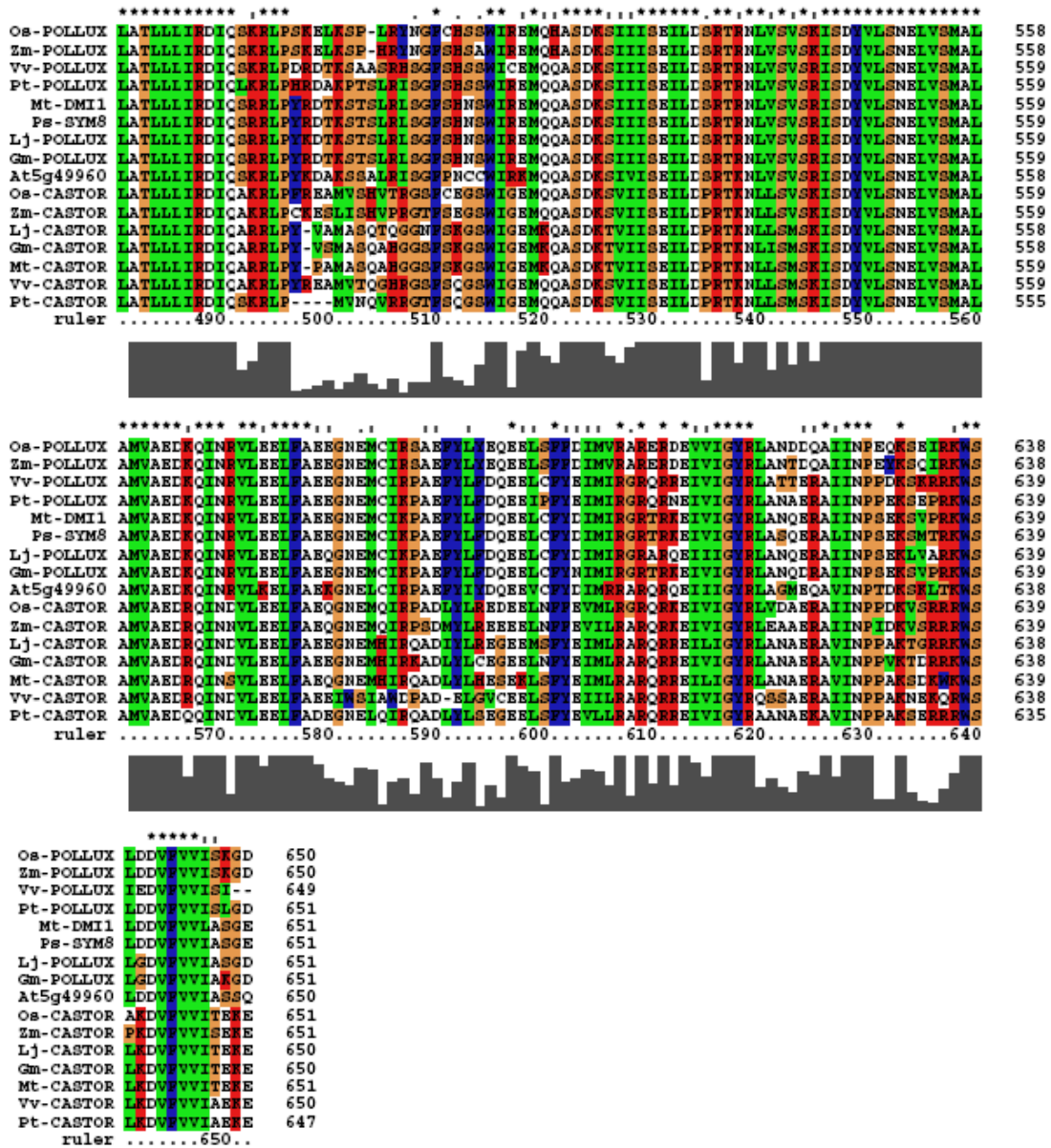


Figure A.1 Clustal X (1.83) multiple sequence alignment

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