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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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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 indispensible 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

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THESIS

Cui Fan

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
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By

Cui Fan

Lexington, Kentucky

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

Lexington, Kentucky

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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 geminate 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).

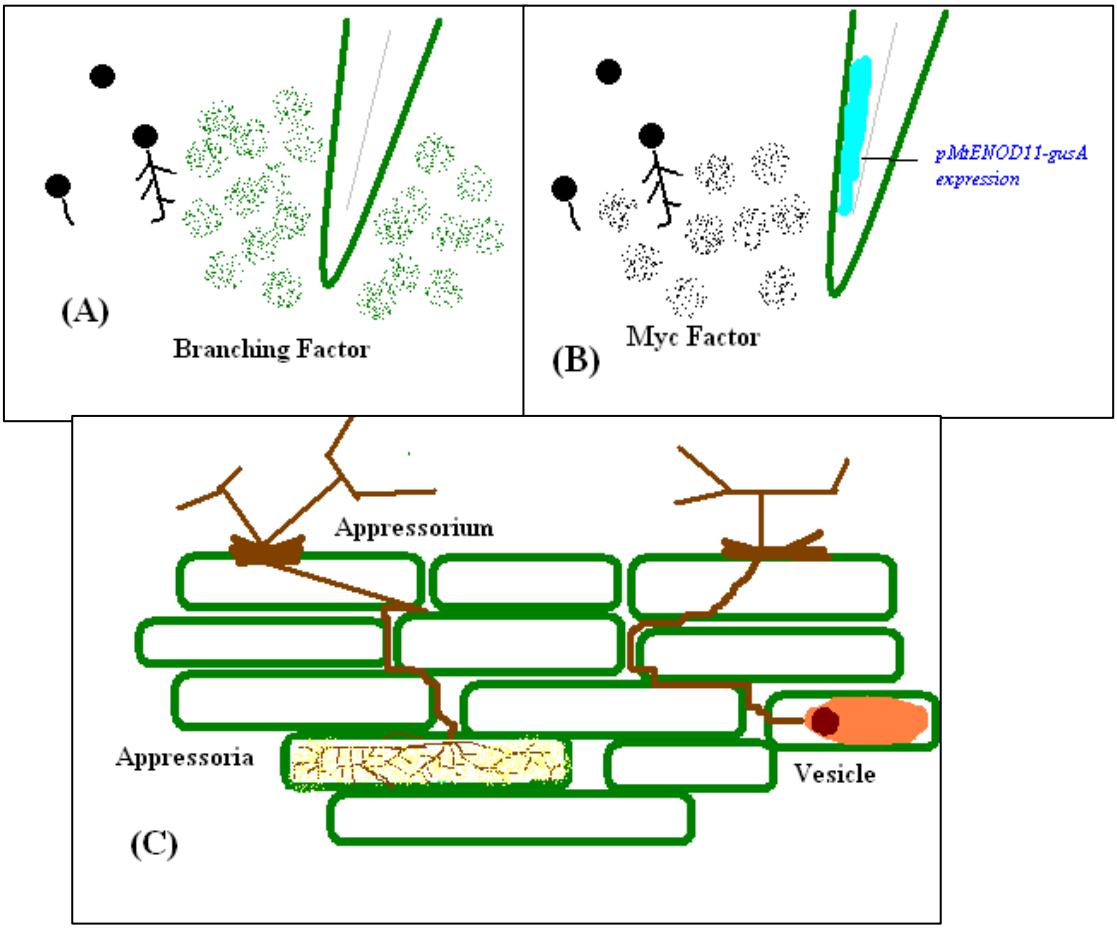

Figure 1. The developmental processes of AM symbiosis**.** (A) AM fungi respond to the plant-derived signals-strigolactone; (B) Plants sense the AM fungi-derived signals-Myc factors. Myc factors induce the p*ENOD11*::GUS expression in plants; (C) Appressorium formation, intercellular hyphae spreading; 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 *Orobanche*, 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.* p*MtENOD11*-gus*A,* 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 p*MtENOD11*-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 bacteriods (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).

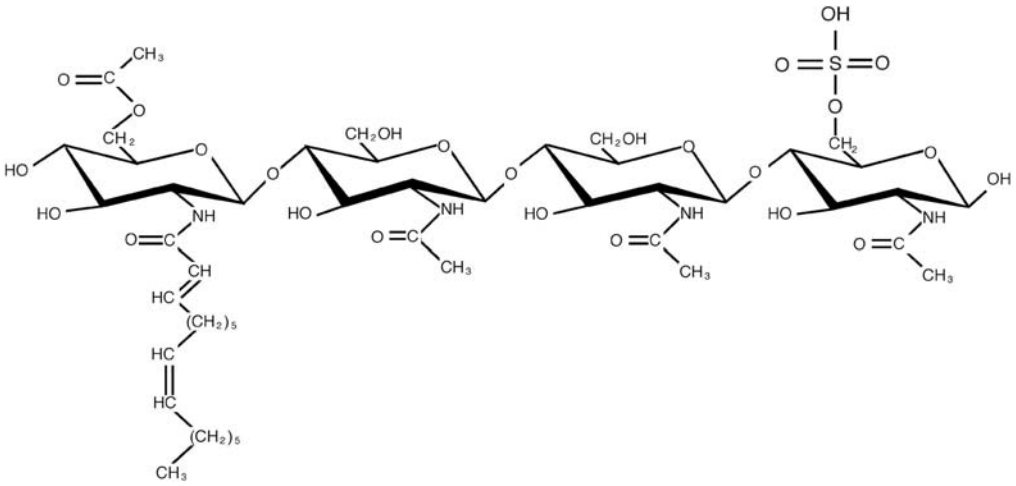

Figure 2. The Nod factor structure of *Sinorhizobium meliloti* (Lerouge et al., 1990). The major Nod factor produced by *Sinorhizobium meliloti,* is tetrameric (four glucosamine units) and consists of a 16-carbon acyl chain with two unsaturated bonds (C16:2). The two specific terminal residues are an *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 Ca^{2+}/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.

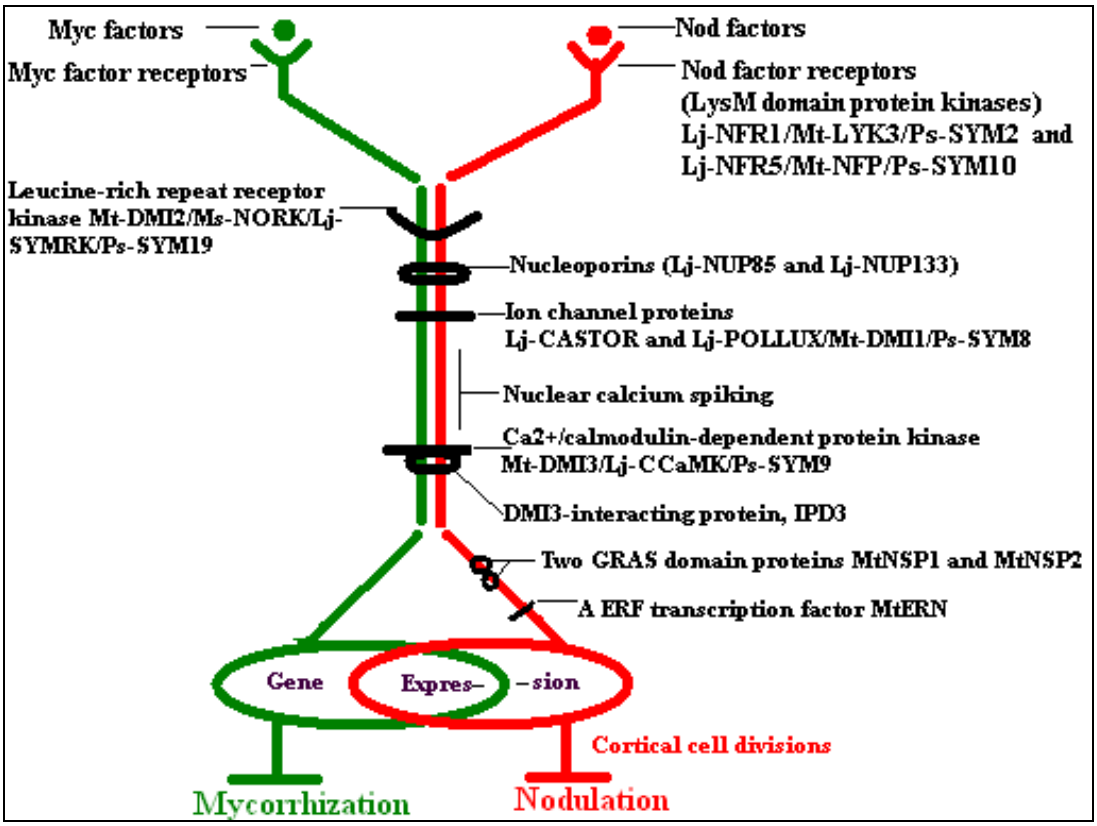

Figure 3. Cross-talk between nodulation and AM signaling pathways. Nod factors and Myc factors are perceived by LysM receptor kinases and other unknown specific receptors, respectively. The two signaling pathways share several components: DMI proteins, nucleoporins, nuclear calcium spiking as well as 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-*DMI1*.

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'-CCACAGTTTTCGCGATCCAGACTG-3'.

Binary vector construction

Two full length cDNA clones of Os-*POLLUX*, AK067564 and AK073102, were cut with *EcoR*I and *Kpn*I 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. *EcoR*I and *Nru*I 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 ARqua1 and transformed into the roots of the *dmi1-1* mutant. Transformed roots were selected on Färhaeus medium (Fahraeus 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 μM 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 $(NO_3)_2$] 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-H_2O, 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^{o}C. After removing the paraplast/Hemo-De solution, melted paraplast was added and incubated at 60 ^{o}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'-GCGATAATGGAACTGGTATG-3' and 5'-CTCCATTTCCTGGTCATAGTC-3'; Os-*POLLUX*, 5'-CGATTTGATCTCTCCCCGTA-3' and 5'-GCTGACAACATAAAGCGCAA-3'; Os-*PT11*, 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 (*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).

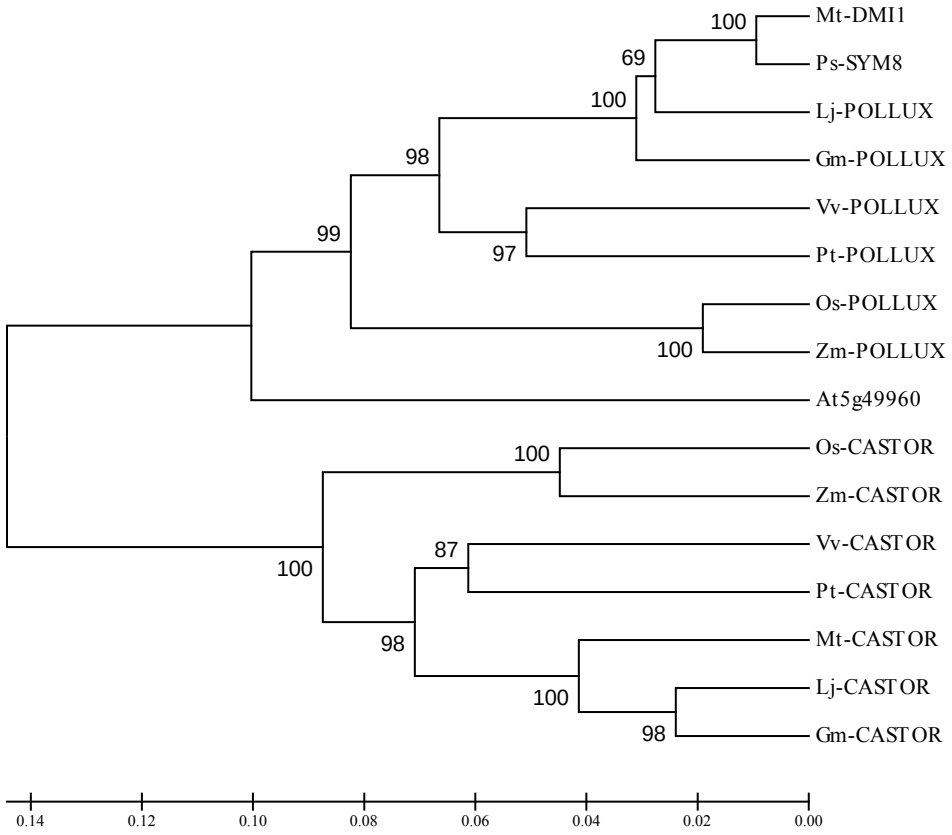

Figure 4. Phylogenetic tree (unrooted) of CASTOR and POLLUX homologs in *M. truncatula* (Mt), *L. japonicas* (Lj), soybean (*Glycine max*, Gm), poplar (*Populus trichocarpa,* Pt), grapevine (*Vitis vinifera,* Vv), *Arabidopsis thaliana* (At), rice (*Oryza sativa,* Os), sorghum (*Sorghum bicolor,* Sb), 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*.

	Ps-SYM8	Lj-POLLUX	Gm-POLLUX	Pt-POLLUX	Vv-POLLUX	Os-POLLUX	Zm-Pollux	Sb-POLLUX	At5g49960	Mt-CASTOR	Lj-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
Ps-SYM8		**94**	**93**	**87**	**87**	**83**	**84**	**84**	**80**	72	75	75	74	75	74	73	74
Lj-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**
Lj-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**

*Percetages of identity were calculated based on C-termial 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-*DMI1* (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).

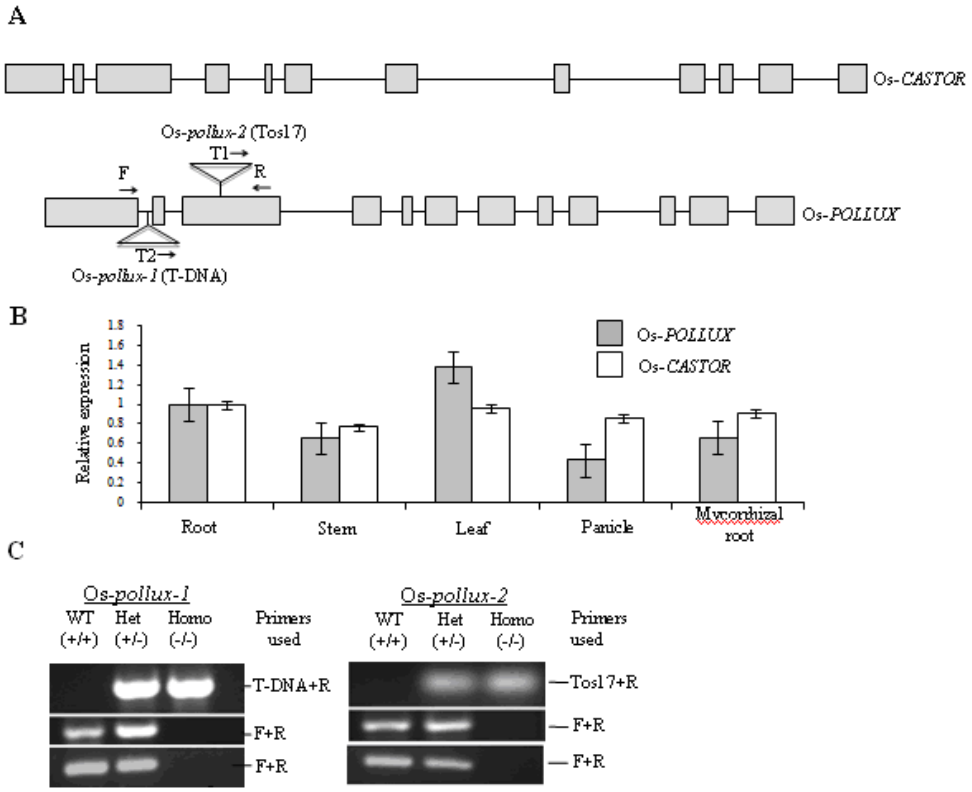

Figure 5. Isolation and characterization of Tos17/T-DNA insertion mutants of Os-*POLLUX*. (A) Gene structure of Os-*CASTOR* and Os-*POLLUX*, and the Tos17/T-DNA insertion sites. The exons and introns are indicated by boxes and lines, respectively. Insertion sites of Tos17/T-DNA are indicated. (B) Os-*CASTOR* 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.

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 serves as a molecular marker for the occurrence of successful symbiotic interaction between the two symbioints. 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.

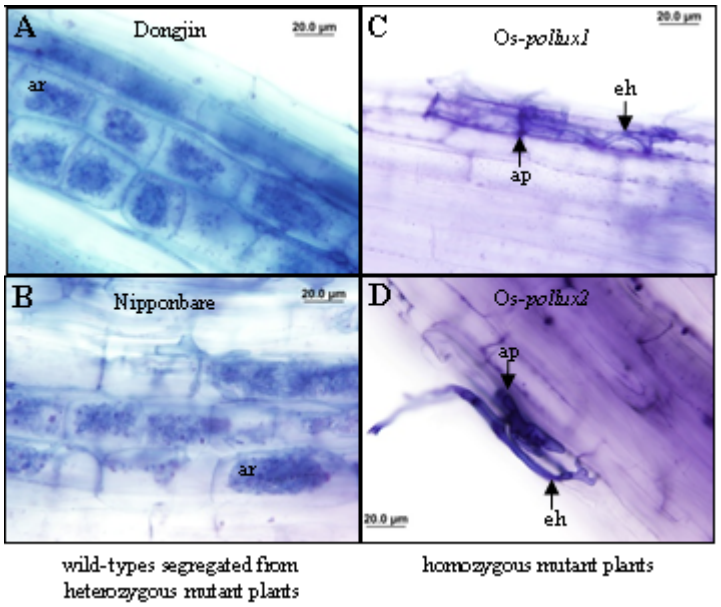

Figure 7. Os-*pollux* is defective in AM symbiosis. (A-B) Roots of wildtype plants segregated from heterozygous mutants formed arbuscules upon inoculation with *G. intraradices*. (C-D) Roots of homozygous mutants failed to form AM symbiosis, despite the presence of fungal hyphae on the root surface. 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.

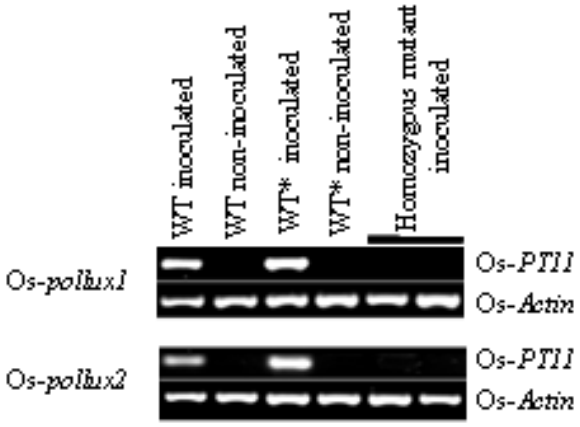

Figure 8. Expression of the rice AM-specific phosphate transporter Os-*PT11* in the mutant lines of Os-*POLLUX* under inoculated and non-inoculated conditions. WT* indicates wild-type plants segregated from a corresponding heterozygous mutant plant.

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.

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AK067564        GAGTGGAGCTTTGAGCTTTGCTAATCTTGGGGCGTTGAGGGAGCTTGAGCTCGCTGCTAA
AK072312        --TGGGAGCTTTGAGCTTTGCTAATCTTGGGGCGTTGAGGGAGCTTGAGCTCGCTGCTAA
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AK067564        TGGCGGAGAGCGATGGCGGCGAGGCTAGCCCGAGCGGTGGCGGCGGCGGAGAGGGGAGCC
AK072312        TGGCGGAGAGCGATGGCGGCGAGGCTAGCCCGAGCGGTGGCGGCGGCGGAGAGGGGAGCC
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AK067564        CCGATCCCCGGCGGCCACCGGCTCGGCCGCAGCTCACCAAGTCGCGCACCATCTCCGGCT
AK072312        CCGATCCCCGGCGGCCACCGGCTCGGCCGCAGCTCACCAAGTCGCGCACCATCTCCGGCT
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AK067564        CCGCCGCGTCCGCGTTCGACAGATGGGGCACCAGCAACAGCAGCAGCAGCATCCTAGTTC
AK072312        CCGCCGCGTCCGCGTTCGACAGATGGGGCACCAGCAACAGCAGCAGCAGCATCCTAGTTC
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AK067564        GCCGCTCCTCCACAGCGCCGCTCCCGCCGGGCGCGGCGCCGCGGGGGCTGCTCACCGTGG
AK072312        GCCGCTCCTCCACAGCGCCGCTCCCGCCGGGCGCGGCGCCGCGGGGGCTGCTCACCGTGG
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AK067564        CCGTGGACGAGCCCTCCTACGCGGCGCCCAACGGCGGGGCGGCCATGCTCGACCGCGACT
AK072312        CCGTGGACGAGCCCTCCTACGCGGCGCCCAACGGCGGGGCGGCCATGCTCGACCGCGACT
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AK067564        GGTGCTACCCGTCGTTCCTCGGCCCGCACGCCTCGCGGCCGCGGCCGCCGCGGTCGCAGC
AK072312        GGTGCTACCCGTCGTTCCTCGGCCCGCACGCCTCGCGGCCGCGGCCGCCGCGGTCGCAGC
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AK067564        AGCAGACGCCGACGACGACGGCTGCTGCTGCTGCCGACAGCCGGAGCCCTACCCCCGCCG
AK072312        AGCAGACGCCGACGACGACGGCTGCTGCTGCTGCCGACAGCCGGAGCCCTACCCCCGCCG
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AK072312        CGCCGCCGCAGACGGCGTCGGTCTCGCAGCGGGAGGAGGAGAAGAGCCTCGCTTCCGTGG
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AK072312        TGAAGCGGCCGATGCTGCTGGATGAGAGGAGATCGCTCTCGCCGCCGCCGCCGCTGCAGC
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AK072312        GCGCCCCGCGATTTGATCTCTCCCCGTATCTTGTACTAATGCTGGTTGTAACTGTCATAA
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AK072312        GTTTTTCCTTGGCTATATGGCAGTGGATGAAAGCTACCGTACTCCAGGAAAAGATCAGAT
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AK067564        CATGCTGTAGTGTTAGTACTGTGGACTGTAAGACAACTACTGAGGCGTTCAAGATTAACG
AK072312        CATGCTGTAGTGTTAGTACTGTGGACTGTAAGACAACTACTGAGGCGTTCAAGATTAACG
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AK072312        GACAGCATGGTTCTGATTTTATCAACTCCGCGGACTGGAATTTAGCTTCCTGCAGCAGAA
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AK072312        TGCTTGTTTTTGCGATACCTGTATTCTTGGTTAAGTACATTGATCAGCTTCGACGAAGGA
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AK072312        ACACAGATTCCATTAGGCTAAGAAGCACCGAGGAAGAAGTGCCTCTGAAGAAGAGGATTG
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AK072312        CTTATAAGGTTGATGTGTTCTTCTCAGGACACCCGTACGCAAAGCTTCTTGCGCTCCTGT
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AK072312        TGTTTGCCACAATGCTTGGGCTTGTGTCAGATGCAATATCAGAGAAGGTAGATTCCTGGC
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AK072312        GACATTCAGTCTAAACGTCTTCCATCAAAGGAACTAAAATCACCTCTGCGTTACAATGGC
                ************************************************************

AK067564        TTCTGTCACAGCTCCTGGATTCGGGAGATGCAGCACGCATCAGATAAATCAATAATTATC
AK072312        TTCTGTCACAGCTCCTGGATTCGGGAGATGCAGCACGCATCAGATAAATCAATAATTATC
                ************************************************************

AK067564        AGTGAAATTCTGGATTCCAGAACCAGAAACCTTGTATCTGTCTCCAAAATCAGCGACTAT
AK072312        AGTGAAATTCTGGATTCCAGAACCAGAAACCTTGTATCTGTCTCCAAAATCAGCGACTAT
                ************************************************************

AK067564        GTTCTATCAAATGAACTTGTCAGCATGGCATTAGCAATGGTAGCAGAGGACAAACAAATC
```

```
AK072312       GTTCTATCAAATGAACTTGTCAGCATGGCATTAGCAATGGTAGCAGAGGACAAACAAATC
               ************************************************************

AK067564       AACAGAGTTCTTGAAGAACTTTTTGCCGAGGAGGGCAACGAGATGTGCATACGTTCAGCT
AK072312       AACAGAGTTCTTGAAGAACTTTTTGCCGAGGAGGGCAACGAGATGTGCATACGTTCAGCT
               ************************************************************

AK067564       GAGTTTTACCTCTATGAGCAAGAGGAATTGAGTTTCTTTGATATAATGGTCAGGGCTCGT
AK072312       GAGTTTTACCTCTATGAGCAAGAGGAATTGAGTTTCTTTGATATAATGGTCAGGGCTCGT
               ************************************************************

AK067564       GAAAGAGACGAGGTTGTCATTGGCTACCGCCTTGCCAACGATGACCAGGCAATCATTAAT
AK072312       GAAAGAGACGAGGTTGTCATTGGCTACCGCCTTGCCAACGATGACCAGGCAATCATTAAT
               ************************************************************

AK067564       CCAGAACAGAAGTCTGAGATTAGGAAGTGGTCGCTAGACGATGTTTTTGTTGTGATCTCA
AK072312       CCAGAACAGAAGTCTGAGATTAGGAAGTGGTCGCTAGACGATGTTTTTGTTGTGATCTCA
               ************************************************************

AK067564       AAAGCGGGCAATGCAACATATTTTGTGAAGACCACGGTGATGCGGTCTAACCCAGTTGTG
AK072312       AAAG--------GTGACTAATTTT------ACAATAGCACCTCACTTTTTTCGAGTTA--
               ****        *  **   *****      ** *  *     *   * *   * ****

AK067564       AAAGCGGGCAATGCAACATATTTTGTGAAGACCACGGTGATGCGGTCTAACCCAGTTGTG
AK072312       AAAG--------GTGACTAATTTT------ACAATAGCACCTCACTTTTTTCGAGTTA--
               ****        *  **    *****     ** *  *    *  * *   * ****

AK067564       TATTCTTCTACATTTTAATTTGACTAATTCTGCT--CCTCCGTTTGCTGTTATTTTTCGA
AK072312       -GTAAGTATATATTCTAAC---ACTAGTGCTGCTAACCAATGCTAACTGAAATGCTTTAA
                *   * ** *** ***    **** * *****  **   * *  ***  **  **  *

AK067564       TTT---AAATTAGTGGGTCCAGGCTCCAG--CAACTCTAATAT-AGATGGCCTAAATTTG
AK072312       GTTGCTAACCAGGCAAACACACGTCACAGTACAGTTCTGATCTCATATGTTGCTAGCTCT
                **   **    *     ** *  ***  **  *** ** * * ***    *  *

AK067564       CCATTGGCCTGTCTGGTTGCCTGGATACTCTCATCCAAGGTTCATATGAATGCTCACCCC
AK072312       GTACATGTTGATTTGCCATCTCCATAGCATTTAGTTGATCTTAACATAAATAATTACACA
                 *   *    * **    *      *  * *    *  ** * ** *** * ** *

AK067564       CAGGCTTGTATGAGGTCATCCTGGATAGAGCTGGCATGGCTAAAGGAATGGTTGCCTGGA
AK072312       TCAGC--ATTTGAAACTGTTGTTACTAA--TTGACATTACAAAATTTTCAGT-GCGTCGA
                  **   * ***    *  *   **    ** ***  * ***     ** ** * **

AK067564       TACTCTCATCCAAGGTTCATATGAATGCTCTACCCCCAGGCTTGTATGAGGGTCTCTACC
AK072312       T----TCAGC-AGAGATAATTTGTAAATTGTAACTACTGTTTCATTTTGAAGAGCATGCT
               *    *** * *  * * ** ** *   * ** *  * *  *  * *  *    *   * *

AK067564       CCCAGGCTTGTATGAGGGCTAAAGGAATGGTTGCCTGGATACTCTCATCCAAGGTTCATA
AK072312       A--ATCATTG----------------------------------------------------
                  *   ***

AK067564       TGAATGCTCTACCCCCAGGCTTGTATGAGGTCATCCTGATAGAGCTGGCCCCCAGGCTTA
AK072312       ------------------------------------------------------------

AK067564       TATGAATGCTCTACCCCCAGGCTTGTATGAGGTCATCCTGATAGAGCTGGCCCCCAGGCT
AK072312       ------------------------------------------------------------

AK067564       TGTATGAGGTCATCCTGGATAGAGCTGGCATGGCTAAAGGAATGCAAAATTGCAAATTGC
AK072312       ------------------------------------------------------------

AK067564       TATGTTTGGCTGTTGCTTGTGGAATTGCAGTTTGTTGGACAGATGTATAGGGCCAGTTTT
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        MAESDGGEASPSGGGGGEGSPDPRRPPARPQLTKSRTISGSAASAFDRWGTSN
AK072312        MAESDGGEASPSGGGGGEGSPDPRRPPARPQLTKSRTISGSAASAFDRWGTSN
                ************************************************

AK067564        SSSSILVRRSSTAPLPPGAAPRGLLTVAVDEPSYAAPNGGAAMLDRDWCYPSFLGPHASR
AK072312        SSSSILVRRSSTAPLPPGAAPRGLLTVAVDEPSYAAPNGGAAMLDRDWCYPSFLGPHASR
                ************************************************************

AK067564        PRPPRSQQQTPTTTAAAAADSRSPTPAAPPQTASVSQREEEKSLASVVKRPMLLDERRSL
AK072312        PRPPRSQQQTPTTTAAAAADSRSPTPAAPPQTASVSQREEEKSLASVVKRPMLLDERRSL
                ************************************************************

AK067564        SPPPPQQRAPRFDLSPYLVLMLVVTVISFSLAIWQWMKATVLQEKIRSCCSVSTVDCKTT
AK072312        SPPPPLQRAPRFDLSPYLVLMLVVTVISFSLAIWQWMKATVLQEKIRSCCSVSTVDCKTT
                *****  ***************************************************

AK067564        TEAFKINGQHGSDFINSADWNLASCSRMLVFAIPVFLVKYIDQLRRRNTDSIRLRSTEEE
AK072312        TEAFKINGQHGSDFINSADWNLASCSRMLVFAIPVFLVKYIDQLRRRNTDSIRLRSTEEE
                ************************************************************

AK067564        VPLKKRIAYKVDVFFSGHPYAKLLALLLATIILIASGGIALYVVSGSGFLEALWLSWTFV
AK072312        VPLKKRIAYKVDVFFSGHPYAKLLALLLATIILIASGGIALYVVSGSGFLEALWLSWTFV
                ************************************************************

AK067564        ADSGNHADQVGLGPRIVSVSISSGGMLVFATMLGLVSDAISEKVDSWRKGKSEVIEVNHI
AK072312        ADSGNHADQVGLGPRIVSVSISSGGMLVFATMLGLVSDAISEKVDSWRKGKSEVIEVNHI
                ************************************************************

AK067564        LILGWSDKLGSLLKQLAIANKSIGGGVVVVLAERDKEEMEMDIGKLEFDFMGTSVICRSG
AK072312        LILGWSDKLGSLLKQLAIANKSIGGGVVVVLAERDKEEMEMDIGKLEFDFMGTSVICRSG
                ************************************************************

AK067564        SPLILADLKKVSVSKARAIIVLASDENADQSDARALRVVLSLTGVKEGLRGHVVVEMSDL
AK072312        SPLILADLKKVSVSKARAIIVLASDENADQSDARALRVVLSLTGVKEGLRGHVVVEMSDL
                ************************************************************

AK067564        DNEPLVKLVGGELIETVVAHDVIGRLMIQCALQPGLAQIWEDILGFENAEFYIKRWPELD
AK072312        DNEPLVKLVGGELIETVVAHDVIGRLMIQCALQPGLAQIWEDILGFENAEFYIKRWPELD
                ************************************************************

AK067564        GMRFGDVLISFPDAVPCGVKIASKAGKILMNPDNDYVLQEGDEVLVIAEDDDTYVPASLP
AK072312        GMRFGDVLISFPDAVPCGVKIASKAGKILMNPDNDYVLQEGDEVLVIAEDDDTYVPASLP
                ************************************************************

AK067564        QVRKGFLPNIPTPPKYPEKILFCGWRRDIHDMIMVLEAFSRSRF----------------
AK072312        QVRKGFLPNIPTPPKYPEKILFCGWRRDIHDMIMVLEAFLAPGSELWMFNEVPEKERERK
                ************************************  .

AK067564        ------------------------------------------------------------
AK072312        LTDGGMDIYGLTNIKLVHKEGNAVIRRHLESLPLETFDSILILADESVEDSIVHSDSRSL

AK067564        ------------------------------------------------------------
AK072312        ATLLLIRDIQSKRLPSKELKSPLRYNGFCHSSWIREMQHASDKSIIISEILDSRTRNLVS

AK067564        ------------------------------------------------------------
AK072312        VSKISDYVLSNELVSMALAMVAEDKQINRVLEELFAEEGNEMCIRSAEFYLYEQEELSFF
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AK067564       ------------------------------------------------
AK072312       DIMVRARERDEVVIGYRLANDDQAIINPEQKSEIRKWSLDDVFVVISKGD
```

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     MAESDGGEASPSGGGGGEGSPDPRRPPARPQLTKSRTISGSAASAFDRWGTSN
AK072312_corrected     MAESDGGEASPSGGGGGEGSPDPRRPPARPQLTKSRTISGSAASAFDRWGTSN
                       ****************************************************

AK067564_corrected     SSSSILVRRSSTAPLPPGAAPRGLLTVAVDEPSYAAPNGGAAMLDRDWCYPSFLGPHASR
AK072312_corrected     SSSSILVRRSSTAPLPPGAAPRGLLTVAVDEPSYAAPNGGAAMLDRDWCYPSFLGPHASR
                       ************************************************************

AK067564_corrected     PRPPRSQQQTPTTTAAAAADSRSPTPAAPPQTASVSQREEEKSLASVVKRPMLLDERRSL
AK072312_corrected     PRPPRSQQQTPTTTAAAAADSRSPTPAAPPQTASVSQREEEKSLASVVKRPMLLDERRSL
                       ************************************************************

AK067564_corrected     SPPPPQQRAPRFDLSPYLVLMLVVTVISFSLAIWQWMKATVLQEKIRSCCSVSTVDCKTT
AK072312_corrected     SPPPPQQRAPRFDLSPYLVLMLVVTVISFSLAIWQWMKATVLQEKIRSCCSVSTVDCKTT
                       ************************************************************

AK067564_corrected     TEAFKINGQHGSDFINSADWNLASCSRMLVFAIPVFLVKYIDQLRRRNTDSIRLRSTEEE
AK072312_corrected     TEAFKINGQHGSDFINSADWNLASCSRMLVFAIPVFLVKYIDQLRRRNTDSIRLRSTEEE
                       ************************************************************

AK067564_corrected     VPLKKRIAYKVDVFFSGHPYAKLLALLLATIILIASGGIALYVVSGSGFLEALWLSWTFV
AK072312_corrected     VPLKKRIAYKVDVFFSGHPYAKLLALLLATIILIASGGIALYVVSGSGFLEALWLSWTFV
                       ************************************************************

AK067564_corrected     ADSGNHADQVGLGPRIVSVSISSGGMLVFATMLGLVSDAISEKVDSWRKGKSEVIEVNHI
AK072312_corrected     ADSGNHADQVGLGPRIVSVSISSGGMLVFATMLGLVSDAISEKVDSWRKGKSEVIEVNHI
                       ************************************************************

AK067564_corrected     LILGWSDKLGSLLKQLAIANKSIGGGVVVVLAERDKEEMEMDIGKLEFDFMGTSVICRSG
AK072312_corrected     LILGWSDKLGSLLKQLAIANKSIGGGVVVVLAERDKEEMEMDIGKLEFDFMGTSVICRSG
                       ************************************************************

AK067564_corrected     SPLILADLKKVSVSKARAIIVLASDENADQSDARALRVVLSLTGVKEGLRGHVVVEMSDL
AK072312_corrected     SPLILADLKKVSVSKARAIIVLASDENADQSDARALRVVLSLTGVKEGLRGHVVVEMSDL
                       ************************************************************

AK067564_corrected     DNEPLVKLVGGELIETVVAHDVIGRLMIQCALQPGLAQIWEDILGFENAEFYIKRWPELD
AK072312_corrected     DNEPLVKLVGGELIETVVAHDVIGRLMIQCALQPGLAQIWEDILGFENAEFYIKRWPELD
                       ************************************************************

AK067564_corrected     GMRFGDVLISFPDAVPCGVKIASKAGKILMNPDNDYVLQEGDEVLVIAEDDDTYVPASLP
AK072312_corrected     GMRFGDVLISFPDAVPCGVKIASKAGKILMNPDNDYVLQEGDEVLVIAEDDDTYVPASLP
                       ************************************************************

AK067564_corrected     QVRKGFLPNIPTPPKYPEKILFCGWRRDIHDMIMVLEAFLAPGSELWMFNEVPEKERERK
AK072312_corrected     QVRKGFLPNIPTPPKYPEKILFCGWRRDIHDMIMVLEAFLAPGSELWMFNEVPEKERERK
                       ************************************************************

AK067564_corrected     LTDGGMDIYGLTNIKLVHKEGNAVIRRHLESLPLETFDSILILADESVEDSIVHSDSRSL
AK072312_corrected     LTDGGMDIYGLTNIKLVHKEGNAVIRRHLESLPLETFDSILILADESVEDSIVHSDSRSL
                       ************************************************************

AK067564_corrected     ATLLLIRDIQSKRLPSKELKSPLRYNGFCHSSWIREMQHASDKSIIISEILDSRTRNLVS
```

```
AK072312_corrected      ATLLLIRDIQSKRLPSKELKSPLRYNGFCHSSWIREMQHASDKSIIISEILDSRTRNLVS
                         ************************************************************

AK067564_corrected      VSKISDYVLSNELVSMALAMVAEDKQINRVLEELFAEEGNEMCIRSAEFYLYEQEELSFF
AK072312_corrected      VSKISDYVLSNELVSMALAMVAEDKQINRVLEELFAEEGNEMCIRSAEFYLYEQEELSFF
                         ************************************************************

AK067564_corrected      DIMVRARERDEVVIGYRLANDDQAIINPEQKSEIRKWSLDDVFVVISKAGNATYFVKTTV
AK072312_corrected      DIMVRARERDEVVIGYRLANDDQAIINPEQKSEIRKWSLDDVFVVISKGD----------
                         ********************************************..

AK067564_corrected      MRSNPVVYSSTF
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).

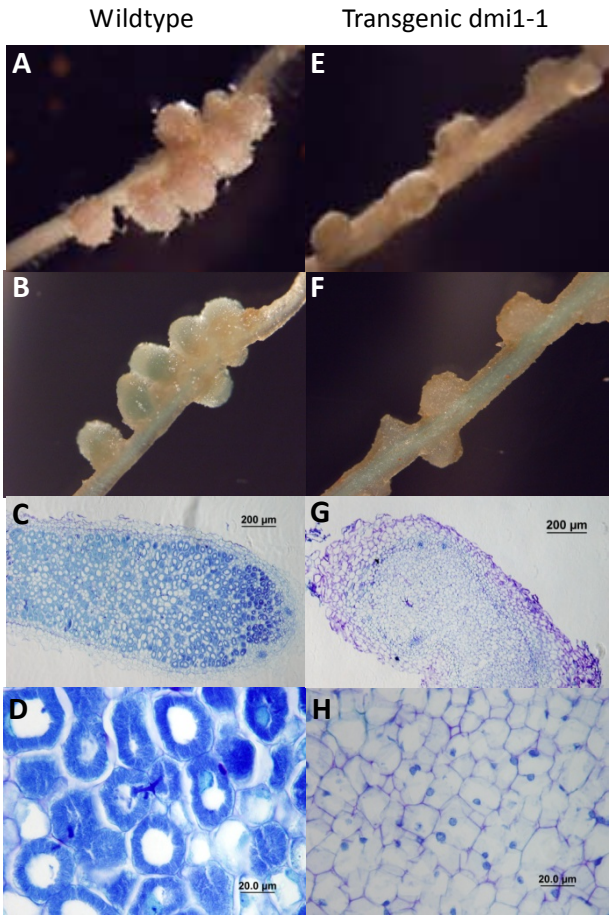


Figure 12. Complementation of the *M. truncatula dmi1-1* mutant (C71) with Os-*POLLUX* using *Agrobacterium. rhizogenes*-mediated hairy root transformation (Boisson-Dernier et al., 2001). A full-length cDNA clone of Os-*POLLUX* (AK067564) was cloned into a binary vector modified from pHellsgate8 driven by the 35S promoter (Helliwell et al., 2002). The binary vector was introduced into the *A. rhizogenes* strain, *ARqua1*, 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.

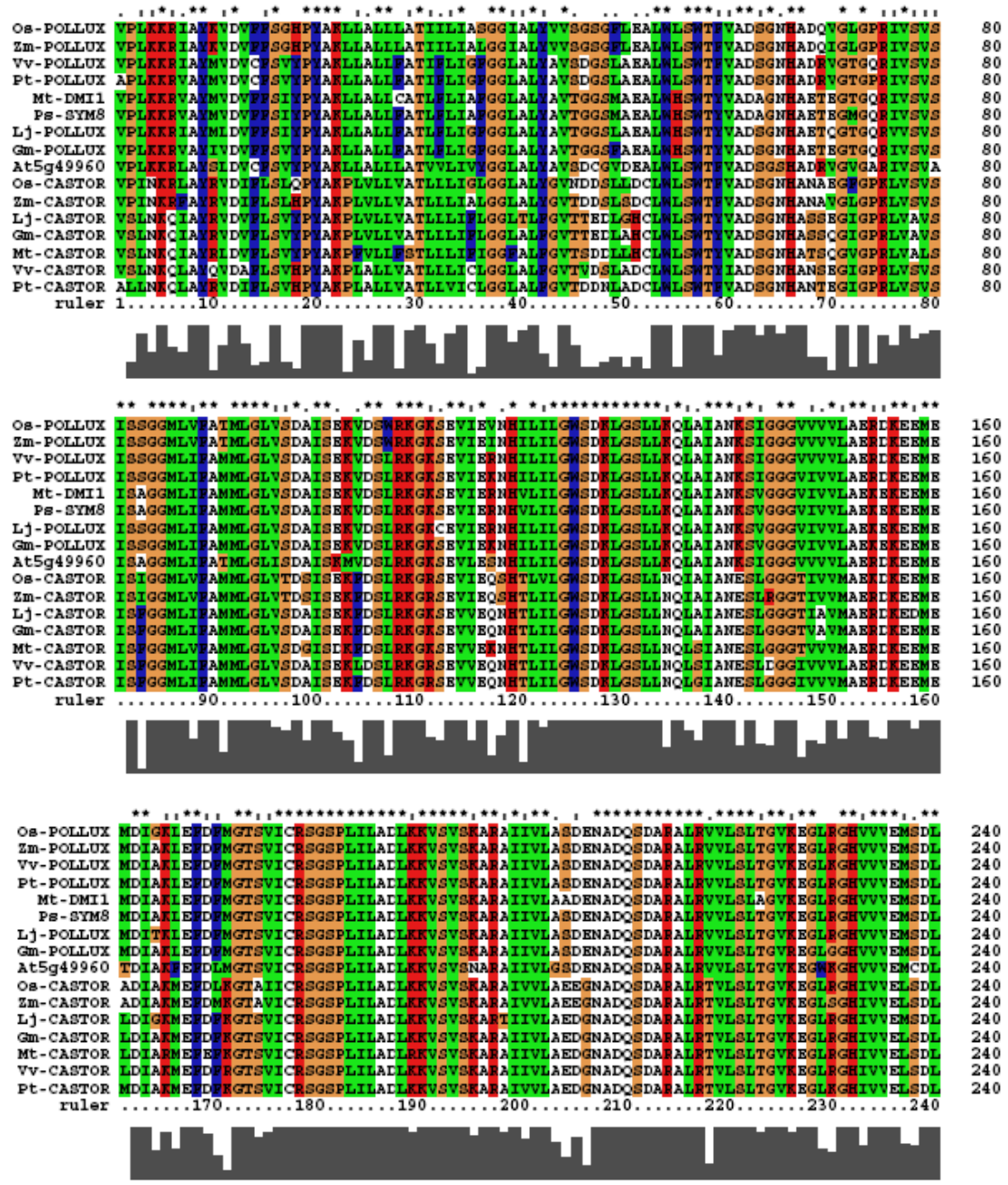
SECTION 4

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 indispensible for mycorrhizal symbiosis in rice. Furthermore, we show that Os-*POLLUX* can restore nodulation, but not rhizobial infection, to a *M. truncatula dmi1* mutant.

APPENDIX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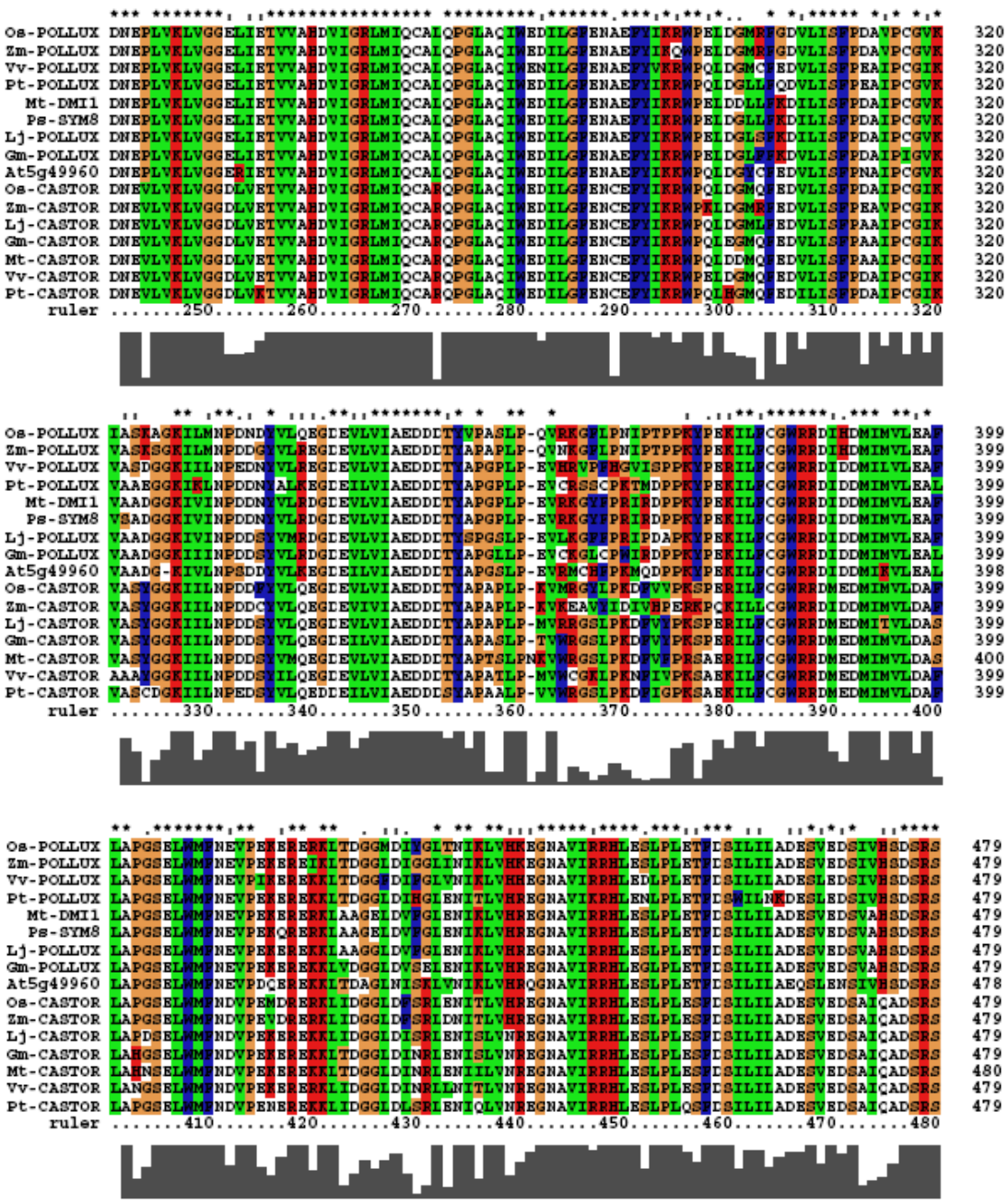
Zm-POLLUX
Vv-POLLUX
Pt-POLLUX
Mt-DMI1
Ps-SYM8
Lj-POLLUX
Gm-POLLUX
At5g49960
Os-CASTOR
Zm-CASTOR
Lj-CASTOR
Gm-CASTOR
Mt-CASTOR
Vv-CASTOR
Pt-CASTOR
ruler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Vv-POLLUX
Pt-POLLUX
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Ps-SYM8
Lj-POLLUX
Gm-POLLUX
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Vv-CASTOR
Pt-CASTOR
ruler

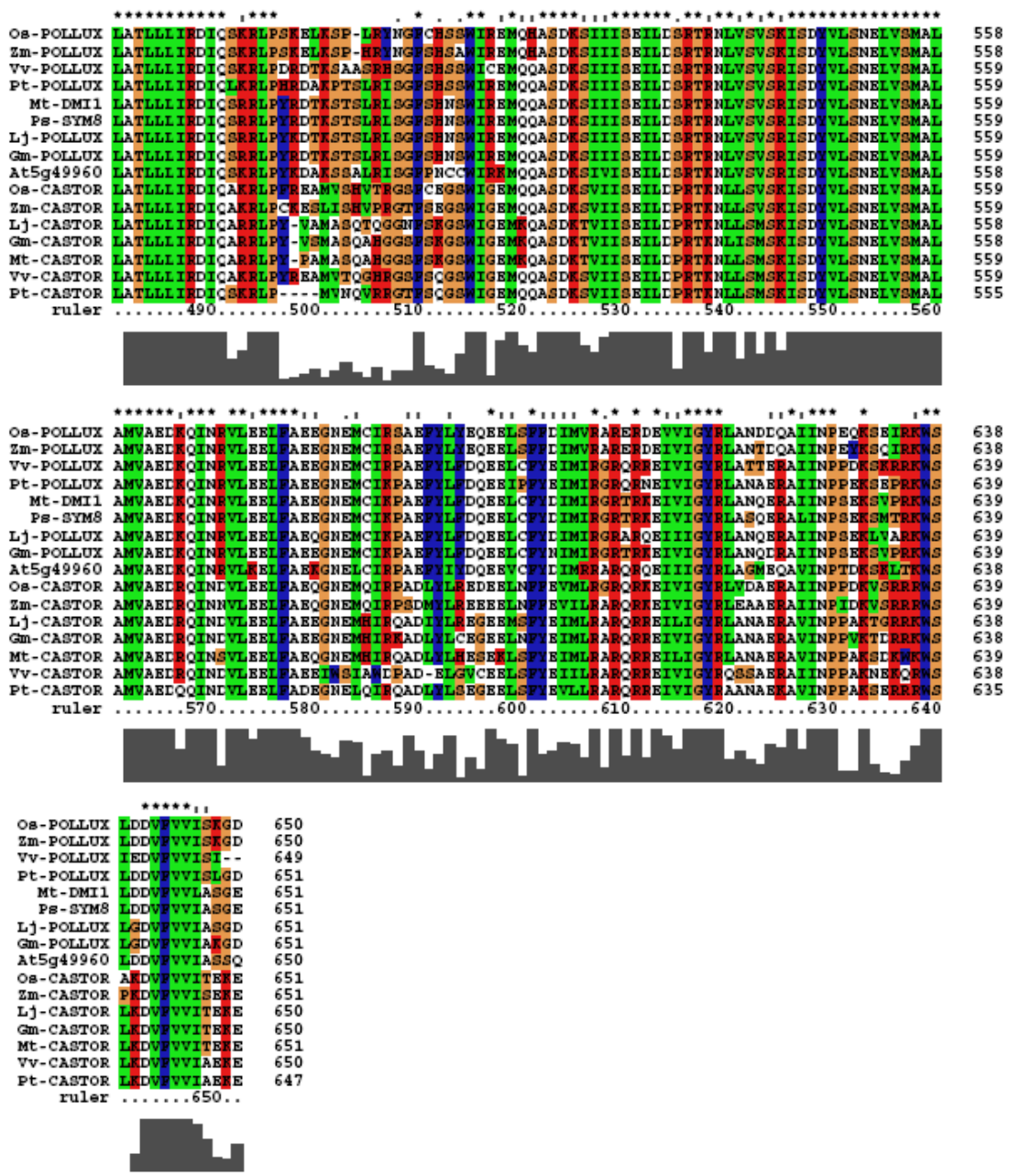

Figure A.1 Clustal X (1.83) multiple sequence alignment

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