Antiquity and Function of *CASTOR* and *POLLUX*, the Twin Ion Channel-Encoding Genes Key to the Evolution of Root Symbioses in Plants[1][W][OA]

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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. Here, we show that the orthologs of *CASTOR* and *POLLUX* are ubiquitously present and highly conserved in both legumes and nonlegumes. Using rice (*Oryza sativa*) as a study system, we employ reverse genetic tools (knockout mutants and RNA interference) to demonstrate that Os-*CASTOR* and Os-*POLLUX* are indispensable for mycorrhizal symbiosis in rice. Furthermore, a cross-species complementation test indicates that Os-*POLLUX* can restore nodulation, but not rhizobial infection, to a *Medicago truncatula dmi1* mutant.

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. 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 and Read, 1997). The AM symbiosis originated more than 400 million years ago and possibly played a key role in helping the first plants to colonize land (Remy et al., 1994; Redecker et al., 2000; Heckman et al., 2001). Initiation of the AM symbiosis is mediated by signal exchanges between the two symbiotic partners. The host plant releases strigolactones, known as ''branching factors,'' into the rhizosphere that induce hyphal branching, thereby increasing the chance of AM fungi contacting the root (Buee et al., 2000; Akiyama et al., 2005; Akiyama and Hayashi, 2006; Besserer et al., 2006). The fungal partner subsequently produces yet unknown diffusible signals, termed ''Myc factors,'' to trigger the host responses (Kosuta et al., 2003; Navazio et al., 2007). During the process, AM fungi enter the plant root and form highly ramified, tree-like fungal structures, called arbuscules, within the inner cortical cells (Harrison, 1997; Smith and Read, 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, particularly inorganic phosphate, from the soil, whereas the intraradical hyphae (arbuscules) serve as an intracellular microbe-plant interface where the soil nutrients move to the plant and plant photosynthates flow to the fungus (Harrison, 1997, 2005). 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; Liu et al., 2007; Pozo and Azcón-Aguilar, 2007).

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 microenvironment 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 and Bisseling, 1996). The majority of leguminous plants as well as a nonleguminous plant, *Parasponia*, can interact with rhizobia, whereas a diverse group of nonleguminous plants, so called actinorhizal plants, are able to enter into an interaction with *Frankia* bacteria. The legume-rhizobia symbiosis is best characterized at the 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

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root and trigger the production of a bacterial signal known as Nod factor. Nod factors are chitin-like lipochitooligosaccharides 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 (for review, see Oldroyd and Downie, 2004, 2006, 2008; Stacey et al., 2006; Jones et al., 2007).

Although the AM and rhizobial symbioses are morphologically distinct, 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 are induced during legume-rhizobia symbiosis also is up-regulated during AM symbioses (Albrecht et al., 1999; Kistner and Parniske, 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 and Weeden, 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. 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., 2003a; 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 Leu-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 two nucleoporins Lj-NUP85 and Lj-NUP133 (Kanamori et al., 2006; Saito 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 the DMI3-interacting protein Mt-IPD3/Lj-CYCLOPS (Messinese et al., 2007; Chen et al., 2008; Yano et al., 2008). Except for Mt-DMI3 and Mt-IPD3, all of these common symbiosis components act upstream of the Nod factor-induced calcium spiking (Ehrhardt et al., 1996; Oldroyd and Downie, 2004, 2006). Mt-DMI3 and Mt-IPD3 function downstream of calcium spiking and are presumably responsible for decoding the calcium spiking signal (Oldroyd and Downie, 2006), which subsequently activates the nodulation-specific transcription factors, such as Mt-NSP1, Mt-NSP2, Mt-ERN1, and Lj-NIN, leading to transcriptional reprogramming of the host symbiotic genes (Schauser et al., 1999; Kalo et al., 2005; Smit et al., 2005; Middleton et al., 2007). In contrast to the Nod factor signaling, the mycorrhiza-specific signaling components beyond the common symbiosis pathway are largely unknown.

Intriguingly, all cloned legume symbiosis genes, including both the common symbiosis genes and genes only required for rhizobial symbiosis, have orthologs in nonlegumes (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 nonlegume boundary. For example, the Lj-*NFR1* ortholog in Arabidopsis (*Arabidopsis thaliana*; At1g21630) has been shown to be essential for chitin signaling, indicating an evolutionary relationship between 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 nonlegume 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 nonlegumes using rice (*Oryza sativa*) 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

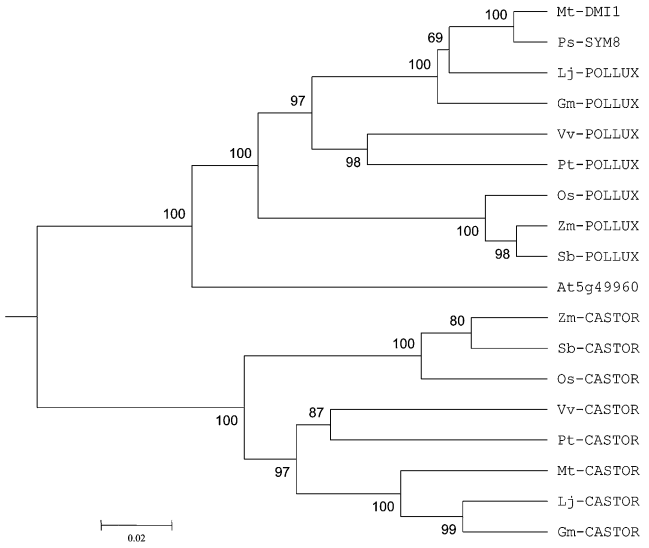

Figure 1. Phylogenetic tree (unrooted) of CASTOR and POLLUX homologs in *M. truncatula* (Mt), *L. japonicus* (Lj), soybean (Gm), poplar (Pt), grapevine (Vv), Arabidopsis (At), rice (Os), sorghum (Sb), and maize (Zm). The tree was based on the C-terminal approximately 650 amino acids of the proteins. 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.

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*. We demonstrate that the rice orthologs of *CASTOR* and *POLLUX*, namely Os-*CASTOR* and Os-*POLLUX*, are indispensable for mycorrhizal symbiosis in rice and that Os-*POLLUX* can restore nodulation, but not rhizobial infection, to a *M. truncatula dmi1* mutant.

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 possibly evolved from an ancient gene duplication event that dated before the monocot-dicot divergence (Zhu et al., 2006; Fig. 1). Under this evolutionary scenario, the duplicated gene copies have degenerated to perform complementary rather than redundant functions. It was speculated that CASTOR and POLLUX, analogous to many other ion channels, may act as heteromultimeric 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, sorghum (*Sorghum bicolor*), and maize (*Zea mays*; Supplemental Sequence Data S1). The between-species orthologous relationship of the *CASTOR-POLLUX* homologs can be readily inferred based on phylogenetic analysis (Fig. 1) and their microsyntenic genomic position (Zhu et al., 2006). The only exception is Arabidopsis, which contains the ortholog of *POLLUX* (At5g49960) but lacks the ortholog of *CASTOR* (Ane et al., 2004; Zhu et al., 2006). Notably, Arabidopsis also lacks the orthologs of Mt-*DMI2*, Mt-*DMI3*, and Mt-*IPD3* (Levy et al., 2004; Zhu et al., 2005, 2006; Messinese et al., 2007). Such gene deletions in Arabidopsis (and likely the lineage leading to the Brassiceae) offer an explanation for the inability of the *Brassica* plants to form root symbioses with mycorrhizal fungi (Ocampo et al., 1980; Glenn et al., 1985).

Table I. *Percentage amino acid sequence identity between CASTOR and POLLUX homologs*

Percentages were calculated based on the C-terminal 650 amino acids of the proteins via the National Center for Biotechnology Information BLAST2 program. Boldface entries indicate percentage identities between orthologs; italic entries indicate percentage identities between within-species paralogs.

	Ps-SYM8 POLLUX	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**

As shown in Figure 1, the phylogenetic position of At-POLLUX (At5g49960) is not congruent with the 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 approximately 650 amino acids, with a sequence identity ranging from 69% to 98% depending on the phylogenetic distances between species (Table I). As shown in Table I, 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 (Supplemental Fig. S1), further supporting the bifurcation of the two orthologous groups in phylogenetic analysis (Fig. 1). 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 (http://www.biochem.ucl.ac.uk/bsm/SIMPLE/) revealed that the N-terminal regions of CASTOR and POLLUX proteins are rich in simple sequence repeats encoded by simple sequence repeats at the DNA level (data not shown), which may have contributed to the fast-evolution feature of this region (Hancock and Simon, 2005).

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

We have selected rice as a model system to assess the function of nonlegume 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 (Jeong et al., 2006; Chen et al., 2007; Miyao et al., 2007). Os-*CASTOR* and Os-*POLLUX* were identified as Os03g62650 and Os01g64980, respectively, in the rice genome (Nipponbare) based on The Institute for Genomic Research Rice Genome Annotation (http://www.tigr.org/tdb/e2k1/osa1/; Zhu et al., 2006). Alignment of full-length cDNAs with the genomic sequences revealed a gene structure of 12 exons for both genes (Fig. 2A), which is conserved with their legume and nonlegume counterparts listed in Table I (Ane 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 (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 (Fig. 2B). This observation was further supported by the analysis of the Rice Gene Index database (http://compbio.dfci.harvard.edu/tgi), 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

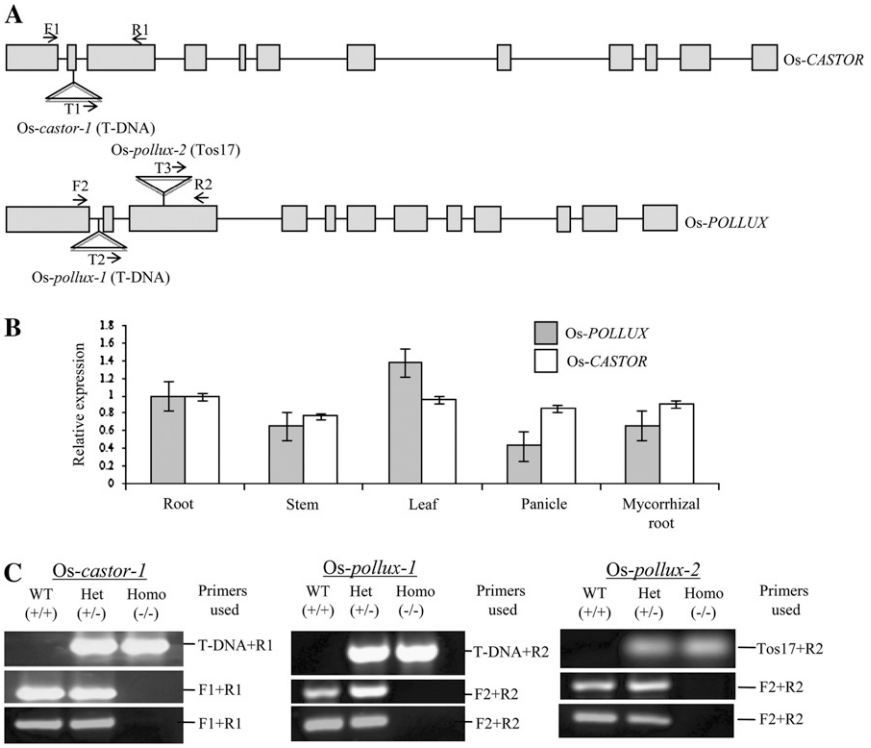

Figure 2. Isolation and characterization of Tos17/T-DNA insertion mutants of Os-*CASTOR* and Os-*POLLUX*. A, Gene structures of Os-*CASTOR* and Os-*POLLUX* and the Tos17/T-DNA insertion sites. The exons and introns are indicated by boxes and lines, respectively. Insertion sites of Tos17/T-DNA are indicated. B, Os-*CASTOR* and Os-*POLLUX* expression levels in roots, stems, leaves, panicles, and mycorrhizal roots. Relative transcript abundance was determined by qRT-PCR and normalized against Os-*ubiquitin1*. Error bars represent SD values 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 (WT) allele under given PCR conditions. Bottom, RT-PCR analysis of Os-*CASTOR* and Os-*POLLUX* expression in the wild-type and mutant plants. The primer positions are indicated in A.

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 (Ane 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 that of their legume orthologs (Ane et al., 2004; Imaizumi-Anraku et al., 2005). Both proteins possess four transmembrane helices, a central region homologous to the RCK (for 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; Fig. 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 more than 91% identical to their maize and sorghum orthologs and 83% to 85% identical to their legume counterparts (Table I).

Isolation of Os-*castor* and 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-*CASTOR* and Os-*POLLUX* in root symbioses (Jeong et al., 2006; Miyao et al., 2007). We identified one T-DNA insertion allele for Os-*CASTOR* in the genetic background of the *japonica* rice cv Hawyoung (line no. C04353), hereafter referred to as Os-*castor-1* (Jeon et al., 2000; Jeong et al., 2002). In the Os-*castor-1* mutant, the T-DNA was inserted into the second 72-bp exon (Fig. 2A). For Os-*POLLUX*, we obtained two mutant alleles, named Os-*pollux-1* and Os-*pollux-2* (Fig. 2A). The Os-*pollux-1* mutant was a T-DNA insertion line in the genetic background of cv Dongjin (line no. B02432), in which the T-DNA was inserted into the first intron. 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 all three mutants, the expression of Os-*CASTOR* or Os-*POLLUX* was disrupted based on RT-PCR analyses (Fig. 2C, 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 (Fig. 2C, top). A second round of PCR analysis 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 (Fig. 2C, middle). Since T-DNA and Tos17 mutant lines may

Figure 3. Sequences and domain structures of Os-CASTOR and Os-POLLUX. The characteristic motifs and domains are underlined, including the four transmembrane (TM) helices and the filter, the pore helix, the hinge, and the RCK (for regulation of conductance of K^+) domain.

A Os-CASTOR

MPLDPDSSPAPPHRDWFFPPAPPFLPSSRARTPRAPFPST

SRSSNPYSFPDRRPPPTPRSRSRSPLPPPEQQKQQQPPPT

TPPPAPRRRDPRYAGVRRGDVRTLTAEKAAAAAAVPTAAQ

VHGSKSAASATTLRWSGMVSVAAIVLCFSSLVRSNSSLHD
TM-1

QVHHLKAQLAEATTKLQSCITESSMDMSSILSYQSNNSTS

QNRGLKNFSLLLSLSTLYAPLLILKYMDLFLKLRSSQDSE
TM-2

EEVPINKRLAYRVDIFLSLQPYAKPLVLLVATLLLIGLGG
TM-3

LALYGVNDDSLLDCLWLSWTFVADSGNHANAEGFGPKLVS
Pore helix Filter

VSISIGGMLVFAMMLGLVTDSISEKFDSLRKGRSEVIEQS
TM-4 Hinge

HTLVLGWSDKLGSLLNQIAIANESLGGGTIVVMAEKDKEE

MEADIAKMEFDLKGTAIICRSGSPLILADLKKVSVSKARA
RCK domain

IVVLAEEGNADQSDARALRTVLSLTGVKEGLRGHIVVELS

DLDNEVLVKLVGGDLVETVVAHDVIGRLMIQCARQPGLAQ

IWEDILGFENCEFYIKRWPQLDGMQFEDVLISFPDAIPCG

IKVASYGGKIILNPDDFYVLQEGDEVLVIAEDDDTYAPAP

LPKVMRGYLPKDFVVPKSPERILFCGWRRDMEDMIMVLDA

FLAPGSELWMFNDVPEMDRERKLIDGGLDFSRLENITLVH

REGNAVIRRHLESLPLESFDSILILADESVEDSAIQADSR

SLATLLLIRDIQAKRLPFREAMVSHVTRGSFCEGSWIGEM

QQASDKSVIISEILDPRTKNLLSVSKISDYVLSNELVSMA

LAMVAEDRQINDVLEELFAEQGNEMQIRPADLYLREDEEL

NFFEVMLRGRQRKEIVIGYRLVDAERAIINPPDKVSRRRW

SAKDVFVVITEKE

B Os-POLLUX

MAESDGGEASPSGGGGGEGSPDPRRPPARPQLTKSRTISG

SAASAFDRWGTSNSSSSILVRRSSTAPLPPGAAPRGLLTV

AVDEPSYAAPNGGAAMLDRDWCYPSFLGPHASRPRPPRSQ

QQTPTTTAAAAADSRSPTPAAPPQTASVSQREEEKSLASV

VKRPMLLDERRSLSPPPPQQRAPRFDLSPYLVLMLVVTVI
TM-1

SFSLAIWQWMKATVLQEKIRSCCSVSTVDCKTTTEAFKIN

GQHGSDFINSADWNLASCSRMLVFAIPVFLVKYIDQLRRR
TM-2

NTDSIRLRSTEEEVPLKKRIAYKVDVFFSGHPYAKLLALL

LATIILIASGGIALYVVSGSGFLEALWLSWTFVADSGNHA
TM-3 Pore helix Filter

DQVGLGPRIVSVSISSSGGMLVFATMLGLVSDAISEKVDSW
TM-4 Hinge

RKGKSEVIEVNHILILGWSDKLGSLLKQLAIANKSIGGGV

VVVLAERDKEEMEMDIGKLEFDFMGTSVICRSGSPLILAD
RCK domain

LKKVSVSKARAIIVLASDENADQSDARALRVVLSLTGVKE

GLRGHVVVEMSDLDNEPLVKLVGGELIETVVAHDVIGRLM

IQCALQPGLAQIWEDILGFENAEFYIKRWPELDGMRFGDV

LISFPDAVPCGVKIASKAGKILMNPDNDYVLQEGDEVLVI

AEDDDTYVPASLPQVRKGFLPNIPTPPKYPEKILFCGWRR

DIHDMIMVLEAFLAPGSELWMFNEVPEKERERKLTDGGMD

IYGLTNIKLVHKEGNAVIRRHLESLPLETFDSILILADES

VEDSIVHSDSRSLATLLLIRDIQSKRLPSKELKSPLRYNG

FCHSSWIREMQHASDKSIIISEILDSRTRNLVSVSKISDY

VLSNELVSMALAMVAEDKQINRVLEELFAEEGNEMCIRSA

EFYLYEQEELSFFDIMVRARERDEVVIGYRLANDDQAIIN

PEQKSEIRKWSLDDVFVVISKGD

comprise multiple insertion sites, the wild-type plants segregated from the progeny of the heterozygous mutant lines were used as additional controls for the experiments described below.

Os-*castor* and Os-*pollux* Mutant Plants Are Defective in Mycorrhizal Symbiosis

To test whether Os-*CASTOR* and Os-*POLLUX* are required for AM symbiosis in rice, we inoculated the mutant and wild-type rice roots with the fungus *Glomus intraradices*. At 35 d after inoculation, wild-type plants were densely colonized by *G. intraradices*, exhibiting the range of symbiotic structures typical of a functional symbiosis, including intercellular and intracellular hyphae, vesicles, and arbuscules. In each of the 60 wild-type plants from the genotypes Nipponbare, Hawyoung, and Dongjin, approximately 60% to 85% of the total root length was colonized. Similar levels of colonization also were observed for wild-type plants segregated from heterozygous mutant plants (Fig. 4). In contrast, intracellular fungal structures, including vesicles and arbuscules, were not observed on roots of 60 Os-*castor-1*, 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 (Fig. 4), but the fungus was unable to penetrate the roots beyond the epidermis. These observations indicate that the knockout of Os-*CASTOR* and 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 *castor* (i.e. Lj-*sym4*, Lj-*sym22*, and Lj-*sym71*; Bonfante et al., 2000; Senoo et al., 2000) and *pollux* (i.e. Lj-*sym23* and Lj-*sym86*; Kistner et al., 2005) mutants in *L. japonicus*, the *dmi1* mutants in *M. truncatula* (Catoira et al., 2000), and the *sym8* mutant in pea (*Pisum sativum*; Balaji et al., 1994). For the weak alleles of the *L. japonicus castor* and *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 et al., 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

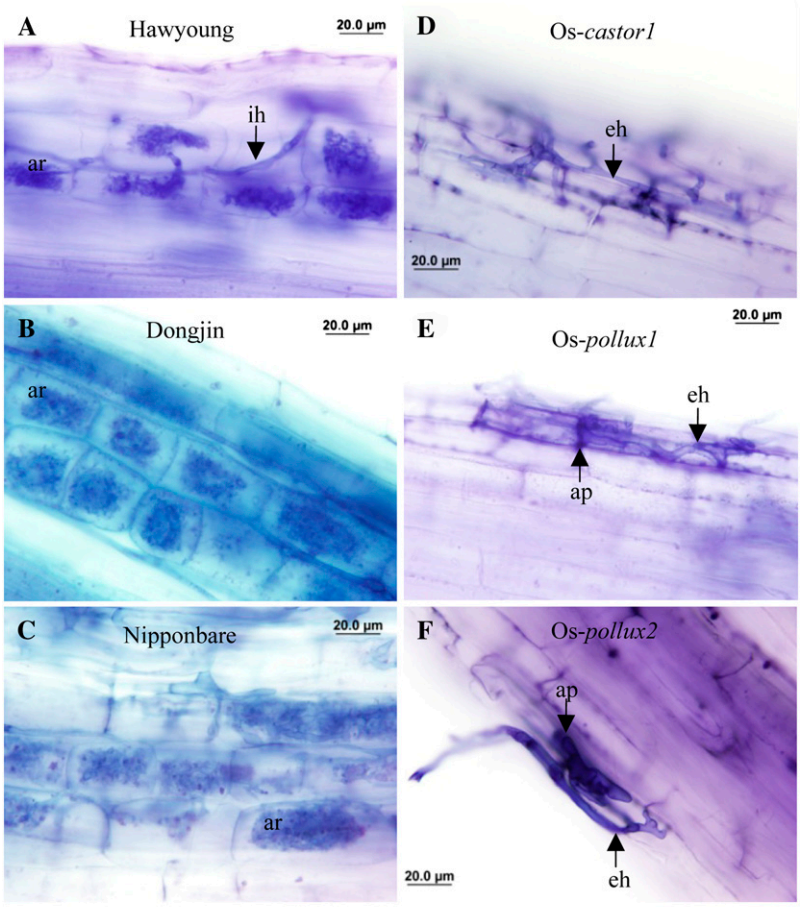

Figure 4. Os-*castor* and Os-*pollux* are defective in AM symbiosis. A to C, Roots of wild-type plants segregated from heterozygous mutants formed arbuscules upon inoculation with *G. intraradices*. D to F, Roots of homozygous mutants failed to form AM symbiosis, despite the presence of fungal hyphae on the root surface. Photographs were taken from roots at 5 weeks after inoculation 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). ap, Appressorium; ar, arbuscule; eh, extraradical hypha; ih, intraradical hypha.

molecular marker for the occurrence of functional 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 noninoculated conditions. The results showed that Os-*PT11* was expressed only in the wild-type roots inoculated with *G. intraradices* and not in the mutant roots (Fig. 5A).

Since only a single mutant allele of Os-*CASTOR* was available for this study, we generated stable transgenic silencing lines (Nipponbare) by RNA interference (RNAi) to provide further evidence for the requirement of Os-*CASTOR* in AM symbiosis. The RNAi silencing cassette consists of a 400-bp inverted-repeat sequence from the third exon of Os-*CASTOR*. We selected two independent transgenic RNAi knockdown lines, designated Os-*CASTORi-1* and Os-*CASTORi-2*, for further analysis. qRT-PCR analysis revealed a 58% to 72% reduction of Os-*CASTOR* mRNA levels in the transgenic T1 plants. Remarkably, symbiotic development in the transgenic RNAi roots was significantly impaired. While all wild-type plants segregated from the T1 progeny were normally colonized by the AM fungus, less than 2% of the root segments of the transgenic plants contained arbuscules (two of 163 from 21 Os-*CASTORi-1* plants and one of 169 from 13 *OsCASTORi-2* plants), despite the frequent presence of extraradical hyphae and appressoria on the root epidermis (data not shown). Again, the observed defective phenotypes were further supported by the differential expression of Os-*PT11* between RNAi and wild-type plants (Fig. 5B). Taken together, cytological and molecular evidence strongly indicates that Os-*CASTOR* and Os-*POLLUX* are required for the establishment of AM symbiosis in rice.

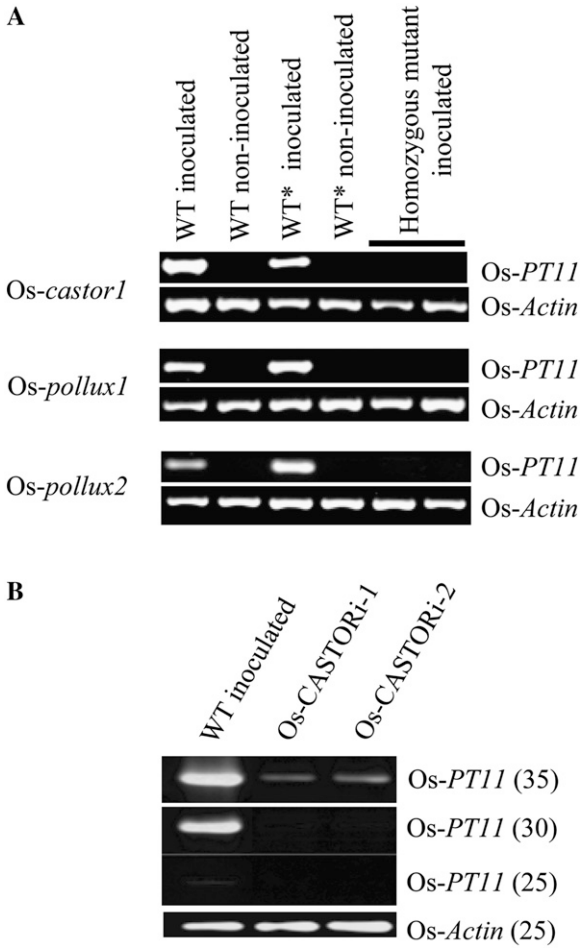

Figure 5. Expression of the rice AM-specific phosphate transporter Os-*PT11* in the mutant and/or RNAi lines of Os-*CASTOR* and Os-*POLLUX* under inoculated and noninoculated conditions. A, Expression of Os-*PT11* in the wild-type (WT) and mutant roots under inoculated and noninoculated conditions. WT* indicates wild-type plants segregated from a corresponding heterozygous mutant plant. B, Expression of Os-*PT11* in the two RNAi lines, Os-*CASTORi-1* and Os-*CASTORi-2*, during AM symbiosis. Numbers in parentheses indicate cycle numbers of the RT-PCR. The rice *Actin* gene was used as a control.

Os-*POLLUX* Can Restore Nodulation, But Not Rhizobial Infection, to a *M. truncatula dmi1* Mutant

To determine whether the nonlegume *CASTOR/POLLUX* orthologs possess an equivalent function to their legume counterparts, we introduced two Os-*POLLUX* full-length cDNAs (AK067564 and AK073102), 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 AK073162, differing at their 3′ ends due to alternative splicing, encode predicted proteins of 943 and 965 amino acids, respectively, of which the 943-amino acid version is canonical based on sequence alignments with other POLLUX orthologs. Nevertheless, the first 941 amino acids are shared between the two isoforms. It is noteworthy that both cDNA clones provided by the Rice Genome Resource Center in Japan contain a point mutation likely resulting from the RT process: there was an insertion of T at position 2,136 of AK067564 and an A-to-T transversion at position 593 of AK073012. Both errors were corrected for the transformation experiments described below. We did not observe any obvious differences resulting from using the two constructs, suggesting that the two protein isoforms are equally functional.

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* was able to complement the nonnodulation phenotype of the *dmi1-1* mutant, we inoculated the composite transgenic plants with *S. meliloti* strain 2011 carrying the *lacZ* reporter gene in plasmid pXLGD4 (Catoira et al., 2000). Two weeks after inoculation, nodule-like organs were observed on roots of 36 of 59 *dmi1-1* plants transformed

with Os-*POLLUX*. However, unlike wild-type plants, which form long, pink, cylindrical nodules, the transgenic mutant roots produce small, white, round 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 revealed that the small white nodules were devoid of bacteria and no infection threads were observed; thus, these nodules were nonfunctional (Fig. 6). The lack of nitrogen-fixing ability of these white nodules also was reflected by the poor growth and chlorotic leaves of the composite transgenic plants under nitrogen-starving conditions. Although Os-*POLLUX* is required for AM symbiosis in rice, the 35S:Os-*POLLUX* failed to complement the *M. truncatula dmi1-1* mutant for AM symbiosis (data not shown).

Our observations are similar to those recently reported for the *L. japonicus pollux-3* mutant complemented by 35S:Os-*POLLUX* (Banba et al., 2008). 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 the fact that the ''full-length'' versions from several other nonlegume 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, 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

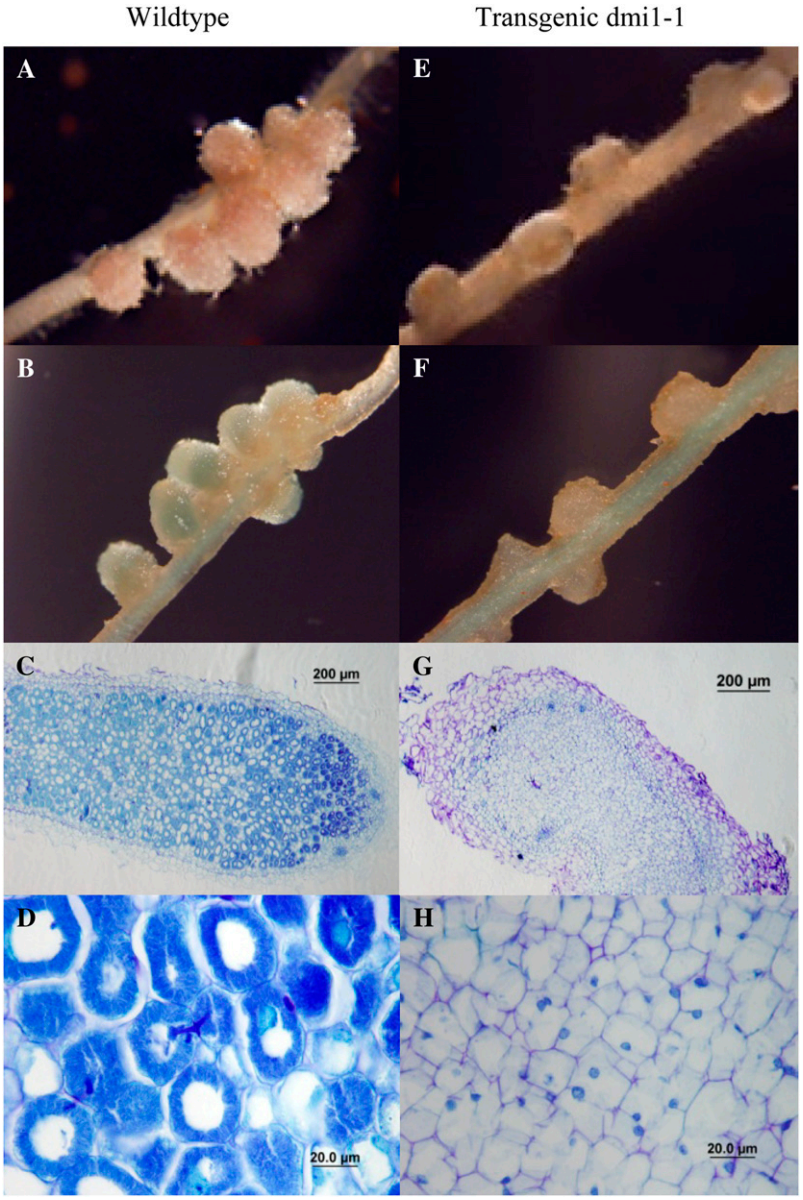

Figure 6. Complementation of the *M. truncatula dmi1-1* mutant (C71) with Os-*POLLUX* using *A. rhizogenes*-mediated hairy root transformation (Boisson-Dernier et al., 2001). A full-length cDNA clone of Os-*POLLUX* (AK067564) was cloned into a binary vector modified from pHellsgate8 driven by the 35S 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 to D, Wild type. E to H, The *dmi1-1* mutant transformed with 35S:Os-*POLLUX*. A and E, Nodule phenotypes of wild-type 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 wild-type and transgenic roots, respectively. D and H, Close-ups of the sectioned nodules.

knockdown mutants of the *DMI2* orthologs maintained the ability to form nodules but failed to form symbiosomes in the legume nodules (Capoen et al., 2005; Limpens et al., 2005). This taxonomy-specific functionality could be due to adaptation through changes in gene regulation and expression or sequence diversification between leguminous and nonleguminous plants.

CONCLUSION

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 and Parniske, 2002). This hypothesis is further supported by the fact that all of the legume common symbiosis genes are present in nonlegumes that have the ability to establish the AM symbiosis (Zhu et al., 2006). However, the function of these nonlegume orthologs needs to be addressed, in order to gain insight into the evolution of the root symbioses in plants. Thus far, five of the seven known common symbiosis genes have been shown to be required for AM symbiosis in nonlegumes (Chen et al., 2007, 2008; Banba et al., 2008; 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 nonlegumes. Interestingly, those genes required only for rhizobial symbiosis but not essential for the AM symbiosis in legumes are also present in nonlegumes. Elucidation of the function of nonlegume orthologs of nodulation-specific genes will provide further insights into the evolution of root symbioses in land plants.

MATERIALS AND METHODS

Rice and *Medicago truncatula* Mutants

The rice (*Oryza sativa*) Tos17 insertion line (NC6423) in the Nipponbare background was provided by the Rice Genome Resource Center of the National Institute of Agrobiological Sciences in Japan. The rice T-DNA insertion mutants, C04353 and B02432, in the genetic backgrounds of Hawyoung and Dongjin, respectively, were provided by the Pohang University of Science and Technology in Korea. The *Medicago truncatula dmi1-1* mutant (C71) was obtained from Dr. Douglas Cook's laboratory at the University of California, Davis.

Isolation of Homozygous Os-*castor* and Os-*pollux* Mutant Lines

Seeds of the Tos17/T-DNA insertion lines from the providers were from the progeny of primary transgenic or tissue culture-derived plants. To isolate homozygous mutants, we carried out two rounds of PCR analyses. The first round of PCR was to identify plants with Tos17/T-DNA insertion using the Tos17/T-DNA-specific and Os-*CASTOR*/Os-*POLLUX*-specific primer pairs. The second round of 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 2A. Primer sequences are as follow: F1, 5′-CGATGGTCAGGGATGGTATC-3′; R1, 5′-CGTGTGGCTTTGCTCTATGA-3′; F2, 5′-CGATTTGATCTCTCCCCGTA-3′; R2, 5′-GCTGACAACATAAAGCGCAA-3′; T1, 5′-ACGCTGAACTTGTGGCCGTT-3′; T2, 5′-CCACAGTTTTCGCGATCCAGACTG-3′; T3, 5′-ATTGTTAGGTTGCAAGTTAGTTAAGA-3′.

Binary Vector Construction and Rice Transformation

For the generation of RNAi knockdown lines of Os-*CASTOR*, a 400-bp inverted-repeat sequence from the third exon of Os-*CASTOR* was cloned into the Gateway-enabled pSTARGATE vector (provided by CSIRO Plant Industry in Australia). The expression of the inverted-repeat sequence in pSTARGATE was driven by a ubiquitin promoter. The construct was introduced into *Agrobacterium tumefaciens* strain EHA105 and transformed to Nipponbare as described by Hiei et al. (1994). Primary transgenic plants (T0) were selfed to obtain the T1 generation for phenotypic analysis of AM symbiosis.

Hairy Root Transformation of *M. truncatula*

The *dmi1-1* mutant of *M. truncatula* was transformed with Os-*POLLUX* using *Agrobacterium 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 (Färhaeus, 1957) containing 20 mg L^{-1} kanamycin for 2 weeks at 20°C. The transgenic roots were further confirmed by PCR analysis.

Inoculation of Rice Roots with *Glomus intraradices*

The AM fungus *Glomus intraradices* was from Premier Tech Biotechnologies. 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 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 KH_2PO_4. 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 approximately 20 min. After rinsing with water, the roots were soaked in 1% HCl at room temperature overnight. The roots were then stained at 90°C for 30 min in an acidic glycerol solution containing 0.1% trypan blue. After destaining in acidic glycerol, the roots were examined using a 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*

The nodulation assay was conducted as described by Limpens et al. (2003b). Three weeks after transformation, composite plants were transferred to sterile Turface saturated with Färhaeus medium [without $Ca(NO_3)_2$] for 3 d at 21°C and 16/8 h of light/dark. Each plant was then inoculated with 1 mL of culture (optical density at 600 nm = 0.1) of *Sinorhizobium 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 (100 mL: 45 mL of 95% ethanol, 40 mL of distilled water, 5 mL of glacial acetic acid, and 10 mL of 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 two changes of 100% ethanol each for 30 min). The samples were then gradually infiltrated with Hemo-De (20%, 50%, and 75% Hemo-De solutions, each for 30 min, then two changes of 100% Hemo-De each for 1 h). Once hydrated with Hemo-De, the samples were infiltrated with Paraplast Plus by successively 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 h. (This step was repeated for at least six changes of Paraplast.) The samples were then embedded and sectioned. The samples were sectioned with a Leica RM2135 microtome.

For light microscopy, 5-μm-thick sections were dried onto glass slides. The slides went through stepwise de-Paraplast and hydration and were stained with 1% (w/v) toluidine blue in 95% ethanol. Photographs were taken with 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 solution. The PCR primers were as follows: Os-*Actin*, 5′-GCGATAATGGAACTGGTATG-3′ and 5′-CTCCATTTCCTGGTCATAGTC-3′; Os-*CASTOR*, 5′-CGATGGTCAGGGATGGTATC-3′ and 5′-CGTGTGGCTTTGCTCTATGA-3′; Os-*POLLUX*, 5′-CGATTTGATCTCTCCCCGTA-3′ and 5′-GCTGACAACATAAAGCGCAA-3′; Os-*PT11*, 5′-ATGGCTCGACGGACAGTAAG-3′ and 5′-GATCAGCTGGATCATGTACCT-3′. qRT-PCR was performed on the Applied Biosystems StepOne Real-Time PCR System using the SYBR Green I detection kit (Bio-Rad). The Os-*ubiquitin* gene was selected as a constitutive internal control. PCR primers used for the real-time PCR experiments were as follows: Os-*ubiquitin*, 5′-TGCACCCTAGGGCTGTCAAC-3′ and 5′-TGACGCTCTAGTTCTTGATCTTCTTC-3′; Os-*CASTOR*, 5′-CAAGAGGGTGATGAGGTGCTAGTA-3′ and 5′-GGTAACCTCTCATAACCTTGGGTAAT-3′; Os-*POLLUX*, 5′-CCTCGGATGGAGCGACAA-3′ and 5′-ACGACACCACCACCAATACTCTT-3′.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Sequence alignment of the CASTOR and POLLUX homologs.

Supplemental Sequence Data S1. The amino acid sequences of the CASTOR and POLLUX homologs.

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