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Rapid paper

Divergence of Evolutionary Ways Among Common *sym* Genes: CASTOR and CCaMK Show Functional Conservation Between Two Symbiosis Systems and Constitute the Root of a Common Signaling Pathway

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In recent years a number of legume genes involved in root nodule (RN) symbiosis have been identified in the model legumes, *Lotus japonicus (Lotus)* and *Medicago truncatula*. Among them, a distinct set of genes has been categorized as a common symbiosis pathway (CSP), because they are also essential for another mutual interaction, the arbuscular mycorrhiza (AM) symbiosis, which is evolutionarily older than the RN symbiosis and is widely distributed in the plant kingdom. Based on the concept that the legume RN symbiosis has evolved from the ancient AM symbiosis, one issue is whether the CSP is functionally conserved between non-nodulating plants, such as rice, and nodulating legumes. We identified three rice CSP gene orthologs, *OsCASTOR*, *OsPOLLUX* and *OsCCaMK*, and demonstrated the indispensable roles of *OsPOLLUX* and *OsCCaMK* in rice AM symbiosis. Interestingly, molecular transfection of either *OsCASTOR* or *OsCCaMK* could fully complement symbiosis defects in the corresponding Lotus mutant lines for both the AM and RN symbioses. Our results not only provide a conserved genetic basis for the AM symbiosis between rice and Lotus, but also indicate that the core of the CSP has been well conserved during the evolution of RN symbiosis. Through evolution, CASTOR and CCaMK have remained as the molecular basis for the maintenance of CSP functions in the two symbiosis systems.

Keywords: Arbuscular mycorrhizal symbiosis — Common symbiosis pathway — Lotus japonicus — Oryza sativa — Root nodule symbiosis — Rice.

Abbreviations: AM, arbuscular mycorrhiza; CaM, calmodulin; CaMV, cauliflower mosaic virus; CCaMK, Ca/calmodulin-dependent protein kinase; CSP, common symbiosis pathway; DsRed, *Discoma* sp. red fluorescent protein; *Ljcastor*, *L. japonicus castor-4*; *Ljccamk*, *L. japonicus ccamk-3*; *Ljpollux*, *L. japonicus pollux-3*; *Ljsymrk*, *L. japonicus symrk-10*; *LjCASTOR*, *L. japonicus CASTOR*; *LjCCaMK*, *L. japonicus CCaMK*; *LjPOLLUX*, *L. japonicus POLLUX*; *Mtdmi3*, *Medicago truncatula dmi3*; *OsCASTOR*, rice *CASTOR*; *OsCCaMK*, rice *CCaMK*; *OsPOLLUX*, rice *POLLUX*; *OsSYMRK*, rice *SYMRK*; RN symbiosis, root nodule symbiosis; TM, transmembrane; wpi, weeks post-inoculation.

Introduction

Nitrogen and phosphate are the primary nutrients for plant growth that have a critical influence on plant survival. Understanding the molecular mechanisms of plant–microbe symbiotic relationships, which have evolved as an effective system for nutrient acquisition, will provide valuable information for plant growth improvement, leading to increases in crop yields. Arbuscular mycorrhizal (AM) and rhizobial root nodule (RN) symbioses represent mutualistic relationships in plant roots. Generally, >80% of land plants are engaged in mycorrhizal symbioses, which was established >400 million years ago (Kistner and Parniske 2002). Fungal spores germinate independently of host plants, but the growth of their hyphae is poor. Upon perception of the branching factors, strigolactones, fungal hyphae branch extensively and come into contact with host roots (Akiyama et al. 2005). Hyphae penetrate into the host roots through appressoria and form arbuscules and vesicles. External hyphae ramify through the soil and acquire nutrients, notably phosphate, which can be utilized by the host plants. In contrast, the rhizobial symbiosis arose about 60 million years ago and is restricted to a clade within the Eurosid, including legumes (Kistner and Parniske 2002). Legume–rhizobia interactions result in the formation of root nodules in which rhizobia reside and fix atmospheric nitrogen. Host-specific flavonoids induce biosynthesis of a bacterial infection signal, Nod factor (Long 1996). Nod factors induce several responses in plants, for example root hair

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deformation and cortical cell division as morphological changes, and Ca influx and Ca spiking at the physiological level (Ehrhardt et al. 1996, Oldroyd and Downie 2006, Oldroyd and Downie 2008). Coupling with these responses, rhizobia enter host roots through infection threads, and a nitrogen-fixing endosymbiosis is established (Kistner and Parniske 2002).

A number of legume mutants blocked at the early stages of rhizobial infection have also been shown to be defective in mycorrhizal colonization. The mutated genes of such Nod$^-$Myc$^-$ mutants are classified into a common symbiosis pathway (CSP) that controls both mycorrhizal and rhizobial endosymbioses. Thus, the legume–rhizobium symbiosis is thought to have its evolutionary origin in the more ancient mycorrhizal symbiosis. In a model legume *Lotus japonicus (Lotus)*, at least seven genes, *SYMRK*, *CASTOR*, *POLLUX*, *NUP133*, *NUP85*, *CCaMK* and *CYCLOPS*, have been reported to be CSP genes. *SYMRK* encodes a protein kinase with three leucine-rich repeat (LRR) domains and is believed to be epistatic to other CSP genes (Stracke et al. 2002, Markmann et al. 2008). Extensive studies on SYMRK suggest its involvement in the generation of Ca signals through autophosphorylation-regulated kinase activation (Yoshida and Parniske 2005, Markmann et al. 2008). CASTOR and POLLUX are predicted to be membrane-integrated channel proteins (Imaizumi-Anraku et al. 2005). They are very similar to each other and show structural homology with Ca-gated potassium channels such as in the *Methanobacterium* MthK. Despite their high similarity, CASTOR and POLLUX do not complement each other. In addition, the temporal and spatial expression patterns of CASTOR and POLLUX are not identical during nodulation, suggesting that they possess functional differentiation and play distinct roles in symbiosis signaling. NUP133 (Kanamori et al. 2006) and NUP85 (Saito et al. 2007) are predicted as nucleoporins, which are the components of the nucleopore complex. Ca spiking is induced around the nucleus, and some of the proteins involved in the early symbiotic signaling pathway are localized on the nucleus. Hence, it is speculated that NUP85 and NUP133 are involved in trafficking and/or localization of factors essential for induction of Ca signals.

Ca spiking is a distinctive physiological response to Nod factor perception (Ehrhardt et al. 1996). The Ca signal is converted to downstream pathway(s), leading to the establishment of endosymbiosis. CCaMK [Ca/calmodulin (CaM)-dependent protein kinase] is possibly a decoder of Ca spiking signals, because of its structural similarity to CaMKII in mammals, which is activated by Ca oscillation in a frequency-dependent manner (De Koninck and Schulman 1998). CCaMK-defective mutants exhibit Nod$^-$Myc$^-$ phenotypes, whereas a Lotus mutant, *snf1*, which harbors a Ca/CaM-independently active form of CCaMK, shows spontaneous nodule formation without rhizobium inoculation (Tirichine et al. 2006). These results indicate a key regulatory position for CCaMK in the CSP, leading to activation of the downstream signaling pathways required for nodule organogenesis. Together with CCaMK, CYCLOPS/IPD3 is also allocated downstream of Ca signaling and is shown to interact with CCaMK (Messinese et al. 2007, Yano et al. 2008). It is likely that upon phosphorylation by CCaMK, CYCLOPS activates the downstream gene cascade(s), leading to successful infection of mycorrhiza and rhizobia (Yano et al. 2008).

Arabidopsis thaliana belongs to the family Brassicaceae, which has no mycorrhization ability. In contrast, a monocotyledonous model plant, rice (*Oryza sativa*), is capable of endosymbiosis with mycorrhiza. A genome database search showed that most CSP gene orthologs were not predicted in the *A. thaliana* genome, while *O. sativa* possesses all CSP ortholog candidates (Godfroy et al. 2006, Chen et al. 2007, Markmann et al. 2008). Well-established rice genome resources, including an endogenous retrotransposon, *Tos17* (Hirochika 1997, Miyao et al. 2003), or a number of mutant panels tagged by T-DNA (Jeong et al. 2002), make it possible to analyze the function of those *O. sativa* CSP (OsCSP) genes in mycorrhizal symbiosis. Among those OsCSP genes, rice *CCaMK* (*OsCCaMK*) and rice *CYCLOPS* (*OsCYCLOPS*) have been certified as essential factors for AM symbiosis in rice using a *Tos17*-tagged mutant panel (Chen et al. 2008, Yano et al. 2008), but engagement of other genes in rice mycorrhization is as yet unproven.

Unlike mycorrhization, the RN symbiosis is accompanied by de novo organogenesis of nodules in the root cortex, in concert with rhizobial infection, indicating that some sort of functional adaptation is required for the evolution of the RN symbiosis from the ancient AM symbiosis system. One way of evolution is the acquisition of additional signal transduction pathways and another is molecular evolution of existing components. It was recently suggested that the domain acquisition in SYMRK plays a pivotal role for the evolution of the RN symbiosis (Markmann et al. 2008). However, a difference in domain structure is only found among SYMRK and its orthologs, whereas other CSP genes show identical domain structures between legume and non-legume plants. Several combinations of cross-species complementation have been shown for legume CSP mutants with corresponding rice ortholog genes. In a combination of the *Medicago truncatula dmi3* (*Mtdmi3*) mutant with *OsCCaMK*, only empty nodules were formed in the transformed roots of *dmi3* and no endosymbiosis occurred (Godfroy et al. 2006). Similarly, nodulation with functional endosymbiosis was not restored in the Lotus *symrk* (*Ljsymrk*) mutant roots expressing rice *SYMRK* (*OsSYMRK*) (Markmann et al. 2008). These results show that OsCSP orthologs do not completely

compensate for rhizobium–legume symbiosis, suggesting that some sort of molecular evolution occurred in the respective legume CSP genes to acquire the RN symbiosis.

In this study, we focus on functional analyses of OsCSP genes in two ways. First, involvement of rice POLLUX (OsPOLLUX) and OsCCaMK in the AM symbiosis was examined by mycorrhization phenotype analysis of *osccamk* and *ospollux* mutant lines, newly isolated from the *Tos17* mutant panel. Secondly, we examined whether functional differentiation of CSP genes occurred during evolution of the RN symbiosis from the AM symbiosis by means of cross-species complementation with rice *CASTOR* (*OsCASTOR*), *OsPOLLUX* and *OsCCaMK* to corresponding Lotus mutants. Our results provide new insights into functional conservation of CSP genes between rice and Lotus.

Results

Structures of CASTOR, POLLUX and CCaMK are conserved between rice and Lotus

Putative rice orthologs of *L. japonicus CASTOR* (*LjCASTOR*), *POLLUX* (*LjPOLLUX*) and *CCaMK* (*LjCCaMK*) have been reported previously. Full-length rice cDNA clones, AK068216 and AK072312, were presumed to be *OsCASTOR* and *OsPOLLUX*, respectively (Imaizumi-Anraku et al. 2005). AK070533 was shown to be a candidate for *OsCCaMK* (Godfroy et al. 2006, Chen et al. 2007).

The structures of proteins predicted for *OsCASTOR*, *OsPOLLUX* and *OsCCaMK* in comparison with those for the corresponding Lotus genes are summarized in Fig. 1. Between rice and Lotus, they shared exactly the same domain structures and highly conserved amino acid sequences. The overall amino acid sequence identities were 69.8 and 63.7% for CASTOR and POLLUX, respectively (Fig. 1A, B). The regions from the third transmembrane (TM) domain to the C-terminus of these proteins were highly conserved between rice and Lotus plants. In particular, the RCK domains, which are only found in potassium channel proteins, showed very high sequence identity. The fourth TM sequences have also been described to be highly conserved between rice and Lotus, and are implicated to constitute the pore and filter region of ion channels (Imaizumi-Anraku et al. 2005). The N-terminal regions, including the first and second TMs, shared much lower sequence identities between rice and Lotus.

OsCCaMK has a kinase domain with a putative autophosphorylation site (Thr263), a CaM-binding (autoinhibitory) domain and three EF hands, in common with LjCCaMK. Overall amino acid sequence identity between OsCCaMK and LjCCaMK was 70.7%. The C-terminal regions containing three EF hands were highly conserved, which potentially trap Ca ions (Fig. 1C).

Screening of rice CSP gene mutants

Using genome resources of rice, we attempted to demonstrate that CSP genes are also crucial for mycorrhization in non-leguminous plants. We screened mutants for putative orthologs of CASTOR, POLLUX, CCaMK, NUP85 and NUP133 from a library of *O. sativa* mutants tagged by an endogenous retrotransposon, *Tos17*. As a consequence, we succeeded in the isolation of two alleles of *Ospollux*, NC6423 and ND5050, and one allele of *Osccamk*, NE1115, among 42,700 mutant lines. In NC6423 and

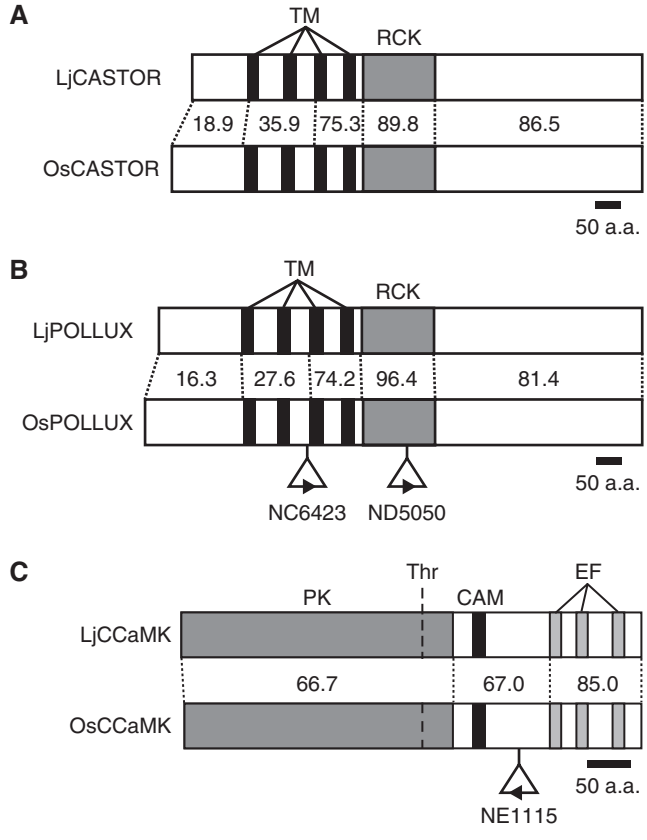

Fig. 1 Structural comparisons of CASTOR, POLLUX and CCAMK between *O. sativa* and *L. japonicus*. The structures of proteins predicted for *OsCastor*, *OsPollux* and *OsCCaMK* are shown in comparison with those for the corresponding Lotus genes. Numerals indicate local amino acid sequence identity (%) between rice and *L. japonicus*. (A) Structures of LjCASTOR and OsCASTOR. Predicted protein domains are: TM, transmembrane helix; RCK, a domain implicated in the regulation of conductance which is only found in potassium channel proteins. (B) Structures of LjPOLLUX and OsPOLLUX. Abbreviations for predicted domains are the same as in (A). The positions of the *Tos17* insertion in two *Ospollux* mutant alleles, NC6423 and ND5050, are shown by triangles with directions of the retroposon. (C) Structures of LjCCaMK and OsCCaMK. Predicted domains are: PK, protein kinase; CAM, calmodulin-binding (autoinhibitory) domain; EF, EF hand. The autophosphorylation site, Thr in the kinase domains is indicated by dashed lines. The position of the *Tos17* insertion in the *Osccamk* mutant NE1115 is shown by a triangle.

Table 1 AM symbiosis phenotypes of *Tos17* insertion lines NE1115, ND5050, NC6423 and wild-type Nipponbare

Line	Location of *Tos17* insertion	AM phenotype		
		Hypha only	Arbuscule	Vesicle
Nipponbare	–	10.42 ± 1.60	19.46 ± 3.52	5.53 ± 1.38
NC6423	Exon 3 of AK072312 (*OsPOLLUX*)	0	0	0
ND5050	Exon 5 of AK072312 (*OsPOLLUX*)	0	0	0
NE1115	Exon 5 of AK070533 (*OsCCaMK*)	0.36 ± 0.14	0.08 ± 0.08	0.04 ± 0.04

AM colonization was quantified at 6 wpi with *G. intraradices*. The data are means (±SE) of root length colonization of 200–400 intersects randomly scored for roots of >80 μm in diameter collected from six plants (NC6423 and ND5050) or 17 plants (NE1115 and Nipponbare).

ND5050, *Tos17* was inserted in front of the third TM domain and into the RCK domain, respectively (Fig. 1B). An insertion between the CaM-binding domain and the first EF hand was defined in the NE1115 line (Fig. 1C). These alleles showed normal growth under the growth conditions we adopted (data not shown).

OsPOLLUX and OsCCaMK are essential for AM symbiosis in rice

To confirm the essentiality of *OsPOLLUX* and *OsCCaMK* in the rice–AM symbiosis, we examined the AM phenotype of rice mutants, NC6423, ND5050 (*Ospollux*) and NE1115 (*Osccamk*).

Internal hyphae, arbuscules and vesicles were counted 6 weeks post-inoculation (wpi) with *Glomus intraradices*, by the line-intersect method (Giovannetti and Mosse 1980) (Table 1). Two *Ospollux* mutant alleles, NC6423 and ND5050, showed no AM infection at all, while abundant AM were colonized in the roots of wild-type Nipponbare (Table 1, Fig. 2C–F). In the roots of the *osccamk* mutant NE1115, mycorrhizal infection was noted only occasionally (Table 1, Fig. 2A, B). A few internal hyphae were observed in seven plants among a total of 17 plants tested, and a few arbuscules and vesicles were observed at very low frequency (1/17 plants). In these plants, however, almost all AM infection processes were blocked at the epidermal cell layer. The AM symbiosis phenotype of the NE1115 roots is consistent with that of NF8513, another *Tos17*-inserted mutant allele of *OsCCaMK* (Chen et al. 2007). Genotyping analysis of F_2 and F_3 seed families of each line was performed by PCR using the Tos17- and gene-specific primer sets, listed in Supplementary Table S1 online. Symbiotic-defective phenotypes of each mutant line were co-segregated with Tos17 insertions (data not shown). Taken together, these results clearly demonstrate that OsPOLLUX and OsCCaMK are both essential for the rice–AM symbiosis.

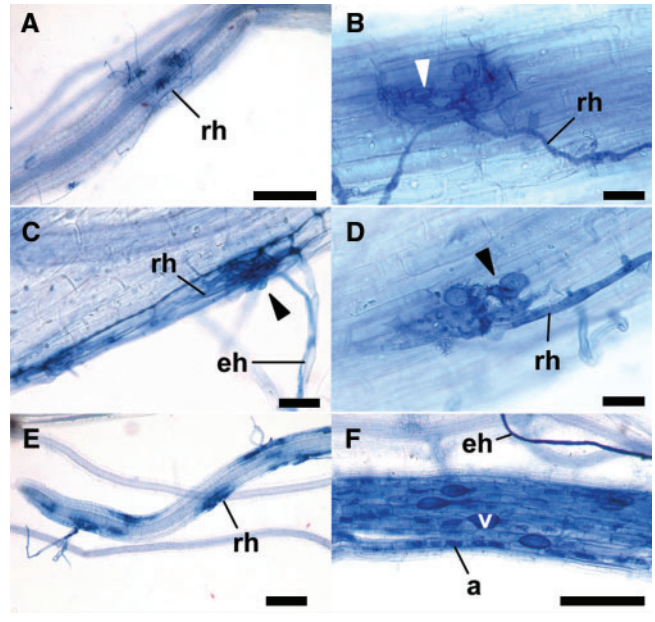

Fig. 2 AM symbiosis phenotypes of *Ospollux* and *Osccamk* mutants. Light micrographs of trypan blue-stained roots 6 wpi with *G. intraradices* are shown. AM structures are indicated by a, arbuscule; eh, external hypha; ih, internal hypha; rh, running hypha; and v, vesicle. Arrowheads indicate aberrant appressoria. Scale bars: A, E and F, 0.2 mm; B, C and D, 0.02 mm. (A and B) *Osccamk* mutant, NE1115. (C and D) *Ospollux* mutant allele. NC6423. (E) *Ospollux* mutant allele, ND5050. (F) Wild-type Nipponbare.

OsCCaMK not only restores both mycorrhizal and rhizobial symbiosis, but also induces spontaneous nodulation in the Ljccamk mutant of Lotus

To examine the interspecific relationship of the OsCSP genes, *OsCCaMK*, *OsCASTOR* and *OsPOLLUX*, between rice and Lotus, we transformed them into the respective Lotus mutants by means of *Agrobacterium*

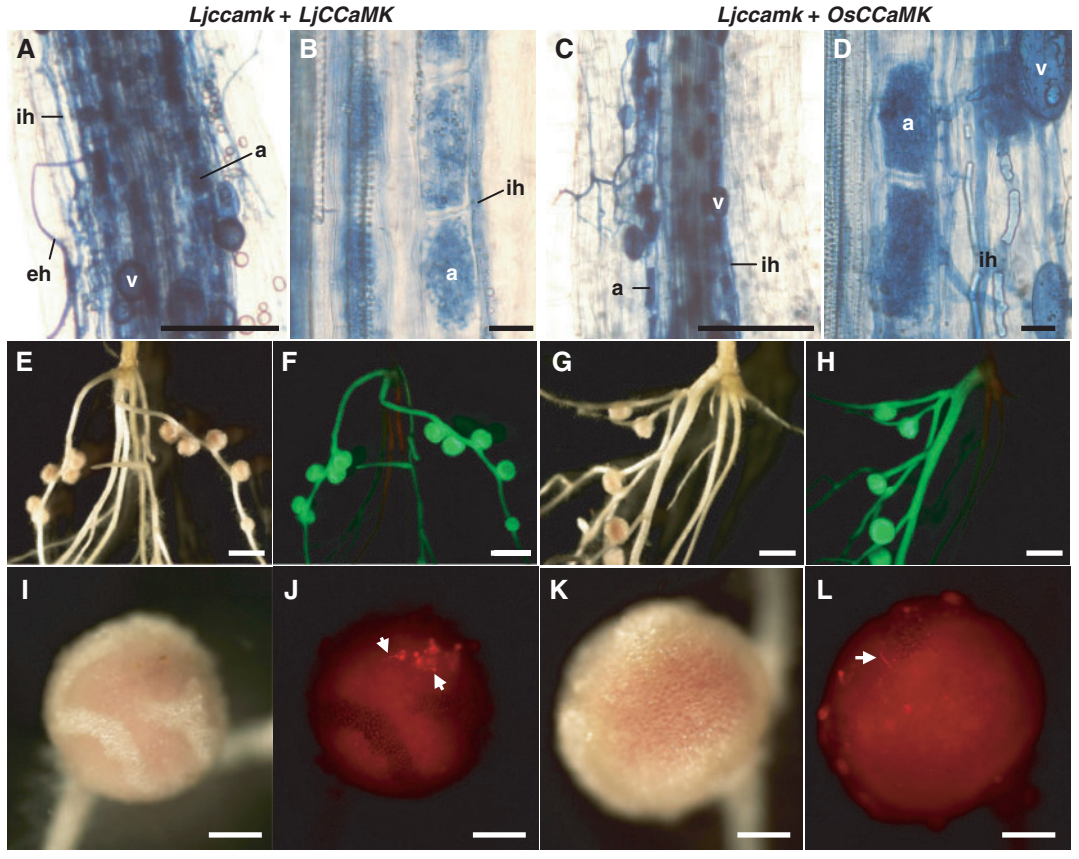

Fig. 3 Restoration of symbiotic defects in *Ljccamk* by *OsCCaMK*. *OsCCaMK* restores both mycorrhizal and rhizobial endosymbioses in *Ljccamk* mutants. Transgenic roots were selected by GFP fluorescence and observed at 4 wpi with *G. intraradices* or DsRed-expressing *M. loti*. AM colonizations are indicated by a, arbuscule; eh, external hypha; ih, internal hypha; and v, vesicle. Scale bars: A and C, 0.1 mm; B and D, 0.02 mm; E–H, 2 mm; I–L, 0.5 mm. (See also Table 2 and Supplementary Fig. 1 online.) (A and B) Trypan blue-stained *Ljccamk* roots transformed with *LjCCaMK*. (C and D) Trypan blue-stained *Ljccamk* roots transformed with *OsCCaMK*. (E) Nodulation phenotype of *Ljccamk* roots transformed with *LjCCaMK*. (F) The same as E showing GFP fluorescence as the marker for transformed roots. (G) Nodulation phenotype of *Ljccamk* roots transformed with *OsCCaMK*. (H) The same as G showing GFP fluorescence as the marker for transformed roots. (I and J) Mature nodule formed on *Ljccamk* roots transformed with *LjCCaMK*. Nodules exhibit a bright pink color, indicating abundant accumulation of leghemoglobin. Red fluorescence in the nodule central zone demonstrates the presence of endosymbiotic bacteria (J). Arrows indicate infection threads. (K and L) A mature nodule formed on *Ljccamk* roots transformed with *OsCCaMK*. The nodule appearance and rhizobial endosymbiosis are comparable with those in I and J.

rhizogenes-mediated hairy root transformation. All mutant lines show severe symbiotic-defective phenotypes, including hyphal penetration block at the root epidermis and non-nodulation (Supplementary Fig. S1).

We introduced the cDNA of *OsCCaMK* under the control of the cauliflower mosaic virus (CaMV) 35S promoter (*P35S:OsCCaMK*) into the *Ljccamk* mutant (*ccamk-3*). The results are shown in Fig. 3 and the quantitative data are summarized in Table 2. AM infection was fully restored in transgenic roots carrying *OsCCaMK*, as well as those with *LjCCaMK* (Fig. 3A–D). Upon inoculation of *Mesorhizobium loti*, *OsCCaMK*-expressing roots formed pink-colored nodules, similar to *LjCCaMK*-expressing roots (Fig. 3E–L). In contrast to the case of *Mtdmi3*, nodules formed on *OsCCaMK*-carrying *Ljccamk* roots appeared to be fully functional and contained endosymbiotic bacteria. This was confirmed further by inoculating DsRed (*Discoma* sp. red fluorescent protein)-labeled *M. loti*. DsRed fluorescence in the central zone of the nodules together with formation of many infection threads was observed, indicating successful infection of rhizobium bacteria (Fig. 3K, L). These results demonstrate that CCaMK function is well conserved between non-nodulating monocot, rice and Lotus, with regards to both mycorrhizal and legume rhizobial symbioses.

It has been reported that a substitution of isoleucine for Thr265 at the autophosphorylation site of the *LjCCaMK* kinase domain results in deregulation (Ca^{2+}/CaM-independent gain of function) of CCaMK and induces spontaneous nodulation in Lotus without rhizobial

Table 2 Complementation of the symbiotic phenotype in Lotus mutants transformed with rice orthologs

Plant genotype	Allele	Transgene	Phenotype-restored plant/GFP-positive plants		
			AM+	Nod+	Inf+
castor-4	*sym71–1*	Empty vector[a]	0/6	0/5	–
castor-4	*sym71–1*	*P35S:LjCASTOR*	6/6	10/10	10/10
castor-4	*sym71–1*	*P35S:OsCASTOR*	14/15	19/19	19/19
pollux-3	*sym86–1*	Empty vector[a]	0/3	0/4	–
pollux-3	*sym86–1*	*P35S:LjPOLLUX*	26/27	34/37	34/37
pollux-3	*sym86–1*	*P35S:OsPOLLUX*	6/32	11/71	0/71
pollux-3	*sym86–1*	*PLjPOL:OsPOLLUX*	0/21	1/49	0/49
ccamk-3	*sym72–1*	Empty vector[a]	0/12	0/19	–
ccamk-3	*sym72–1*	*P35S:LjCCaMK*	17/17	32/33	32/33
ccamk-3	*sym72–1*	*P35S:OsCCaMK*	23/24	72/73	72/73

Symbiotic phenotypes were examined at 4 wpi with *M. loti* or *G. intraradices*. Data are compiled results of more than two independent experiments. AM, arbuscular mycorrhiza infection; Nod, nodule formation; Inf, rhizobial infection into nodule cells.
[a] Plants were transformed with a binary vector harboring the GFP marker only.

inoculation (Tirichine et al. 2006). In general, substitution of the autophosphorylated threonine residue of the kinase domain by an acidic amino acid mimics the effect of autophosphorylated threonine, and the mutagenized kinase showed constitutive activity without Ca dependence (Rasmussen 1994). Based on these reports, site-directed mutagenesis was performed on the kinase domain of CCaMK, in which aspartic acid (Asp, D) substituted for threonine (Thr, T). The resultant $CCaMK^{T265D}$ (TD) showed evidence of its gain-of-function activity by formation of spontaneous nodules in the roots of transformants (Fig. 4A, B). It is noteworthy, however, that wild-type *OsCCaMK* could also induce spontaneous nodulation in Lotus (Table 3, Fig. 4C, D). This implies that OsCCaMK is able to drive the downstream pathway(s) required for nodule organogenesis in Lotus, but might be not completely consistent as a component of the legume CSP, which is supposed to be triggered in response to rhizobial infection.

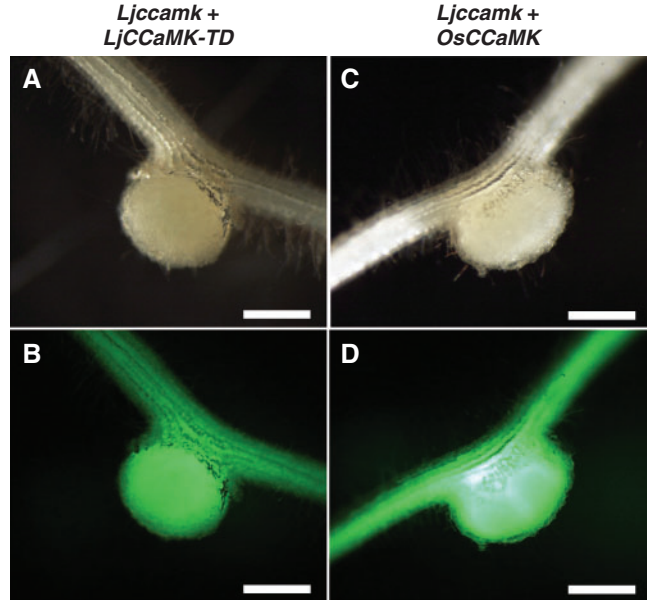

Fig. 4 Spontaneous nodulation in *Ljccamk* transformed with *OsCCaMK*. Transformation of *Ljccamk* roots with *OsCCaMK* induced spontaneous nodulation similar to those with *LjCCaMK*-TD without rhizobium inoculation. Scale bars = 0.5 mm. (A) A spontaneous nodule formed on *Ljccamk* roots transformed with *LjCCaMK*-TD. (B) The same as A showing GFP fluorescence as the marker for transformed roots. (C) A spontaneous nodule formed on *Ljccamk* roots transformed with *OsCCaMK*. (D) The same as C showing GFP fluorescence as the marker for transformed roots.

OsCASTOR restores mycorrhizal and rhizobial symbiosis in the Ljcastor mutant of Lotus

To test functional complementation of *LjCASTOR* with *OsCASTOR*, we transformed an *L. japonicus castor-4* mutant (*Ljcastor*) with *OsCASTOR* cDNA driven by the CaMV 35S promoter (*P35S:OsCASTOR*). As in the case of OsCCaMK described above, OsCASTOR perfectly restored the symbiotic defects of *Ljcastor*. Both AM symbiosis and nodulation with successful endosymbiosis of rhizobium bacteria were fully restored in transgenic hairy roots carrying *P35S:OsCASTOR* (Table 2, Fig. 5), which is evidence for the functional conservation of CASTOR between non-nodulating rice and Lotus.

OsPOLLUX is not fully compatible with LjPOLLUX

In contrast to the fact that OsCCaMK and OsCASTOR are fully functional in Lotus, OsPOLLUX was not able to complement symbiotic defects of the

Table 3 Spontaneous nodulation in *Ljccamk* transformed with *LjCCaMK*-TD and *OsCCaMK*

Plant genotype	Transgene	Spontaneously nodulated plants/GFP-positive plants
ccamk-3	Empty vector[a]	0/7
ccamk-3	*P35S:LjCCaMK*	0/19
ccamk-3	*P35S:LjCCaMK-TD*	18/20
ccamk-3	*P35S:OsCCaMK*	43/48

Seedlings with hairy roots transformed with each construct were transplanted in pre-autoclaved vermiculite pots and grown for 4 weeks under aseptic conditions followed by examination of spontaneous nodulation on GFP-positive roots. Data are the compiled results of two independent experiments.
[a] Plants were transformed with the respective binary vector lacking a *CCaMK* expression cassette.

L. japonicus pollux-3 mutant (*Ljpollux*). Transformation of the *Ljpollux* mutants with *P35S:OsPOLLUX* did not restore mycorrhizal or rhizobial symbiosis (Table 2, Fig. 6). In transformed roots carrying *P35S:OsPOLLUX*, neither internal hyphae nor arbuscule formation was observed (Fig. 6G, H) except for rare occasions where a few internal hyphae and/or arbuscules developed (Fig. 6K, L). When inoculated with *M. loti*, almost all the *OsPOLLUX*-expressing roots did not form nodules (Fig. 6I). Only a few transformants formed small bumps without rhizobial infection and infection threads (Fig. 6M–P). To avoid the possibility that ectopic expression of *OsPOLLUX* caused some adverse effects, we also transformed the coding region of the *OsPOLLUX* gene under the control of the native promoter of *LjPOLLUX* (*pLjPOL:OsPOLLUX*) into the *Ljpollux* mutant (Table 2); symbiosis-defective phenotypes of *Ljpollux* were not

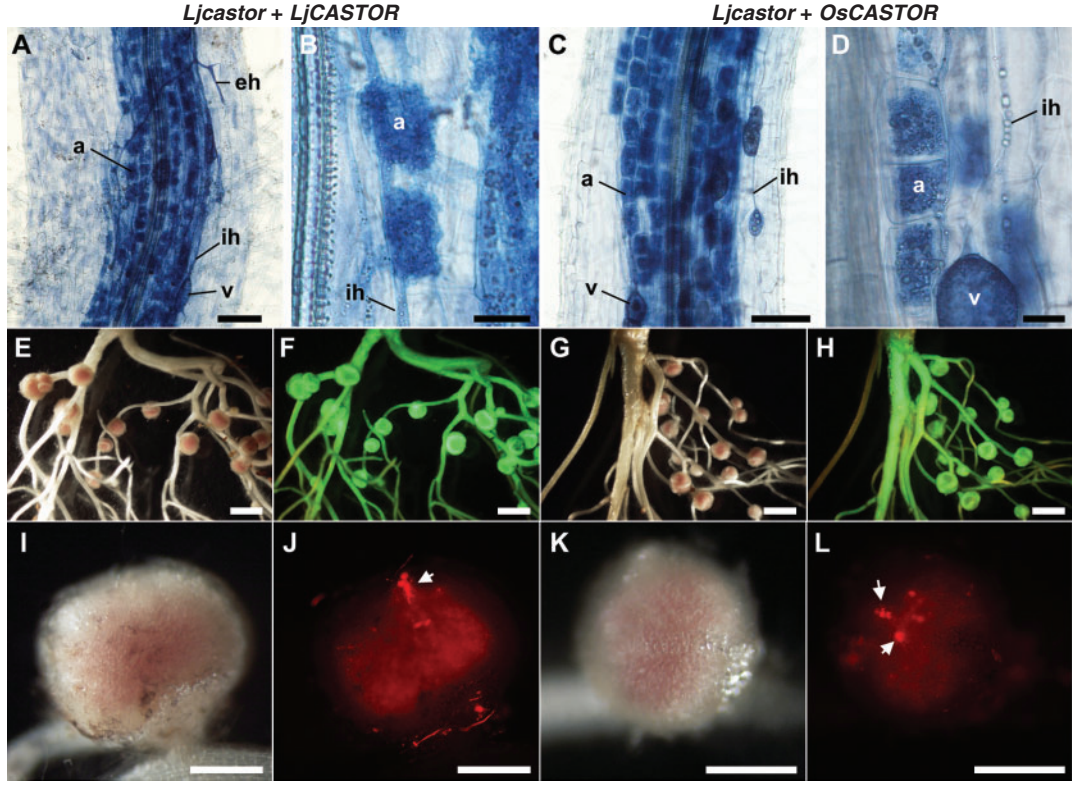

Fig. 5 Restoration of symbiotic defects in *Ljcastor* by *OsCASTOR*. *OsCASTOR* restores both mycorrhizal and rhizobial endosymbioses in *Ljcastor* mutants. Transgenic roots were selected by GFP fluorescence and observed at 4 wpi with *G. intraradices* or DsRed-expressing *M. loti*. For AM colonizations, see abbreviations in Fig. 3. Scale bars: A and C, 0.1 mm; B and D, 0.02 mm; E–H, 2 mm; I–L, 0.5 mm. (See also Table 2 and Supplementary Fig. S1 online.) (A and B) Mycorrhization of *Ljcastor* roots transformed with *LjCASTOR*. (C and D) Mycorrhization of *Ljcastor* roots transformed with *OsCASTOR*. (E) Nodulation phenotype of *Ljcastor* roots transformed with *LjCASTOR*. (F) The same as E showing GFP fluorescence as the marker for transformed roots. (G) Nodulation phenotype of *Ljcastor* roots transformed with *OsCASTOR*. (H) The same as G showing GFP fluorescence as the marker for transformed roots. (I and J) A mature nodule formed on *Ljcastor* roots transformed with *LjCASTOR*. The presence of endosymbiotic rhizobia is shown by red fluorescence in the nodule central tissue (J). An arrow indicates infection thread penetration. (K and L) A mature nodule formed on *Ljcastor* roots transformed with *OsCASTOR*. The nodule appearance and rhizobial endosymbiosis are comparable with those in I and J.

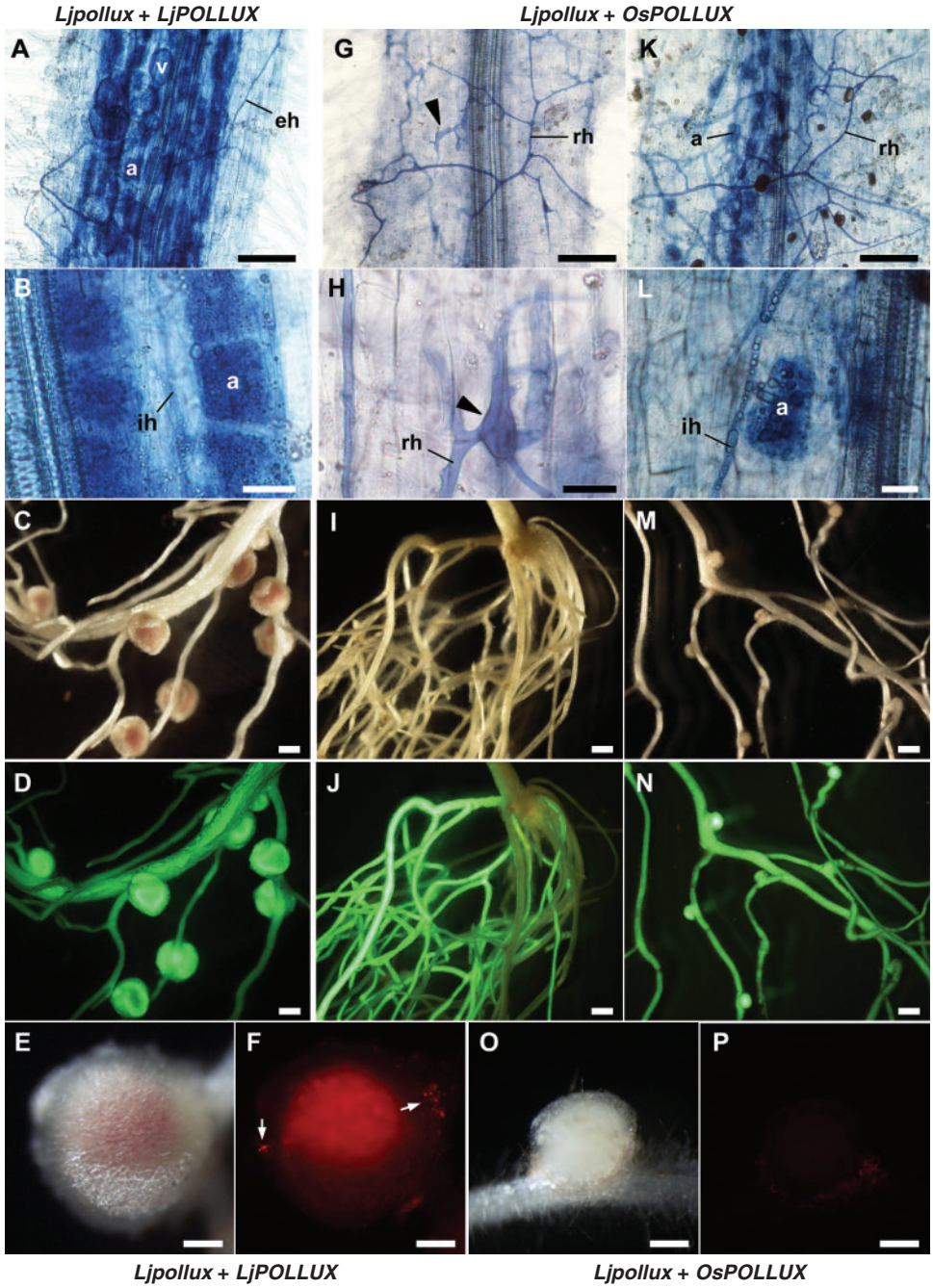

Fig. 6 OsPOLLUX is not able fully to restore the symbiotic defects in *Ljpollux*. *OsPOLLUX* could not restore both mycorrhizal and rhizobial endosymbioses in *Ljpollux* mutants. Transgenic roots were observed at 4 wpi with *G. intraradices* or DsRed-expressing *M. loti*. For AM colonizations, see abbreviations in Fig. 3. Scale bars: A, G and K, 0.1 mm; B, H and L, 0.02 mm; C, D, I, J, M and N, 2 mm; E, F, O and P, 0.5 mm. (See also Table 2 and Supplementary Fig. 1 online.) (A and B) Full restoration of mycorrhiza infection in *Ljpollux* roots by transformation with *P35S:LjPOLLUX*. (C–F) Nodulation of *Ljpollux* roots carrying *P35S:LjPOLLUX* (C and D) with rhizobial endosymbiosis as shown by infection with DsRed-labeled *M. loti* (E and F). The arrows in F indicate the infection threads. (G–J) Transfection of *P35S:OsPOLLUX* confers neither AM symbiosis nor nodulation capacity. Running hyphae with aberrant appressorium and hyphal swelling (arrowheads in G and H) indicate that AM infection is aborted at the epidermis. (K–P) On rare occasions, a few AM infections and arbuscule formation were observed in *Ljpollux* roots carrying *OsPOLLUX* (K and L), and nodulation appeared as small bumps (M and N) in which, however, endosymbiotic rhizobia were never detected (O and P).

complemented, as shown in Supplementary Fig. 2. These results indicate that OsPOLLUX does not act as an alternative for LjPOLLUX in the symbiosis system of Lotus.

Discussion

Identification and characterization of CSP genes have been mainly studied using model leguminous plants, *L. japonicus* and *M. truncatula*. To prove the function of the CSP for mycorrhization in non-leguminous plants, we selected rice (*O. sativa* cv. Nipponbare), a monocot model plant for which the whole genome sequence and many resources for molecular genetic studies are available. In silico analysis showed that all seven CSP orthologs were predicted in the rice genome. We screened for mutant lines of *OsCASTOR*, *OsPOLLUX*, *OsCCaMK*, rice *NUP85* (*OsNUP85*) and rice *NUP133* (*OsNUP133*) from a library of *O. sativa* mutants tagged by *Tos17*, resulting in the isolation of two alleles of *ospollux* and one allele of *osccamk*, and verification that *OsPOLLUX* and *OsCCaMK* are essential for the AM symbiosis. Tagged lines of either *OsNUP85* or *OsNUP133* could not be identified by screening the three-dimensional pool of the *Tos17* mutant panel. Moreover, no tagged lines for them were found in any other rice mutant panels, in contrast to *OsCASTOR*, where a few tagged lines were found in T-DNA mutant panels. Collectively, these results suggest that *OsNUP85* and *OsNUP133* both act as housekeeping genes in rice. In addition to *OsPOLLUX* and *OsCCaMK*, *OsCYCLOPS* was also shown to be essential for mycorrhizal symbiosis in rice (Chen et al. 2008, Yano et al. 2008). In addition, the restoration of an AM-defective phenotype of *Ljsymrk* by introduced *OsSYMRK* has been reported (Markmann et al. 2008). Giving an overview of recent advances, we propose that the CSP is a ubiquitous signaling pathway for AM symbiosis in higher plants.

Three lines of rice CSP mutants, including *Ospollux*, *Osccamk* and *Oscyclops*, with several independent alleles have so far been analyzed (Chen et al. 2007, Chen et al. 2008, Yano et al. 2008). In contrast to variations in nodulation phenotypes of Lotus mutants for corresponding CSP genes, namely the extent of root hair deformation and/or curling, elongation of infection threads and formation of small bumps, all rice mutants showed high similarity in AM phenotypes, including running hyphae with aberrant appressoria, and absence of internal fungal colonization. These phenotypes show that AM fungal infection is mainly aborted at the stage of hyphal penetration at the root epidermis in rice CSP mutants. Comparable AM phenotypes have been reported in all seven Lotus CSP mutants (Senoo et al. 2000, Kistner et al. 2005; see also Supplementary Fig. S1). Judging from these results, the CSP may regulate the epidermal response for AM fungal invasion, and this function has been conserved in both rice and legumes.

In Lotus, CASTOR and POLLUX are prerequisites for the elicitation of Ca spiking in response to Nod factors (Imaizumi-Anraku et al. 2005). In silico analysis has also indicated the existence of these orthologs in rice. Like Lotus, the single mutation of *OsPOLLUX* leads to a mycorrhization-defective phenotype, suggesting their functional differentiation in AM symbiosis signaling.

It is generally accepted that legumes acquired the RN symbiosis system by recruiting signaling pathways and/or components required for the AM symbiosis system, which is widely distributed in the plant kingdom (Kistner and Parniske 2002). To examine the functional evolution of CSP genes, cross-species complementation tests between non-nodulating mycorrhizal plants and legumes provide clues to solving the evolutionary route of RN symbiosis from the ancestral AM symbiosis system. Cross-species complementation assays in this study produced different results for each gene. *OsCASTOR* could fully complement the symbiosis-defective phenotypes of *Ljcastor*, including nodule formation, rhizobial infection and mycorrhizal infection. Comparable results were also observed in combination with *OsCCaMK* and *Ljccamk* mutants. Together with our results, previous reports based on cross-species complementation assays are listed in Fig. 7, according to the extent of restoration of mutant phenotypes. In combination with *OsCCaMK* transformation for the *Mtdmi3* mutant (Godfroy et al. 2006, Chen et al. 2007), however, the rhizobial infection phenotype was not recovered. A similar result was also reported for *Ljsymrk* transformed with *OsSYMRK* (Markmann et al. 2008). In both cases, however, the AM symbiosis-defective phenotype was fully restored, implying some kind of functional differences between rice and legumes. In contrast, our results clearly show full reversion of symbiotic defects in mutant lines with regard to both mycorrhizal and rhizobial symbioses by corresponding rice orthologs, and provide evidence for functional conservation of CASTOR and CCaMK between rice and Lotus.

OsPOLLUX could not restore the symbiosis-defective phenotypes of *Ljpollux* regardless of the promoters used for the experiments. Occasional AM infection or nodule organogenesis was observed. However, endosymbiosis with rhizobia or AM fungi was not fully complemented. Notably, both RN- and AM-defective phenotypes of *Ljpollux* were not restored by *OsPOLLUX*. In view of the fact that the CSP is involved in the induction and decoding of Ca spiking in Nod factor signaling (Imaizumi-Anraku et al. 2005, Miwa et al. 2006, Saito et al. 2007), the Ca signal probably acts as a second messenger in the mycorrhizal signaling pathway as well. Indeed, another Ca signal with

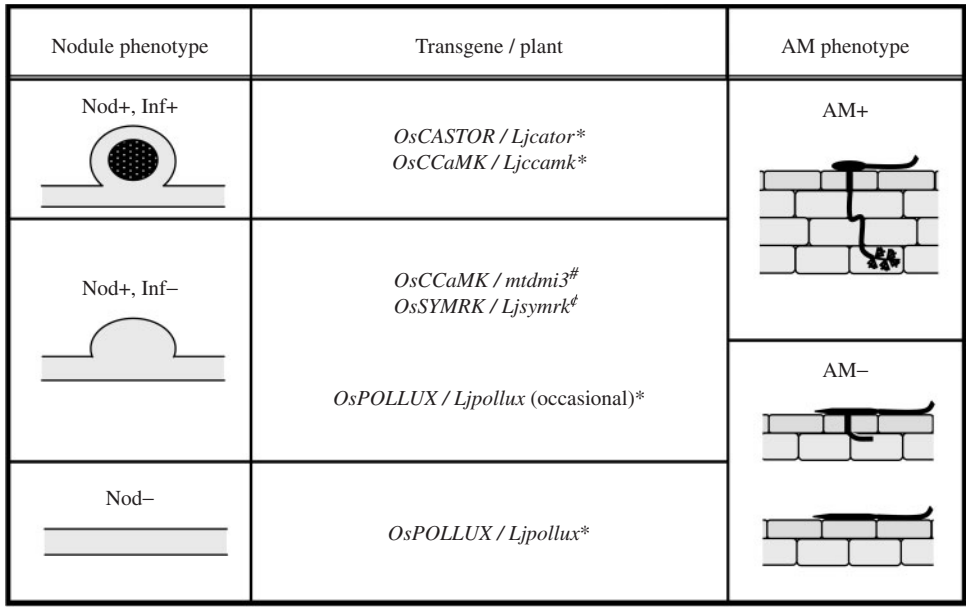

Fig. 7 Summary of the cross-species complementation tests. The results of cross-species complementation tests between a non-nodulating mycorrhizal plant, rice and legumes obtained in the present work and previous reports are categorized in regards to AM and RN symbiosis phenotypes. *Results in this work; #Godfroy et al. (2007); ¢Markmann et al. (2008).

a different signature was induced by mycorrhizal infection with DMI1 and DMI2 in a dependent manner (Kosuta et al. 2008). These reports lead to the speculation that acceptance criteria of endosymbioses depend on the nature of the Ca signature or the decoding ability of Ca signals induced by transfected heterologous genes. From this point of view, OsCASTOR and OsCCaMK are replaceable in the symbiosis signaling pathway of Lotus, resulting in the full activation of downstream pathways. In contrast, OsPOLLUX did not act like LjPOLLUX and is supposed to fail to elicit appropriate Ca signals sufficient for infection of symbiotic partners. The presence of the 'non-conformity' of OsCSP genes with the signaling pathways of heterologous legume plants may provide intriguing issues for future studies on the evolution of CSP from ancient mycorrhizal plants to legumes which acquired RN symbiosis.

CCaMK is assumed to be the decoder of symbiotic Ca signaling and a key component in activating the downstream pathway(s) (Tirichine et al. 2006, Yano et al. 2008). *OsCCaMK* was unable to restore the rhizobial infection in *Mtdmi3*, whereas it could complement fully the symbiotic-defective phenotypes of *Ljccamk*. These conflicting results may also be caused by the 'non-conformity' of *OsCCaMK* with the genetic background of *M. truncatula*. In this combination, introduction of *OsCCaMK* may cause failure in decoding of Ca spiking or activation of downstream components. One possible explanation is that heterologous CCaMK causes impaired interaction with other components of downstream symbiosis signaling pathways. This incomplete interaction may end in unsuccessful infection of rhizobia in *M. truncatula*. OsCCaMK appears to act as a substitute for LjCCaMK, and, furthermore, it could also act as a gain-of-function CCaMK and induce spontaneous nodules. OsCCaMK seems to act in an appropriate manner in Lotus, but the interspecific difference leads to reduction in Ca dependency of OsCCaMK in Lotus roots. Elucidation of molecular mechanisms underling these 'non-conformities' is also an issue to be solved in the future. Co-transformation with those proteins that probably interact with CCaMK is a possible future approach to explain the elusive nature of such 'non-conformities'.

Considering the evolution of the two different symbiosis systems, efforts have been made to determine how the RN symbiosis has evolved by recruiting the pre-existing CSP pathway of the AM symbiosis. Distinctive versions of SYMRK with different domain compositions have been isolated from a number of plant species. LjSYMRK is a 'full-length' version and shows the potential to manage both AM and RN symbioses. In contrast, 'shorter length' SYMRKs, isolated from non-leguminous mycorrhizal plants, manage only the AM symbiosis, suggesting that stepwise domain acquisition of SYMRK has played a crucial role in the evolution of the RN symbiosis (Markmann et al. 2008). However, a difference in domain structure is only found among SYMRK and its orthologs, whereas other CSP genes show identical domain structures

between legumes and non-legumes. During the evolution of the RN symbiosis, the CSP has also retained its conventional function which mediates encoding and decoding of Ca signals for establishment of the AM symbiosis. Such constraints may affect the evolutionary direction of the RN symbiosis system, resulting in molecular evolution of only a few gene(s) among the CSP components, as represented by SYMRK. At the same time, most of the CSP genes, including CASTOR, CCaMK and CYCLOPS (Yano et al. 2008), have conserved their functions without drastic evolution at the molecular level. Recently, two interactors of SYMRK, MtHMGR and SIP1 have been reported (Kevei et al. 2007, Zhu et al. 2008). Although involvement of these genes for mycorrhization is not yet clear, they may also provide important clues for understanding the acquisition of the signaling pathway for the evolution of the RN symbiosis.

In conclusion, our results provide a conserved genetic basis for the AM symbiosis between the monocot rice and a legume, Lotus. Taken together with previous reports, we also demonstrate divergent evolutionary ways among CSP genes based on cross-species complementation analyses. SYMRK exemplifies its distinctive position in the CSP as an adaptive factor which confers the ability of the RN symbiosis on leguminous plants, while CASTOR and CCaMK represent the conserved components, which have retained both their functions and domain structures during the evolution of the RN symbiosis. In other words, CASTOR and CCaMK have stayed essentially the same in their functions and constitute the root of the CSP.

Materials and Methods

Plant materials

A *Tos17* retrotransposon-tagged mutant library of rice (*O. sativa* cv. Nipponbare, Miyao et al. 2003), which is composed of 42,700 independent mutant lines, was used to isolate OsCSP mutants. For cross-complementation analyses, we used the mutant lines of *L. japonicus* B-129 'Gifu', *Ljcastor-4* (*sym71-1*), *Ljpollux-3* (*sym86-1*) and *Ljccamk-3* (*sym72-1*), described previously (Imaizumi-Anraku et al. 2005, Kistner et al. 2005, Tirichine et al. 2006).

Screening of the rice Tos17 mutant panel

Three-dimensional pooled DNAs prepared from a mutant panel of rice (*O. sativa* var. Nipponbare) generated by *Tos17* insertional mutagenesis (Hirochika 1997) were screened by PCR using the pairs of a *Tos17*-specific primer (Tos17-L1 or Tos17-R1) and primers specific for the respective OsCSP genes of interest. When amplified DNA fragments were detected, nested PCR was performed with primer pairs of *Tos17*-specific primer (Tos17-L1 or Tos17-R2) and gene-specific primers designed inside the first amplified fragments to confirm and isolate each individual mutant line.

Rice genomic DNA was isolated from leaf tissue using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's manual and used for sequencing analysis and genotyping. We confirmed the position of *Tos17* insertion by sequencing of the amplified PCR fragments. Genotyping of the descendant mutant lines was performed by PCR using the *Tos17*- and gene-specific primer sets. The primer sequences used are listed in Supplementary Table S1 online.

Microbial strains, inoculation methods and plant growth conditions

Glomus intraradices DAOM 197198 (Premier Tech, Quebec, Canada) was used for inoculation of both *O. sativa* and *L. japonicus* with approximately 200 spores per plant. Surface-sterilized seeds were germinated on 0.6% agar plates in a Eyelatron FL1-301N growth cabinet (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) on a 16 h light/8 h dark cycle at 28°C for 3 d and then transplanted in an autoclaved 1: 1 (v/v) mixture of vermiculite and commercial soil for turf grass (Shibametuchi, Sunbellex, Tokyo, Japan) followed by AM inoculation. After inoculation, plants were watered every 2 d with 1/2 Hoagland solution supplemented with 100 μM KH_2PO_4 and grown in a growth cabinet at a 14 h day, 28°C/10 h night, 24°C cycle for 6 weeks. For inoculation of *A. rhizogenes*-induced hairy roots of *L. japonicus*, transformants were grown in an autoclaved 1: 1 mixture of Shibametuchi and a nutrient-rich commercial horticulture soil (Kureha, Tokyo, Japan). AM-inoculated Lotus plants were grown in a growth chamber at 24°C with a 16 h (day)/8 h (night) cycle for 4 weeks. The plants were watered every 3 d.

To examine the extent of rhizobial infection, *A. rhizogenes*-induced hairy roots of *L. japonicus* were transplanted in vermiculite pots supplied with B&D medium (Broughton and Dilworth 1971) supplemented with 0.5 μM ammonium nitrate and inoculated with *M. loti* MAFF303099 constitutively expressing DsRed at 3 d after transplanting. The plants were grown in a growth cabinet with a 16 h day/8 h night cycle at 24°C for 4 weeks.

Evaluation of mycorrhizal colonization

Rice roots were harvested and cleared with 2% KOH, and stained with 0.05% trypan blue according to Saito et al. (2007). Hyphal, arbuscular or vesicular colonization was determined as the percentage of root length colonization using a line-intersect method (Giovannetti and Mosse 1980). Data were derived from 200–400 intersects per plant randomly scored for roots of >80 μm in diameter. Trypan blue-stained AM colonies were observed under a bright-field microscope (Leitz DMRB; Leica), and images were acquired using a CCD camera system (Penguin 600CL, Pixera).

Complementation tests of Lotus mutants by rice orthologs

For the construction of OsCASTOR, OsPOLLUX, OsCCaMK, LjCASTOR, LjPOLLUX, LjCCaMK and $LjCCaMK^{T265D}$, cDNA sequences of the respective genes were amplified and cloned in pENTR-D Topo (Invitrogen, Carlsbad, CA, USA). The resulting entry clones were converged with Gateway-compatible destination vectors of P35S:GFP-gw, which were modified vectors of P35S:GFP (Yano et al. 2008) encoding P35S:GFP as a selection marker and a Gateway reading frame cassette RfC.1. For construction of PLjPOL:OsPOLLUX, which confers the expression of OsPOLLUX under the control of the *LjPOLLUX* promoter/terminator, genomic regions of the *LjPOLLUX* promoter (3,013 bp) and terminator (1,256 bp) were amplified by PCR with primer pairs including overlapping sequences of *OsPOLLUX* coding regions and an *Asc*I recognition site. Amplicons of the *LjPOLLUX* promoter, terminator and the coding region of *OsPOLLUX* cDNA were fused by joint PCR with the outermost primer pair of AscI-LjPOLLUX-f1 and

LjPOLLUX-AscI-r3. The resulting fragment was digested with *Asc*I and cloned into the *Asc*I restriction site of pHKN29, which was constructed by replacement of HPT (hygromycin phosphotransferase) by GFP (green fluorescent protein) (Kumagai and Kouchi, 2003). Information of the primers and vectors used for construction are listed in Supplementary Table S2 online.

The constructs carrying Lotus or rice *CASTOR*, *POLLUX* and *CCaMK* genes were transformed into the corresponding *L. japonicus* mutants, *Ljcastor-4*, *Ljpollux-3* and *Ljccamk-3*, by hairy root transformation with *A. rhizogenes* LBA 1334 as described by Maeda et al. (2006). Plants with GFP-positive hairy roots were inoculated with *G. intraradices* or DsRed-labeled *M. loti* as described above. Plants were harvested 4 weeks after inoculation, and transformed hairy roots were selected again by GFP fluorescence with a Leica MZFLIII stereomicroscope. *Glomus intraradices*-inoculated roots were stained with trypan blue as described above, and the number of mycorrhiza-infected plants was counted. For characterization of nodulation phenotypes, the number of nodulated plants was counted. In addition, the extent of rhizobial infection and nodule organogenesis was examined with a Leica MZFLIII stereomicroscope. DsRed-expressing *M. loti* were observed with a 565/595 nm bandpass filter and a CCD camera system (Penguin 600CL, Pixera). The grayscale images were pseudo-colored using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

Computer analysis

Multiple alignment and calculation of amino acid sequence identity were performed using the default settings of Clustal W (http://www.ebi.ac.uk/clustalw/). The TM regions were predicted using both TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) and TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Domain structure analyses were performed by Pfam 22.0 (http://pfam.sanger.ac.uk/).

Supplementary data

Supplementary data are available at PCP online.

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