

# Nuclear-Localized and Deregulated Calcium- and Calmodulin-Dependent Protein Kinase Activates Rhizobial and Mycorrhizal Responses in *Lotus japonicus* <sup>W</sup>

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**The common symbiosis pathway is at the core of symbiosis signaling between plants and soil microbes. In this pathway, calcium- and calmodulin-dependent protein kinase (CCaMK) plays a crucial role in integrating the signals both in arbuscular mycorrhizal symbiosis (AMS) and in root nodule symbiosis (RNS). However, the molecular mechanism by which CCaMK coordinates AMS and RNS is largely unknown. Here, we report that the gain-of-function (GOF) variants of CCaMK without the regulatory domains activate both AMS and RNS signaling pathways in the absence of symbiotic partners. This activation requires nuclear localization of CCaMK. Enforced nuclear localization of the GOF-CCaMK variants by fusion with a canonical nuclear localization signal enhances signaling activity of AMS and RNS. The GOF-CCaMK variant triggers formation of a structure similar to the prepenetration apparatus, which guides infection of arbuscular mycorrhizal fungi to host root cells. In addition, the GOF-CCaMK variants without the regulatory domains partly restore AMS but fail to support rhizobial infection in *ccamk* mutants. These data indicate that AMS, the more ancient type of symbiosis, can be mainly regulated by the kinase activity of CCaMK, whereas RNS, which evolved more recently, requires complex regulation performed by the regulatory domains of CCaMK.**

## INTRODUCTION

Legume plants benefit from two major mutualistic plant–microbe interactions: arbuscular mycorrhizal symbiosis (AMS) and root nodule symbiosis (RNS). AMS and RNS provide inorganic materials (mainly phosphate and nitrogen sources, respectively) to host plants. In return, the host plant supplies photosynthetic products to the symbionts and an anaerobic environment for RNS. These symbiotic nutrient supplies have contributed to survival and fitness of the host plants on land.

Arbuscular mycorrhizal (AM) fungi absorb inorganic materials and water from the rhizosphere through extraradical hyphae, which attach to and penetrate into the host root (Harrison, 2005). The intraradical hyphae elongate longitudinally in the apoplastic spaces between cortical cells, in which a tree-like symbiotic structure called the arbuscule is surrounded by the plant membrane (Bonfante and Perotto, 1995; Gianinazzi-Pearson, 1996).

In the arbuscule, nutrients are exchanged across the fungal and plant membranes by transporters (Harrison and van Buuren, 1995; Bago et al., 2003).

In RNS, the root nodule is a symbiotic secondary organ that develops on the host roots for accommodation of the symbiont. Symbiotic partner rhizobia colonize root hairs and secrete rhizobial symbiosis–signaling molecules called Nod factors (Lerouge et al., 1990; Dénarié and Cullimore, 1993). Cortical cell division is stimulated by recognition of Nod factors, resulting in nodule formation. The host plant takes rhizobia into the nodule cells through an infection thread, a structure that allows bacterial entry into the host roots (Gage, 2004). In the nodules, rhizobia produce nitrogenase to fix (convert) atmospheric nitrogen into ammonia, which provides a usable nitrogen source for the host plants (Udvardi and Day, 1997). The host plant produces the oxygen binding protein leghemoglobin in the infected cells and maintains the anaerobic environment necessary for nitrogenase function.

AMS and RNS share several similarities despite the differences between fungal and bacterial symbionts. These similarities have presumably originated from a common evolutionary origin. RNS, which is the more recent type of symbiosis, has retained some of the same symbiosis factors and mechanisms as the more ancient AMS type of symbiosis (Parniske, 2008; Markmann and Parniske, 2009). Indeed, previous studies have identified symbiosis mutants in legume plants that abolish both AMS and RNS. Several such symbiosis (*Sym*) genes have been identified through studies of these mutants (Endre et al., 2002;

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Stracke et al., 2002; Ané et al., 2004; Lévy et al., 2004; Imaizumi-Anraku et al., 2005; Kanamori et al., 2006; Tirichine et al., 2006; Saito et al., 2007; Yano et al., 2008; Groth et al., 2010; Murray et al., 2011). A signaling pathway composed of these symbiosis genes is termed the “common Sym pathway” (Parniske, 2008).

One of the common Sym proteins, calcium- and calmodulin-dependent protein kinase (CCaMK), has an essential role in transduction of both AMS and RNS signals (Lévy et al., 2004; Gleason et al., 2006; Tirichine et al., 2006). CCaMK is composed of a kinase domain, a calmodulin binding domain (CaM BD), and the EF-hand domain (Patil et al., 1995; Takezawa et al., 1996). The regulatory domains, including the CaM BD and the EF hands, are believed to function as sensors for changes in calcium ion ( $\text{Ca}^{2+}$ ) concentration generated by upstream symbiosis signaling. According to the model, the binding of  $\text{Ca}^{2+}$  to CCaMK causes a conformational change of the protein and deregulates the kinase activity, which in turn activates a downstream signaling pathway by phosphorylation of substrates (Sathyanarayanan et al., 2000).

Previously, a gain-of-function (GOF) type of CCaMK mutant, *spontaneous nodule formation1* (*snf1*), was isolated in *Lotus japonicus* (Tirichine et al., 2006). The kinase domain of the mutated CCaMK contains a Thr-to-Ile amino acid substitution at residue 265 (T265I), which is believed to mimic autophosphorylation for deregulation/activation of the CCaMK kinase domain. In *snf1* mutants, deregulation of CCaMK appears to activate the RNS signaling pathway, which results in formation of a nodule-like structure called the spontaneous nodule (SPN) in the absence of rhizobia. In addition, another type of GOF-CCaMK has been identified in *Medicago truncatula* by truncation of the regulatory domains, resulting in similar phenotypes (Gleason et al., 2006). This indicates that the common Sym pathway is sufficient for activation of cortical cell division and consequent nodule formation in RNS (Hayashi et al., 2010; Madsen et al., 2010). However, the functions of GOF-CCaMK in AMS are largely unknown. Since AMS does not trigger obvious structural changes, such as nodule formation, induction of AMS-related genes can be used as a proxy to examine the functions of GOF-CCaMK in AMS.

In *L. japonicus*, expression of the subtilisin-like Ser protease (subtilase) gene *SbtM1* and the phosphate transporter gene *PT4* is induced by AM fungal infection (Takeda et al., 2009, 2011). Expression of *SbtM1* is exclusively induced in AMS not in RNS, which is under the regulation of the common Sym pathway. RNA interference silencing of *SbtM1* reduces hyphal elongation and arbuscule formation in the host roots (Takeda et al., 2009). Lj *PT4* is an apparent ortholog of the Mt *PT4* gene isolated from *M. truncatula* (Liu et al., 1998; Harrison et al., 2002; Guether et al., 2009). Mt *PT4* is required for AMS, essential for maintenance of arbuscules in the later stages of AMS (Javot et al., 2007). In addition to the changes in gene expression, development of the prepenetration apparatus (PPA), an intracellular preinfection structure produced during AM fungal colonization, has been reported in carrot (*Daucus carota*) and *M. truncatula* roots (Genre et al., 2005, 2008). During PPA formation, the epidermal and cortical cells that are proximate to the fungal hyphae show dense endoplasmic reticulum (ER) and migration of the nuclei. Formation of the PPA is likely to facilitate fungal penetration into the

host cells by reorganization of cytoplasmic structure. Since the PPA is abolished in the common *sym* mutants, formation of the PPA requires a trigger through the common Sym pathway (Genre et al., 2005). Despite the importance of CCaMK in AMS, the functions of GOF-CCaMK in induction of AMS-specific genes or formation of the PPA are unclear.

Similar to expression of *SbtM1* in AMS, expression of *NIN* (for *Nodule inception*) is specifically induced in RNS (Radutoiu et al., 2003). It is expressed in epidermal cells early in the infection stage of RNS and subsequently in dividing cortical cells and mature nodules in the later stages of nodule development (Schäuser et al., 1999; Grønlund et al., 2005). In *snf1* mutants, *NIN* expression is detected merely in dividing cortical cells and in SPNs (Tirichine et al., 2006), whereas early spontaneous *NIN* expression in *snf1* mutants is undetectable at epidermal cells. Expression of *Early Nodulin11* (*ENOD11*) in *M. truncatula* is induced both by RNS and by AMS (Journet et al., 2001). Similar to *snf1* in *L. japonicus*, GOF-CCaMK in *M. truncatula* constitutively induces *ENOD11* expression in the absence of rhizobia (Gleason et al., 2006). In *L. japonicus*, expression of subtilase genes *SbtM4* and *SbtS* is induced 2 to 4 d after inoculation of either rhizobia or AM fungi (Takeda et al., 2009). Whereas *SbtM4* expression is maintained throughout nodule development, *SbtS* expression is induced only in the early stage of nodule development and is hardly detected in nodules. In *snf1* mutants, *SbtM4* expression is observed only in the late stage of SPN formation, whereas *SbtS* is not detected (Takeda et al., 2011). These facts indicate that RNS responses are not fully activated in *snf1*. This led us to hypothesize that another form of GOF-CCaMK (i.e., truncation of the regulatory domains) may have a different spectrum in symbiosis signaling.

In this study, we examined the functions of GOF-CCaMK both in AMS and in RNS. We constructed a GOF-CCaMK by truncation of the regulatory domains and examined whether downstream gene expression was affected by its nuclear localization. Fusion of GOF-CCaMK with a nuclear localization signal (NLS) enhanced symbiosis gene expression in the absence of the symbionts. Furthermore, a cytoplasmic structure similar to the PPA was induced by nuclear-localized GOF-CCaMK.

## RESULTS

### Nuclear Localization of Deregulated CCaMK Strongly Induced Both RNS- and AMS-Dependent Genes

To analyze the role of CCaMK in AMS and RNS, we constructed a series of vectors containing the GOF-CCaMK variants expressed under the control of the cauliflower mosaic virus 35S promoter (see Supplemental Figure 1 online). In previous studies, CCaMK<sup>TD</sup>, which has the amino acid substitution Thr265Asp (T265D) in wild-type CCaMK (CCaMK<sup>WT</sup>), was shown to trigger SPN formation in *L. japonicus* (Banba et al., 2008; Hayashi et al., 2010), but regulation of symbiosis gene expression downstream of CCaMK<sup>TD</sup> was not examined. In addition, the functions of truncated CCaMK lacking the regulatory domains were not tested in *L. japonicus*. Therefore, we constructed CCaMK<sub>314</sub>, a C-terminally truncated CCaMK containing amino acids 1 to 314 that corresponded to the DMI3

(CCaMK of *M. truncatula*) kinase domain used by Gleason et al. (2006). We also tested the T265D substituted form of CCaMK<sub>314</sub>, designated CCaMK<sub>314</sub><sup>TD</sup>. Analysis in *L. japonicus* revealed that green fluorescent protein (GFP)-fused CCaMK is localized in the nucleus (Yano et al., 2008), although there is no canonical NLS in CCaMK. However, GFP-fused CCaMK<sub>314</sub><sup>TD</sup> was found both in the cytosol and in the nucleus of leaf epidermal cells of *Nicotiana benthamiana* (see Supplemental Figures 2A to 2C online), indicating that these proteins had lost the ability to specifically localize to the nucleus. To address whether nuclear activity of CCaMK is essential for symbiosis signaling, we fused an NLS to CCaMK<sub>314</sub><sup>TD</sup>. The GFP-fused CCaMK<sub>314</sub><sup>TD</sup>-NLS protein was predominantly retained in the nucleus in *N. benthamiana* leaves (see Supplemental Figures 2D to 2F online). We also constructed a CCaMK<sub>314</sub><sup>TD</sup> fusion with a nuclear export signal (NES), and localization of GFP-CCaMK<sub>314</sub><sup>TD</sup>-NES was analyzed in *N. benthamiana* leaf epidermal cells. Although weak fluorescence of GFP-CCaMK<sub>314</sub><sup>TD</sup>-NES could be seen in the nucleus (see Supplemental Figure 2G online), a significant reduction in nuclear localization of the fusion protein was detected (see Supplemental Figures 2G to 2I online), compared with GFP-CCaMK<sub>314</sub><sup>TD</sup> (see Supplemental Figure 2A online) or GFP-CCaMK<sub>314</sub><sup>TD</sup>-NLS (see Supplemental Figure 2D online).

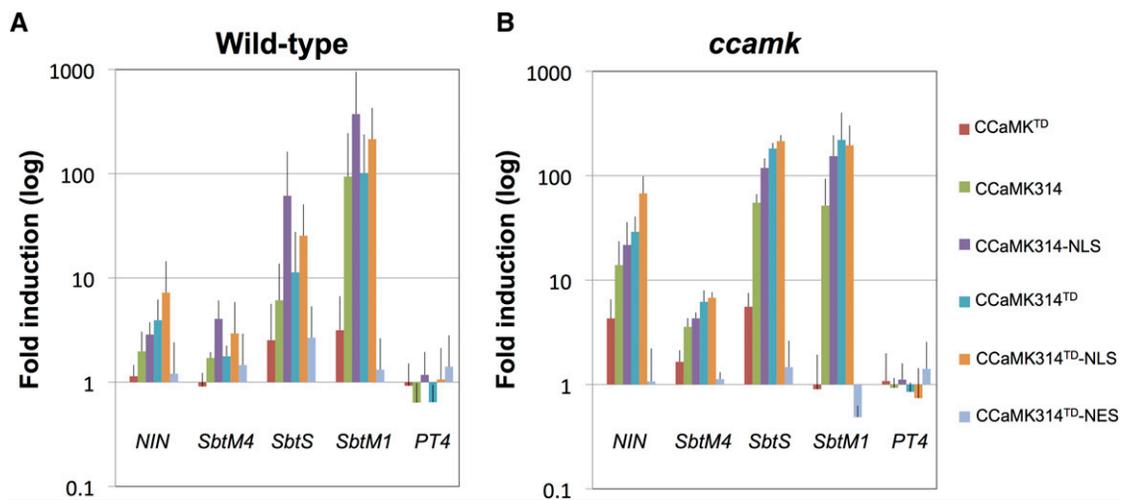
We introduced full-length CCaMK<sup>TD</sup> and the kinase-only GOF-CCaMK variants (CCaMK<sub>314</sub>, CCaMK<sub>314</sub>-NLS, CCaMK<sub>314</sub><sup>TD</sup>, CCaMK<sub>314</sub><sup>TD</sup>-NLS, and CCaMK<sub>314</sub><sup>TD</sup>-NES) into *L. japonicus* by hairy root transformation and analyzed expression of symbiosis genes. Among the series of GOF-CCaMK transformants, hairy roots carrying CCaMK<sub>314</sub>, CCaMK<sub>314</sub>-NLS, CCaMK<sub>314</sub><sup>TD</sup>, and CCaMK<sub>314</sub><sup>TD</sup>-NLS showed induction of RNS-induced genes *NIN*, *SbtM4*, and *SbtS* at 2 weeks after generation of hairy roots (Figure 1A). As we expected, the enforced nuclear localization and the T265D substitution of CCaMK<sub>314</sub> enhanced its effect on the induction of the RNS-dependent marker genes (*NIN*, *SbtM4*, and *SbtS*). On the other hand, the nuclear-exported variant

(CCaMK<sub>314</sub><sup>TD</sup>-NES) triggered only weak induction of those genes (Figure 1A). These results indicate that nuclear localization of GOF-CCaMK is important for its ability to induce expression of RNS-induced genes. Expression of RNS-induced genes was not statistically significant in hairy roots carrying the full-length CCaMK<sup>TD</sup> variant (Figure 1A). In *snf1* mutants, *NIN* expression is not observed before cortical cell division (Tirichine et al., 2006). The lack of induction of *NIN* by full-length CCaMK<sup>TD</sup> agrees with the results previously obtained in *snf1* mutants (Tirichine et al., 2006).

CCaMK is known to be a key factor both in AMS and in RNS because deficiency of a CCaMK function abolishes both types of symbioses (Catoira et al., 2000; Senoo et al., 2000). Therefore, we also analyzed expression of two AMS-specific genes in *L. japonicus*, *SbtM1* and *PT4*. The GOF-CCaMK variants were introduced into wild-type plants to examine AMS-specific gene expression (Figure 1A). The expression analysis revealed that all of the kinase-only GOF-CCaMK variants except for the NES fusion could induce expression of *SbtM1* but not that of *PT4*, whereas full-length CCaMK<sup>TD</sup> and *snf1* could not induce expression of either gene (Figure 1A; Takeda et al., 2011). Because *SbtM1* is involved in an early stage of AM fungal colonization (Takeda et al., 2009), these results showed that kinase-only GOF-CCaMK could activate the early signaling pathway in AMS. Failure of induction by full-length CCaMK<sup>TD</sup> indicates that the regulatory domains play an inhibitory role in the activation of the AMS signaling pathway upstream of the AMS-induced gene *SbtM1*.

### Histochemical Examination of the RNS and AMS Genes Induced by GOF-CCaMK

Since some symbiosis genes, such as *NIN*, express in epidermis as well as in cortex in RNS, a similar regulation mechanism is



**Figure 1.** Symbiosis-Induced Gene Expression in Wild-Type and *ccamk* Mutant Roots Induced by Transformation with GOF-CCaMK Variants.

Induction of *NIN*, *SbtM4*, *SbtS*, *SbtM1*, and *PT4* in 2-week-old hairy roots carrying each of the GOF-CCaMK variants was analyzed. Fold induction levels in wild-type Gifu (A) and in *ccamk* (B) were calculated compared with that of transgenic hairy roots carrying a control vector (p35S:GFP) (expression level = 1). Total RNA was extracted from more than 10 roots per experiment. Error bars indicate SD ( $n = 3$  to 6 independent experiments).

supposed in AMS. Therefore, we wondered whether our GOF-CCaMK could mimic tissue expression pattern of symbiosis genes. The tissue-specific activation of the RNS and AMS signaling pathways was examined histochemically using transgenic plants carrying  $\beta$ -glucuronidase (*GUS*) genes driven by the *NIN* and *SbtM1* promoters, respectively. In *snf1* mutants, *NIN* promoter-GUS (*NIN<sub>pro</sub>:GUS*) expression is limited to dividing cortical cells and SPNs, and no *GUS* activity is detected in epidermal cells (Tirichine et al., 2006). The series of GOF-CCaMK variants was introduced into the *NIN<sub>pro</sub>:GUS* plants to examine the activation of the RNS pathway. At 1 week after transfer of the plants to sterilized soil, *GUS* activity was not detected in hairy roots carrying full-length CCaMK<sup>TD</sup> (0/22; *GUS*-positive roots/all transgenic roots examined), in agreement with the results in *snf1* mutants. However, hairy roots carrying any of the kinase-only GOF-CCaMK variants (CCaMK<sub>314</sub>, 12/29; CCaMK<sub>314</sub>-NLS, 16/20; CCaMK<sub>314</sub><sup>TD</sup>, 24/36; CCaMK<sub>314</sub><sup>TD</sup>-NLS, 37/47; *GUS*-positive roots/all transgenic roots examined) except for the NES fusion (CCaMK<sub>314</sub><sup>TD</sup>-NES, 0/9; *GUS*-positive roots/all transgenic roots examined) showed *GUS* activity. The *GUS* activity was initially detected in the root epidermal layer (Figures 2A and 2B) and shifted later to dividing cortical cells (Figure 2C). The spatial expression pattern was similar to *NIN* expression induced upon rhizobial infection (Grønlund et al., 2005). The fact that no induction of *GUS* activity can be observed in *NIN<sub>pro</sub>:GUS* roots carrying CCaMK<sub>314</sub><sup>TD</sup>-NES is consistent with the results of the expression analysis by real-time PCR (Figure 1A). The histochemical expression analysis demonstrates that the nuclear-localized kinase-only GOF-CCaMK variants, but not full-length CCaMK<sup>TD</sup>, activate the RNS signaling pathway in epidermal cells at the time points examined.

The series of GOF-CCaMK variants was introduced into *SbtM1* promoter-GUS (*SbtM1<sub>pro</sub>:GUS*) plants by means of hairy root transformation, and the transgenic roots were grown in the absence of AM fungal infection. The roots showed *GUS* staining induced by the GOF-CCaMK variants consistent with the real-time PCR analysis of *SbtM1* expression (Figures 1A). All of the kinase-only GOF-CCaMK variants activated the AMS signaling pathway since *GUS* staining was observed in the uninfected roots (CCaMK<sub>314</sub>, 12/40; CCaMK<sub>314</sub>-NLS, 30/50; CCaMK<sub>314</sub><sup>TD</sup>, 46/87; CCaMK<sub>314</sub><sup>TD</sup>-NLS, 57/97; *GUS*-positive roots/all transgenic roots examined) except for the NES fusion (CCaMK<sub>314</sub><sup>TD</sup>-NES, 0/49; *GUS*-positive roots/all transgenic roots examined). Full-length CCaMK<sup>TD</sup> (0/75; *GUS*-positive roots/all transgenic roots examined) did not activate this pathway. The *GUS* activity was detected both in epidermal and in cortical cells at 1 week after transferring to sterilized soil (Figures 2D and 2E), whereas the *GUS* staining was confined only to cortical cells in 2-week-old hairy roots (Figure 2F). Interestingly, discrete rows of the *GUS*-positive cortical cells showed higher *GUS* activity than that seen in neighboring cells (Figure 2F). This type of *GUS* staining pattern was similar to that induced by AM fungal infection (Figures 2G to 2I). In *SbtM1<sub>pro</sub>:GUS* roots infected with AM fungi, the intense *GUS* staining corresponded to cells that adjoined elongated fungal hyphae or that contained arbuscules (Figures 2H and 2I; Takeda et al., 2009). These results indicate that the position of the cortical cells where fungal hyphae elongate in between and/or where arbuscules develop is likely to be deter-

mined downstream of CCaMK and independent of actual infection by AM fungi.

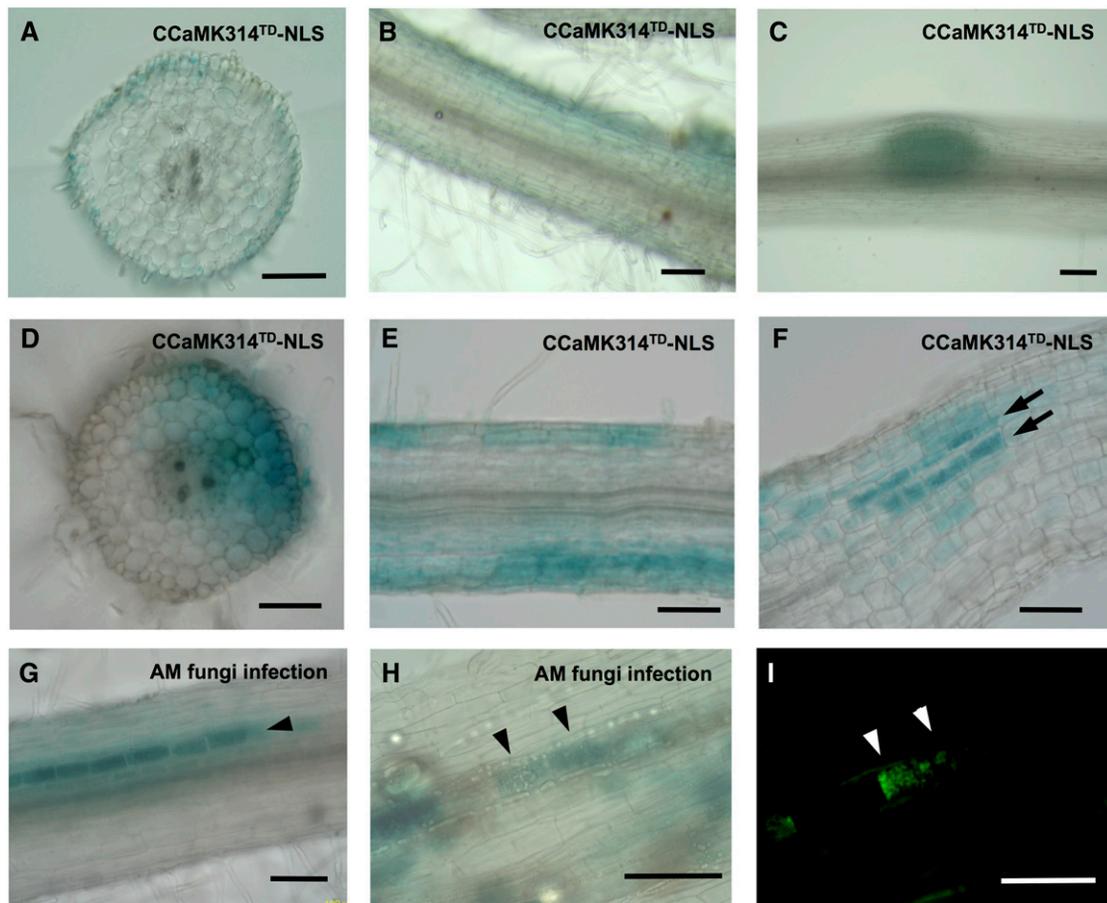
### Activation of SPN Formation by Nuclear-Localized GOF-CCaMK Variants

All of the GOF-CCaMK variants induced SPN formation in transgenic hairy roots, in contrast with wild-type CCaMK (CCaMK<sup>TT</sup>) (Table 1; see Supplemental Figure 3 online). The SPNs induced by GOF-CCaMK variants were structurally comparable to those in *snf1* (Tirichine et al., 2006). The frequency of SPN formation induced by kinase-only GOF-CCaMK was lower than that induced by full-length CCaMK<sup>TD</sup> (Table 1), despite the fact that hairy roots carrying full-length CCaMK<sup>TD</sup> showed lower expression of RNS-induced genes than those carrying the nuclear-localized kinase-only GOF-CCaMK variants (Figure 1A). The difference in frequency of SPN formation between full-length CCaMK<sup>TD</sup> and kinase-only GOF-CCaMK is likely to be caused by the presence of the regulatory domains of CCaMK (i.e., the CaM BD and EF hands). RNS-induced gene expression in cortical cells appears to be necessary and sufficient for SPN formation, indicating that epidermal expression may not correlate with SPN formation. The transgenic roots carrying the NES fusion formed small, distorted SPNs (see Supplemental Figure 3 online), but the frequency was very low (Table 1). This SPN formation might have been induced by residual nuclear localization of CCaMK<sub>314</sub><sup>TD</sup>-NES (see Supplemental Figure 2G online). These results show that the nuclear localization of GOF-CCaMK is required for SPN formation.

Both RNS and AMS gene induction as well as SPN formation might be caused by the activity of endogenous CCaMK in wild-type plants. To test this possibility, we introduced the series of GOF-CCaMK variants into *ccamk* mutant roots and examined the symbiotic responses. In *ccamk* transgenic hairy roots, nuclear-localized GOF-CCaMK triggered expression of RNS- and AMS-induced genes and SPN formation similar to those in wild-type plants (Figure 1B, Table 1). These results suggest that activation of the RNS/AMS signaling pathway and induction of the downstream symbiosis genes by the nuclear-localized kinase-only GOF-CCaMK variants are independent of endogenous wild-type CCaMK, strengthening the idea that the regulatory domains are not essential for RNS/AMS gene induction and SPN formation. In the expression analysis, the level of RNS/AMS gene induction in *ccamk* mutants was generally higher than that in wild-type plants, and induction of RNS-induced genes was also observed in hairy roots carrying full-length CCaMK<sup>TD</sup> (Figure 1B). Relatively higher expression in *ccamk* mutants could be caused by the difference in basal gene expression in uninfected roots carrying the vector control between *ccamk* mutants and wild-type plants.

### Complementation of Symbiont Infection Defects in *ccamk* mutants by the GOF-CCaMK Variants

To further examine the functional features of the GOF-CCaMK variants, we attempted to complement the defect in bacterial entry in *ccamk* mutants by transformation with each of the variants. Among the *ccamk* mutants transformed with GOF-CCaMK, only



**Figure 2.** Histochemical Detection of *NIN* and *SbtM1* Induction by GOF-CCaMK.

(A) to (F) *CCaMK314<sup>TD</sup>-NLS* was introduced into transgenic plants carrying *NIN<sub>pro</sub>:GUS* [(A) to (C)] or *SbtM1<sub>pro</sub>:GUS* fusions [(D) to (F)] by hairy root transformation. Activity of GUS was detected 1 week [(D) and (E)] or 2 weeks after transferring 10-d-old hairy roots to sterilized soil [(A) to (C) and (F)]. (A) and (B) Cross section (A) and longitudinal section (B) (100- $\mu$ m thick) of the hairy root carrying *CCaMK314<sup>TD</sup>-NLS* showed the *NIN<sub>pro</sub>:GUS* activity in epidermal cells.

(C) The activity was also detected in dividing cortical cells which would develop into a SPN (whole mount).

(D) to (F) Cross section (D) and longitudinal sections [(E) and (F)] (100- $\mu$ m thick) of the hairy root containing *CCaMK314<sup>TD</sup>-NLS* showed the *SbtM1<sub>pro</sub>:GUS* activity in epidermal and cortical cells. Arrows indicate cortical cells with strong GUS activity (F).

(G) to (I) *SbtM1* promoter GUS plants without GOF-CCaMK were infected with *G. intraradices* for 2 weeks and examined for the GUS activity (whole mount). The AM infected roots were stained for the GUS activity [(G) and (H)], and the AM fungi were visualized with WGA Alexa Fluor 488 (I). Arrowheads indicate cortical cells with strong GUS activity [(G) and (H)]. The GUS-positive cells in (H) correspond to the arbuscule-containing cells (indicated by white arrowheads in [I]).

Bars = 100  $\mu$ m.

hairy roots carrying full-length *CCaMK<sup>TD</sup>* recovered the ability to form infection threads and to allow entry of rhizobia into nodules, comparable to that of the wild-type *CCaMK<sup>TT</sup>* (Table 1; see Supplemental Figure 3 online). On the other hand, the nuclear-localized kinase-only GOF-CCaMK variants did not complement the bacterial entry process in *ccamk* mutants (Table 1; see Supplemental Figure 3 online) even though they induced expression of RNS genes to higher levels than did full-length *CCaMK<sup>TD</sup>* (Figure 1B) and caused SPN formation. (We presume that the nodule-like structures observed in hairy roots transformed with the GOF-CCaMK variants are SPNs induced by the variants independent of bacterial infection.) These results sug-

gest that the regulatory domains of *CCaMK* play a crucial role in infection thread formation and bacterial entry into nodules, as shown by Gleason et al. (2006).

We subsequently examined whether any of the GOF-CCaMK variants could complement the AMS-deficient phenotypes in *ccamk* mutant roots. In this analysis, the defect in AM fungal colonization in the *ccamk* mutants was complemented by all of the GOF-CCaMK constructs except for the NES fusion (Table 1; see Supplemental Figure 3 online). Hyphal entry into the host root as well as formation of arbuscules and vesicles was observed in the transformed hairy roots. The very low complementation frequency obtained with the NES fusion (Table 1) was consistent

**Table 1.** SPN Formation and Complementation of *ccamk* Mutants by GOF-CCaMK

CCaMK Variant	Spontaneous Nodule Formation <sup>a</sup>		Symbiont Entry into <i>ccamk</i> Mutants <sup>a</sup>	
	Wild Type	<i>ccamk</i>	Rhizobia	Mycorrhiza
Vector control	0% (0/64)	0% (0/35)	0% (0/16)	0% (0/55)
CCaMK <sup>TT</sup> (wild type)	0% (0/40)	0% (0/45)	86% (32/37)	79% (27/34)
CCaMK <sup>TD</sup>	87% (41/47)	41% (14/34)	75% (15/20)	64% (18/28)
CCaMK <sub>314</sub>	16% (5/32)	32% (11/34)	0% (0/14)	29% (9/31)
CCaMK <sub>314</sub> -NLS	39% (9/23)	26% (6/23)	0% (0/27)	17% (5/30)
CCaMK <sub>314</sub> <sup>TD</sup>	17% (5/29)	33% (9/27)	0% (0/24)	18% (9/50)
CCaMK <sub>314</sub> <sup>TD</sup> -NLS	15% (3/19)	15% (6/39)	0% (0/25)	57% (16/28)
CCaMK <sub>314</sub> <sup>TD</sup> -NES	2% (1/48)	2% (1/53)	0% (0/35)	2% (1/43)

Values in parentheses indicate numbers of SPN formed or complemented roots/total transformed roots.

<sup>a</sup>SPN formation was observed at 4 weeks after transferring to a sterilized soil. Infection of rhizobia or arbuscular mycorrhiza was observed at 4 and 3 weeks after inoculation, respectively.

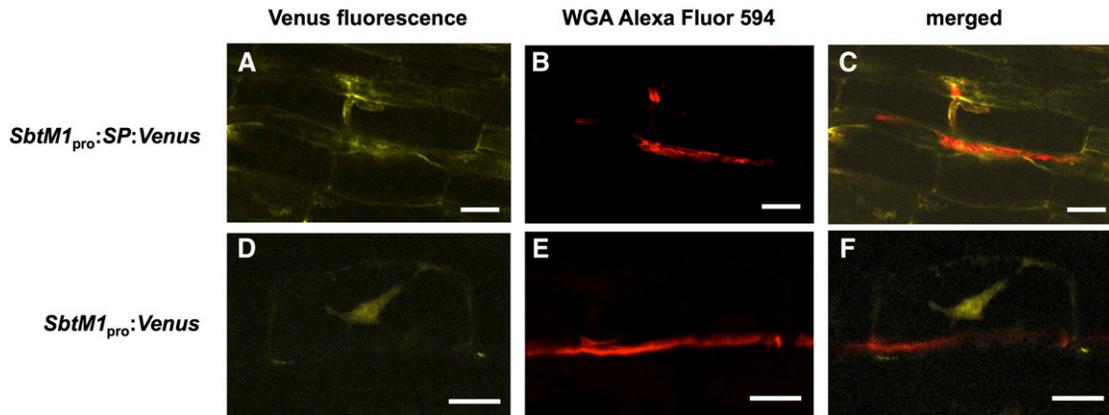
with the result that AMS-induced gene expression was not significant in inoculated hairy roots carrying the NES fusion (see Supplemental Figure 4 online), again suggesting that the nuclear localization of GOF-CCaMK is also important for its signaling activity in AMS. Induction of *PT4* expression was also restored by the series of GOF-CCaMK variants, except for the NES fusion, upon infection by AM fungi (see Supplemental Figure 4 online). *PT4* expression was not induced in hairy roots carrying GOF-CCaMK in the absence of AM fungi, despite the presence of *SbtM1* expression (Figure 1). These results indicate either that infection by AM fungi and activation by CCaMK are both required for *PT4* induction or that fungal infection suppresses a mechanism that inhibits *PT4* expression downstream of CCaMK.

### Remodeling of a Cytoplasmic Structure by GOF-CCaMK Variants

In addition to AMS-induced gene expression, we examined cytological changes induced by the GOF-CCaMK variants at the preinfection stage of AMS. *SbtM1* is expressed in and secreted from arbuscule-containing cells (Takeda et al., 2009). In addition, a gene encoding a fluorescent protein (Venus) fused with the signal peptide of *SbtM1* under the control of the *SbtM1* promoter (*SbtM1*<sub>pro</sub>:*SP:Venus*; Takeda et al., 2009) showed that hyphal penetration into cortical layers induced expression of *SbtM1* (Figures 3A to 3C). The result indicates that the cortical cells expressing *SbtM1* correspond to cells penetrated by hyphae. To examine the cytoplasmic remodeling in the cells before fungal penetration, the cytoplasm was visualized with Venus fusion protein driven by the *SbtM1* promoter (*SbtM1*<sub>pro</sub>:*Venus*; Takeda et al., 2009). PPA formation was expected to appear as a dense cytoplasmic bridge in cortical cells. The *SbtM1*<sub>pro</sub>:*Venus* construct was introduced by means of hairy root transformation, and AM fungi were allowed to infect the roots for 2 weeks. Cortical cells along which the AM hyphae elongated expressed *SbtM1*<sub>pro</sub>:*Venus*, and the fluorescence revealed development of a cytoplasmic bridge before fungal penetration into the cells (Figures 3D to 3F). The cytoplasmic bridges visualized by Venus fluorescence were dense and often showed a particular pattern: Strands of the cytoplasm elongated from the nucleus to the

opposite side of the cell membrane (Figure 3D). This polar distribution of the dense cytoplasm was not typically observed in cortical cells without fungal infection, in which normal transvacuolar strands, if any, are very thin and spread randomly from the nucleus (see Supplemental Figures 5A and 5B online). These results demonstrate that PPAs are also formed in *L. japonicus*, in which expression of *SbtM1* is a suitable marker for the PPA-containing cells.

Using this test system, we investigated whether remodeling of a cytoplasmic structure was induced by the kinase-only GOF-CCaMK variants. We examined transgenic roots carrying both CCaMK<sub>314</sub><sup>TD</sup>-NLS and *SbtM1*<sub>pro</sub>:*Venus* in the absence of AM fungal infection. In the transgenic roots, the *SbtM1* promoter was activated by CCaMK<sub>314</sub><sup>TD</sup>-NLS, and Venus fluorescence was observed in cortical cells (Figure 4). As we observed in the histochemical expression analysis using the GUS fusion (Figure 2F), various strengths of fluorescence were detected in cortical cells (Figure 4A). The AMS signaling pathway appeared to be activated in the fluorescent cells. Although a majority of the cells showed weak Venus fluorescence in the nucleus and in thin transvacuolar strands, a few numbers of cells exhibiting strong fluorescence (>2% of detectable Venus-expressing cells, *n* = 122) showed dense cytoplasmic bridges (Figures 4A and 4B) in the cortical layers but not in the epidermal layer. Development of the cytoplasm was especially obvious around the nucleus, and the structures were nearly identical to the PPAs induced by AM fungal infection (Figures 3D to 3F; Genre et al., 2005, 2008). Formation of the PPA-like structure was observed independent of the developmental stage of cortical cells in which *SbtM1* expression was highly induced, but the well-developed PPA-like structures tended to be found more often in the young cortical cells (Figures 4A and 4B; see Supplemental Figures 5C and 5D online). The cells with the PPA-like structures were often found to be next each other (Figure 4A) and adjacent to each other (Figure 4B; see Supplemental Figures 5C and 5D online). The other neighboring cells without Venus fluorescence did not show such a dense cytoplasmic bridge (Figures 4C to 4E). Formation of the PPA-like structure was also observed using GFP fused with the ER retention signal (HDEL = HisAspGluLeu) under the control of the *SbtM1* promoter (*SbtM1*<sub>pro</sub>:GFP-HDEL) (see Supplemental



**Figure 3.** Induction of a PPA by AM Fungal Infection.

Transgenic hairy roots carrying *SbtM1<sub>pro</sub>:SP:Venus* (**A**) to (**C**) or *SbtM1<sub>pro</sub>:Venus* (**D**) to (**F**) were infected with *G. intraradices* for 2 weeks. The images of Venus fluorescence (**A**) and (**D**) and fungal structure visualized by WGA Alexa Fluor 594 (**B**) and (**E**) are merged (**C**) and (**F**). Secreted Venus fluorescent protein was detected in the apoplastic space where the AM fungal hypha had penetrated into the host cell (**C**). A dense cytoplasmic bridge visualized by Venus fluorescence was detected in a cortical cell adjacent to an AM hypha (**F**). Bars = 25  $\mu$ m.

Figure 6 online). The PPAs were induced in GFP-HDEL-expressing cortical cells by AM fungal infection (see Supplemental Figure 6A online). In the PPA-like structure, tubular ER structures induced by CCaMK<sub>314</sub><sup>TD</sup>-NLS were formed in the cytosol (see Supplemental Figure 6B online; Genre et al., 2005, 2008). However, the ER structures were not evident when compared with those induced by AM fungal infection, which indicates that actual infection is necessary for developing the extensive ER structures.

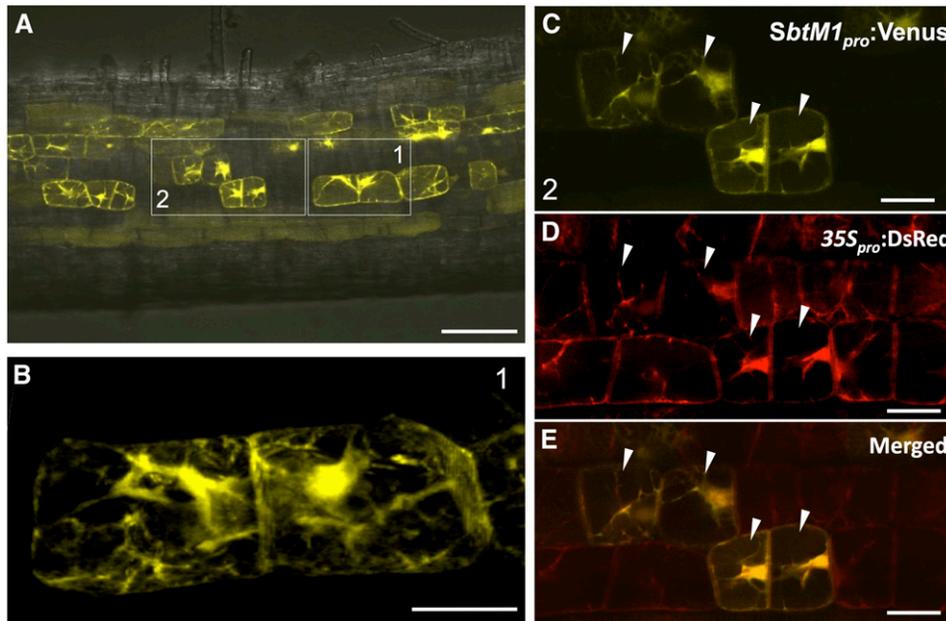
It was reported that the PPA-containing cells exhibit enlargement of the nucleus (Genre et al., 2008). To confirm whether the PPA-like structure accompanied enlarged nuclei, the nuclear size was measured in cortical cells (Table 2; see Supplemental Figure 7 online). With AM fungal infection, the PPAs were induced in *SbtM1<sub>pro</sub>:Venus*-expressing cells, in which the nucleus was significantly enlarged compared with that in cortical cells of uninfected roots or cortical cells without Venus fluorescence neighboring to the PPA formed cells. Cells with Venus fluorescence induced by CCaMK<sub>314</sub><sup>TD</sup>-NLS also showed statistically significant enlargement of the nucleus, although the degree of enlargement was less than that induced by AM fungal infection. Enlargement of the nucleus was not observed in cortical cells without Venus fluorescence in the same region. Above observations confirm that the PPA-like structure induced by the nuclear-localized kinase-only GOF-CCaMK variants alone appears to have similar identity to the PPA that is formed in response to AM fungi.

## DISCUSSION

### Regulation of CCaMK by Ca<sup>2+</sup> Signals

In this study, we investigated the functions of CCaMK in symbiosis signaling by conferring a GOF to the protein (a summary of the results is shown in Table 3). The nuclear-localized kinase-only GOF-CCaMK variants, in contrast with full-length CCaMK<sup>TD</sup>,

triggered strong RNS-induced gene expression in epidermal cells of *L. japonicus* (Figures 1 and 2). In *M. truncatula*, there is no substantial difference in spontaneous activation of one of the early nodulins *ENOD11* expression between the kinase-only and the full-length GOF-CCaMK variants (Gleason et al., 2006). The available information on tissue-specific expression of *NIN* led us to hypothesize that epidermal expression of *NIN* by kinase-only GOF-CCaMK would be sufficient for infection thread formation and bacterial entry into the host, leading to complementation of *ccamk* mutants. However, our analysis revealed that epidermal activation of the RNS signaling pathway by the nuclear-localized kinase-only GOF-CCaMK variants was not sufficient for complementation of the infection defect in *ccamk* mutants (Table 1). This result indicates importance of the regulatory domains at the C terminus of CCaMK for bacterial entry (see Supplemental Figure 8 online). It has been suggested that different Nod factor molecular structures are required between bacterial entry and cortical cell division, resulting in bacterial accommodation (Ardourel et al., 1994). Our analyses showed that CCaMK integrates both events. According to our model (see Supplemental Figure 8 online), three functions of CCaMK (nuclear localization, kinase activity, and regulation by the C-terminal domains) regulate symbiosis signaling for expression of symbiosis-induced genes, cortical cell division/SPN formation, and bacterial entry. Importantly, whereas the regulatory domains play both a negative role in kinase activity and a positive role in bacterial entry in RNS, they are less important in AMS, in which the regulatory domains are not strictly required for successful AMS. AMS is an evolutionarily older type of symbiosis than RNS (Parniske, 2008), so that the signaling pathway in AMS is expected to be simpler than that in RNS. This may explain why the nuclear-localized kinase-only CCaMK variants are able to complement the defective AMS phenotype in the *ccamk* mutant but are unable to complement part of the defective RNS phenotype in the same mutant. Activation of the AMS signaling pathway is likely to be one of the fundamental aspects of the CCaMK functions in the symbioses.



**Figure 4.** Induction of a PPA-Like Structure by GOF-CCaMK.

(A) Hairy roots cotransformed with CCaMK<sub>314</sub><sup>TD</sup>-NLS and *SbtM1<sub>pro</sub>::Venus* were observed after 3 weeks of growth under aseptic conditions. Laser transmission and Venus fluorescence images were merged.

(B) A stack of images obtained along the z axis of cells corresponding to box 1 region in (A) strongly expressed *SbtM1<sub>pro</sub>::Venus* and showed dense cytoplasmic bridges.

(C) to (E) Venus, DsRed, and merged images in the box 2 region in (A).

(C) Cortical cells expressing *SbtM1<sub>pro</sub>::Venus* showed the PPA-like structure.

(D) Cytosol was visualized with a transformation marker DsRed under the control of the 35S promoter.

(E) Merged image of Venus (C) and DsRed (D) showed cells (arrowheads) with dense cytoplasmic bridges compared with the neighboring cells.

Bars = 100 μm in (A) and 25 μm in (B) to (E).

The deregulation of symbiosis signaling by the truncated CCaMK protein emphasizes the negative regulatory role of the C-terminal domains, that is, the CaM BD and the three EF hands (Gleason et al., 2006). The C-terminal domains have been reported to possess an autoinhibitory activity through their ability to change conformation (Sathyanarayanan et al., 2000; Harper et al., 2004). In the inactive form, the folded C-terminal region covers the kinase domain. The physical barrier created by the C-terminal region inhibits the activation of the kinase domain. Activation of the symbiosis signaling pathway leads to unfolding of the protein, which enables the kinase domain either to make contact with an upstream activator or to be self-activated through autophosphorylation, or a combination of both. The presence of the CaM BD and

EF hands in the C terminus of CCaMK indicates that Ca<sup>2+</sup> is a trigger for the conformational change of CCaMK. Alteration of Ca<sup>2+</sup> concentration is sensed by the EF hands or the CaM BD through calmodulin, or both. Thus, Ca<sup>2+</sup> binding affects the conformation of CCaMK. In RNS, Ca<sup>2+</sup> influx and Ca<sup>2+</sup> spiking are known to occur early in the symbiotic response. The Ca<sup>2+</sup> influx, which is a transient flux of Ca<sup>2+</sup> into a root hair cell, is observed several minutes after application of Nod factors (Cárdenas et al., 1999; Felle et al., 1999). Periodic Ca<sup>2+</sup> oscillation known as Ca<sup>2+</sup> spiking is observed in the root hair ~10 min after treatment with Nod factor (Ehrhardt et al., 1996). The largest amplitude of Ca<sup>2+</sup> spiking is associated with the nucleus, in which a characteristic fluctuation of Ca<sup>2+</sup> is thought to encode a

**Table 2.** Nuclear Size in Cortical Cells

Treatment	Nuclear Diameter (μm)	Minimum (μm)	Maximum (μm)	n
Uninfected root (vector control)	5.60 (0.79) <sup>a</sup>	4.05	7.54	56
AM infected root (no fluorescence)	6.28 (1.10) <sup>b</sup>	4.38	9.24	53
AM infected root (Venus fluorescence)	9.18 (1.58) <sup>c</sup>	6.54	13.19	25
CCaMK <sub>314</sub> <sup>TD</sup> -NLS (no fluorescence)	6.68 (1.43) <sup>b</sup>	4.55	10.41	84
CCaMK <sub>314</sub> <sup>TD</sup> -NLS (Venus fluorescence)	7.85 (1.44) <sup>d</sup>	5.64	11.12	71

Values in parentheses indicate standard deviation. Letters indicate statistically significant differences at P < 0.05 (Tukey-Kramer's multiple comparison).

**Table 3.** Symbiotic Functions of the CCaMK Variants in RNS and AMS

CCaMK Variants	GOF (without Symbionts)				Complementation			
	Structural Change		Gene Induction		Symbiont Entry			
			RNS ( <i>N/N</i> )					
SPN	PPA	Epi	Cor	<i>SbtM1</i>	<i>PT4</i>	RNS	AMS	
CCaMK <sup>TT</sup> (wild type)	–	–	–	–	–	–	+	+
CCaMK <sup>TD</sup>	+	–	–	+	–	–	+	+
CCaMK <sub>314</sub> <sup>TD</sup> -NLS	+	+	+	+	+	–	–	+
CCaMK <sub>314</sub> <sup>TD</sup> -NES	–	–	–	–	+	–	–	–

Cor, expression in cortical cells; Epi, expression in epidermal cells; +, detected, –, not or scarcely detected.

symbiosis signal (Sieberer et al., 2009). Furthermore, Ca<sup>2+</sup> spiking is also observed in AMS, indicating that Ca<sup>2+</sup> spiking is a signaling factor in the common Sym pathway (Kosuta et al., 2008; Chabaud et al., 2011). These facts suggest that Ca<sup>2+</sup> spiking is a candidate for an activator of CCaMK. The importance of the nuclear localization of CCaMK demonstrated in this work further supports the relationship of CCaMK with Ca<sup>2+</sup> spiking. Although it has not yet been proven by direct evidence, it is worth considering the possibility that CCaMK is a decoder of the Ca<sup>2+</sup> spiking signal.

### Importance of Nuclear Localization of CCaMK for Downstream Signaling

The exclusion of GOF-CCaMK from the nucleus abolished or reduced its signaling activity, which indicates that the nuclear localization of CCaMK is essential for the proper activation of the signaling pathway both in AMS and in RNS. Activation of downstream signaling events such as that of transcription factors is likely to occur in the nucleus. It is still unknown whether the C terminus of CCaMK contains a functional NLS or interacts with a protein containing an NLS. CYCLOPS, a common Sym protein and a CCaMK interactor, is predicted to possess two NLS motifs and is localized in the nucleus (Yano et al., 2008). It is therefore possible that the association with CYCLOPS may enhance the nuclear localization of CCaMK. In addition, kinase-only CCaMK fails to interact with CYCLOPS (Yano et al., 2008). This is in agreement with the observation that epidermal expression of *N/N*, which is normally triggered by rhizobial infection, seems severely compromised in *cyclops* mutants (Yano et al., 2008). However, because *ccamk* mutant roots transformed with the nuclear-localized kinase-only GOF-CCaMK variants by NLS fusion still show defects in infection similar to those seen in *cyclops* mutants, it is unlikely that the function of CYCLOPS is solely to confer nuclear localization to CCaMK.

The proper CCaMK function requires its nuclear localization, indicating that the target protein is probably also nuclear localized. CYCLOPS is a target of phosphorylation by CCaMK (Yano et al., 2008); however, its loss-of-function phenotype differs from that of CCaMK. In *cyclops*, infection by rhizobia induces formation of nodule primordia in the cortex and colonization of rhizobia on root hairs. On the other hand, *ccamk* mutants show no distinct

symbiotic phenotypes in RNS other than root hair deformation and Ca<sup>2+</sup> spiking. These facts indicate that CYCLOPS functions as a secondary target of CCaMK. Initial phosphorylation targets of CCaMK are likely to be symbiosis-related transcriptional regulators, such as NSP1, NSP2, or NIN (Schauser et al., 1999; Hirsch et al., 2009). Since the loss-of-function phenotypes of these three regulators are partially similar to those of CCaMK (i.e., no cortical cell division upon infection), it is plausible that these regulators are directly or indirectly phosphorylated by CCaMK and then activate transcription of a number of symbiosis-induced genes. The AMS-specific pathway may be regulated by a similar mechanism, although no such AMS-specific transcriptional regulators have yet been identified from genetic analyses.

### AMS Signaling and Downstream Events Regulated by CCaMK

The interaction of *L. japonicus* and *Glomus intraradices* shows Arum-type hyphal elongation, in which intercellular hyphae develop to form intracellular arbuscules, as occurs in *M. truncatula* (Dickson, 2004; Genre et al., 2005, 2008). AM hyphae elongate longitudinally between cortical cells and often penetrate into the cell when they cross the cell transversely or form an arbuscule. Our results revealed that PPA formation coincided with *SbtM1* expression, suggesting that PPA formation would be observed not only in cortical layers where fungal penetration occurs, but also in arbuscule-containing cells where *SbtM1* is strongly induced (Figure 3). The system for fungal penetration into the host cell may be shared by the processes of hyphal elongation and arbuscule formation, as indicated by Genre et al. (2008). Our experimental method was unable to show whether AM fungi could enter the cells containing the PPA-like structures that had been induced by kinase-only GOF-CCaMK. However, Genre et al. (2005) have shown that PPA formation precedes fungal invasion into the host cell, which requires the common *Sym* genes. Our results have confirmed that the PPA forms before fungal penetration, which is likely to be regulated by CCaMK in the common *Sym* pathway. The PPA-like structures we observed here are confirmed by two characters: cytoplasmic remodeling and nuclear enlargement (Figure 4, Table 2; see Supplemental Figure 6 online). The PPAs induced by AM fungal infection have three characteristics: cytoplasmic remodeling that

is visualized by cytoskeletons or ER, nuclear migration, and nuclear enlargement (Genre et al., 2005, 2008). Nuclear migration is triggered by AM fungal infection even in common *Sym* mutants (Genre et al., 2005), so that we regard two characteristics (i.e., cytoplasmic remodeling and nuclear enlargement) as being sufficient for the identity of the PPAs, which are also observed in the PPA-like structures. The PPA-like structures were not formed at the epidermal layer despite of *SbtM1* expression in this layer. This can be due to the fact that in *L. japonicus*, the majority of AM fungal invasion in the epidermal layer is apoplastic and requires epidermal opening (Demchenko et al., 2004; Parniske, 2004; Kistner et al., 2005). The identity and/or responses of the epidermal layer to AM fungi may differ between *L. japonicus* and *M. truncatula*.

We found that expression of *L. japonicus* *PT4*, an AMS-specific gene, responded differently to the GOF-CCaMK variants and AM fungal infection. The lack of *PT4* induction by the GOF-CCaMK variants in the absence of fungal infection indicates that one or more other factors, possibly triggered by the presence of AM fungi, are required (see Supplemental Figure 8 online). In addition, the regulatory domains, which are required for bacterial entry in RNS, are not required for induction of *PT4* expression. These facts indicate that other signals may be produced after fungal entry that additionally activate the kinase domain of CCaMK, which is not conferred by the GOF mutation. Alternatively, it is possible that a completely different pathway exists for signaling of symbiosis responses downstream of fungal entry. One candidate for the triggering factor is lysophosphatidylcholine, identified by Drissner et al. (2007) as an inducer of the phosphate transporter in maize (*Zea mays*). After PPA formation and fungal entry, this factor may induce late symbiotic responses, including *PT4* expression. Another, but not exclusive, idea is that supply of phosphorus by AM fungi directly induces *PT4* expression. In RNS, symbiosis-induced genes have been categorized as early-nodulin and late-nodulin genes (Mylona et al., 1995). AMS-induced responses can also be categorized as early and late; for example, *SbtM1* and *PT4* can be considered as early and late AMS-induced genes, respectively.

Our analyses of the GOF-CCaMK variants revealed that alterations in the regulatory domains and nuclear localization resulted in different degrees of activation of CCaMK and, as a consequence, different symbiotic responses. This feature of CCaMK suggests the possibility that CCaMK may function in sorting AMS and RNS signals within the common *Sym* pathway. The detailed regulation mechanism of the regulatory domains of CCaMK is currently under investigation. Such analysis will provide further insights into the mechanisms of CCaMK signaling and possibly serve to identify upstream activators and downstream signaling factors.

## METHODS

### Plant Growth

Seeds of *Lotus japonicus* accession Gifu B-129, *ccamk-3* (Tirichine et al., 2006), *NIN<sub>pro</sub>:GUS* (Radutoiu et al., 2003), or *SbtM1<sub>pro</sub>:GUS* (Takeda et al., 2009) transgenic plants were scarified, germinated on 0.8% agar plates, and sterilized with 0.1% sodium hypochlorite. Seedlings were grown in a growth chamber (24°C, 16 h light/8 h dark). For SPN formation,

the transgenic plants carrying GOF-CCaMK were grown in sterilized expanded vermiculite (Nittai) supplied with water for 4 weeks. The *ccamk-3* plants transformed with GOF-CCaMK variants were inoculated with *Mesorhizobium loti* carrying *DsRED* (Maekawa et al., 2009) and then grown under the same conditions as above. For induction of the PPA-like structure, plants transformed with the GOF-CCaMK variants were grown in sterilized 1:1 (v/v) mixture of vermiculite and commercial soil for turf grass (Sunbellex) according to Banba et al. (2008) and supplied with water. The AM fungus *Glomus intraradices* (DAOM197198; PremierTech) was propagated in a chive (*Allium schoenoprasum*) nurse pot system (Demchenko et al., 2004) made using the same soil as induction of the PPA-like structure. The pot was used for AM fungi inoculation after removal of the chive plants.

### Construction of Binary Vectors

The kinase-only GOF-CCaMK variants were amplified by PCR (forward primer, 5'-CACCATGGGATATGATCAAACCAGAA-3'; reverse primer, 5'-TTAAATCTCAGGGTCCATTGCT-3') from CCaMK<sup>TT</sup> or CCaMK<sup>TD</sup> constructs (Banba et al., 2008). NLS (ProLysLysLysArgLysValGluAsp) and NES (AsnGluLeuAlaLeuLysLeuAlaGlyLeuAspIleAsnLysThr) (Wen et al., 1995; Shen et al., 2007) were C-terminally joined to the coding sequence for the truncated CCaMK protein by PCR (NLS coding sequence, 5'-CCTAA GAAGAAGAGAAAAGTTGAGGAT-3'; NES coding sequence, 5'-AACG AGCTTGCTTTGAAATTGGCAGGATTAGATATTAACAAGACG-3'). The amplified fragments were cloned into pENTR D/TOPO (pENTR D/TOPO cloning kit; Invitrogen). The resulting entry clones were converted with the destination vector p35S-GW-GFP (Yano et al. 2008) or p35S-GW-DsRed (K. Yano and M. Hayashi, unpublished data) by the LR reaction (Gateway LR clonase II Enzyme Mix; Invitrogen). The destination vector without the Gateway cassette (p35S-GFP or p35S-DsRed) was used for the vector control.

### Plant Transformation

*Agrobacterium tumefaciens* AGL1 was used for the tobacco (*Nicotiana benthamiana*) infiltration transformation. The infiltration was performed as described by Yano et al. (2008). Transgenic hairy roots were induced by *Agrobacterium rhizogenes* LBA1334 (Offringa et al., 1986) following the method described by Diaz et al. (2005).

### Expression Analysis

Total RNA was extracted from roots using the RNeasy plant mini kit (Qiagen). Reverse transcription (RT) and real-time PCR were performed using the QuantiTect reverse transcription kit (Qiagen) and Light Cycler FastStart DNA Master<sup>PLUS</sup> SYBR Green I (Roche) with Light Cycler (Roche) according to the manufacturer's instructions. cDNA was synthesized from 100 ng total RNA in a 20- $\mu$ L RT reaction mixture. The RT product (0.8  $\mu$ L) was added to a total of 20  $\mu$ L real-time PCR reaction mixture. Real-time PCR primer sets and thermal cycler conditions were as described by Takeda et al. (2009). Ubiquitin was used as a reference gene (Takeda et al., 2005). The relative expression level of each gene was calculated with the delta cycle threshold method (Takeda et al., 2005) and normalized to the transcription level of ubiquitin. Three to six biologically independent experiments were performed to calculate the mean and SD values.

### Histochemistry and Microscopy Observation

Transgenic roots carrying *NIN<sub>pro</sub>:GUS* or *SbtM1<sub>pro</sub>:GUS* were treated with GUS staining buffer (0.5 mg/mL X-Gluc, 100 mM phosphate buffer, pH 7.0, 100 mM EDTA, 0.5 mM K<sub>4</sub>[Fe(CN)<sub>6</sub>], 0.5 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>], and 0.1% Triton X-100) at 37°C for 1 to 6 h.

Wheat germ agglutinin (WGA) Alexa Fluor 488 or 594 (Invitrogen) was used for visualization of fungal cell walls (Harrison et al., 2002). The transgenic roots infected with AM fungi were boiled with 2% KOH at 95°C for 20 min and washed three times with PBS. Infected roots were then stained with WGA Alexa Fluor (1 µg/mL final concentration) and kept at room temperature for more than 1 h. For the staining of living cells, transgenic roots carrying the Venus fusion constructs were embedded in 5% low-melting agarose (SeaPlaque Agarose; CAMBREX) without fixing, kept at 30°C during embedding, and sectioned with a VT1200S vibrating-blade microtome (Leica). The root sections (100 to 120 µm) were stained with 1 µg/mL WGA Alexa Fluor 594 or 488 in PBS for more than 1 h at room temperature. Nuclear staining in root cells was performed with 4',6-diamidino-2-phenylindole (Dojindo). Roots were soaked in 10 µg/mL 4',6-diamidino-2-phenylindole in half-strength PBS and vacuumed for 30 min. The roots were incubated for 3 h at room temperature and washed three times with half-strength PBS.

Bright-field and fluorescence microscopy were performed with a stereomicroscope (SZX12; Olympus) or an inverted microscope (BZ-9000; Keyence) equipped with ×10 dry (numerical aperture 0.45) and ×20 dry (numerical aperture 0.75) objectives. Venus fluorescence was detected with a confocal microscope (TCS SP5; Leica, Nikon A1) equipped with ×20 or ×40 dry objective lenses. Images were acquired and analyzed using the LAS-AF software package (Leica), NIS elements (Nikon), and Adobe Photoshop (Adobe Systems).

#### Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: AJ239041.1 (*NIN*), AP009542 (*SbtM4*), AP006864 (*SbtS*), AP009544 (*SbtM1*), AP010874 (*PT4*), and DQ249171.1 (*Ubiquitin*).

#### Supplemental Data

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

**Supplemental Figure 1.** Wild-Type CCaMK and GOF-CCaMK Variants Used in This Study.

**Supplemental Figure 2.** Localization of GOF-CCaMK.

**Supplemental Figure 3.** SPN Formation and Complementation of *ccamk* Mutants by GOF-CCaMK.

**Supplemental Figure 4.** Activation of AMS-Induced Genes by GOF-CCaMK in *ccamk* Mutants.

**Supplemental Figure 5.** Transvacuolar Strands and PPA-Like Structures Induced by GOF-CCaMK.

**Supplemental Figure 6.** PPA-Like Structures Visualized by ER-Retained GFP.

**Supplemental Figure 7.** Histograms of Nuclear Sizes in Cortical Cells.

**Supplemental Figure 8.** Model of CCaMK-Regulated AMS and RNS Signaling.

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#### AUTHOR CONTRIBUTIONS

N.T., T.M., and M.H. conceived and designed the experiments. N.T. and T.M. performed the experiments. N.T., T.M., and M.H. analyzed the data. N.T. and M.H. wrote the article with the assistance of T.M.

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**Nuclear-Localized and Deregulated Calcium- and Calmodulin-Dependent Protein Kinase  
Activates Rhizobial and Mycorrhizal Responses in *Lotus japonicus***

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