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The Genotype of the Calcium/Calmodulin-Dependent Protein Kinase Gene (*CCaMK*) Determines Bacterial Community Diversity in Rice Roots under Paddy and Upland Field Conditions▽†

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The effects of the *Oryza sativa* calcium/calmodulin-dependent protein kinase *OsCCaMK* genotype (dominant homozygous [D], heterozygous [H], recessive homozygous [R]) on rice root-associated bacteria, including endophytes and epiphytes, were examined by using a *Tos17* rice mutant line under paddy and upland field conditions. Roots were sampled at the flowering stage and were subjected to clone library analyses. The relative abundance of *Alphaproteobacteria* was noticeably decreased in R plants under both paddy and upland conditions (0.8% and 3.0%, respectively) relative to those in D plants (10.3% and 17.4%, respectively). Population shifts of the *Sphingomonadales* and *Rhizobiales* were mainly responsible for this low abundance in R plants. The abundance of *Anaerolineae* (*Chloroflexi*) and *Clostridia* (*Firmicutes*) was increased in R plants under paddy conditions. The abundance of a subpopulation of *Actinobacteria* (*Saccharothrix* spp. and unclassified *Actinosynnemataceae*) was increased in R plants under upland conditions. Principal coordinate analysis revealed unidirectional community shifts in relation to *OsCCaMK* gene dosage under both conditions. In addition, shoot length, tiller number, and plant weight decreased as the *OsCCaMK* gene dosage decreased under upland conditions. These results suggest significant impacts of *OsCCaMK* on both the diversity of root-associated bacteria and rice plant growth under both paddy and upland field conditions.

Legumes developed systems to attain mutual symbiosis with rhizobia and mycorrhizae during their evolution. The genetic requirements for rhizobial and arbuscular mycorrhizal (AM) fungal interactions in plants overlap in a common symbiosis pathway (CSP) that leads to successful root nodule (RN) and AM symbioses (21, 24, 46). Similarly, the negative control of the degree of nodulation and mycorrhization of roots is also regulated through a common signaling system, so-called autoregulation of nodulation (42). These findings raise the question of whether molecular components regulating RN and AM symbioses also affect other symbiotic microbes in the phytosphere.

Diverse microorganisms reside in and on plants as endophytes and epiphytes (11, 29, 35, 48). These symbiotic microbes assist plants in the uptake of nutrients (22), scavenge toxic compounds (5), and exert considerable influence upon the overall health of host plants (6). However, many questions remain about the driving forces and ecological rules underlying the relationships between these microbes and plants (12, 36).

Recently, it was shown that symbiosis-defective mutants of *Medicago truncatula* (30) and soybean (16, 32) possess bacterial and fungal communities in their roots different from those in wild-type host plants and that certain bacteria preferentially associate with mycorrhizal roots (41). These findings indicate that genetic alteration in RN/AM signaling pathways can also alter the microflora of the rhizosphere. Interestingly, analyses of the rhizosphere of soybeans indicated that the bacterial community structures of nonnodulated soybeans were more similar to those of hypernodulated soybeans than to those of wild-type soybeans (16). Since nodulation is autoregulated by signal transduction between root and shoot tissues (31), it is of

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interest to compare bacterial communities in shoots between wild-type and symbiosis-defective mutants. Indeed, the results of our previous study of stem- and leaf-associated bacteria suggested that a subpopulation of *Proteobacteria* in soybean was controlled through the system that regulates RN symbiosis (18–20).

Thus, it is worthwhile examining whether plant CSP mutants also change the microbial community in the phytosphere. The CSP plays an important role in accommodating RN and AM symbionts, by which plant cells actively decompose their cell wall structures to facilitate microbial colonization and endosymbiosis (34). It would be interesting to examine the intracellular and intercellular niches of the endophytic microbial communities that respond to CSP genes (19).

Orthologs of CSP are also well conserved in nonlegumes (50), and the equivalent functionality of these orthologs in nodulation and mycorrhization has been reported (3). Using rice mutant lines with a *Tos17* insertion, the essentiality of calcium/calmodulin (CaM)-dependent protein kinase (*CCaMK*), a central factor of CSP, for mycorrhization has been proven (3).

In this study, the impacts of the CSP on plant-associated microbes in nonleguminous plants were examined. Strong expression of *Oryza sativa CCaMK* (*OsCCaMK*) was detected in rice roots not only under aerobic conditions, where mycorrhization is expected to occur (3), but also under the anaerobic conditions of a paddy field throughout the growth stages of rice (39). The stable and strong expression of *OsCCaMK* in rice roots (see Fig. S1 in the supplemental material) implies its importance in rice. CCaMK is thought to be a decoder of Ca spiking signals, a distinctive physiological response to endosymbioses (13, 24), because of its structural similarity to CaMKII, which is activated by Ca oscillation in a frequency-dependent manner in animals (4).

The impacts of *OsCCaMK* genotype (dominant homozygous [D], heterozygous [H], and recessive homozygous [R]) on the root-associated bacterial community in rice plants were examined under both paddy and upland field conditions. Clone libraries of the 16S rRNA genes of bacteria were constructed for each *OsCCaMK* genotype, and community analyses were performed. The results clearly indicate that *OsCCaMK* has considerable impacts on both the diversity of root-associated bacteria and the growth of rice plants under both paddy and upland field conditions.

MATERIALS AND METHODS

Plant materials and field experimental design. Mutants for a putative ortholog of CCaMK were screened from a library of *O. sativa* mutants tagged by an endogenous retrotransposon, *Tos17* (14). Descendant seeds of a *Tos17* mutant line (NE1115, H genotype) of *Oryza sativa* subsp. *japonica* cv. Nipponbare were sown. NE1115 has a *Tos17* insertion mutation in the coding region of *OsCCaMK* (3). Seeds were placed on two layers of filter paper in a petri dish (diameter, 6 cm) containing 4 ml tap water on 15 April 2008, and the petri dishes were placed in an incubator at 30°C. After 2 days, the germinated seeds were sown in a commercial soil (No. 3; Mitsui-Toatsu, Tokyo, Japan) in a 60-cm by 30-cm cell tray (cell diameter, 1.5 cm; depth, 3 cm) and grown in a greenhouse under natural light for 4 weeks. During the seedling stage, DNA was isolated from leaf tissue by using a DNeasy plant minikit (Qiagen, Hilden, Germany) according to the manufacturer's manual, and the *OsCCaMK* gene was genotyped as previously reported (3). Seedlings of each genotype were planted in both paddy fields (dominant homozygous [PD], heterozygous [PH], recessive homozygous [PR]) and upland fields (dominant homozygous [UD], heterozygous [UH], recessive homozygous [UR]) at the National Agricultural Research Center, Tsukuba City, Japan, on 15 May 2008. These fields have been used to test the effects of N, P, and K on rice and winter wheat grown continuously for more than 30 years. Before the seedlings were planted, soil samples were collected, air dried, sieved through a 2-mm-mesh-size mesh, and analyzed (Table 1). Basal fertilizer [70 kg N/ha supplied as $(NH_4)_2SO_4$, 150 kg/ha P_2O_5 supplied as superphosphate, 100 kg/ha K_2O supplied as KCl] had been applied to the paddy field in May 2008 and to the upland field in October 2007, before seedlings were planted.

TABLE 1. Soil characteristics used in the present study

Field	pH		Truog P[a]	% total		CN ratio	CEC[b]
	H_2O	KCl		N	C		
Paddy	6.1	5.6	28.8	0.3	4.3	14.3	27.0
Upland	6.9	6.2	22.6	0.3	2.7	10.8	26.4

[a] Soil phosphate content (mg P_2O_5 kg^{-1}), determined by the Truog method.
[b] CEC, cation exchange capacity in centimoles kg^{-1}.

Growth evaluation and sampling. To define the factors relevant to any changes in bacterial community structure, shoot length, tiller number, and shoot and root fresh weights were measured. Three plants per genotype were harvested on 6 August 2008 and immediately transported on ice to the laboratory. The roots were washed well with tap water and then stored at −80°C. Examination of the roots under a microscope showed no mycorrhizal infection in either field.

Clone library construction and sequencing. Root tissues were ground to a powder in liquid nitrogen with a mortar and pestle. DNA was extracted from 0.5 g powdered tissue as described previously (15). The final DNA samples were resuspended in 100 μl sterilized water. The quality and quantity of DNA were assessed spectrophotometrically from the absorbance at 260 nm (A_{260}) and from the A_{260}/A_{230} and A_{260}/A_{280} ratios. DNA extraction and PCR amplification were independently carried out for individual plants of each genotype in triplicate. PCR clone libraries for 16S rRNA genes were constructed as follows. Briefly, 25 ng total bacterial DNA was used as a template in a final reaction volume of 12.5 μl, including 25 pmol of each primer and 1 U of Ex *Taq* DNA polymerase (Takara Bio, Otsu, Japan) with the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGWTCCARCC-3′) (25). The cycling conditions were an initial denaturation for 2 min at 94°C; 25 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C; and a final extension for 10 min at 72°C. The three PCR products derived from the triplicate DNA samples were combined, and the PCR products were resolved by 1% agarose gel electrophoresis in 0.5× TBE (44.5 mM Tris-borate, 0.1 mM EDTA) buffer. PCR products of the predicted size (~1,500 bp) were extracted from the gels by using a NucleoSpin Extract II extractor (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and ligated into the pGEM-T Easy plasmid vector (Promega Japan, Tokyo, Japan) at 25°C for 1 h. A partial sequence of the 16S rRNA gene from 192 randomly selected clones was determined by Takara Bio Inc. using the 27F forward primer as a sequencing primer. Sequences were manually edited to eliminate primer sequences and low-quality regions. Approximately 650 bases of the 16S rRNA gene (corresponding to bases 160 to 793 of the *Escherichia coli* 16S rRNA gene) were then used for the sequence analyses.

Sequence analysis. Sequences were examined for orientation and non-16S rRNA gene sequences by using OrientationChecker software (2). The presence of chimeras was assessed by Mallard software (2). A sequence identified at the 99.9% threshold was discarded as chimeric. The remaining sequences were aligned by using the CLUSTAL W program (44). A distance matrix based on the alignment was constructed by using the DNADIST program from the PHYLIP (version 3.66) package (http://evolution.genetics.washington.edu/phylip.html) with the default parameters. The resulting matrices were run in Mothur software (40) to generate diversity indexes. Library coverage was calculated with the nonparametric estimator *C* (9). The reciprocal of Simpson's index (1/*D*) was used as a measure of diversity to evaluate the level of dominance in a community (49). The UniFrac software tool (26) was used to examine similarities between clone libraries. Principal coordinate analysis (PCA) was performed in UniFrac with the abundance-weighted option.

Phylogenetic analysis. The phylogenetic composition of the sequences in each library was evaluated by using the Classifier program of the RDP-II (release 10) package (47) with confidence levels of 80%. The BLASTN (1) program was also used to classify the clones and to identify the closest relatives in the GenBank database. For the phylogenetic analysis, sequences were aligned by CLUSTAL

TABLE 2. Accession numbers of sequences deposited in DDBJ

OsCCaMK genotype	Paddy field	Upland field
D	AB598995 to AB599101 (PD)	AB599321 to AB599452 (UD)
H	AB599102 to AB599201 (PH)	AB599453 to AB599584 (UH)
R	AB599202 to AB599320 (PR)	AB599585 to AB599685 (UR)

W (44). The neighbor-joining method was used to build the trees (38). The PHYLIP format tree output was obtained by using the bootstrapping procedure (7) with 1,000 bootstrap trials. The trees were constructed in TreeView software (33).

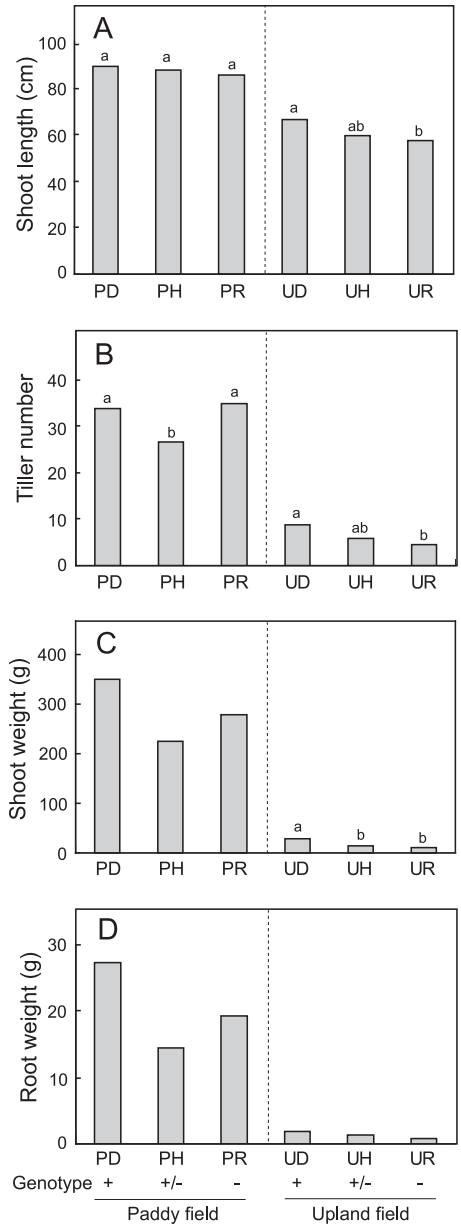

FIG. 1. Growth measurements for rice plants under paddy (P) and upland (U) conditions. The growth of rice plants was measured at 84 days after they were planted. Values are the means of 3 replicate plants of each *OsCCaMK* genotype. Different letters indicate significant difference between columns at 5% by Tukey's multiple comparison test.

TABLE 3. Statistical characteristics of 16S rRNA gene clone libraries derived from rice roots

Statistical parameter	Paddy field			Upland field		
	PD	PH	PR	UD	UH	UR
No. of sequences	107	100	119	132	132	101
% library coverage[a]	41.1	45	49.6	61.4	56.1	67.3
No. of OTUs[b] (≥95% identity)	79	72	77	74	82	53
Diversity indices						
Chao1[c]	256.5	220.5	273.7	189.9	232.3	93.6
ACE[c]	329.6	207.7	262.4	197.5	216.1	133.6
Shannon index (H')	4.2	4.1	4.0	4.0	4.2	3.7
Simpson index ($1/D$)	101.3	115.1	46.2	62.7	87.3	37.1

[a] Coverage (C_x) = $1 - (n_x/N)$, where n_x is the number of singletons that are encountered only once in a library and N is the total number of clones.
[b] OTUs were defined at ≥97% sequence identity.
[c] Chao1 and ACE (abundance-based coverage estimator) are nonparametric estimators of species richness (40).

Nucleotide sequence accession numbers. The nucleotide sequences of the 16S rRNA genes in the clone libraries have been deposited in DDBJ under the accession numbers shown in Table 2.

RESULTS

Plant growth. In the paddy field, shoot and root weights tended to be lower in PH and PR plants than in PD plants (Fig. 1C and D). In the upland field, all growth measurements were significantly lower in UR plants than in UD plants (Fig. 1). The growth of UH plants was intermediate, suggesting a gene-dosage-dependent effect on growth by *OsCCaMK*.

Statistics on clone libraries. The clone library coverage of all three genotypes in the paddy field (41.1% to 49.6%) was lower than that in the upland field (56.1% to 67.3%) (Table 3). Among paddy field genotypes, there were no noticeable differences in three of the four diversity indexes. Among upland field genotypes, UR plants tended to have lower values of diversity indexes than the other genotypes. Interestingly, both PR and UR plants had noticeably lower $1/D$ values than the other plants.

Phylogenetic diversities of bacteria with different *OsCCaMK* genotypes. The phylogenetic diversity of rice root-associated bacteria, including endophytes and epiphytes, was evaluated under the impacts of different genotypes for *OsCCaMK*. The relative abundance of *Alphaproteobacteria* was significantly lower in PR (0.8%) and UR (3.0%) plants than in PD (10.3%) and UD (17.4%) plants (Table 4). The abundance of *Betaproteobacteria* was also noticeably (but not significantly) lower in PR (19.3%) and UR (6.9%) plants than in PD (26.2%) and UD (13.6%) plants. Conversely, the abundance of *Actinobacteria* and *Chloroflexi* was greater in PR and UR plants than in PD and UD plants (Table 4). In addition, the abundance of *Firmicutes* was significantly greater in PR plants (5.9%) than in PD plants (0.9%).

Further analyses at lower taxonomic levels revealed that population shifts of the *Sphingomonadales* and *Rhizobiales* were mainly responsible for the low abundance of *Alphaproteobacteria* in PR and UR plants (Fig. 2A). Population shifts of the *Rhodocyclales* and *Burkholderiales* were responsible for the slight reduction of betaproteobacterial abundance in PR and

TABLE 4. Phylogenetic compositions of 16S rRNA gene clone libraries

Library	Relative abundance (%)[a]					
	Paddy field			Upland field		
	PD	PH	PR	UD	UH	UR
Acidobacteria	9.3	5.0	5.0	5.3	8.3	9.9
Actinobacteria		2.0	5.9*	24.2	26.5	35.6
Bacteria incertae sedis				2.3	2.3	2.0
Bacteroidetes	9.3	8.0	9.2	13.6	11.4	8.9
Chloroflexi	2.8	5.0	5.9	0.8	1.5	4.0
Cyanobacteria			0.8		0.8	
Fibrobacteres	0.9	2.0	0.8			
Firmicutes	0.9	4.0	5.9*		0.8	
Gemmatimonadetes			0.8	0.8	4.5	1.0
Nitrospira					0.8	
Planctomycetes	0.9					
Proteobacteria	57.9	54.0	54.6	46.2	34.8	27.7*
Alphaproteobacteria	10.3	1.0	0.8**	17.4	12.9	3.0**
Betaproteobacteria	26.2	26.0	19.3	13.6	11.4	6.9
Gammaproteobacteria	13.1	11.0	21.8	13.6	8.3	15.8
Deltaproteobacteria	8.4	16.0	10.9	1.5	2.3	2.0
Epsilonproteobacteria			1.7			
Spirochaetes	0.9	1.0	0.8			
TM7	0.9			0.8		
Verrucomicrobia	1.9	2.0	1.7	1.5	0.8	2.0
Unclassified bacteria	14.0	17.0	8.4	4.5	7.6	8.9

[a] * and ** indicate statistical significance at the 1 and 5% levels ($P < 0.01$ and $P < 0.05$), respectively, calculated with Fisher's exact test, between dominant homozygous (PD and UD) plants and heterozygous or recessive (PH-PR and UH-UR) plants. Shading indicates the relative abundances that were changed according to *OsCCaMK* genotypes (see the text).

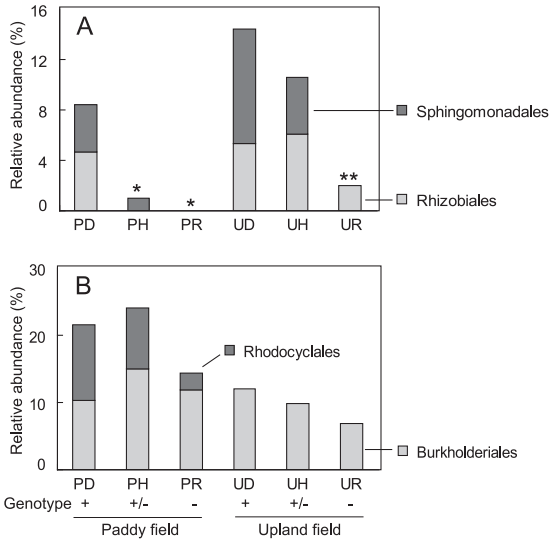

FIG. 2. Relative abundances of major subpopulations of *Alphaproteobacteria* and *Betaproteobacteria* in 16S rRNA gene clone libraries of rice root-associated bacteria by *OsCCaMK* genotype under paddy (P) and upland (U) conditions. (A) *Sphingomonadales* and *Rhizobiales*; (B) *Rhodocyclales* and *Burkholderiales*. ** and *, significant difference between D plants and H or R plants at $P < 0.01$ and $P < 0.05$, respectively, by Fisher's exact test.

UR plants, respectively (Fig. 2B). The abundance of *Clostridia* (*Firmicutes*) was significantly greater in PR plants than in PD plants, and the abundance of *Anaerolineae* (*Chloroflexi*) was greater in PR and UR plants than in PD and UD plants (Fig. 3A). In contrast, the population shift of the *Actinosynnemataceae* (9.9% for *Saccharothrix* spp. and 5% for unclassified *Actinosynnemataceae* in UR plants) was the main cause of the higher abundance of *Actinomycetes* in UR plants than in UD and UH plants (Fig. 3B). Clustering analyses at the species level did not identify a specific operational taxonomic unit (OTU) that could explain the significance of community shifts among genotypes in most of the taxonomic groups described above owing to the low library coverage (Table 3), except among the *Actinosynnemataceae* (Fig. 4). All three OTUs in the *Actinosynnemataceae* (in particular, ACT3) were more abundant in UR plants than in UD plants (Fig. 4). The representative sequences of these OTUs showed high similarity to *Lentzea* spp. (99% for ACT1 and 98% for ACT2) or to *Saccharothrix espanaensis* (97% for ACT3). Interestingly, the relative abundances of *Alphaproteobacteria*, *Betaproteobacteria*, *Anaerolineae*, and *Actinosynnemataceae* in UH plants were intermediate between those of the other genotypes, suggesting a gene-dosage-dependent effect of the *OsCCaMK* gene (Fig. 2 and 3).

The results of principal coordinate analysis (PCoA) clearly showed that the field environment (paddy versus upland) was the dominant force defining bacterial community structures in rice roots, as explained by principal coordinate 1 (PC1; 55.8%; Fig. 5). However, unidirectional shifts responding to the *OsCCaMK* genotype were also evident under both field conditions, as explained by PC2 (18.1%). The results also revealed a greater impact of *OsCCaMK* genotype on UR plants than on UD plants (Fig. 5).

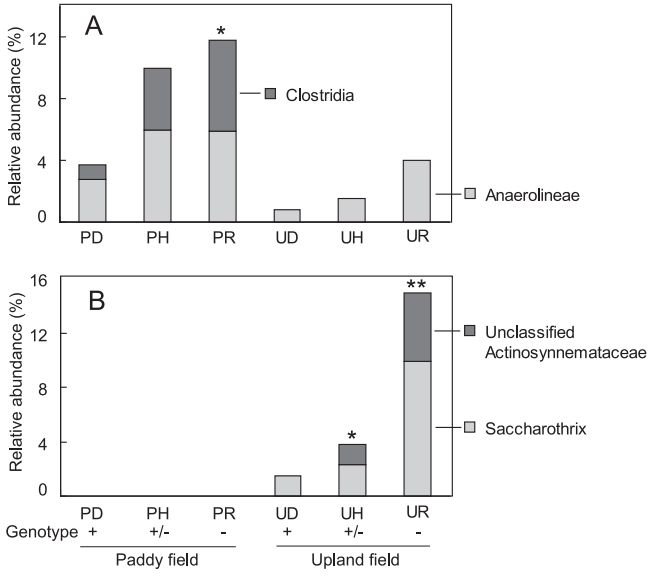

FIG. 3. Relative abundances of major subpopulations of *Chloroflexi*, *Firmicutes*, and *Actinobacteria* in 16S rRNA gene clone libraries of rice root-associated bacteria by *OsCCaMK* genotype under paddy (P) and upland (U) conditions. (A) *Anaerolineae* and *Clostridia*; (B) *Actinosynnemataceae*. ** and *, significant difference between D plants and H or R plants at $P < 0.01$ and $P < 0.05$, respectively, by Fisher's exact test.

	Paddy field			Upland fileld					
Libraries	PD	PH	PR	UD	UH	UR			
OTUs	+	+/-	-	+	+/-	-	Closest known species	Acc. No.	Identity (%)
ACT1	0	0	0	1	3	4	*Lentzea kentuckyensis*	DQ291145	99
ACT2	0	0	1	0	5*	4*	*Lentzea violacea*	EU570364	98
ACT3	0	0	0	2	3	10**	*Saccharothrix espanaensis*	AF114807	97
Subtotal	0	0	1	3	11*	18**			

FIG. 4. Phylogenetic distribution of OTUs of the *Actinosynnemataceae* responding to the *OsCCaMK* genotype in the 16S rRNA gene clone libraries of rice root-associated bacteria under paddy (P) and upland (U) conditions. The dendrogram indicates the phylogenetic relationships among the representative sequences of OTUs (defined by ≥97% identity). The table indicates the relative abundance of clones belonging to each OTU in each library and the results of a BLAST search using the representative sequences. ** and *, significant difference between D plants and H or R plants at $P < 0.01$ and $P < 0.05$, respectively, by Fisher's exact test.

DISCUSSION

Recent advances in plant genomics reveal that a series of genes required for both RN and AM symbioses in legumes are also conserved in a wide range of nonlegume species, including rice (50). The equivalent functionality of these orthologous genes between legumes and nonlegumes has been confirmed (3, 10): among CSP genes of rice, *OsCASTOR*, *OsPOLLUX*, and *OsCCaMK* are involved in mycorrhization in rice roots (3, 10). Nonlegumes are ideal materials for examining the effects of CSP genes on plant-associated bacterial communities because they obviate the disturbance of roots by rhizobial infection.

Preliminary experiments conducted in a phytotron under both paddy and upland field conditions produced almost identical growth among plant genotypes and revealed no significant differences in root-associated microbial community structure, as assessed by DNA fingerprinting (data not shown). In contrast, field experiments showed considerable effects of the *OsCCaMK* genotype on both rice plant growth and root-associated bacterial communities; in particular, plant growth under upland conditions showed a gene dosage effect (Fig. 1A, B, and D). Under paddy field conditions, the impacts of *OsCCaMK* genotypes were less clear, but shoot and root fresh weights of PH and PR plants tended to be less than those of PD plants (Fig. 1C and D). The diminished growth of the mutants suggests the important role of CCaMK in plants in the response to environmental stresses, since the growth of mutants was diminished more severely under field and upland conditions than under phytotron and paddy conditions. In plant physiology, it has been speculated that putative orthologs of CaMKII, such as CCaMK, play an important role in the response to environmental stresses (28). The possibility of the involvement of plant-associated microbes in the diminished growth of the *OsCCaMK* mutants should be examined in future studies, in light of the data from the present study.

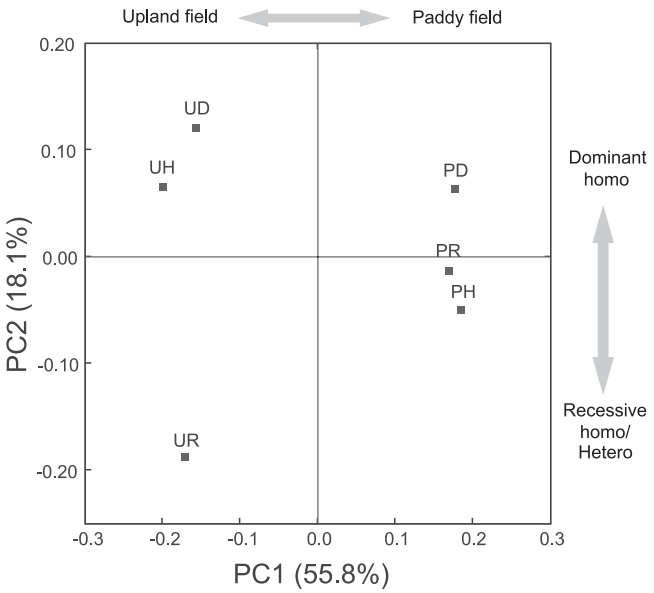

FIG. 5. Principal coordinate analysis of the 16S rRNA gene clone libraries of rice root-associated bacteria by *OsCCaMK* genotype under paddy (P) and upland (U) conditions. The positions of heterozygous (UH and PH) and recessive homozygous (UR and RP) plants relative to dominant homozygous (UD and PD) plants along the PC2 axis show the unidirectional community shifts relative to *OsCCaMK* gene dosage under both field conditions.

The statistics for the clone libraries also showed the clear effects of genotype on the diversity indexes of UR plants, implying the importance of *OsCCaMK* to bacterial community structure. The results make more sense if *OsCCaMK* is less important under paddy conditions, since *CCaMK* is essential for arbuscular mycorrhization, which is more likely to occur under upland conditions (3, 10).

Surprisingly, phylogenetic analyses revealed almost no *Alphaproteobacteria* in PR or UR plants (Table 4), despite their being a ubiquitous bacterial group in common environments, including the rhizosphere and phytosphere (8, 19, 48), as observed in PD and UD plants (Table 4). This low abundance of *Alphaproteobacteria* in R plants was mainly caused by population shifts of the *Sphingomonadales* and *Rhizobiales* among the genotypes (Fig. 2A); no clone belonging to the *Sphingomonadales* was found in PR or UR plants. These two orders are generally considered to be the dominant taxonomic groups in the phytosphere, so their absence is unusual (17–20, 27, 43) and might imply the importance of *OsCCaMK* for interactions with plant-associated *Alphaproteobacteria*.

In contrast, under both field conditions, the abundance of obligate anaerobic bacteria in *Clostridia* or the *Anaerolineae* was noticeably greater in R plants than in D plants (Fig. 3A). Obligate anaerobic bacteria have been found as endophytes in several plant species (37). Under upland conditions, an actinobacterial population belonging to the *Actinosynnemataceae* was greatly increased in UR plants (Fig. 3B). These results suggest that *OsCCaMK* significantly affects the community structures of root-associated bacteria under both paddy and upland conditions at the level of phylum or class. Moreover, clustering analyses of *Actinosynnemataceae* species revealed that the abundance of specific OTUs clearly responded to the

OsCCaMK genotype under upland conditions (Fig. 4). Detailed phylogenetic analysis of this family indicated that a specific population of *Saccharothrix* was highly sensitive to the *OsCCaMK* genotype at the species level, and distinct taxonomic groups in *Lechevalieria* and *Lentzea* were also sensitive (see Fig. S2 in the supplemental material). Interestingly, species in these genera have been reported to be potential sources of antimicrobial compounds (23, 45).

As expected, PCoA indicated that the field environmental conditions are the dominant force in shaping the communities of root-associated bacteria. However, the results also revealed that the *OsCCaMK* genotype caused similar unidirectional shifts in community structure under both paddy and upland conditions (PC2, 18.1%; Fig. 5). Thus, the *OsCCaMK* genotype affects both the species richness and species abundance of similar taxonomic bacterial groups in the roots of rice plants under both field conditions, regardless of the wide environmental differences between the conditions. The effect of genotype was partly reflected by the fact that the population of *Alphaproteobacteria* was drastically diminished in PR and UR plants (Fig. 2A). The results of PCoA also suggest that the *OsCCaMK* genotype more severely affected bacteria under upland conditions, as shown by a broader cluster in the upland field (UD, UH, and UR in Fig. 5) than in the paddy field (PD, PR, and PH). This difference suggests that *OsCCaMK* is more important under aerobic conditions than under anaerobic conditions. These results are again consistent with the fact that both RN and AM symbioses are generally considered to occur under aerobic conditions.

Another point of interest in the results of PCoA is that under upland conditions, the three genotypes were well correlated with PC2, showing a gene-dosage-dependent effect (UD, UH, and UR in Fig. 5). The effect was also reflected in plant growth under upland conditions (Fig. 1) and was correspondingly shown in the abundance of several taxonomic groups (Fig. 2A and B and 3A and B). The CCaMK protein has a dominant function in nodule organogenesis and in successful infection for both RN and AM symbioses in legumes (13). Therefore, the *OsCCaMK* heterozygous genotype may have less of an impact on rhizobial and arbuscular mycorrhizal infections, but it may have some effects on other plant-associated bacteria. Alternatively, for unknown reasons it may be critical when plants are grown under natural conditions, as observed in the differences in plant growth between field and phytotron conditions.

These results show the importance of *OsCCaMK* for regulating both plant growth and the community structure of root-associated bacteria in rice plants under both paddy field and upland conditions. The results and previous data (18–20) strongly suggest that the manipulation of genetic factors underlying RN and AM symbioses would have significant effects on the diversity of plant-associated microbes. To investigate the roles of such plant genes in interacting with plant-associated *Alphaproteobacteria* such as the *Sphingomonadales*, the significance of the diversity and functionality of plant-associated *Alphaproteobacteria* in relation to rice plant growth should be examined. Such studies will reveal novel plant-microbe interactions and could ultimately allow ecological engineering of plant symbiotic microbes to introduce beneficial traits into host plants.

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OsCCaMK genotype determines bacterial communities in rice roots under paddy and upland field conditions

Supplemental materials

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Running title: Impacts of *OsCCaMK* gene on rice root bacteria

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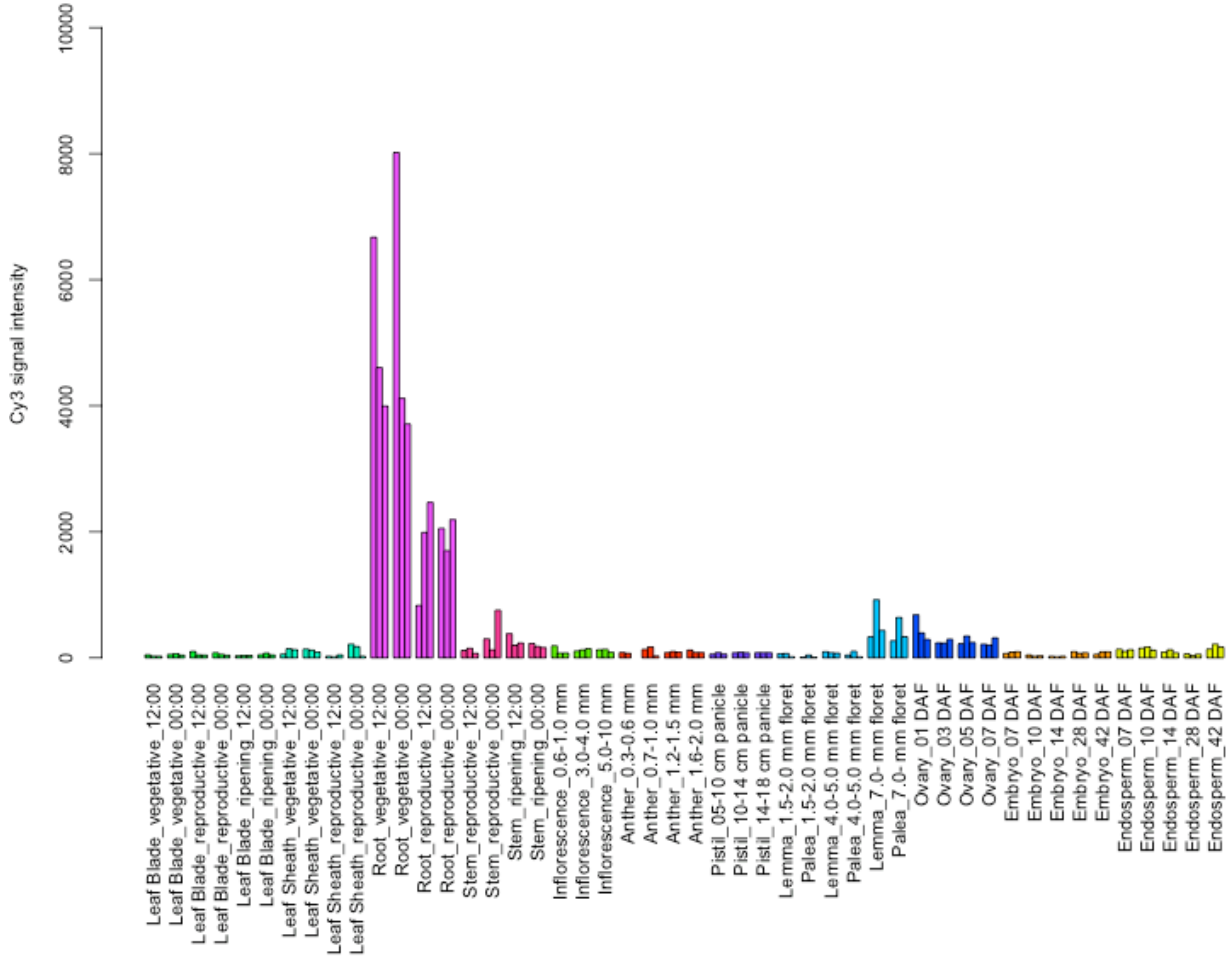

Fig. S1. Expression profile of *OsCCaMK* (Os05g0489900) by RiceXPro ver. 1.5 (1)

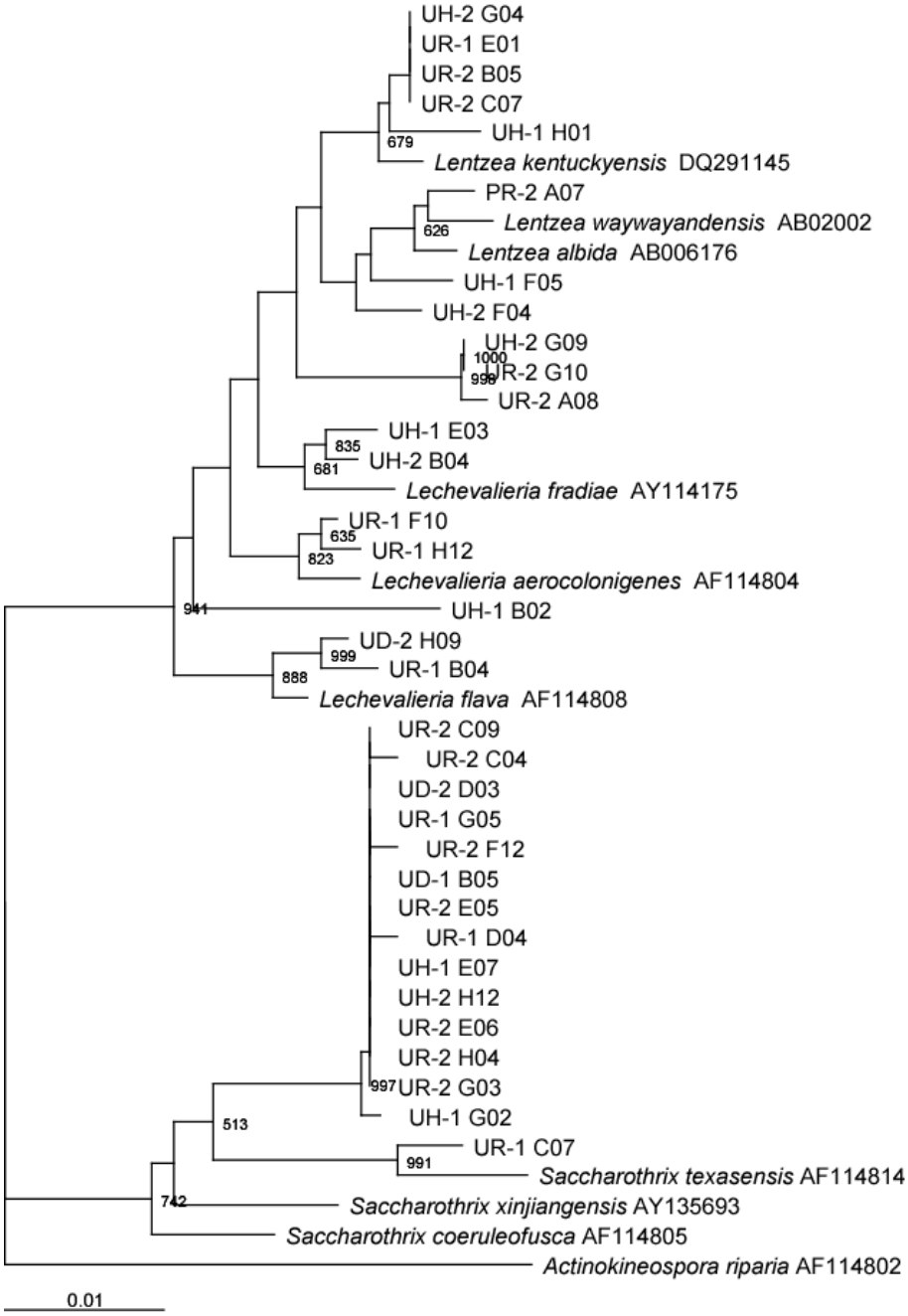

Fig. S2. Phylogenetic tree of 16S rRNA genes of OTUs of the Actinosynnemataceae. A region of the 16S rRNA gene (corresponding to bp 109–665 of the *E. coli* 16S rRNA gene) was used for the sequence analyses. The tree was constructed by the neighbor-joining method. The scale represents 0.01 substitutions per site. The numbers at nodes are the percentage of 1000 bootstrap replicates; values < 500 are not shown. PR: clones derived from recessive homozygous plants under paddy condition; UD, UH, UR: clones derived from dominant homozygous, heterozygous, and recessive homozygous plants, respectively, under upland condition.

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