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Genome-wide Identification of the Rice Calcium-dependent Protein Kinase and its Closely Related Kinase Gene Families: Comprehensive Analysis of the *CDPKs* Gene Family in Rice

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In plants, calcium acts as a universal second messenger in various signal transduction pathways. The plant-specific calcium-dependent protein kinases (CDPKs) play important roles regulating downstream components of calcium signaling. We conducted a genome-wide analysis of rice CDPKs and identified 29 *CDPK* genes and eight closely related kinase genes, including five CDPK-related kinases (CRKs), one calcium and calmodulin-dependent protein kinase (CCaMK) and two phosphoenolpyruvate (PEP) carboxylase kinase-related kinases (PEPRKs). The mRNA splicing sites of the rice *CDPKs*, *CRKs* and *PEPRKs* (but not *OsCCaMK*) are highly conserved, suggesting that these kinases are derived from a common ancestor. RNA gel blot analyses revealed that the majority of rice *CDPK* genes exhibited tissue-specific expression. Expression of *OsCPK9* was elevated in seedlings infected by rice blast, indicating that this gene plays an important role in signaling in response to rice blast treatment. Our genomic and bioinformatic analyses will provide an important foundation for further functional dissection of the rice *CDPK* gene family.

Keywords: Calcium-dependent protein kinase — Gene expression — Gene structure — Rice.

Abbreviations: CCaMK, calcium and calmodulin-dependent protein kinase; CDPK, calcium-dependent protein kinase; CRK, CDPK-related kinase; EST, expressed sequence tag; PEPRK, phosphoenolpyruvate (PEP) carboxylase kinase-related kinase; RT–PCR, reverse transcription–polymerase chain reaction; SPK, seed-specific protein kinase; UTR, untranslated region.

Introduction

All organisms use a network of signal transduction pathways to control their metabolism and to adapt to their environment. Among these pathways, calcium plays an important role as a universal second messenger (Trewavas and Malho 1998, Sanders et al. 1999, Berridge et al. 2000). In plants, the intracellular Ca^{2+} concentration is changed in response to various stimuli, including hormones, pathogens, light and abiotic stresses (Evans et al. 2001, Harper 2001, Knight and Knight 2001, Sanders et al. 2002). Several Ca^{2+} sensors or Ca^{2+}-binding proteins recognize transient Ca^{2+} elevations and induce downstream effects such as altered protein phosphorylation and gene expression patterns (Sanders et al. 1999).

Three major classes of Ca^{2+}-binding proteins have been characterized in plants: calcium-dependent protein kinases (CDPKs), calmodulins (Zielinski 1998) and calcineurin B-like proteins (Kolukisaoglu et al. 2004). Of these three classes, CDPKs are of particular interest because they represent a novel type of Ca^{2+} sensors and effectors. CDPKs have been identified throughout the plant kingdom and in some protozoans, but not in animals.

CDPKs possess a variable N-terminal domain and several functional domains, including a protein kinase domain, an autoinhibitory region and a calmodulin-like domain (Harper et al. 1991, Harmon et al. 2000, Cheng et al. 2002, Hrabak et al. 2003). CDPKs are activated by the binding of Ca^{2+} to their calmodulin-like domain, but are not stimulated by calmodulins. It has been reported that CDPKs from various plants are induced by a variety of stimuli, including both hormones and/or stresses (Urao et al. 1994, Botella et al. 1996, Sheen 1996, Patharkar and Cushman 2000, Saijo et al. 2000, Yang and Komatsu 2000, Murillo et al. 2001, Romeis et al. 2001, Chico et al. 2002), elicitor (Romeis et al. 2001) and light (Frattini et al. 1999). Furthermore, research using transgenic plants has revealed the biological function of a few *CDPK* genes in higher plants. In rice, transgenic plants with disrupted SPK (calcium dependent seed-specific protein kinase) function showed reduced accumulation of storage starch and proteins in immature seeds (Asano et al. 2002a). Transgenic rice constitutively overexpressing *OsCDPK7* had enhanced tolerance of cold, salt and drought stress (Saijo et al. 2000). In tobacco, CDPK-silenced plants showed a reduced and delayed hypersensitive response to fungal Avr9 elicitor (Romeis et al. 2001). However, the biological function of most of the CDPKs in plants and their target proteins is still unclear.

Recently, a genome-wide analysis of *Arabidopsis* CDPKs identified 34 *CDPK* genes (Cheng et al. 2002, Hrabak et al. 2003). Based on research on the *Arabidopsis CDPK* gene family, it is thought that CDPKs constitute a large multigene family in higher plants. However, to date, this idea had not been confirmed by genome-wide identification of *CDPK* gene family members from other plant species or analysis of the functional divergence of the *CDPK* gene family in plants.

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Table 1 Characteristics of CDPKs from rice

Name (previous name)	Chromosome	Accession no.	Genomic locus (cM)	cDNA/EST clones	cDNA length	Amino acids	Mol. wt (kDa) [a]	No. of EF hands [b]	Amino acids at N-terminus	Myristoylation motif [c]
OsCPK1	1	AP002819	102.3	EST				4	MGNRTSRH	Yes
OsCPK2	1	AP003073	137.2	AK112112	1853	515	57.0	4	MGN**CC**PGS	No
		AP003260	138							
OsCPK3	1	AP004366	142.4	EST				4	MGN**CC**RSP	No
OsCPK4	2	AP005311	6.9	AK060738	2043	522	58.5	4	MGA**C**FSSH	Yes
(CDPK1; AF194413)				AK067146	2023	520	58.3	4		Yes
OsCPK5	2	AP004071	114.0–118.1	EST				3	MGNT**C**GVT	Yes
OsCPK6	2	AP004082	157.9	AK069127	2092	508	54.9	1	MGNYYSCG	Yes
OsCPK7	3	AC099739	7.6	AK066500	2822	542	61.1	4	MGNQ**C**QNG	No
(CDPK1; AY158077)		AP000615	7.6							
(OsCDPK11; X81393) [d]		AC144426	6.3–7.6							
(CDPK12; AF048691)										
(OsCDPK13; AB078634) [e]										
OsCPK8	3	AC135595	152	AK066615	2252	538	60.6	4	MGN**CC**GTP	Yes
		AC145384	151.5–152.0							
OsCPK9	3	AC097277	122.3	AK105102	2163	574	63.9	4	MGNT**CC**VA	Yes
OsCPK10	3	AC084296	146.4	AK072204	2641	623	68.5	4	MGNT**C**VGP	No
OsCPK11	3	AC084296	146.4					4	MGNN**C**VGP	No
		AC087096	146.8							
OsCPK12	4	AL606687	87.1	EST				4	MGN**C**FTKT	Yes
OsCPK13	4	AL662957	97.7	AK061881	2254	551	60.9	4	MGNA**C**GGS	Yes
(OsCDPK7; AB042550) [f]		AL662987	97.4	AK066495	2218	516	57.4	4		Yes
OsCPK14	5	AC129718	101.5–102.8	EST				4	MGN**CC**PPG	No
OsCPK15	5	AC098836		AK070346	1937	542	61.3	4	MGARASRH	Yes
		AC137608	120.6							
OsCPK16	5	AC108503	95.3	AK101942	2127	547	61.0	4	MGN**CC**RSP	No
		AC121361	95.3							
OsCPK17	7	AP003847	24.8	AK068414	2391	568	62.7	4	MGNT**C**VGP	No
OsCPK18	7	AP005325	50	AK121471	1906	512	57.5	4	MGL**C**SSSS	Yes
(CDPK5; AF194414)		AP004305	49.7							
OsCPK19	7	AP003954		AK074028	2044	533	59.5	4	MGS**CC**SRA	Yes
(OsCDPK2; X81394) [d]		AP004269	69.2	AK069341	2313	533	59.5	4		Yes
OsCPK20	7	AP003866	80.5	AK068315	2366	550	62.3	4	MGN**CC**VTP	No
OsCPK21	8	AP003948	112.6	AK103306	2026	565	61.9	4	MGS**C**YSAY	Yes
OsCPK22	9	AC108755	77.7–78.0	AK100474	2172	577	63.7	4	MGG**C**SSAF	Yes
		AP004138								
		AC108760								
OsCPK23	10	AC073166	68.6–71.4	AK065456	2078	534	60.5	4	MGNS**C**QNG	No
(SPK; D13436) [g]		AE017116								
OsCPK24	11	AC128643	19	AK102308	2524	513	56.7	4	MQPDPSGS	No
OsCPK25	11	AC123528	7.6					3	MGQ**CC**TGG	Yes
OsCPK26	12	BX000508	10.8					4	MGQ**CC**TGG	Yes
OsCPK27	12	AL845343	62.2	EST				4	MGNV**C**IGP	No
OsCPK28	12	AL954825	27.6					4	MQPDPQPH	No
OsCPK29	12	BX842241	42.7	AK072981	2390	563	62.8	4	MGN**CC**VSR	No

[a] Molecular mass were calculated using Genetyx software (Genetyx, Tokyo, Japan).
[b, c] The number of EF hands and myristoylation motifs were predicted by PROSITE scan (Falquet et al. 2002).
[d] Breviario et al. (1995) and Frattini et al. (1999).
[e] Yang et al. (2003).
[f] Saijo et al. (2000).
[g] Kawasaki et al. (1993) and Asano et al. (2002a).

In this study, we carried out a genome-wide analysis of rice *CDPK* genes, and identified 29 rice *CDPK* genes and eight genes encoding related kinases such as CDPK-related kinases (CRKs), calcium and calmodulin-dependent protein kinases (CCaMKs) and phosphoenolpyruvate (PEP) carboxylase kinase-related kinases (PEPRKs). Phylogenetic analysis was performed and intron positions compared to reveal the evolutionary relationships among these kinases. For representative

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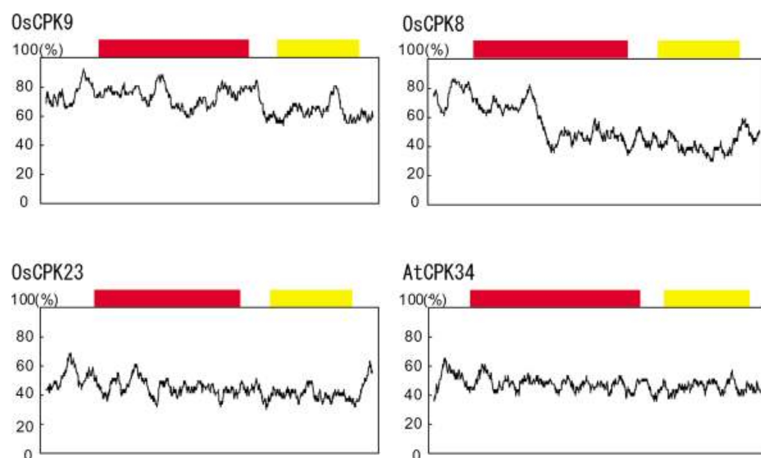

Fig. 1 GC contents of representative rice (*OsCPK8*, *OsCPK9* and *OsCPK23*) and *Arabidopsis* (*AtCPK34*) *CDPK* genes. The protein kinase domain and calmodulin-like domain are indicated by red boxes and yellow boxes, respectively. The GC contents were analyzed using GENETYX software. A 51 bp sliding window was used to filter out the fluctuations in the sequence.

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rice CDPKs, gene expression was analyzed in specific tissues or in plants infected with rice blast. Our study provides fundamental information on the phylogeny, gene structure and gene expression of the rice CDPKs.

Results and Discussion

Identification of 29 rice CDPK genes

CDPKs are widespread in the plant kingdom and constitute a multigene family. It was hypothesized that CDPKs would be encoded by a large multigene family in rice because of the 34 *CDPK* genes identified by a genome-wide analysis of *Arabidopsis* CDPKs (Cheng et al. 2002, Hrabak et al. 2003). However, only a limited number of rice CDPK family members have been characterized. Now that the draft sequence of the rice genome is almost finished, we undertook a database search to identify *CDPK* family genes in the rice genome using entire amino acid sequences from previously identified rice CDPKs and the full-length rice cDNA clones encoding CDPKs. We selected the genomic sequences as candidates which showed scores of >90 by the TBLASTN algorithm. Subsequently the candidate sequences were analyzed by the BLASTX program and motif scanning in order to distinguish CDPKs from other related protein kinases. Through this search, we identified 29 rice *CDPKs*, designated as *OsCPK1–OsCPK29* according to the proposed nomenclature for *CDPK* genes (Hrabak et al. 1996) (Table 1). All rice CDPKs analyzed in this study possess the structures typical of the CDPK family, including an N-terminal variable domain, a protein kinase domain, an autoinhibitory domain and a calmodulin-like domain. All of the rice CDPKs, except OsCPK5, OsCPK6 and OsCPK25, also possess four EF hands as predicted by a motif search. In contrast, OsCPK5 and OsCPK25 each have three EF hands, and OsCPK6 has only one (Table 1).

In *Arabidopsis*, some CDPKs are predicted to be myristoylated at their N-terminus, a modification that is thought to promote protein–membrane and protein–protein interactions (Towler et al. 1988, Johnson et al. 1994). Fifteen of the rice CDPKs were found to contain myristoylation sites at their N-terminus (Table 1). In addition to N-myristoylation, 12 CDPKs in the rice genome showed high probability of a second type of lipid modification, also known as palmitoylation (Table 1) (Resh 1994, Milligan et al. 1995, Martin and Busconi 2000). However, all the myristoylation sites or palmitoylation sites except for that of OsCPK19 (OsCDPK2) (Martin and Busconi 2000) are putative or potential sites; whether the sequences are involved in subcellular localization has not been proved experimentally.

Comparative analysis of the GC content of rice and Arabidopsis CDPK genes

We analyzed the GC content of the coding region of rice and *Arabidopsis CDPK* genes. Based on this analysis, the rice *CDPK* genes were classified into three types as follows: (i) the majority of rice *CDPK* genes (such as *OsCPK2*, *4*, *8*, *10*, *11*, *12*, *13*, *15*, *16*, *17*, *18*, *20*, *22*, *24* and *29*) had GC-rich nucleotide sequences (average 68.6% G + C) in the region corresponding to the N-terminal variable domain and a part of the protein kinase domain; (ii) the *OsCPK6*, *OsCPK9* and *OsCPK21* genes had GC-rich sequences (65.8, 70.1 and 66.8%, respectively) throughout their coding region; and (iii) the *OsCPK7*, *OsCPK19* and *OsCPK23* genes were composed of relatively low GC content sequences (45.7, 46.3 and 44.4%, respectively) (Fig. 1). In contrast, all *Arabidopsis CDPK* genes are composed of relatively low GC content sequences (average 42.1%) (Fig. 1). The results indicate that the GC contents of the N-terminal sequences of rice *CDPK* genes are quite different from those of *Arabidopsis CDPK* genes. Consistent with this finding, previous studies found that the 5′ ends of rice genes were up to 25% richer in GC content than the 3′ ends, although different genes exhibited different gradients (Wong et al. 2002, Yu et al. 2002). It is not known whether the GC-rich sequence corresponding to the N-terminal variable domain and a part of the protein kinase domain of the rice CDPKs has any functional significance.

Table 2 Characteristics of rice CRKs, CCaMK and PEPRKs

Gene family	Name (Previous name)	Chromosome	Accession no.	Genomic locus (cM)	cDNA/EST clones	cDNA length	Amino acids	Mol. wt (kDa) [a]	No. of EF hands [b]	Amino acids at N-terminus	Myristoylation motif [c]
CRK or CaMK	OsCRK1	3	AC125784	64.9	AF368282	2800	597	65.9		MGL**C**HGKS	Yes
	OsCRK2	6	AP004678	120.1–121.7	EST					MGQ**C**YGKG	Yes
	OsCRK3	7	AP004309	94.7	EST					MGG**C**HAKP	Yes
			AP004380	94.7							
	OsCRK4	7	AP005292	99.3	EST					MGL**C**HGKP	Yes
			AP004671	99.6							
	OsCRK5	10	AE017111							MGQ**C**YARN	Yes
			AC087192	55.6–57.5							
CCaMK	OsCCaMK1	5	AC097175	101.5	AK070533	1968	516	57.4	3	MSKTESRK	No
PEPRKs	OsPEPRK1	6	AP002838	6.0–6.3	EST					MAAAGGGG	No
			AP001168	6							
	OsPEPRK2	9	AP005676	63.0–65.1	AK122120	2035	453	49.0		MESSLPRK	No
					AK109479	2059	372	40.3			

[a] Molecular mass were calculated using Genetyx software (Genetyx, Tokyo, Japan).
[b, c] The number of EF hands and myristoylation motifs were predicted by PROSITE scan (Falquet et al. 2002).

Protein kinases closely related to the CDPKs

It was reported that CRKs, CCaMKs and PEPRKs are closely related to the CDPKs (Hrabak et al. 2003). Like the CDPKs, the CRKs and PEPRKs are unique to plants. The *Arabidopsis* genome contains eight *CRKs* and two *PEPRKs*, but no *CCaMK* (Hrabak et al. 2003).

We identified a rice *CCaMK* and some rice *CRKs* and *PEPRKs* through a rice *CDPK* search by the BLAST algorithm. To uncover the full complement of kinase genes closely related to the CDPKs in the rice genome, we conducted a database analysis using the full length of amino acid sequences of previously identified CRKs and PEPRKs by the BLAST algorithm. The identified genes were classified further with respect to protein kinase family by sequence homology. The results showed that five putative *CRKs*, one putative *CCaMK* and two putative *PEPRKs* are present in the rice genome (Table 2).

Although the structures of CRKs and CDPKs are similar, their enzymatic properties differ because the calmodulin-like domain of the CRKs is degenerate (Lindzen and Choi 1995). All the rice CRKs identified in this study have potential myristoylation sites and palmitoylation sites at their N-terminus (Table 2). However, CRKs have never been demonstrated to be acylated experimentally.

The rice CCaMK has a kinase domain and a calcium-binding regulatory domain, which contains three EF hands and is similar to the visinin-like domain (Takezawa et al. 1996). The activity of CCaMKs is regulated by both calcium and calmodulin because of conserved structural features (Takezawa et al. 1996). These kinases have been isolated from tobacco, lily and a legume, but are absent from the *Arabidopsis* genome (Patil et al. 1995, Liu et al. 1998, Levy et al. 2004). It was reported that the legume CCaMK acts immediately downstream of calcium spiking in the signal transduction pathway leading to nodule development and mycorrhizal infection (Levy et al. 2004).

Phylogenetic relationships and chromosomal distribution

For phylogenetic analysis, rice CCaMK, and rice and *Arabidopsis* CDPKs, CRKs and PEPRKs were aligned using the ClustalW program. As shown in Fig. 2, the phylogenetic tree of these kinase sequences forms seven subgroups: CDPKs I–IV, CRKs, CCaMK and PEPRKs. Furthermore, the 29 rice CDPKs were divided into four distinct classes.

The phylogenetic analysis showed that 18 rice and 20 *Arabidopsis* CDPKs belong to group I, III-b and IV. In contrast, the remaining CDPKs are distributed as follows: group II-a, two rice and 10 *Arabidopsis* CDPKs; group II-b, six rice and three *Arabidopsis* CDPKs; and group III-a, three rice and one *Arabidopsis* CDPK.

Phylogenetic analysis based on amino acid sequences could distinguish 11 closely related pairs of rice CDPKs: OsCPK1/15 (86.2% identity), OsCPK2/14 (86.8% identity), OsCPK3/16 (91.9% identity), OsCPK4/18 (82.3% identity), OsCPK5/13 (81.3% identity), OsCPK7/23 (70.8% identity), OsCPK8/20 (75.3% identity), OsCPK11/17 (78.6% identity), OsCPK21/22 (71.3% identity), OsCPK24/28 (86.5% identity) and OsCPK25/26 (99.6% identity). In *Arabidopsis*, CPK7 and CPK8 are closely related, and are localized in the plasma membrane (Dammann et al. 2003).

For OsCPK25 and OsCPK26, the genomic sequences extending from the initiation codons to the termination codons showed high identity at the nucleotide level (99.3%) to each other. *OsCPK25* and *OsCPK26* mapped to duplicated regions of chromosomes 11 and 12, respectively, which are reported to have a similar expected physical length of 2.5 Mb (Wu et al. 1998). Therefore, *OsCPK25* and *OsCPK26* may have arisen through a recent duplication of the rice genome. Furthermore, seven out of the 11 closely related pairs of *CDPK* genes (*OsCPK1*/*15*, *OsCPK2*/*14*, *OsCPK3*/*16*, *OsCPK5*/*13*, *OsCPK11*/*17*, *OsCPK21*/*22* and *OsCPK25*/*26*) and one closely related pair of *CRK* genes (*OsCRK1*/*OsCRK4*) were also

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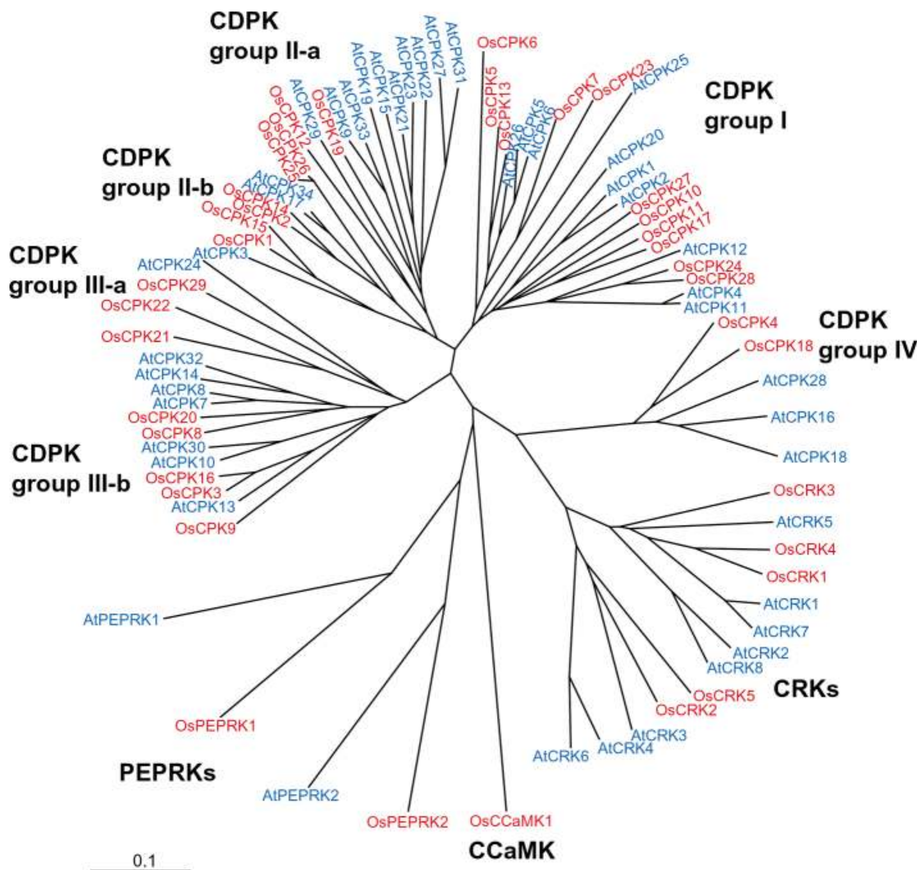

Fig. 2 Phylogenetic relationships among rice CCaMK, and rice and *Arabidopsis* CDPKs, CRKs and PEPRKs. A phylogenetic tree was created using the ClustalW program, based on the predicted amino acid sequences of the rice and *Arabidopsis* kinases, which are indicated by red and blue type, respectively.

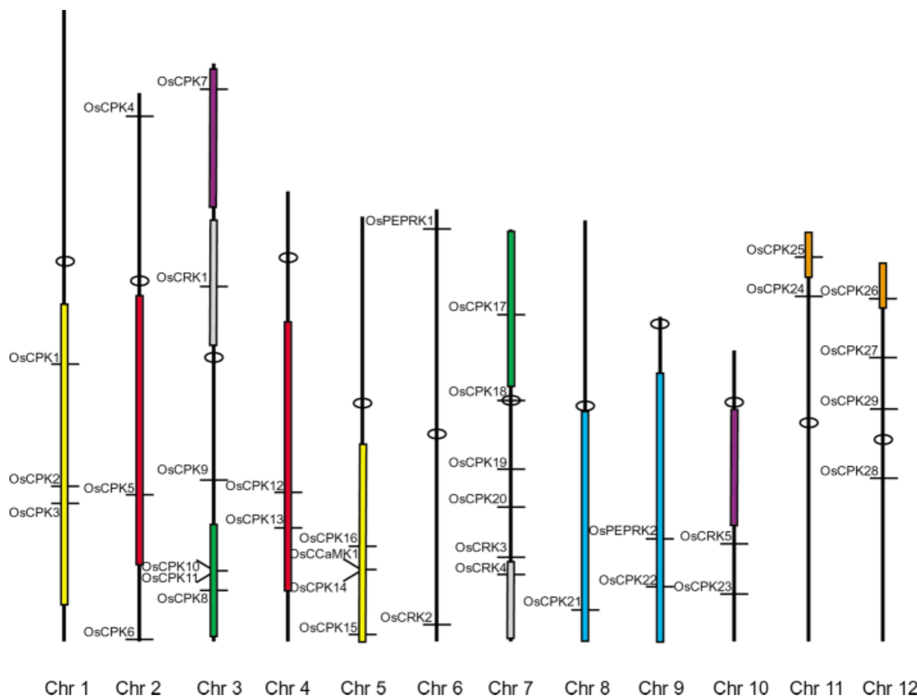

Fig. 3 Chromosomal distribution of *CDPK*, *CRK*, *CCaMK* and *PEPRK* genes in the rice genome. The chromosome numbers are shown at the bottom. Circles mark the centromere of each chromosome. Duplicated genome segments, as previously reported by Paterson et al. (2004), are indicated by colored boxes.

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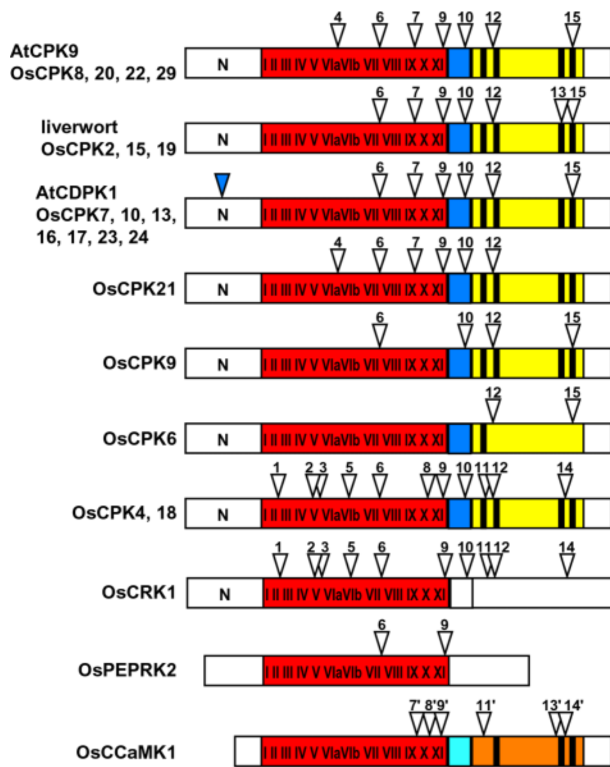

Fig. 4 Splicing sites of the rice *CDPK*, *CRK*, *PEPRK* and *CCaMK* genes. Introns located within the open reading frame of the *CDPKs* are shown by triangles. A blue triangle indicates the alternative splicing site in *OsCPK13* (Accession no. AK066495). The splicing site positions are numbered from the N-terminus to the C-terminus and are depicted above the triangles. Red boxes, protein kinase domains; yellow boxes, calmodulin-like domains; orange box, visinin-like domain; black boxes, EF hands; blue boxes, junction domains; light-blue box, calmodulin-binding domain; N, N-terminal variable region; I–XI, subdomains of the protein kinase domain.

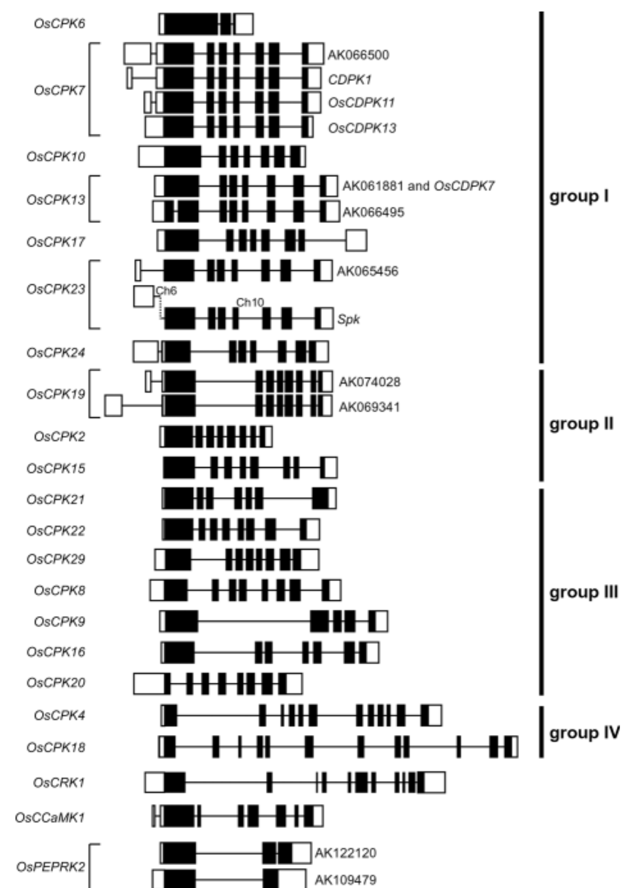

Fig. 5 Schematic representation of the rice *CDPK* genes. Boxes, exons; black boxes, open reading frames; lines, introns. The number to the right of the gene structures indicates the gene accession number.

located within duplicated genome segments (Fig. 3). Hence, these kinase genes may have diverged via genome segmental duplication events. In contrast, four other closely related pairs of *CDPK* genes (*OsCPK4*/*18*, *OsCPK7*/*23*, *OsCPK8*/*20* and *OsCPK24*/*28*) were not found on any of the duplicated genome segments identified by Paterson et al. (2004). The evolution of these rice *CDPK* gene pairs in relation to the shaping of the rice genome is unclear.

The rice *CDPK* genes are distributed among all the rice chromosomes except for chromosome 6 (Fig. 3). In the case of *Arabidopsis*, the 34 *CDPK* genes are distributed among all five chromosomes. *AtCPK31*, *AtCPK27*, *AtCPK22*, *AtCPK21* and *AtCPK23* genes are located in tandem orientation on chromosome 4 and are all classified into group II-a of the phylogenetic tree (Fig. 2) (Cheng et al. 2002, Hrabak et al. 2003). However, no tandemly oriented cluster of *CDPK* genes was observed in the rice genome. Therefore, tandem duplication of *CDPK* genes has not occurred in the rice genome.

Structure of the rice CDPKs

In order to reveal the gene structure of the rice CDPKs, mRNA splicing sites were mapped by comparing the full-length cDNAs with their corresponding genomic sequences. Full-length cDNAs were available for 19 out of the 29 *OsCPK* genes (Table 1). The predicted splicing sites of the rice *CDPK* genes correspond to those of liverwort and *Arabidopsis* (Fig. 4) (Nishiyama et al. 1999, Cheng et al. 2002, Hrabak et al. 2003), suggesting that *CDPK* gene structure is highly conserved from the psilotophyta to the higher plants. Although most of the rice *CDPK* genes consisted of seven or eight exons, the genes fell into seven different pattern types based on the locations of the splicing sites (Fig. 4, 5). This result shows that the structures of rice *CDPK* genes are more variable than those of the *Arabidopsis CDPK* genes.

Unlike most of the rice *CDPKs*, *OsCPK9* and *OsCPK6* have four and two splicing sites, respectively (Fig. 4, 5), fewer than the number typical for *CDPKs*. This finding suggests that intron loss events have occurred within these rice *CDPK* genes during evolution.

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Table 3 Rice *CDPK* genes known to be regulated in response to various stimuli, and their inferred functions

Gene name (previous gene name)	Functions	Experimental treatment	Reference
OsCPK7		Jasmonic acid	Akimoto-Tomiyama et al. (2003)
(CDPK1)			
(OsCDPK11)			
(CDPK12)			
(OsCDPK13)		Gibberellin	Yang et al. (2003)
OsCPK9		Rice blast	This study
OsCPK13		Elicitor	Akimoto-Tomiyama et al. (2003)
(OsCDPK7)	Cold and salt/drought tolerance	Cold and salt stress	Saijo et al. (2000)
OsCPK15		Elicitor	Akimoto-Tomiyama et al. (2003)
OsCPK19			
(OsCDPK2)		Light	Breviario et al. (1995)
OsCPK20		Elicitor	Akimoto-Tomiyama et al. (2003)
OsCPK23		Jasmonic acid	Akimoto-Tomiyama et al. (2003)
(SPK)	Seed development (storage product accumulation)		Asano et al. (2002a)
OsCPK24		Elicitor	Akimoto-Tomiyama et al. (2003)

OsCPK4 and *OsCPK18* each consist of 12 exons, and all of their splicing sites correspond to those of *AtCPK16*, *AtCPK18*, *AtCPK28* and the *CRKs* of *Arabidopsis*. Moreover, within the phylogenetic tree, the subgroup IV CDPKs are found in the branch that is most closely related to the CRKs. Therefore, the *CDPK* genes of subgroup IV and the *CRKs* are thought to be evolved from a common ancestor. The gene structure and phylogenetic analysis in this study reveal that the rice CDPK family is constituted from two types of genes: (i) general *CDPK* genes (subgroups I–III); and (ii) *CDPK* genes showing similarity in amino acid sequence and splicing pattern to the *CRKs* (subgroup IV).

The splicing site positions are highly conserved in the rice *CDPKs*, *CRKs* and *PEPRKs*, as also predicted in *Arabidopsis* (Hrabak et al. 2003), even though the number of splicing sites varied among these kinases. Hence, these kinases may have evolved from a common ancestor. In contrast, none of the splicing sites of the *OsCCaMK1* gene, which otherwise has a similar structure to the CDPKs, coincide with those of the *CDPK* genes or the closely related kinases, although the differences in splicing position are slight (Fig. 4). Results of phylogenetic and splicing position analyses suggest that *OsCCaMK1* diverged a very long time ago from the common ancestor of the *CDPK* family or originated from a distinct ancestor.

Alternative splicing

In eukaryotes, such as mammals and higher plants, some genes are spliced alternatively during various developmental stages or in response to stress (Lopez 1998, Reddy 2001, Kazan 2003). In fact, some plant genes are known to encode multiple proteins with different functions and/or different cellular or subcellular localization (Reddy 2001, Eckardt 2002). One example is the *Arabidopsis FCA* gene, which has been linked to the promotion of the floral transition (Macknight et al. 2002).

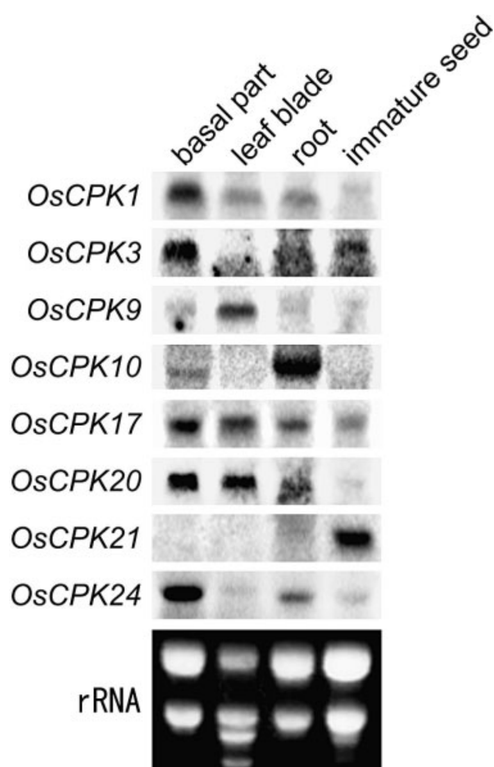

Fig. 6 Tissue-specific gene expression of rice *CDPKs*. Total RNA was isolated from the basal 30 mm parts, leaf blades, roots and immature seeds including panicles of rice. The gene-specific probes were used for RNA gel blot analysis of rice *CDPK* genes.

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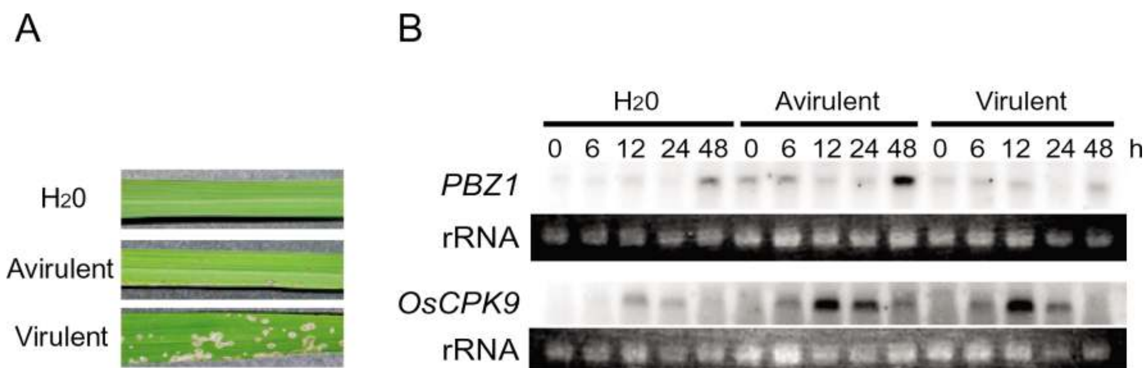

Fig. 7 Expression analysis of *CDPK* genes following infection with avirulent or virulent races of rice blast. (A) Rice leaves were infected with avirulent or virulent races of rice blast. Flecks indicative of the hypersensitive response were observed at 4 d after inoculation with the avirulent race of rice blast. Typical disease symptoms on leaves were found at 4 d after inoculation with the virulent race of rice blast. (B) Total RNA was extracted from leaf blades at each time point after infection. The *PBZ1* and *OsCPK9* probes were used for RNA gel blot analysis. Expression of *PBZ1*, which is a rice probenazole-inducible gene encoding an intracellular pathogenesis-related protein, was tested in order to confirm the experimental condition as control.

In the present study, database searches uncovered differential splicing of some rice *CDPK* transcripts. In the case of *OsCPK13*, we detected two alternatively spliced forms (accession nos AB042550 and AK061881 encoding 551 amino acids; accession no. AK066495 encoding 516 amino acids), differing in the N-terminal variable region (Fig. 5). Specifically, the AK066495 cDNA clone had an additional splicing site in the N-terminal variable region, resulting in deletion of a nucleotide sequence encoding 35 amino acids. We also found two alternatively spliced cDNA clones (accession nos AK060738 and AK067146) for OsCPK4; these differed at the eleventh splicing site where an insertion/deletion of 6 bp occurred in the sequence between the third and fourth EF hands in the C-terminal region (data not shown). These results suggest that two potential protein isoforms each of OsCPK4 and OsCPK13 are translated. Whether these potential isoforms have the same properties or have different biological functions in rice cells is unknown.

Four alternatively spliced mRNAs corresponding to *OsCPK7* were found in the database search (Fig. 5). Moreover, two alternatively spliced mRNAs were found for *OsCPK19* and *OsCPK23* (Fig. 5). In the case of these three genes, the splicing differences are in the 5′ untranslated region (UTR) of the transcripts and affect the final sequence of the processed 5′ UTR (Fig. 5). Two kinds of transcripts encoding SPK (OsCPK23) have been isolated, whose first and second exons were joined by either *trans*- or *cis*-splicing (Kawasaki et al. 1999). These observations may reflect the mechanism for regulating expression of these rice *CDPK* genes, although there are as yet no experimental data supporting this hypothesis.

Gene expression analysis of rice CDPK genes

Expression data can help predict a gene's function. Table 3 lists the rice *CDPK* genes for which there is evidence of regulation in response to certain stimuli, and the functions inferred from these data. Information available from the nucleotide [expressed sequence tag (EST) or cDNA] database and our own reverse transcription–polymerase chain reaction (RT–PCR) results for *OsCPK11* and *OsCPK28* (data not shown) indicate that all rice *CDPK* genes, except for *OsCPK25* and *OsCPK26*, are expressed in rice plants (Table 1). Although a cDNA corresponding to either *OsCPK25* or *OsCPK26* exists in the databases, it is not possible to determine which of these two genes gave rise to the cDNA. To investigate the tissue-specific expression pattern of each member of the rice *CDPK* gene family, we performed RNA gel blot analysis using RNA isolated from the following rice sample: the basal parts, including the meristems; leaf blades; roots; and immature seeds. The rice *CDPK* genes used in this study showed diverse gene expression patterns, and the majority were preferentially expressed in a specific tissue (Fig. 6).

The rice *CDPK* genes showed six different patterns of tissue-specific expression: (i) predominant expression in basal parts of rice plants including meristems (*OsCPK1*, *OsCPK3* and *OsCPK24*); (ii) predominant expression in leaf blade (*OsCPK9*); (iii) predominant expression in root (*OsCPK10*); (iv) immature seed-specific expression (*OsCPK21*); (v) predominant expression in both leaf blade and basal parts (*OsCPK20*); and (vi) expressed in all tissues (*OsCPK17*).

Previous studies reported the gene expression of *OsCPK7*, *13*, *19* and *23* as follows. *Spk* (*OsCPK23*) was expressed specifically in immature seeds and its expression pattern is very similar to those of genes encoding starch-synthesizing enzymes such as *sbe1* and *waxy* (Kawasaki et al. 1993). Antisense expression of *Spk* (*OsCPK23*) resulted in defective accumulation of storage starch and proteins in immature seeds (Asano et al. 2002a). Expression of the *OsCDPK7* (*OsCPK13*) gene was increased by cold and salt stress in both shoots and roots of 10-day-old seedling (Saijo et al. 2000) and the gene was preferentially expressed in vascular bundles of crowns and central cyl-

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inder of the root elongation zone under cold and salt stress (Saijo et al. 2001). Furthermore, transgenic rice constitutively overexpressing *OsCDPK7* (*OsCPK13*) had enhanced tolerance of cold, salt and drought stress (Saijo et al. 2000). It was also shown that *OsCDPK13* (*OsCPK7*) was expressed in rice leaf sheath and roots and is induced in response to cold and gibberellin (Yang et al. 2003). *OsCDPK2* (*OsCPK19*) mRNAs were equally abundant in rice roots and coleoptiles (Breviario et al. 1995).

No transcripts could be detected for *OsCPK2*, *4*, *5*, *6*, *8*, *11*, *12*, *14*, *15*, *16*, *18*, *22*, *27*, *28* or *29* in any of the tissues analyzed in this study (data not shown). These genes may be expressed in the tissues analyzed but at a level below the limits of detection, or may be expressed only in response to certain stimuli, at a specific developmental stage, or in limited cell types.

Rice blast-induced gene expression

Some rice *CDPK* genes were expected to function in the signal transduction pathway of disease resistance because Ca^{2+} acts as a universal second messenger and the enzymatic activity of rice CDPKs was induced by probenazole treatment (Komatsu et al. 2004). The probenazole (3-allyloxy-1,2-benzisothiazole-1,1-dioxide) is known as an effective agricultural chemical against rice blast disease (Midoh and Iwata 1996). However, the rice *CDPK* genes which are involved in the signal transduction pathway of disease resistance have not been identified and characterized. In order to investigate the expression of rice *CDPK* genes in response to infection with avirulent or virulent races of rice blast (Fig. 7A), we initially analyzed mRNA levels by RT–PCR. For this analysis, we selected *OsCPK1*, *3*, *9*, *10*, *17*, *20*, *21* and *24*, because these were the genes for which we could detect transcripts in this study (Fig. 6). In the RT–PCR analysis, expression of *OsCPK9* increased in response to rice blast treatment. Expression of the other genes either was not changed by infection with rice blast or showed the same response to mock treatment (data not shown).

To confirm the expression patterns seen in the RT–PCR analysis, we carried out RNA gel blot analysis using gene-specific probes. The results of this analysis were consistent with those of the RT–PCR. In other words, *OsCPK9* expression was increased after 12–24 h of both avirulent and virulent rice blast treatment (Fig. 7B). These results suggest that OsCPK9 is involved in decoding the rice blast-induced Ca^{2+} signal pathway. However, further experiments (e.g. gene expression analysis in response to phytohormone and gain- or loss-of-function experiments) are required to investigate the function of *OsCPK9* in rice blast infection.

In rice suspension culture cells, the transcripts of *OsCPK15*, *OsCPK20* and *OsCPK24* were strongly induced by the addition of *N*-acetylchitooligosaccharide elicitor (Table 3) (Akimoto-Tomiyama et al. 2003). However, these rice *CDPK* genes did not respond to rice blast infection under our experimental conditions (data not shown).

In summary, we identified 29 *CDPK* genes, five *CRK* genes, one *CCaMK* gene and two *PEPRK* genes in the rice genome. The functions of most of the CDPKs and their related protein kinases remain elusive. Further studies using multiple approaches will be required to resolve functional divergence in this gene family. The genomic and bioinformatic analyses of rice *CDPK* genes in this work can provide a solid foundation for the further functional dissection of rice *CDPK* genes.

Materials and Methods

Identification of rice CDPK genes and this structural analysis

CDPK genes from the rice genome sequence were searched using the BLAST algorithm at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/) and the Rice Genome Research Program (RGP; http://rgp.dna.affrc.go.jp/). The sequences of cDNA or EST clones were identified by BLAST analysis from the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) and Knowledge-based Oryza Molecular biological Encyclopedia (KOME; http://cdna01.dna.affrc.go.jp/cDNA/). The full-length cDNAs obtained were mapped on chromosomes by identifying their corresponding genomic sequences using the BLASTN algorithm at the Rice Genome Research Program (RGP; http://rgp.dna.affrc.go.jp/). Genomic sequences containing putative *CDPK* genes were annotated further by using the Rice Genome Automated Annotation System (RiceGAAS) (Sakata et al. 2002). Motif scanning in CDPK was done by PROSITE scan (Falquet et al. 2002) or NCBI-CD searches. Predicted molecular masses of CDPKs protein were calculated using Genetyx software (Genetyx, Tokyo, Japan).

Phylogenetic analysis of rice CDPK proteins

The deduced protein sequence was aligned with ClustalW (Thompson et al. 1994). A phylogenetic tree was created according to the neighbor-joining method clustering strategy (Saitou and Nei 1987) using the Clustalw program.

Infection of rice blast

Three-week-old rice seedlings (*Oryza sativa* L. cv. Nipponbare) were sprayed with *Magnophorthe grisea* (races 102S and 003) at a concentration of 200,000 spores ml^{-1} containing 0.02% Tween-20. After inoculation, seedlings were placed in a moist chamber at 25°C for 28 h in the dark, and then moved to a greenhouse at 28°C.

RT–PCR and RNA gel blot analysis

Isolation of RNAs, RNA gel blot analysis and RT–PCR were conducted essentially as described previously (Asano et al. 2002a, Asano et al. 2002b). In brief, rice total RNA was extracted from the basal parts of rice including meristems, leaf blades, roots and immature seeds using an RNeasy plant mini kit (Qiagen, Germany). A 10 µg aliquot of total RNA was separated on a 1.2% agarose/formaldehyde gel and blotted onto a nylon membrane according to the standard protocol. Primers used for the probe preparation in RNA gel blot analyses were OsCPK1a (5′-AATTAGACAGCTGATGGAGG-3′) and OsCPK1y (5′-AGCCCTGGCAACAGGTGTAT-3′) for *OsCPK1*, OsCPK3d (5′-GCCTAAGGGTGATAATTTTATGCG-3′) and OsCPK3z (5′-CACGAGGGCTAAGCAGGCGC-3′) for *OsCPK3*, OsCPK9b (5′-AATGGGTACCTGGACTACGG-3′) and OsCPK9× (5′-TCTCAAGCCTGAATCGACTG-3′) for *OsCPK9*, OsCPK10a (5′-ACCTCCAAGAATCCGAGATC-3′) and OsCPK10× (5′-TCGAACCAATGACCCAAGAG-3′) for *OsCPK10*, OsCPK17c (5′-TCTGGAGGATATCATCAAAG-3′) and OsCPK17z (5′-AAGCGTGTCAAAGCTTCACC-3′)

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for *OsCPK17*, OsCPK20b (5′-GGCAACGATGAGCATCTGCA-3′) and OsCPK20y (5′-CCGTCATCTATCTATTGGGTAG-3′) for *OsCPK20*, OsCPK21b (5′-AAGGACGGCAGCGGGTTCAT-3′) and OsCPK21y (5′-CTCATTTCACAATCCAAGCGCA-3′) for *OsCPK21*, OsCPK24d (5′-TCTTGAACCCCAGCAACAGC-3′) and OsCPK24z (5′-TACTATCATCTCCAGCATCA-3′) for *OsCPK24*. cDNAs or rice genomic DNAs were used as a template. Hybridization and washing conditions were performed as described by Ausubel et al. (1987). cDNA synthesis was performed using SuperScript first-strand synthesis system for RT–PCR (Invitrogen, CA, U.S.A.) according to the manifucturer's instructions. PCR was performed with 20 pmol of each primer set by 1 U of AccuPrime *Taq* DNA polymerase (Invitrogen, CA, U.S.A.). A 20 ng aliquot of rice genomic DNA or 100 μg of cDNAs were used as template. Other conditions were followed according to the manufacturer's instructions.

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