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ORIGINAL ARTICLE

Calcium-dependent protein kinase (CDPK) and CDPK-related kinase (CRK) gene families in tomato: genome-wide identification and functional analyses in disease resistance

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Abstract Calcium-dependent protein kinases (CDPKs) and CDPK-related kinases (CRKs) play multiple roles in plant. Nevertheless, genome-wide identification of these two families is limited to several plant species, and role of CRKs in disease resistance remains unclear. In this study, we identified the *CDPK* and *CRK* gene families in genome of the economically important crop tomato (*Solanum lycopersicum* L.) and analyzed their function in resistance to various pathogens. Twenty-nine *CDPK* and six *CRK* genes were identified in tomato genome. Both SlCDPK and SlCRK proteins harbored an STKc_CAMK type protein kinase domain, while only SlCDPKs contained EF-hand type Ca^{2+} binding domain(s). Phylogenetic analysis revealed that plant CRK family diverged early from CDPKs, and shared a common ancestor gene with subgroup IV CDPKs. Subgroup IV SlCDPK proteins were basic and their genes contained 11 introns, which were distinguished from other subgroups but similar to CRKs. Subgroup I SlCDPKs generally did not carry an N-terminal myristoylation motif while those of the remaining subgroups and SlCRKs universally did. *SlCDPK* and *SlCRK* genes were differently responsive to pathogenic stimuli. Furthermore, silencing analyses demonstrated that *SlCDPK18* and *SlCDPK10* positively regulated nonhost resistance to *Xanthomonas oryzae* pv. *oryzae* and host resistance to *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000, respectively, while *SlCRK6* positively regulated resistance to both *Pst* DC3000 and *Sclerotinia sclerotiorum* in tomato. In conclusion, *CRK*s apparently evolved from CDPK lineage, *SlCDPK* and *SlCRK* genes regulate a wide range of resistance and *SlCRK6* is the first *CRK* gene proved to function in plant disease resistance.

Keywords Calcium-dependent protein kinase (CDPK) · CDPK-related kinase (CRK) · Genome-wide identification · Phylogeny · Resistance · Tomato

Introduction

Calcium-dependent protein kinases (CDPKs) are main receptors of Ca^{2+} signal (Sanders et al. 2002; Kudla et al. 2010; Reddy et al. 2011). They are multifunctional in plants, including regulation of plant growth, development as well as abiotic and biotic stress resistance (Boudsocq and Sheen 2013; Romeis and Herde 2014). CDPK-related kinase (CRK) is another type of protein kinase closely related to CDPKs (Harmon et al. 2000). Some CRKs are involved in plant development or abiotic stress tolerance (Leclercq et al. 2005; Li et al. 2006; Liu et al. 2008; Rigo et al. 2013; Tao and Lu 2013). Currently, genome-wide identification of these two families is mainly conducted in model plant species. Moreover, role of CRKs in plant disease resistance remains unclear. The aim of this study is to identify *CDPK* and *CRK* gene families in genome of the economically important crop tomato (*Solanum*

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lycopersicum L.), and analyze their function in resistance to various pathogens. The results provide new insights into composition, phylogeny and function of plant CDPK and CRK families.

CDPKs and CRKs are two structurally related families of protein kinases. CDPKs carry two kinds of key domains, Ser/Thr kinase domain and EF-hand type calcium-binding domain. In addition, they contain an N-terminal variable domain, an auto-inhibitory junction region and a C-terminus (Cheng et al. 2002; Harper et al. 2004; Ludwig et al. 2004; Harper and Harmon 2005). However, CRKs solely harbor a Ser/Thr kinase domain at their N-termini of protein sequences. Their C-termini only contain degenerated EF-hand-like sequences (Harmon et al. 2000). Therefore, CDPKs function in a calcium-dependent way, whereas CRKs are thought to act in a calcium-independent way (Harmon et al. 2000).

CDPK genes have been identified in plant as well as in green algae, oomycetes and protists, but are not found in fungi and animals (Hamel et al. 2014; Valmonte et al. 2014). Genome-wide identification of *CDPK* genes has been conducted individually in a number of plant species, such as *Arabidopsis thaliana* (Cheng et al. 2002), *Oryza sativa* (Asano et al. 2005; Ray et al. 2007; Boudsocq and Sheen 2013), *Triticum aestivum* (Li et al. 2008), *Gossypium raimondii* (Liu et al. 2014), *Brassica napus* (Zhang et al. 2014), *Populus trichocarpa* (Zuo et al. 2013), *Selaginella moellendorffii*, and *Physcomitrella patens* (Hamel et al. 2014), as well as comprehensively in many species (Valmonte et al. 2014). Results of these studies reveal that CDPK proteins in a plant species are typically encoded by a gene family, which is usually classified into four distinct subgroups (Hamel et al. 2014; Valmonte et al. 2014). There are 34 and 31 *CDPK* genes in *Arabidopsis* (Cheng et al. 2002) and rice genome (Ray et al. 2007; Boudsocq and Sheen 2013), respectively. Compared with CDPKs, CRKs are relatively less studied. Genome-wide analysis has identified eight CRKs in *Arabidopsis* (Hrabak et al. 2003), five CRKs in rice (Asano et al. 2005) and nine CRKs in *P. trichocarpa* (Zuo et al. 2013), respectively. Nevertheless, genome-wide identification of the *CDPK* and *CRK* gene families in many economically important crop plant species such as tomato (*Solanum lycopersicum* L.) has not yet been conducted.

CDPK substrates have increasingly been identified. CDPK may directly phosphorylate an MAPK to compromise MAPK signaling to regulate stress responses and disease resistance (Ludwig et al. 2005; Xie et al. 2014). LeCDPK2 directly phosphorylates LeACS2 to regulate ethylene biosynthesis in response to wound signaling (Kamiyoshihara et al. 2010), while AtCDPK4 and AtCDPK11 and StCDPK2 may target ABA-responsive transcription factors ABF1 and ABF4 to regulate ABA signaling (Zhu et al. 2007). StCDPK5 and AtCDPK1/2/4/5/11 phosphorylate and thereby activate NADPH oxidase to promote ROS production in response to abiotic and biotic stimuli (Kobayashi et al. 2007; Gao et al. 2013). AtCDPK32 interacts with AtCNGC18 to confer severe depolarization of pollen tube growth in tobacco (Zhou et al. 2013). OsCDPK4 plays a positive role in rice tolerance to salt and drought stress by protection of cellular membranes from lipid peroxidation (Campo et al. 2014). CDPKs are widely involved in the regulation of various types of disease resistance (Boudsocq et al. 2010; Boudsocq and Sheen 2013; Romeis and Herde 2014). AtCDPK28 phosphorylates BIK1 to attenuate PTI and antibacterial immunity (Monaghan et al. 2014), while AtCDPK1 plays a positive role in *Arabidopsis* resistance to various pathogens by promoting salicylic acid (SA) signaling pathway (Coca and San Segundo 2010). Six AtCDPKs are involved in the *Arabidopsis* NLR immune signaling via distinct functions, AtCDPK1/2 regulating the initiation of programmed cell death, AtCDPK4/5/6/11 phosphorylating specific WRKY transcription factors to regulate the immune gene expression, while AtCDPK1/2/4/11 phosphorylate NADPH oxidases to induce the production of ROS (Gao et al. 2013). NtCDPK2 is involved in *Cf-4/Avr4* and *Cf-9/Avr9* dependent hypersensitive response (HR) induction (Romeis et al. 2001). Additionally, CDPKs not only locally but also systemically regulate plant defense (Romeis and Herde 2014). Moreover, a *CDPK* gene may simultaneously regulate several biological processes. For example, *OsCDPK12* positively regulates salt tolerance while negatively affects the blast resistance by affecting ABA signaling and suppressing ROS production (Asano et al. 2012).

Unlike CDPKs, whose functions have been widely studied, functional analysis of CRKs has been mainly conducted in *Arabidopsis*. AtCRK1 binds CaM in a Ca^{2+}-dependent manner but phosphorylates itself and substrates such as histone IIIS and syntide-2 in a Ca^{2+}-independent manner (Wang et al. 2004). It positively regulates plant tolerance to salt and heat stresses (Liu et al. 2008; Tao and Lu 2013). AtCRK3 interacts with a cytosolic glutamine synthetase AtGLN1;1 to regulate nitrogen remobilization during leaf senescence (Li et al. 2006), while AtCRK5 functions in primary root elongation and gravitropic bending of shoots and roots in *Arabidopsis* (Rigo et al. 2013). Additionally, a tomato CRK (LeCRK1) was found to play a role in the fruit ripening process (Leclercq et al. 2005). The role of CRKs in plant disease resistance remains unclear.

In this study, we conducted a genome-wide identification of the CDPK and CRK families in tomato and analyzed the function of a set of these genes in disease resistance. Our data demonstrated that the structural and biochemical features of SlCDPK (previously LeCDPK) family are obviously subgroup dependent. The SlCRK family shared

the same ancestor with subgroup IV SlCDPKs. Additionally, our results revealed that *SlCDPK* genes regulate a wide range of resistance in tomato but effectiveness against individual pathogen is *CDPK* gene dependent. *SlCRK6* was proved to function in tomato resistance to both *Sclerotinia sclerotiorum* and *Pseudomonas syringae* pv. *tomato* DC3000. This is the first report that demonstrates a role of a *CRK* gene in plant disease resistance.

Materials and methods

Identification of *CDPK* and *CRK* genes in tomato genome and *CRK* genes in *Selaginella moellendorffii* and *Physcomitrella patens*

To identify *CDPK* and *CRK* genes in tomato (*Solanum lycopersicum* L.), all 34 *Arabidopsis* CDPK protein sequences were collected through searching the genome sequence databases TAIR (The Arabidopsis Information Resource, http://www.arabidopsis.org/). All retrieved AtCDPK protein sequences were used to BLASTp search the tomato genome database (http://solgenomics.net/). All non-redundant sequences were collected, and subjected to domain analysis using the Pfam (http://pfam.sanger.ac.uk/), SMART (http://smart.embl-heidelberg.de/), COG and Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/cdd) programs. The proteins containing both an STKc_CAMK kinase domain and two EF-hand type domains (four EF-hand motifs) were considered as prototypical CDPK proteins. The remaining full length proteins that possessed an STKc_CAMK kinase domain were further subjected to phylogenetic tree construction together with the *Arabidopsis* and rice CRKs. Those clustered with *Arabidopsis* and rice CRKs were recognized as tomato CRKs. Similar approaches were used to identify CRKs in *Selaginella moellendorffii*, *Physcomitrella patens* and algal species (*Chlamydomonas reinhardtii*, *Volvox carteri*, *Coccomyxa subellipsoidea* C-169, *Micromonas pusilla* CCMP1545, *Micromonas* sp. RCC299, *Ostreococcus lucimarinus*) whose genomes are deposited in Phytozome database version 10 (http://phytozome.jgi.doe.gov/pz/portal.html). The pI value and molecular weight of CDPK and CRK proteins were predicted by DNAStar software.

Sequence comparison, gene structure and phylogenetic analyses of *SlCDPK* and *SlCRK* genes

CDPK protein sequences from *Arabidopsis* and tomato and CRK protein sequences from *Arabidopsis*, tomato, rice, *Populus trichocarpa*, *Selaginella moellendorffii*, *Physomitrella patens* and representatives of algal CDPKs were aligned using MUSCLE program (Edgar 2004). The phylogenetic trees were constructed based on the alignments using MEGA 5.0 by maximum likelihood (ML) with the JTT model (Jones et al. 1992; Tamura et al. 2011). One thousand bootstrap replicates were performed to evaluate the support of clusters and nodes. Three apicomplexan CDPKs, TgCDPK1 (ToxoDB ID TGME49_301440), PfCDPK3 (PlasmoDB ID PF3D7_0310100) and CpCDPK1 (CryptoDB ID cgd3_920), were included as outgroup for rooted tree construction (Valmonte et al. 2014). The exon/intron structure of *SlCDPK* and *SlCRK* genes was analyzed online using the Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/) with default settings (Guo et al. 2007). For comparison of EF-hands of tomato CDPKs with the corresponding region of tomato CRKs, the sequences were aligned using MUSCLE program (Edgar 2004) and visualized using GeneDoc 2.6 software.

N-terminal myristoylation prediction

The N-terminal myristoylation of all SlCDPK, SlCRK, SmCRK and PpCRK proteins was predicted using the Myristoylator program in ExPASy (http://web.expasy.org/myristoylator/) with default settings (Bologna et al. 2004).

Plant materials for expression analysis

Tomato plants were grown in growth chambers at 28 °C with a 16 h/8 h light/dark daily cycle. For fungi pathogen inoculation, *Sclerotinia sclerotiorum* was grown at 25 °C on potato dextrose agar (PDA) plates for 2 days. PDA plugs of 5 mm in diameter containing actively growing *S. sclerotiorum* mycelia were placed on the fully developed leaves of the 7- to 8-week-old tomato plants. For bacterial inoculation, *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) were incubated overnight at 28 °C on King's B plates containing rifampicin (50 μg/ml) and NA medium plates containing carbenicillin (50 μg/ml), respectively. After overnight shaking, the bacterial cells were collected by centrifugation, resuspended in 10 mM $MgCl_2$ buffer or sterilized ddH_2O and diluted to an OD_{600} of 0.002 and 0.5, respectively. The prepared bacterial suspensions (with 10 mM $MgCl_2$ buffer or sterilized ddH_2O as controls) were infiltrated into leaves of tomato plants (Zhao et al. 2013). For oxalic acid (OA) treatment, tomato leaves were infiltrated with 500 μM of OA (Kim et al. 2011). Samples were collected for gene expression analysis at two time points after inoculation or treatment; 0 and 12 h for *S. sclerotiorum*, 0 and 8 h for *Xoo*, 0 and 4 h for *Pst* DC3000 as well as 0 and 4 h for OA treatment.

Gene expression analyses by RT-qPCR

Real-time quantitative RT-PCR (RT-qPCR) analyses and subsequent statistical analyses of the gene expression data were conducted as described (Zhao et al. 2013). The primers used in RT-qPCR analyses are listed in Table S1.

VIGS manipulation procedure and plant disease resistance analysis

SlCDPK10/12/13/18 and *SlCRK4/6* were selected for virus-induced gene silencing (VIGS) analysis. VIGS analysis in tomato was conducted as described (Wang et al. 2006; Cai et al. 2007; Zhao et al. 2013) except using the recombinant pTRV2 with insertion of an eGFP fragment instead of empty pTRV2 as a negative control vector to repress the viral symptom efficiently (Cheng et al. 2012). Gene-specific VIGS-targeted fragments from CDS regions of *SlCDPK10*, *SlCDPK12* and *SlCDPK18* and 5′ UTR regions of *SlCDPK13* as well as *SlCRK4* and *SlCRK6* were cloned and ligated into the VIGS vector PYL156 (pTRV2), which were immediately electroporated into *Agrobacterium tumefaciens* strain GV3101 for VIGS analyses (Saand et al. 2015). Primers used in the VIGS experiments are listed in Table S1. VIGS analyses were conducted with the vacuum-infiltration delivery approach. The agro-inoculated plants were grown in a plant growth chamber at 21 °C with a 16 h/8 h light/dark regime. Three weeks later, the plants were subjected to disease resistance analyses (Zhao et al. 2013; Saand et al. 2015). They were inoculated with nonhost pathogen *Xoo* and host pathogens *S. sclerotiorum* and *Pst* DC3000 as described above. For each pathogen, at least six silenced plants were examined and the experiments were conducted three times independently. Data were analyzed using SPSS (verson19.0) by Student's *t* test and Duncan's multiple range test (DMRT) (P value ≤ 0.05).

Results

Identification of *CDPK* and *CRK* genes in tomato (*Solanum lycopersicum* L.) genome and *CRK* genes in spikemoss (*Selaginella moellendorffii*) and moss (*Physcomitrella patens*) genomes

BLASTp searches of the tomato genome using all 34 *Arabidopsis* CDPK protein sequences as templates retrieved 197 non-redundant sequences. After domain composition analysis of these sequences using Pfam, SMART, COG and CDD programs (Table S2), 29 of them were found to possess both STKc_CAMK protein kinase and EF-hand domains and were thus recognized as tomato CDPKs. To identify the tomato CRKs, a phylogenetic tree was constructed for the remaining 15 full length sequences containing solely an STKc_CAMK kinase domain together with the known *Arabidopsis* and rice CRKs. Six of them clustered with the known *Arabidopsis* and rice CRKs into a distinct clade, separated from the remaining tomato sequences, and were thus identified as tomato CRKs (Fig. S1). Following similar BLASTp searching, domain composition and phylogenetic analyses, two and five CRKs were identified in spikemoss (*Selaginella moellendorffii*) and moss (*Physcomitrella patens*) genomes, respectively (Table 1). However, no CRK was identified from algal species (data not shown). We assigned names of all individual SlCDPK and CRK members in ascending order in accordance with group numbers on the basis of phylogenetic tree for easy recognition.

Phylogeny of CDPK and CRK families

To examine the phylogenetic relationship among the CDPKs and CRKs, kinase domains of CDPKs from *Arabidopsis* and tomato, and CRKs from four higher flowering plant species, one primitive lycophyte and one moss were subjected to multiple sequence alignment along with representatives of algal CDPKs. Subsequently, a rooted maximum likelihood (ML) phylogenetic tree was constructed using three apicomplexan CDPKs as outgroup (Fig. 1). The ML tree demonstrated that the CDPK family of both tomato and *Arabidopsis* and all CRKs clustered distinctly from those of algal CDPKs. CDPKs formed four subgroups, while the CRK family of all six plant species including both higher and lower plant species was divided into two subgroups. All CRKs of lower plant species clustered in subgroup I along with a subclade of CRKs of higher plant species, while the remaining CRKs of higher plant species formed subgroup II which was split into two clades (Fig. 1). Together with the finding that CRK family does not exist in algal species (data not shown), it is obvious that CRK family may have emerged very early in land plant species before the divergence of nonvascular and vascular plant species, and the diversification of CRKs in land plant species apparently resulted from gene duplications and rearrangement. Remarkably, the CRK family clustered with the subgroup IV CDPKs, which clearly separated from the other three subgroups of CDPK family with a strong bootstrap support (Fig. 1). This result reveals that the CRK family shares ancestral CDPK gene with subgroup IV CDPKs, which is distinct from the remaining three subgroups of CDPK family. Collectively, CRK gene family is most likely to have arisen from a CDPK ancestor and its appearance possibly predated diversification of CDPKs and separation of land plant into nonvascular and vascular plants, but after the split of algae and the ancestor of plant lineage.

In the SlCDPK family, 13 members, SlCDPK1 to SlCDPK13, clustered into subgroup I; eight members, SlCDPK14 to SlCDPK21, comprised subgroup II; six

Table 1 The CDPKs and CRKs identified in this study

Protein	Locus	Protein kinase domain	Ca^{2+} binding domains (number)	Protein size (aa)	Mol Wt (kDa)	pI	Introns (number)	Subgroup	N terminus	*N*-Myristoylation
Solanum lycopersicum L.										
SlCDPK1	Solyc11g006370.1.1	STKc_CAMK	EF-hands(2)	578	64.68	5.47	6	I	MGNTCVGP	No
SlCDPK2	Solyc04g009800.2.1	STKc_CAMK	EF-hands(2)	581	64.60	5.60	6	I	MGNTCVGP	No
SlCDPK3	Solyc10g081740.1.1	STKc_CAMK	EF-hands(2)	499	55.76	5.01	7	I	MLSAGLQV	No
SlCDPK4	Solyc01g006840.2.1	STKc_CAMK	EF-hands(2)	598	67.55	5.34	6	I	MGNNCVHA	No
SlCDPK5	Solyc01g006730.2.1	STKc_CAMK	EF-hands(2)	582	64.62	5.92	7	I	MGNTCIGP	No
SlCDPK6	Solyc10g076900.1.1	STKc_CAMK	EF-hands(2)	501	55.61	5.27	7	I	MGNTCSGP	Yes
SlCDPK7	Solyc10g081640.1.1	STKc_CAMK	EF-hands(2)	579	63.52	4.94	6	I	MGNNCVGP	No
SlCDPK8	Solyc05g056570.2.1	STKc_CAMK	EF-hands(2)	503	56.43	4.95	6	I	MDSSDLTK	No
SlCDPK9	Solyc04g049160.2.1	STKc_CAMK	EF-hands(2)	508	57.23	5.05	6	I	MASETDRK	No
SlCDPK10	Solyc11g018610.1.1	STKc_CAMK	EF-hands(2)	505	56.89	5.39	6	I	MEIPKSEN	No
SlCDPK11	Solyc06g065380.2.1	STKc_CAMK	EF-hands(2)	507	57.14	5.83	7	I	MAQVVAKK	No
SlCDPK12	Solyc10g074570.1.1	STKc_CAMK	EF-hands(2)	557	62.25	5.67	6	I	MGNTCRGS	No
SlCDPK13	Solyc01g112250.2.1	STKc_CAMK	EF-hands(2)	534	59.92	5.73	6	I	MGNACRGS	No
SlCDPK14	Solyc01g008740.1.1	STKc_CAMK	EF-hands(2)	541	61.10	5.56	7	II	MGNCCSSG	Yes
SlCDPK15	Solyc12g099790.1.1	STKc_CAMK	EF-hands(2)	535	59.63	5.42	8	II	MGGCCSKA	Yes
SlCDPK16	Solyc11g064900.1.1	STKc_CAMK	EF-hands(2)	529	59.53	5.93	7	II	MGNCCSRG	Yes
SlCDPK17	Solyc08g008170.2.1	STKc_CAMK	EF-hands(2)	516	57.70	5.92	7	II	MGNCNSLS	Yes
SlCDPK18	Solyc04g081910.2.1	STKc_CAMK	EF-hands(2)	521	58.92	5.24	7	II	MGLCFTKE	Yes
SlCDPK19	Solyc07g064610.2.1	STKc_CAMK	EF-hands(2)	521	57.82	6.87	7	II	MGICASKG	Yes
SlCDPK20	Solyc02g032820.2.1	STKc_CAMK	EF-hands(2)	527	60.03	6.56	7	II	MGGCFSKN	Yes
SlCDPK21	Solyc03g031670.2.1	STKc_CAMK	EF-hands(2)	553	62.99	6.58	7	II	MGGCFSKK	Yes
SlCDPK22	Solyc03g113390.2.1	STKc_CAMK	EF-hands(2)	538	60.94	6.69	6	III	MGNCNACI	No
SlCDPK23	Solyc09g005550.2.1	STKc_CAMK	EF-hands(2)	529	59.62	6.25	6	III	MGNCCRSP	No
SlCDPK24	Solyc06g073350.2.1	STKc_CAMK	EF-hands(2)	536	61.06	5.84	8	III	MGTCMSVQ	Yes
SlCDPK25	Solyc10g079130.1.1	STKc_CAMK	EF-hands(2)	525	59.63	6.12	7	III	MGNCCAVP	Yes
SlCDPK26	Solyc01g008440.2.1	STKc_CAMK	EF-hands(2)	533	59.99	6.84	7	III	MGNCCVKP	Yes

Table 1 continued

Protein	Locus	Protein kinase domain	Ca^{2+} binding domains (number)	Protein size (aa)	Mol Wt (kDa)	pI	Introns (number)	Subgroup	N terminus	*N*-Myristoylation
SlCDPK27	Solyc11g065660.1.1	STKc_CAMK	EF-hands(2)	533	59.66	6.25	7	III	MGNCCGTP	Yes
SlCDPK28	Solyc03g033540.2.1	STKc_CAMK	EF-hands(2)	565	63.88	8.95	11	IV	MGSCFSSS	Yes
SlCDPK29	Solyc02g083850.2.1	STKc_CAMK	EF-hands(2)	570	64.20	9.28	11	IV	MGNICFSS	No
SlCRK1	Solyc03g082500.2.1	STKc_CAMK		598	66.84	8.89	10	I	MGQCCSKG	Yes
SlCRK2	Solyc01g096350.2.1	STKc_CAMK		589	66.10	7.77	10	I	MGQ-CYGKT	Yes
SlCRK3	Solyc10g078390.1.1	STKc_CAMK		574	64.32	8.57	10	II	MGLCHGKP	Yes
SlCRK4	Solyc01g108400.2.1	STKc_CAMK	FRQ1(1)	588	65.84	8.90	10	II	MGACTSKP	Yes
SlCRK5	Solyc02g065070.2.1	STKc_CAMK	FRQ1(1)	591	66.30	8.37	10	II	MGGCTSKP	Yes
SlCRK6	Solyc02g090510.2.1	STKc_CAMK	EH(1)	607	67.92	9.07	10	II	MGACTSRP	Yes
Selaginella moellendorffii										
SmCRK1	173444	STKc_CAMK		579	64.80	9.17	10	I	MGQ-CYGKV	Yes
SmCRK2	73433[a]	STKc_CAMK		586	65.36	9.33	10	I	MGQ-CYGKT	Yes
Physcomitrella patens										
PpCRK1	Phpat.026G018100	STKc_CAMK		598	67.27	9.18	10	I	MGQ-CYGKH	Yes
PpCRK2	Phpat.004G095200	STKc_CAMK		597	67.28	9.16	10	I	MGQ-CYGKF	Yes
PpCRK3	Phpat.003G024100	STKc_CAMK		592	67.18	9.28	10	I	MGQ-CYGKY	Yes
PpCRK4	Phpat.003G024200	STKc_CAMK	FRQ1(1)	598	67.22	7.93	11	I	MGQ-CYGKY	Yes
PpCRK5	Phpat.013G012900	STKc_CAMK	FRQ1(1)	595	67.00	9.27	10	I	MGQ-CYGKY	Yes

[a] This protein sequence was incomplete and a complete version was obtained from NCBI under accession number XP_002960835.1

members, SlCDPK22 to SlCDPK27, constituted subgroup III, while the remaining two members SlCDPK28 and SlCDPK29 formed subgroup IV (Fig. 1). Two members of the SlCRK family, SlCRK1 and SlCRK2, belong to subgroup I, while four members, SlCRK3 to SlCRK6, represent subgroup II (Figs. 1, 2, 3). Regarding the CRK family in the lower plants *S. moellendorffii* and *P. patens*, all two and five members, respectively, converged in subgroup I (Fig. 1).

Prediction of biochemical characteristics of SlCDPKs and plant CRKs

All SlCDPK proteins were composed of approximately 500–600 amino acids (Table 1). As a result, the molecular weight of the SlCDPK proteins was around 60 kDa. Additionally, the predicted pI value of the SlCDPKs was from 4.94 (SlCDPK7) to 9.28 (SlCDPK29) and apparently varied among the different subgroups of the SlCDPKs. It seems to increase from subgroup I to IV as shown by both the averaged pI and the range of pI of the subgroups. The predicted averaged pI of the subgroups I–IV was 5.40, 6.01, 6.33 and 9.12, respectively, while the range of pI of the subgroups I–IV was 4.94–5.92, 5.24–6.87, 5.84–6.84 and 8.95–9.28, respectively (Table 1). The predicted protein pI data demonstrated that the two SlCDPKs of subgroup IV are basic proteins while all the remaining 27 SlCDPKs are either acidic or neutral proteins. The biochemical features of CRKs from three species with different evolutionary positions were compared. The six

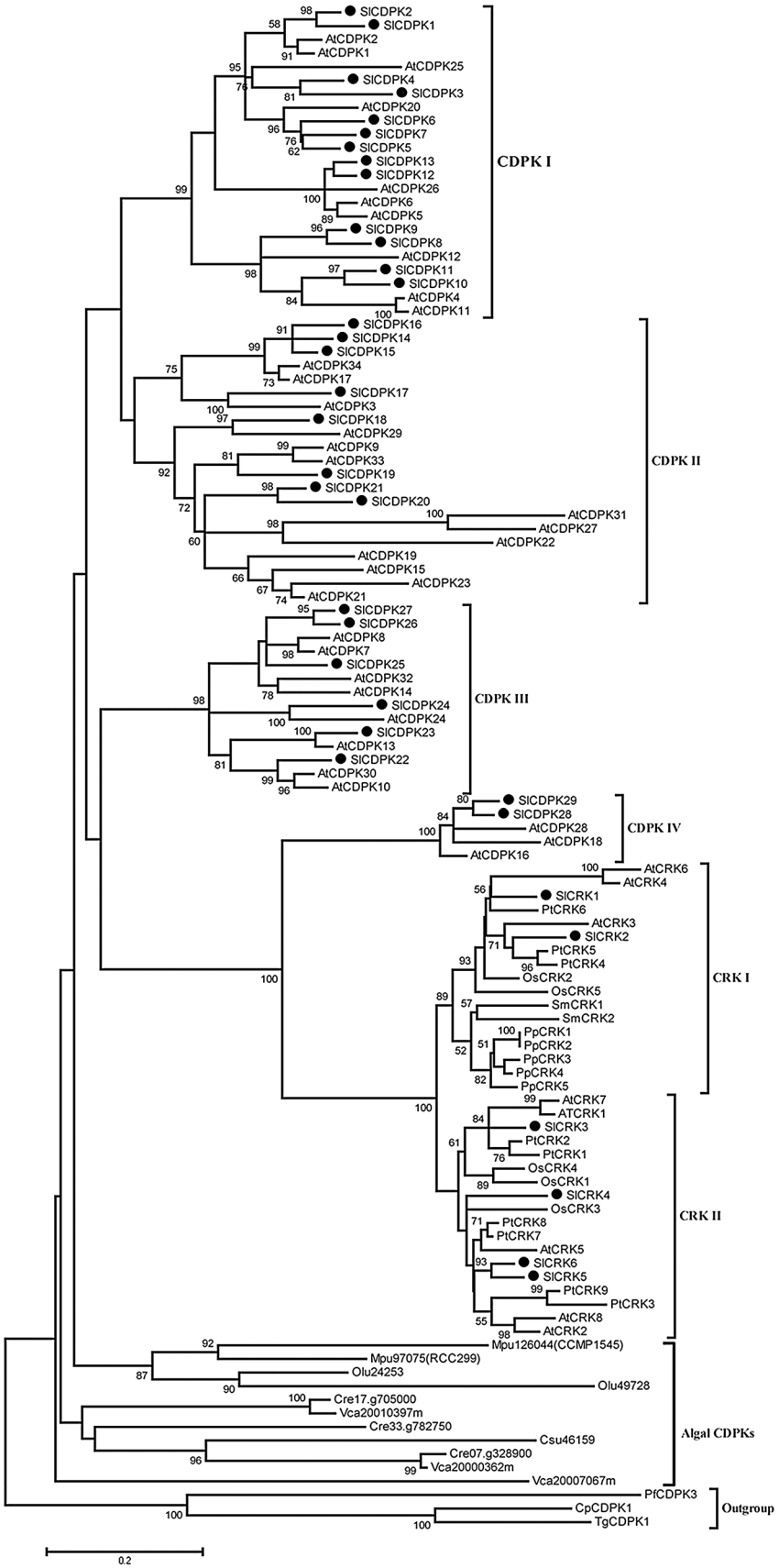

Fig. 1 Phylogenetic tree of plant CDPK and CRK proteins. The tree was created based on the alignment of kinase domain of protein sequences using MUSCLE program. Maximum likelihood (ML) method was used with bootstrap of 1000 in MEGA 5.0. CDPK sequences from *Arabidopsis*, tomato and algae as well as CRK sequences from *Arabidopsis*, tomato, rice, *Populus trichocarpa*, *Selaginella moellendorffii* and *Physcomitrella patens* were subjected to tree construction. Three apicomplexan CDPKs, TgCDPK1 (ToxoDB ID TGME49_301440), PfCDPK3 (PlasmoDB ID PF3D7_0310100) and CpCDPK1 (CryptoDB ID cgd3_920), were also included as outgroup. Tomato CDPKs and CRKs are indicated as a *solid circle*

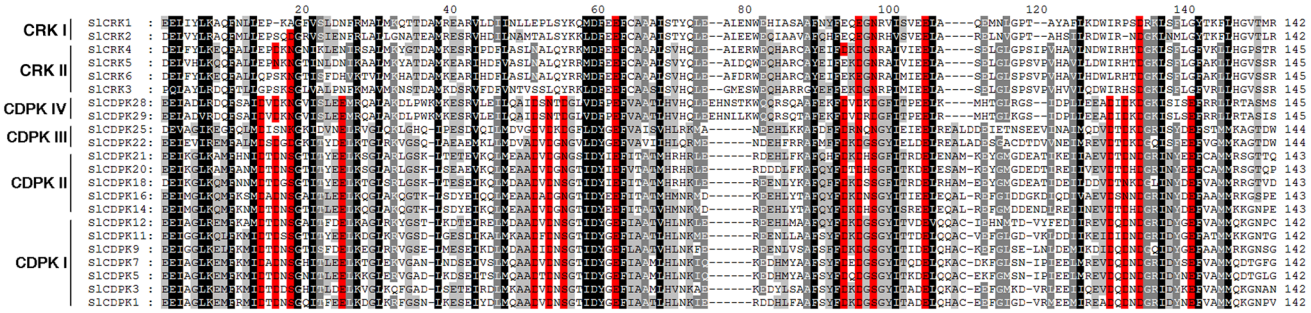

Fig. 2 Multiple sequence alignment of EF-hands of tomato CDPKs with the corresponding region of tomato CRKs. Sequence alignment was conducted using MUSCLE program and visualized using GeneDoc 2.6 software. Conserved residues are shaded *gray* and *black*, while predicted functional Ca^{2+} binding residues are highlighted in *red*

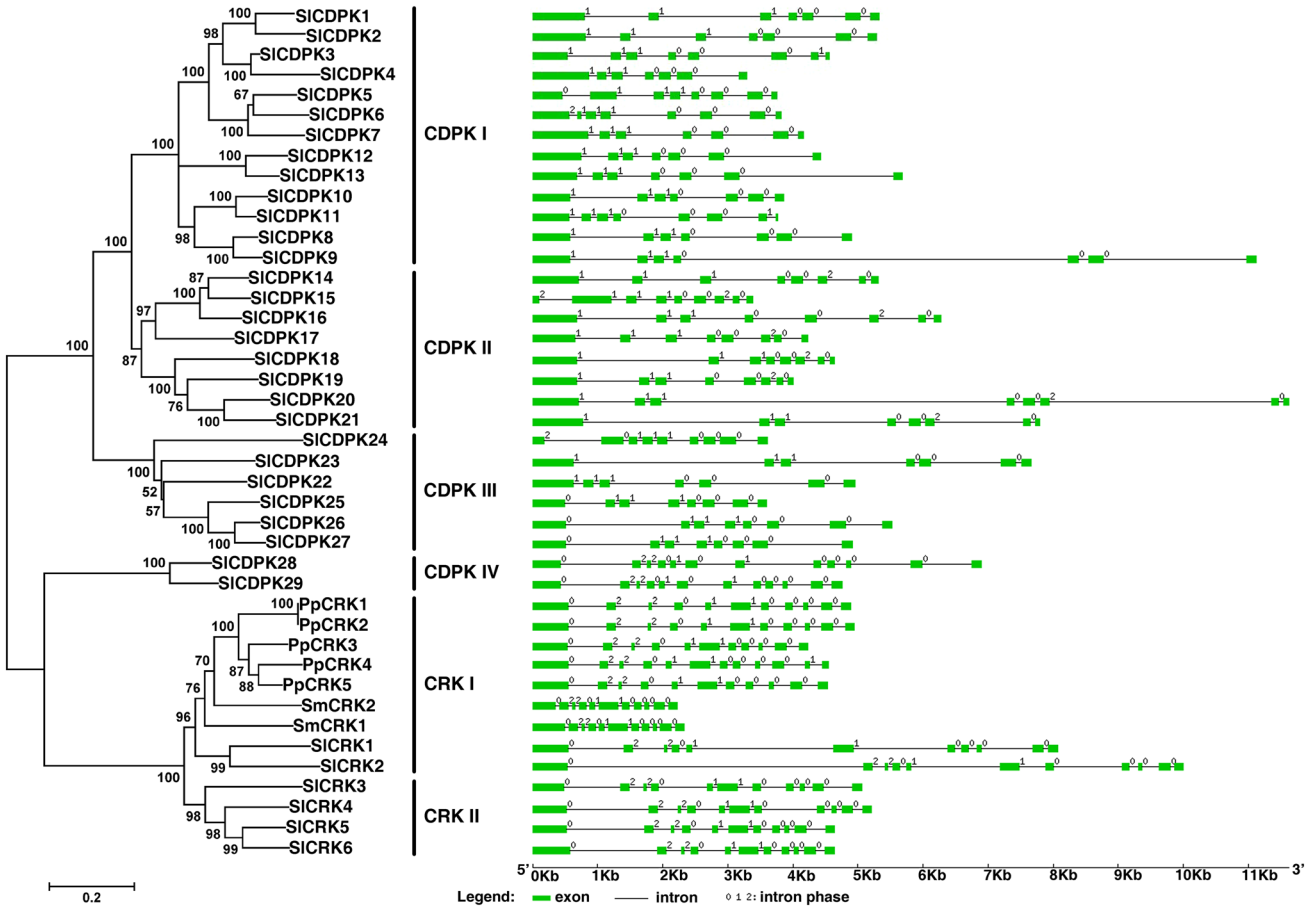

Fig. 3 Schematic diagram indicating the exon/intron structure of *CDPK* and *CRK* genes. Exon/intron configuration was analyzed with tomato *CDPK* genes and *CRK* genes from tomato, *Selaginella moellendorffii* and *Physcomitrella patens*. The locus number of these *CDPK* and *CRK* genes is listed in Table 1. Exons and introns are indicated as *green boxes* and *black lines*, respectively. The intron phase numbers *0*, *1* and *2* are labeled at the beginning of each intron. The diagram is drawn to scale. An unrooted tree for their protein sequences is also shown in the *left side*

SlCRK proteins contained 574–607 amino acids and their corresponding predicted molecular weight was 64.32–67.92 kDa. They were all basic proteins with a predicted pI value of 7.77–9.07 (Table 1). All the five CRKs from the nonvascular plant *P. patens* (PpCRKs) were found to have around 600 amino acids with predicted molecular weight of about 67 kDa. They also appeared to be basic proteins with a pI value of 7.93–9.28 (Table 1), while the two CRK

genes from the vascular nonflowering plant *S. moellendorffii* (SmCRKs) encode proteins with around 580 amino acids with a molecular weight of about 65 kDa. These were basic proteins as well as their pI value was estimated to be 9.17 and 9.33, respectively (Table 1). These data reveal that plant CRKs are highly conserved with respect to their biochemical characteristics, which are highly similar to those of subgroup IV CDPKs. This supports the clustering of CRKs and CDPKs in the phylogenetic tree (Figs. 1, 3).

Domain and motif composition of SlCDPKs and plant CRKs

CDPKs should contain at least a protein kinase domain and a calcium-binding domain. As expected, all SlCDPKs harbored an STKc_CAMK type protein kinase domain and a calcium-binding domain, which consisted of two pairs of EF-hands (Table 1; Fig. S2). CRKs from tomato, *S. moellendorffii* and *P. patens* also contained an STKc_CAMK type protein kinase domain at a similar position of CDPK proteins. However, they did not contain a canonical complete EF-hand domain. Instead, they possessed degenerated EF-hand-like sequences. For example, SlCRK4, SlCRK5, PpCRK4 and PpCRK5 carried a FRQ1-like domain, while SlCRK6 possessed an incomplete EH-like domain (Table 1; Fig. S3). FRQ1 and EH are two members of the EF-hand superfamily (Confalonieri and Di Fiore 2002; Huttner et al. 2003). To further clarify the differences in the C terminal region of the SlCDPK and SlCRK protein sequences, pairwise comparison of this region in the two protein families was performed. Results revealed low similarities among SlCDPK and SlCRK protein sequences in this region. Importantly, most of the calcium-binding sites in EF-hand motifs of the SlCRKs were substituted by physico-chemically distinct amino acids except the third EF-hand. SlCRK4 displayed a complete third EF-hand motif, while for the other SlCRKs, three of the four calcium-binding residues of this EF-hand were conserved, whereas the remaining one was a D–E substitution. Hence, this EF-hand of SlCRKs might be still functional. In addition, this sequence analysis also further reflected closeness between subgroup IV SlCDPKs and SlCRKs. For example, SlCRK4 exhibited up to 39 % similarity to SlCDPK28 and SlCDPK29 each, while the SlCRK protein sequences generally showed only less than 28 % similarity to SlCDPKs of other subgroups. These results unveiled that degeneration of EF-hand motifs of SlCRKs during the emergence of this family from CDPK involved mutational substitution of residues required for Ca^{2+} binding with eventual activity reduction or even loss of the EF-hand domains (Fig. 2).

Additionally, the N-terminal myristoylation motif of the SlCDPK and CRK proteins was predicted by a myristoylator program in ExPASy (http://web.expasy.org/myristoylator/) (Bologna et al. 2004). Results showed that 14 SlCDPKs were predicted to bear an N-terminal myristoylation motif for membrane association (Table 1). The presence of an N-terminal myristoylation motif in SlCDPKs was subgroup-dependent. All members of subgroup II, the majority of subgroups III and one of the two subgroup IV SlCDPKs contained an N-terminal myristoylation motif whereas all but one member of subgroup I did not (Table 1). All CRKs from tomato, *S. moellendorffii* and *P. patens* were predicted to have the N-terminal myristoylation motif (Table 1), suggesting that the N-terminal myristoylation was highly conserved during the evolution of the plant CRKs.

Gene structure and chromosome location of tomato *CDPK* and plant *CRK* genes

To further understand the relationship among the members of the *SlCDPK* and plant *CRK* gene families, we examined the exon/intron gene structure of all *SlCDPK* genes and *CRK* genes from tomato, *S. moellendorffii* and *P. patens*. Results of comparison of *SlCDPK* genomic coding sequences showed that the intron number and phase pattern of the *SlCDPK* genes varied obviously in a subgroup-dependent manner (Table 1; Fig. 3). The majority (9 out of 13) of the subgroup I *SlCDPK* genes contained six introns with a phase pattern of 111000. The remaining four genes of this subgroup bore seven introns with an additional one in the 5′ or 3′ end. All subgroup II *SlCDPK* genes carried seven introns with a phase pattern of 1110020 except *SlCDPK15*, which had eight introns with an extra intron gain in the 5′ end of the gene. Subgroup III *SlCDPK* genes possessed 6–8 introns. Among them, three (*SlCDPK25*, *SlCDPK26* and *SlCDPK27*) were constituted of seven introns with a phase pattern of 0111000; two (*SlCDPK22* and *SlCDPK23*) contained six introns with a phase pattern of 111000, while the remaining one (*SlCDPK24*) carried eight introns with a phase pattern of 20111000. Strikingly, the subgroup IV *SlCDPK* genes were composed of 11 introns, which were significantly more than those found in *SlCDPK* genes of any other subgroups. Their phase pattern was 02201010000 (Table 1; Fig. 3). The exon/intron structure of the *CRK* genes from tomato, *S. moellendorffii* and *P. patens* was similar to each other. All of them carried ten introns with a phase pattern of 0220110000 except the PpCRK4 gene, which contained 11 introns with an extra phase 1 intron in the 3′ end. Thus, except for loss of one intron of phase 0 in the middle of the genes, the exon/intron structure of the *SlCRK* genes was highly similar to that of the subgroup IV *SlCDPK* genes (Table 1; Fig. 3). Collectively, these gene structural data fit the classification of the SlCDPK and SlCRK families based on the phylogenetic

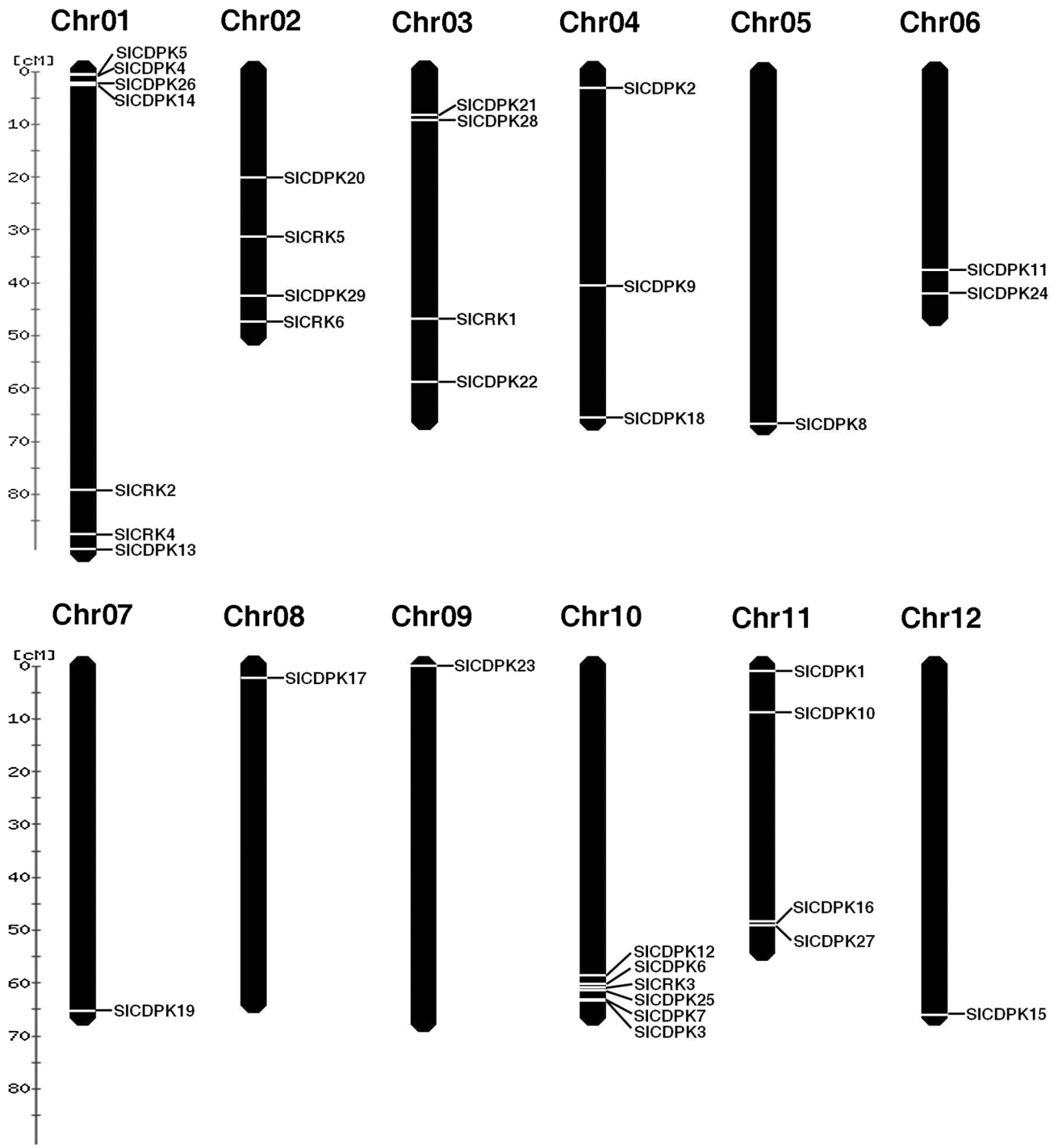

Fig. 4 Chromosomal localization of tomato *CDPK* and *CRK* genes

tree of protein sequences, and indicate the possible diversification in gene expression and functions among the different subgroups.

Chromosomal localization analysis showed that the *SlCDPKs* were distributed in all 12 chromosomes of the tomato genome. However, this distribution in each chromosome was unequal. Chromosomes 5, 7, 8, 9 and 12 each contained only a single *SlCDPK* gene copy, while chromosomes 1, 10 and 11 carried 5, 5 and 4 *SlCDPK* genes, respectively. Moreover, many *SlCDPK* genes were found to form clusters on their respective chromosomes (Fig. 4), suggesting repeated gene duplication events and thus the expansion of this gene family. The six SlCRKs are scattered on four chromosomes. Interestingly, *SlCRK3* is located in

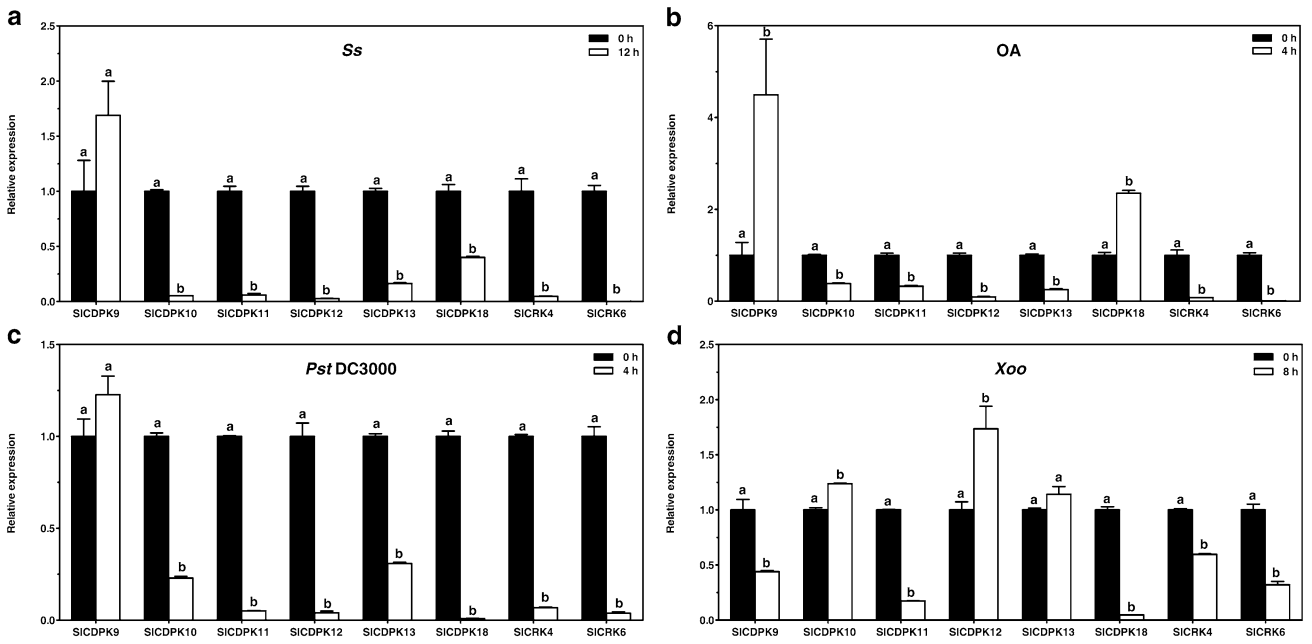

Fig. 5 The expression patterns of selected *SlCDPK* and *SlCRK* genes in response to pathogen inoculation and pathogenecity factor treatment. Gene expression was analyzed at 12 h after *S. sclerotiorum* inoculation (**a**), 4 h after OA treatment (**b**), 4 h after *Pst* DC3000 infiltration (**c**) and 8 h after *Xoo* infiltration (**d**). The *small letters* indicate the significance in expression of *SlCDPK* and *SlCRK* genes under each stimulus ($P \leq 0.05$, by Student's *t* test)

the middle of a cluster of five SlCDPKs on chromosome 10 (Fig. 4), implying that these SlCDPKs and SlCRK3 might have similar functions.

Expression of *SlCDPK* and *SlCRK* genes was highly responsive to diverse stimuli

To gain information about potential gene function in plant disease resistance, expression patterns of a set of six *SlCDPK* genes (*SlCDPK9/10/11/12/13/18*) and two *SlCRK* (*SlCRK4/6*) genes in response to host pathogens [*Sclerotinia sclerotiorum*, *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000] and a non-host pathogen (*Xanthomonas oryzae* pv. *oryzae*, *Xoo*) as well as a pathogenicity factor of the pathogen *S. sclerotiorum*, oxalic acid (OA) in tomato were investigated. These *SlCDPK* genes were selected because they might be the orthologs of *Arabidopsis* CDPK4/5/6/11 and rice CDPK12 (Fig. 1, this study; Boudsocq and Sheen 2013), which have been reported to function in plant disease resistance (Boudsocq et al. 2010; Asano et al. 2012).

At 12 h post-inoculation (hpi) of *S. sclerotiorum*, expression of the majority (5 out of 6) of the *SlCDPK* genes was dramatically down-regulated. However, the expression of *SlCDPK9* of subgroup I was slightly up-regulated (Fig. 5a). The expression pattern of the *SlCDPK* genes in response to OA treatment was very similar to that in response to inoculation with *S. sclerotiorum*, which produces OA during plant infection, except *SlCDPK18*, a gene of subgroup II, which was in contrast in response to *S. sclerotiorum* inoculation and OA treatment (Fig. 5b). Upon inoculation with the bacterial host pathogen *Pst* DC3000, the expression pattern of the *SlCDPK* genes was similar to that of inoculation with *S. sclerotiorum* (Fig. 5c). When inoculated with a non-host pathogen *Xoo*, expression of *SlCDPK10*, *SlCDPK12* and *SlCDPK13* was induced at 8 hpi, while that of *SlCDPK9* was reduced significantly, which was opposite to its expression after inoculation with *S. sclerotiorum*. Expression of all the remaining *SlCDPK* genes was reduced as observed for their expression after inoculation with *S. sclerotiorum* (Fig. 5d). These data indicate that expression of the *SlCDPK* genes is diverse in a gene- and stimulus-dependent manner. However, unlike *SlCDPK* genes, the two *SlCRK* genes *SlCRK4* and *SlCRK6* exhibited similar response to all stimuli in this study. They were all down-regulated by all pathogen inoculations and OA treatment. This indicates that these *SlCRK* genes might be involved in plant resistance to a wide range of pathogens.

Knock-down of a set of *SlCDPK* and *SlCRK* genes altered the resistance to *S. scelrotiorum*, *Pst* DC3000 and *Xoo* in tomato

To understand the function of *SlCDPKs* and *SlCRKs* in plant disease resistance, virus-induced gene silencing (VIGS) was performed for the *SlCDPK10/12/13/18* and *SlCRK4/6* genes. These genes were selected for VIGS

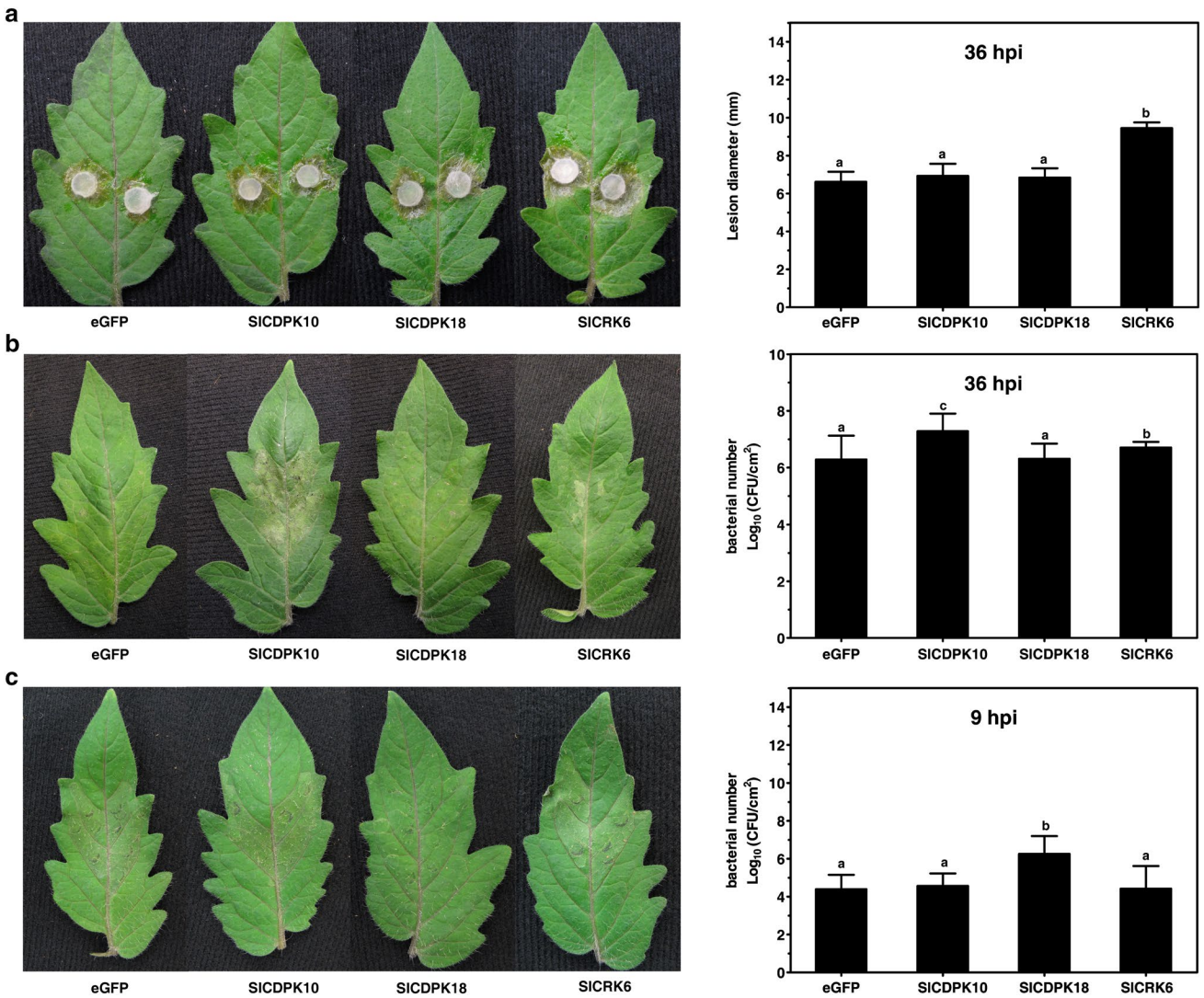

Fig. 6 Knock-down of a set of *SlCDPK* and *SlCRK* genes by VIGS decreased the tomato disease resistance. **a** The necrosis symptoms caused by *S. sclerotiorum* inoculation and statistical analysis of lesion diameter at 36 hpi. **b** The necrosis symptoms and bacterial numbers in the areas infiltrated with *Pst* DC3000 at 36 hpi. **c** The HR symptoms and bacterial numbers in the areas infiltrated with *Xoo* at 9 hpi. Significant differences of bacterial numbers and lesion diameter are indicated as *different lowercase letters* ($P \leq 0.05$, by Student's *t* test and DMRT)

analysis because they are highly responsive to various pathogen inoculations (Fig. 5). Moreover, orthologs of these *SlCDPK* genes are involved in plant disease resistance (Boudsocq et al. 2010; Asano et al. 2012). A vector containing a fragment of eGFP was used as the control in agro-infiltrated plants (Zhao et al. 2013). Three weeks post-agro-infiltration, the VIGS-treated (VT) tomato plants were inoculated with the host pathogens *S. sclerotiorum* and *Pst* DC3000 and the nonhost pathogen *Xoo*, and thereafter the resistance was evaluated.

When inoculated with *S. sclerotiorum*, the *SlCRK6*-VT plants displayed more severe disease symptom than the eGFP-control plants. The lesion diameter of these plants was 9.4 mm at 36 hpi, which was significantly larger than that of eGFP-control plants (6.6 mm) ($P \leq 0.05$). However, the *SlCDPK10*-VT and *SlCDPK18*-VT plants did not show significant difference from the control plants (Fig. 6a). This result indicated that *SlCRK6* plays a positive role in basal resistance to *S. sclerotiorum*. In case of inoculation with *Pst* DC3000, the *SlCDPK10*-VT and *SlCRK6*-VT plants exhibited highly and weakly stronger necrosis disease symptom, respectively, than the eGFP-control plants. Meanwhile, the bacterial number on these plants was 1.0 and 0.5 orders of magnitude, respectively, higher than the control plants. However, the *SlCDPK18*-VT plants did not show obvious difference from the control plants (Fig. 6b). This result showed that *SlCDPK10* and *SlCRK6* are positively involved in resistance to *Pst* DC3000. When inoculated

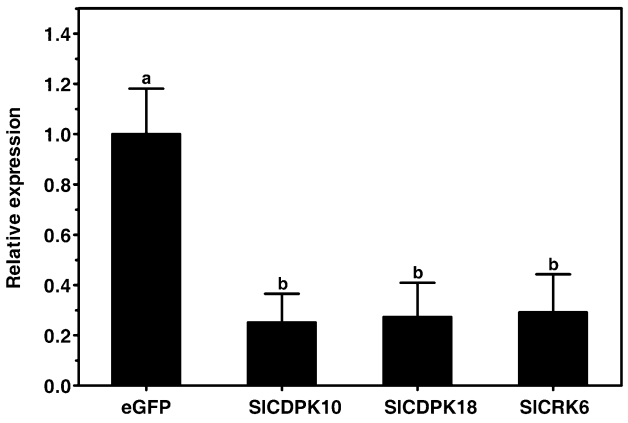

Fig. 7 Evaluation of gene silencing efficiency. Expression levels of the *SlCDPK10*, *SlCDPK18* and *SlCRK6* genes in tomato plants were examined by RT-qPCR. Significant difference between expression values within the target genes is indicated as *different lowercase letters* ($P \leq 0.05$, DMRT)

with *Xoo*, the HR necrosis was significantly weaker in the *Xoo*-infiltrated areas of the *SlCDPK18*-VT plants at 9 hpi when compared with the eGFP-control plants. Coincidently, the bacterial number on these plants was 1.8 orders of magnitude higher than the control plants. However, the *SlCDPK10*-VT and *SlCRK6*-VT plants did not show obvious difference in either HR symptom or bacterial number in the *Xoo*-infiltrated areas in comparison with the control plants (Fig. 6c). This result demonstrated that *SlCDPK18* plays a positive role in tomato nonhost resistance to *Xoo*. In addition, VIGS treatment of the *SlCDPK12*, *SlCDPK13* and *SlCRK4* showed no influence on tomato resistance to these three pathogens (data not shown), suggesting that these genes are not involved in these resistances or that other members of the *SlCDPK* and *SlCRK* families act redundantly.

To ensure the silencing efficiency of the *SlCDPK* and *SlCRK* genes, the expression of the target genes in the VIGS-treated and non-silenced eGFP control plants was compared. Results of RT-qPCR analysis showed that transcript of all the *SlCDPK* and *SlCRK* genes in the VIGS-treated plants accumulated to less than 30 % of that of control plants (Fig. 7), indicating that they were effectively knocked down, and the observed alteration in disease resistance is attributed to the *SlCDPK* and *SlCRK* genes.

Taken together, these results revealed that *SlCDPK* and *SlCRK* genes play roles in a wide range of resistance in tomato but their effectiveness against individual pathogen is gene-dependent. *SlCDPK18* is required for nonhost resistance to *Xoo*, *SlCDPK10* for basal resistance to *Pst* DC3000, while *SlCRK6* for basal resistance to both *S. sclerotiorum* and *Pst* DC3000.

Discussion

The phylogeny of *CDPK* and *CRK* gene families and their function in plant disease resistance are not well understood. In the present study, this issue was addressed through different approaches. Following identification of the *CDPK* family in tomato and the *CRK* family in tomato, a primitive lycophyte (*Selaginella moellendorffii*) and a moss (*Physcomitrella patens*), we performed various bioinformatics analyses including prediction of protein domain composition and physico-chemical characteristics, dissection of gene exon/intron structure, pairwise comparison of protein sequences and construction of rooted phylogenetic tree. As a result, we clarify that the plant CRK family evolved from the CDPK family and emerged very early in land plant species before the divergence of nonvascular and vascular plant species, sharing the same ancestor gene with the subgroup IV CDPKs. Moreover, we report for the first time the role of a CRK (SlCRK6) in plant disease resistance and the function of plant *CDPK* genes in nonhost resistance.

Phylogenetic relationship between the CDPK and CRK families

CDPK and CRK are two types of closely related protein kinases. They differ in presence or absence of EF-hand motifs. CRKs are thought to lack canonical EF-hand motifs and thus can not bind calcium (Harmon et al. 2000). The evolution of these two protein kinase families has been studied, but their phylogenetic relationship has not yet been well understood. Phylogenetic trees for CDPK and CRK families have been constructed previously based on the alignment of kinase catalytic domains (Harmon et al. 2000; Hrabak et al. 2003). It has been suggested that the plant *CDPK* and *CRK* genes shared a single common origin (Harmon et al. 2000) and protist and plant CDPKs have a monophyletic origin (Zhang and Choi 2001). In another study, it has been proposed that CRKs have arisen relatively recently in evolution from a distinct subgroup of CDPKs and had been only identified in angiosperm until that moment (Hrabak et al. 2003). In this study, we identified the complete *CDPK* and *CRK* gene families in tomato genome and the *CRK* genes in genomes of a primitive lycophyte (*S. moellendorffii*) and a moss (*P. patens*) (Table 1) and their existence in algal species was searched. We found no *CRK* gene in genomes of algal species, but did identify two and five CRKs in *S. moellendorffii* and *P. patens* genomes, respectively (Table 1), which is different from CDPKs as they have been previously identified in all these ancient green plant species (Hamel et al. 2014). Furthermore, we constructed the rooted phylogenetic tree for CDPK family of both tomato and *Arabidopsis* and CRK

family from six plant species including both higher and lower plant species using apicomplexan CDPKs as outgroup based on the alignment of kinase domains of protein sequences. In this tree, all CRKs clustered along with mainstream CDPK sequences and were found to be the closest relatives of subgroup IV CDPKs as they shared node with 100 % bootstrap support (Fig. 1). Since plant CRKs did not cluster with any algal CRK, it is suggestive of their early emergence from the ancestral CDPK which is common to that of group IV CDPKs. In addition, it is highly probable that CRK and group IV ancestors separated after the split of green algae and the last common ancestor of land plant lineage, which is in agreement with the absence of CRKs in green algae. However, the CRK expansion and diversification seem to be lineage independent like those of CDPKs that are estimated to have taken place after the split of land plants into vascular and non-vascular plants as recently revealed (Valmonte et al. 2014). Here, we reveal that the plant CRK family evolved from the CDPK family and emerged very early in land plant species before the divergence of nonvascular and vascular plant species, sharing the same ancestor with the subgroup IV CDPKs.

In addition to the phylogenetic tree, data on sequence similarity, prediction of biochemical characteristics and exon/intron structure of the CDPKs and CRKs also support our conclusion. Protein sequences of the CRKs are much more similar to those of the subgroup IV CDPKs than to those of any other CDPK subgroup (Fig. 2). Additionally, all identified CRKs are basic proteins, which are highly similar to the subgroup IV SlCDPKs, but distinguished from SlCDPKs of the remaining subgroups (Table 1). Moreover, all but one of the identified *CRK* genes possessed ten introns, which was similar to the subgroup IV *CDPKs* (11 introns), but distinct to *CDPK* genes of the other subgroups (6–8 introns) (Table 1; Fig. 3, this study; Hamel et al. 2014; Valmonte et al. 2014).

Collectively, these results clarify that the CRK lineage appeared very early from the last common ancestor and shares the immediate ancestral gene with subgroup IV CDPKs. Besides, it is discernible that the degeneration of EF-hand motifs of CRKs involved events such as mutational substitution of residues required for Ca^{2+} binding that occurred during the emergence of this family from CDPK gene of the last common ancestor of all land plant species.

Functions of SlCDPKs and SlCRKs in plant disease resistance

There has been increasing evidences supporting the involvement of CDPKs in plant disease resistance (Boudsocq and Sheen 2013). Nevertheless, the role of CDPKs in plant disease resistance has been studied only in the limited phytopathosystems. Additionally, whether CRKs play a role in plant disease resistance remains unknown. To gain more information about potential gene function in plant disease resistance, we checked expression patterns of a set of *SlCDPK* and *SlCRK* genes and performed their VIGS functional analyses in three pathosystems. These include tomato-*Sclerotinia sclerotiorum*, tomato-*Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and tomato-*Xanthomonas oryzae* pv. *oryzae* (*Xoo*), representing three different types of resistance: host basal resistance to necrotrophic fungal pathogen, host basal resistance to biotrophic bacterial pathogen and nonhost resistance to bacterial pathogen, respectively. Expression data demonstrated that different *SlCDPK* genes display diverse expression in response to the same pathogen, and the same *SlCDPK* gene exhibits various expression patterns in response to different pathogens such as host and nonhost pathogens (Fig. 5), indicating that expression of the *SlCDPK* genes is diverse in a gene- and stimulus-dependent manner. This is similar to what have been reported in other systems (Valmonte et al. 2014). Furthermore, our VIGS functional analyses revealed that different *SlCDPK* and *SlCRK* genes are involved in different resistance to various pathogens. For example, *SlCDPK18* is required for tomato nonhost resistance to the rice pathogen *Xoo*, *SlCDPK10* is involved in tomato basal resistance to *Pst* DC3000, while *SlCRK6* affects basal resistance to both *S. sclerotiorum* and *Pst* DC3000 (Fig. 6). According to the phylogenetic tree, *SlCDPK18* is the ortholog of *AtCDPK29* and *OsCDPK12*. These genes have identical intron phase pattern of 1110020 (Figs. 1, 3 of this study; Fig. S13 of Valmonte et al. 2014). *OsCDPK12* was found to negatively regulate the blast resistance in rice (Asano et al. 2012). Similarly, *SlCDPK10* is phylogenetically very close to *AtCDPK4/11* (Fig. 1) which were found to play a positive role in *Arabidopsis* resistance to *Pst* DC3000 (Boudsocq et al. 2010). These observations indicate that the function in disease resistance is conserved in othologs of *SlCDPK10/AtCDPK4/11* and *SlCDPK18/AtCDPK29/OsCDPK12* in various plant species. In addition, to our knowledge, this is the first report on the role of *CRK* in plant disease resistance, and *SlCRK6* is the first plant *CRK* gene that is proved to function in disease resistance. This is also the first report on function of tomato *CDPK* genes in disease resistance and is the first finding that plant *CDPK* genes are involved in nonhost resistance. Our results extended the spectrum of resistance and pathogens that are regulated by the plant *CDPK* genes, and revealed that plant *CDPK* genes play roles in a wide range of resistance with effectiveness against individual pathogen being CDPK gene dependent.

The functional mechanism of these *SlCDPKs* to regulate plant resistance remains unclear. Some CDPKs target NADPH oxidase to regulate reactive oxygen species (ROS)

production. For example, the orthologs of *SlCDPK10*, *AtCDPK4/11* were found to play a positive role in *Arabidopsis* resistance to *Pst* DC3000 through promoting ROS production, potentially by directly phosphorylating NADPH oxidase RBOHB (Boudsocq et al. 2010). On the contrary, the ortholog of *SlCDPK18*, *OsCDPK12*, negatively modulates blast resistance through reducing ROS accumulation (Asano et al. 2012). We wondered whether *SlCDPK10* and *SlCDPK18* function similarly. However, DAB staining analysis shows that the ROS accumulation level of the *SlCDPK*-knock-down plants both before and after pathogen inoculation does not alter significantly when compared with non-silenced control plants (data not shown). This result suggests that *SlCDPK10* and *SlCDPK18* might be not involved in ROS production. Alternatively, their function is likely overlapped by other functionally redundant *SlCDPK* gene(s) such as the phylogenetically closest paralog *SlCDPK11* (Fig. 1).

In addition to alter ROS accumulation, CDPKs may target MAPKs (Xie et al. 2014), BIK1 (Monaghan et al. 2014), WRKY transcription factors (Gao et al. 2013), and/or affect defense hormones (Coca and San Segundo 2010) locally or systemically (Romeis and Herde 2014) in response to pathogen infection. Whether *SlCDPK10* and *SlCDPK18* target similar substrates awaits further analyses. Unlike CDPKs, no target of CRKs has yet been identified under pathogenic conditions. Therefore, identification of the targets of *SlCRK6* will provide new insights into the molecular mechanism of *SlCRK6* to regulate plant resistance. Additionally, whether the function of SlCRK6 depends on Ca^{2+} is worth clarifying, considering that this protein only carries degenerated EF-hand motifs (Fig. 2).

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Asano T, Tanaka N, Yang G, Hayashi N, Komatsu S (2005) 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. Plant Cell Physiol 46:356–366

Asano T, Hayashi N, Kobayashi M, Aoki N, Miyao A, Mitsuhara I, Ichikawa H, Komatsu S, Hirochika H, Kikuchi S (2012) A rice calcium-dependent protein kinase OsCPK12 oppositely modulates salt-stress tolerance and blast disease resistance. Plant J 69:26–36

Bologna G, Yvon C, Duvaud S, Veuthey AL (2004) N-terminal myristoylation predictions by ensembles of neural networks. Proteomics 4:1626–1632

Boudsocq M, Sheen J (2013) CDPKs in immune and stress signaling. Trends Plant Sci 18:30–40

Boudsocq M, Willmann MR, McCormack M, Lee H, Shan L, He P, Bush J, Cheng SH, Sheen J (2010) Differential innate immune signalling via Ca^{2+} sensor protein kinases. Nature 464:418–422

Cai X, Wang C, Xu Y, Xu Q, Zheng Z, Zhou X (2007) Efficient gene silencing induction in tomato by a viral satellite DNA vector. Virus Res 125:169–175

Campo S, Baldrich P, Messeguer J, Lalanne E, Coca M, San Segundo B (2014) Overexpression of a Calcium-dependent protein kinase confers salt and drought tolerance in rice by preventing membrane lipid peroxidation. Plant Physiol 165:688–704

Cheng SH, Willmann MR, Chen HC, Sheen J (2002) Calcium signaling through protein kinases. The *Arabidopsis* calcium-dependent protein kinase gene family. Plant Physiol 129:469–485

Cheng WS, Xu QF, Li F, Xu YP, Cai XZ (2012) Establishment of a suitable control vector for Tobacco rattle virus-induced gene silencing analysis in *Nicotiana benthamiana*. J Zhejiang Univ (Agric Life Sci) 38:10–20

Coca M, San Segundo B (2010) AtCPK1 calcium-dependent protein kinase mediates pathogen resistance in Arabidopsis. Plant J 63:526–540

Confalonieri S, Di Fiore PP (2002) The Eps15 homology (EH) domain. FEBS Lett 513:24–29

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797

Gao X, Chen X, Lin W, Chen S, Lu D, Niu Y, Li L, Cheng C, McCormack M, Sheen J (2013) Bifurcation of *Arabidopsis* NLR immune signaling via Ca^{2+}-dependent protein kinases. PLoS Pathog 9:e1003127

Guo A, Zhu Q, Chen X, Luo J (2007) GSDS: a gene structure display server. Yi chuan 29:1023–1026

Hamel LP, Sheen J, Séguin A (2014) Ancient signals: comparative genomics of green plant CDPKs. Trends Plant Sci 19:79–89

Harmon AC, Gribskov M, Harper JF (2000) CDPKs–a kinase for every Ca^{2+} signal? Trends Plant Sci 5:154–159

Harper JF, Harmon A (2005) Plants, symbiosis and parasites: a calcium signalling connection. Nat Rev Mol Cell Biol 6:555–566

Harper JF, Breton G, Harmon A (2004) Decoding Ca^{2+} signals through plant protein kinases. Annu Rev Plant Biol 55:263–288

Hrabak EM, Chan CWM, Gribskov M, Harper JF, Choi JH, Halford N, Kudla J, Luan S, Nimmo HG, Sussman MR, Thomas M, Walker-Simmons K, Zhu JK, Harmon AC (2003) The Arabidopsis CDPK-SnRK superfamily of protein kinases. Plant Physiol 132:666–680

Huttner IG, Strahl T, Osawa M, King DS, Ames JB, Thorner J (2003) Molecular interactions of yeast frequenin (Frq1) with the phosphatidylinositol 4-kinase isoform, Pik1. J Biol Chem 278:4862–4874

Jones DT, Taylor WR, Thornton JM (1992) The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci 8:275–282

Kamiyoshihara Y, Iwata M, Fukaya T, Tatsuki M, Mori H (2010) Turnover of LeACS2, a wound-inducible 1-aminocyclopropane-1-carboxylic acid synthase in tomato, is regulated by phosphorylation/dephosphorylation. Plant J 64:140–150

Kim H-J, Chen C, Kabbage M, Dickman MB (2011) Identification and characterization of *Sclerotinia sclerotiorum* NADPH oxidases. Appl Environ Microbiol 77(21):7721–7729

Kobayashi M, Ohura I, Kawakita K, Yokota N, Fujiwara M, Shimamoto K, Doke N, Yoshioka H (2007) Calcium-dependent protein

kinases regulate the production of reactive oxygen species by potato NADPH oxidase. Plant Cell 19:1065–1080

Kudla J, Batistic O, Hashimoto K (2010) Calcium signals: the lead currency of plant information processing. Plant Cell 22:541–563

Leclercq J, Ranty B, Sanchez-Ballesta MT, Li ZG, Jones B, Jauneau A, Pech JC, Latche A, Ranjeva R, Bouzayen M (2005) Molecular and biochemical characterization of LeCRK1, a ripening-associated tomato CDPK-related kinase. J Exp Bot 56:25–35

Li RJ, Hua W, Lu YT (2006) Arabidopsis cytosolic glutamine synthetase AtGLN1;1 is a potential substrate of AtCRK3 involved in leaf senescence. Biochem Biophys Res Commun 342:119–126

Li AL, Zhu YF, Tan XM, Wang X, Wei B, Guo HZ, Zhang ZL, Chen XB, Zhao GY, Kong XY (2008) Evolutionary and functional study of the CDPK gene family in wheat (*Triticum aestivum* L.). Plant Mol Biol 66:429–443

Liu HT, Gao F, Li GL, Han JL, Liu DL, Sun DY, Zhou RG (2008) The calmodulin-binding protein kinase 3 is part of heat-shock signal transduction in *Arabidopsis thaliana*. Plant J 55:760–773

Liu W, Li W, He Q, Daud MK, Chen J, Zhu S (2014) Genome-wide survey and expression analysis of calcium-dependent protein kinase in *Gossypium raimondii*. PLoS One 9:e98189

Ludwig AA, Romeis T, Jones JD (2004) CDPK-mediated signalling pathways: specificity and cross-talk. J Exp Bot 55:181–188

Ludwig AA, Saitoh H, Felix G, Freymark G, Miersch O, Wasternack C, Boller T, Jones JD, Romeis T (2005) Ethylene-mediated cross-talk between calcium-dependent protein kinase and MAPK signaling controls stress responses in plants. Proc Natl Acad Sci USA 102:10736–10741

Monaghan J, Matschi S, Shorinola O, Rovenich H, Matei A, Segonzac C, Malinovsky FG, Rathjen JP, MacLean D, Romeis T (2014) The calcium-dependent protein kinase CPK28 buffers plant immunity and regulates BIK1 turnover. Cell Host Microbe 16:605–615

Ray S, Agarwal P, Arora R, Kapoor S, Tyagi AK (2007) Expression analysis of calcium-dependent protein kinase gene family during reproductive development and abiotic stress conditions in rice (*Oryza sativa* L. ssp. *indica*). Mol Genet Genomics 278:493–505

Reddy ASN, Ali GS, Celesnik H, Day IS (2011) Coping with stresses: roles of calcium-and calcium/calmodulin-regulated gene expression. Plant Cell 23:2010–2032

Rigo G, Ayaydin F, Tietz O, Zsigmond L, Kovacs H, Pay A, Salchert K, Darula Z, Medzihradszky KF, Szabados L, Palme K, Koncz C, Cseplo A (2013) Inactivation of plasma membrane-localized CDPK-RELATED KINASE5 decelerates PIN2 exocytosis and root gravitropic response in Arabidopsis. Plant Cell 25:1592–1608

Romeis T, Herde M (2014) From local to global: CDPKs in systemic defense signaling upon microbial and herbivore attack. Curr Opin Plant Biol 20:1–10

Romeis T, Ludwig AA, Martin R, Jones JD (2001) Calcium-dependent protein kinases play an essential role in a plant defence response. EMBO J 20:5556–5567

Saand MA, Xu YP, Li W, Wang JP, Cai XZ (2015) Cyclic nucleotide gated channel gene family in tomato:genome-wide identification and functional analyses in disease resistance. Front Plant Sci 6:303

Sanders D, Pelloux J, Brownlee C, Harper JF (2002) Calcium at the crossroads of signaling. Plant Cell 14(suppl.):S401–S417

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Tao XC, Lu YT (2013) Loss of *AtCRK1* gene function in *Arabidopsis thaliana* decreases tolerance to salt. J Plant Biol 56:306–314

Valmonte GR, Arthur K, Higgins CM, MacDiarmid RM (2014) Calcium-dependent protein kinases in plants: evolution, expression and function. Plant Cell Physiol 55:551–569

Wang Y, Liang SP, Xie QG, Lu YT (2004) Characterization of a calmodulin-regulated Ca^{2+}-dependent-protein-kinase-related protein kinase, AtCRK1, from Arabidopsis. Biochem J 383:73–81

Wang C, Cai X, Wang X, Zheng Z (2006) Optimisation of tobacco rattle virus-induced gene silencing in *Arabidopsis*. Funct Plant Biol 33:347–355

Xie K, Chen J, Wang Q, Yang Y (2014) Direct phosphorylation and activation of a mitogen-activated protein kinase by a calcium-dependent protein kinase in rice. Plant Cell 26:3077–3089

Zhang XS, Choi JH (2001) Molecular evolution of calmodulin-like domain protein kinases (CDPKs) in plants and protists. J Mol Evol 53:214–224

Zhang H, Liu WZ, Zhang Y, Deng M, Niu F, Yang B, Wang X, Wang B, Liang W, Deyholos MK (2014) Identification, expression and interaction analyses of calcium-dependent protein kinase (CPK) genes in canola (*Brassica napus* L.). BMC Genom 15:211

Zhao Y, Liu W, Xu YP, Cao JY, Braam J, Cai XZ (2013) Genome-wide identification and functional analyses of calmodulin genes in *Solanaceous* species. BMC Plant Biol 13:70

Zhou L, Lan W, Jiang Y, Fang W, Luan S (2013) A calcium-dependent protein kinase interacts with and activates a calcium channel to regulate pollen tube growth. Mol Plant 7:369–376

Zhu SY, Yu XC, Wang XJ, Zhao R, Li Y, Fan RC, Shang Y, Du SY, Wang XF, Wu FQ (2007) Two calcium-dependent protein kinases, CPK4 and CPK11, regulate abscisic acid signal transduction in *Arabidopsis*. Plant Cell 19:3019–3036

Zuo R, Hu R, Chai G, Xu M, Qi G, Kong Y, Zhou G (2013) Genome-wide identification, classification, and expression analysis of CDPK and its closely related gene families in poplar (*Populus trichocarpa*). Mol Biol Rep 40:2645–2662