TaCPK2-A, a calcium-dependent protein kinase gene that is required for wheat powdery mildew resistance enhances bacterial blight resistance in transgenic rice

Shuaifeng Geng1,2,*, Aili Li2,*, Lichuan Tang2,*, Lingjie Yin2, Liang Wu2, Cailin Lei2, Xiuping Guo2, Xin Zhang2, Guanghuai Jiang3, Wenxue Zhai3, Yuming Wei1, Youliang Zheng1, Xiujin Lan1,† and Long Mao2,†

1 Triticeae Research Institute, Sichuan Agricultural University, 211 Huimin Road, Wenjiang, Chengdu, Sichuan, 611130, PR China
2 National Key Facility of Crop Gene Resources and Genetic Improvement and Institute of Crop Sciences, MOA Key Laboratory for Germplasm and Biotechnology, Chinese Academy of Agricultural Sciences (CAAS), Beijing 100081, PR China
3 Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, PR China

* These authors contributed equally to this work.
† To whom correspondence should be addressed. E-mail: maolong@caas.net.cn; lanxiujin@yahoo.com.cn

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Abstract

Calcium-dependent protein kinases (CPKs) are important Ca2+ signalling components involved in complex immune and stress signalling networks; but the knowledge of CPK gene functions in the hexaploid wheat is limited. Previously, TaCPK2 was shown to be inducible by powdery mildew (Blumeria graminis tritici, Bgt) infection in wheat. Here, its functions in disease resistance are characterized further. This study shows the presence of defence-response and cold-response cis-elements on the promoters of the A subgenome homoeologue (TaCPK2-A) and D subgenome homoeologue (TaCPK2-D), respectively. Their expression patterns were then confirmed by quantitative real-time PCR (qRT-PCR) using genome-specific primers, where TaCPK2-A was induced by Bgt treatment while TaCPK2-D mainly responded to cold treatment. Downregulation of TaCPK2-A by virus-induced gene silencing (VIGS) causes loss of resistance to Bgt in resistant wheat lines, indicating that TaCPK2-A is required for powdery mildew resistance. Furthermore, overexpression of TaCPK2-A in rice enhanced bacterial blight (Xanthomonas oryzae pv. oryzae, Xoo) resistance. qRT-PCR analysis showed that overexpression of TaCPK2-A in rice promoted the expression of OsWRKY45-1, a transcription factor involved in both fungal and bacterial resistance by regulating jasmonic acid and salicylic acid signalling genes. The opposite effect was found in wheat TaCPK2-A VIGS plants, where the homologue of OsWRKY45-1 was significantly repressed. These data suggest that modulation of WRKY45-1 and associated defence-response genes by CPK2 genes may be the common mechanism for multiple disease resistance in grass species, which may have undergone subfunctionalization in promoters before the formation of hexaploid wheat.

Key words: CPK, defence signalling, disease resistance, jasmonic acid, wheat.

Introduction

Plant resistance to pathogens is achieved either through cell-surface pattern-recognition receptors that recognize microbe-associated molecular patterns or via intracellular nucleotide-binding leucine-rich repeat immune sensors as effector proteins. These processes initiate overlapping and distinct signalling pathways that involve protein kinase activation, Ca2+ influx, hormone biosynthesis, oxidative burst, and transcriptional reprogramming (Boudsocq and Sheen, 2013). Among them, Ca2+ plays an important role as a secondary messenger which can be sensed by calcium-dependent protein kinases (CDPKs, or lately
named CPKs). CPK genes are broadly distributed in the plant kingdom and protists (Harmon et al., 2000; Cheng et al., 2002). Studies have shown that they act downstream of cytosolic calcium concentration changes to mediate various physiological responses (Harper et al., 1991; Harmon et al., 2000; Asano et al., 2005; Ray et al., 2007). In eudicots, several cases have shown that transient and sustained transcriptional reprogramming in plant innate immune responses are mediated by various members of the CPK gene family, where they play key roles in the defence-induced oxidative burst by activating NADPH oxidases through direct phosphorylation and with either a positive or a negative effect, such as Arabidopsis AtCPK4/5/6 and AtCPK11 (Boudsocq et al., 2010), tobacco NtCPK2 (Ludwig et al., 2005), and potato StCPK4 and StCPK5 (Kobayashi et al., 2007). Similar reactive oxygen species regulation was also observed in monocot plants. For instance, the rice OsCPK13 induces cell death and accumulation of pathogenesis-related proteins when ectopically expressed in sorghum (Sorghum bicolor; Mall et al., 2011). On the other hand, overexpressing OsCPK12 renders susceptibility to both virulent and avirulent blast fungus in rice plants, potentially through abscisic acid hypersensitivity and, hence, the reduction in reactive oxygen species production (Asano et al., 2012). Similarly, the active form of the barley CPK gene HvCPK4 also has the capability to trigger cell death, whereas HvCPK3 promotes host cell entry of powdery mildew during both compatible and incompatible interactions (Freymerc et al., 2007).

An alternative functional mode of CPKs in disease resistance is the regulation of hormonal signalling. Hormones such as salicylic acid (SA) and jasmonic acid (JA) are closely related to resistance against both biotrophic and hemi-biotrophic pathogens. They are essential in defence-response initiation and subsequent defence signals transmission (Glazebrook, 2001). Evidence for the involvement of CPKs in most of these signalling events is accruing (Glazebrook et al., 2003; Beckers and Spoel, 2006; Boudsocq and Sheen, 2013). In Arabidopsis, for example, overexpressing AtCPK1 elevated the expression of SA-regulatory and -biosynthesis genes PAD4 (phytoalexin-deficient 4) and SID2/ICS1 (SA induction-deficient 2 isochorismate synthase 1; Coca and San Segundo, 2010) and hence the accumulation of SA and subsequently SA-regulated genes, without affecting JA or ethylene. Interestingly, unlike AtCPK1, the tobacco CPK gene NiCPK2 triggers JA and ethylene accumulation when its constitutively active form is transiently expressed, which then induces the expression of JA- and ethylene-regulated genes (Ludwig et al., 2005). Thus, CPKs work as important modulators for both SA and JA signalling.

Wheat is an allohexaploid species with the genome constitution as AABBDD: the A genome came from Triticum urartu, the B genome from Aegilops speltoides or another species classified in the Sitopsis section, and the D genome from Aegilops tauschii (Feldman and Levy, 2005). Before the formation of hexaploid common wheat ~8000 years ago, the three genomes diverged for 2.5-4.5 million years. Functional evolution did occur in the polyploidy wheat (e.g. Q gene; Zhang et al., 2011), but most diversification such as subfunctionalization, neo-functionalization, and pseudogenization should have existed in the diploid ancestors (Lynch and Force, 2000; Wendel, 2000). Despite extensive study of CPK genes in different plant species or in different biological contexts, the biological function of wheat CPKs are largely unknown, especially the functional divergence of CPK homoeologues.

Previously, the expression of TaCPK2 was shown to be inducible by powdery mildew (Blumeria graminis tritici, Bgt) infection in wheat (Li et al., 2008). This work expanded the study on the functions of TaCPK2-A in disease resistance. It shows that silencing the TaCPK2-A homoeologue compromises the resistance capability of powdery mildew-resistant wheat lines. In the meantime, overexpression of TaCPK2-A in rice enhanced resistance to bacterial blight. The data also suggest that the capability of TaCPK2-A to confer both fungal and bacterial resistance is probably achieved by its modulation on the disease-resistance-related transcription factor WRKY45-1 and, hence, a subset of defence-response genes. In addition, this study provides evidence for TaCPK2 gene promoter subfunctionalization in the A and D subgenomes. Thus, TaCPK2 may represent a common disease-resistance signalling pathway in grass species and, in the meantime, should be considered as another good example of gene sub-functionalization by promoter divergence.

Materials and methods

Plant materials and treatments

Wheat cv. Chinese Spring (Sears, 1961), diploid wheat T. urartu (accession UR206), Ae. speltoides (Y2006), and Ae. tauschii (Y2282) were grown in a growth chamber at 22 °C and a 16/8 light/dark cycle (60 μmol m−2 s−1 photon flux density). For powdery mildew inoculation, Bgt conidia) on heavily diseased plants were shaken off over a settling tower onto the PmAm6/Beijing837 BCSF3 (PmA) primary leaves. For cold treatment, 7-day-old seedlings were transferred from 22 °C to a 4 °C refrigerator and kept for 24 h. Leaves were harvested at 0, 3, 6, 12, 24, and 48 h and 0, 3, 6, 12, and 24 h for the above two treatments, respectively. Collected samples were stored at −70 °C until RNA extraction. Am6 is a synthetic amphiplant derived from a cross between Triticum durum (AABB) accession DR147 and Ae. tauschii (Szyrko et al., 2001) accession Ae39 (Zhou et al., 2005). The line used for this study (PmA) is a BCSF3 progeny between Am6 and the cultivar Beijing837 with a novel powdery mildew resistant gene. PmA is resistant to the popular Bgt race no. 15 in the Beijing area, whose virulence type is E09, while Beijing837 is susceptible.

Isolation of complete open reading frame of three TaCPK2 members

Total RNA was extracted from wheat leaves using an RNaseasy plant extraction Mini Kit (Tian-wei-shi-dai, China) according to the manufacturer’s instructions. First-round cDNA was synthesized using the SuperScript first-strand synthesis kit (Invitrogen, CA, USA). cDNA (containing the complete open reading frame) was amplified by PCR using nested primers. Primers for the first and second rounds of PCRs are shown in Supplementary Table S5 (available at JXB online). PCR products were subcloned into pGEM-T Easy vector and sequenced.

Chromosomal locations of three TaCPK2 members

Genomic DNA samples were prepared from the Chinese Spring nullisomic-tetrasomic lines and served as templates for the PCR
amplifications. Primers are shown in Supplementary Table S5. The PCR parameters were as follows: 94 °C for 10 min, 32 cycles of 94 °C for 30 s, annealing (56 °C for TaCPK2-A, 55 °C for TaCPK2-B and TaCPK2-D primers) for 30 s, and 72 °C for 45 s, and a final step at 72 °C for 10 min. For visualization, amplification products were separated by electrophoresis on a 2% agarose gel.

Virus-induced gene silencing assays of TaCPK2-A

The plasmids utilized in these experiments were based on the constructions described (Holzberg et al., 2002). A 190-bp fragment of TaCPK2-A was amplified from the plasmid pTaCPK2-A with the forward primer 5’-GCTAGCTCGTCCCTTGTAGGCAACGC-3’ and the reverse primer 5’-GCTAGCCCGCTGTCCTCGCTTT-3’. The underlined bases are NcoI restriction sites and were used for cloning the fragment into the vector pSS031-1. Capped transcripts were prepared from three linearized plasmids that contain the tr-partite barley stripe mosaic virus (BSMV) genome using the mMessage mMachine T7 in-vitro transcription kit (Ambion, Austin, TX), typically resulting in a final concentration of 1–1.5 mg/ml RNA. Plants were infected with BSMV RNA using a modified protocol (Holzberg et al., 2002; Scofield et al., 2005).

Bgt–wheat interaction assays

The method to estimate Bgt infection efficiency was largely in accordance with Li et al. (2005). Dwarf wheat (3 cm) were aligned on the surface of 0.5% agarose with 50 mg l⁻¹ 2-[4-(chlorophenyl) methyl]-1H-benzimidazol and sprayed with Bgt sporals (isolate E09) using an air compressor and nozzle. After 5 days, the leaves were bleached with trichloroacetic acid (1.5 g l⁻¹) in ethanol/chloroform (4:1 v/v), stained with aniline blue (1 mg l⁻¹) and observed under a light microscope for the formation of elongated secondary hyphae. A segment of the same leaf was kept at −80 °C for mRNA extraction and subsequent quantitative real-time PCR (qRT-PCR).

qRT-PCR

RNA was extracted using Trizol reagent (TIANGEN, China) and qRT-PCR experiments were performed on an ABI Prism 7300 (Applied Biosystems, USA) with SYBR Green as a fluorescent reporter. Numbers of transcripts were normalized with the mRNA level of the glyceraldehyde-3-phosphate dehydrogenase gene, which is constitutively expressed in wheat (Hong et al., 2008; Li et al., 2011). For virus-induced gene silencing (VIGS) plants, newly emerging leaves of 14 d after the viral inoculation (usually the fourth leaves) were used. At least eight VIGS plants were tested for each VIGS vector. To detect the overall expression level of all three homoeologues, regions identical across all three homoeoloalleles were used to design primers for PCR amplification (Supplementary Table S5). All experiments were performed with three independent biological replicates.

Construction of plant expression vectors and rice transformation

Full-length TaCPK2-A cDNA was produced by reverse-transcription PCR from total RNA and was cloned into the binary vector pCUBi1390 to generate the plasmids pCUBi:TaCPK2-A:nos. The constructs were introduced into Agrobacterium tumefaciens EHA105 by electroporation. Rice transformation was performed as described (Hiei et al., 1994). Kuiku 131, a Xoo-susceptible japonica rice cultivar was used as a transformation acceptor. Transgenic rice plants were selected on media containing 50 mg l⁻¹ hygromycin. Primers used for positive plant detection are listed in Supplementary Table S5. Three lines of T2 plants from three resistant T0 transgenic plants (67–8, 68-3, 74-13) were inoculated with the Philippines race (P6) of Xoo at the seedling stage as well as the booting stage. Bacteria were grown on plants for 2–14 days after inoculation when the development of lesion area was evaluated.

Bacterial populations were determined for inoculated leaves harvested at 0, 5, and 15 d after Xoo inoculation according to the methods of Yamaguchi et al. (2009).

Promoter isolation and cis-element prediction

To isolate the promoter region sequences of the TaCPK2-A genes, the cDNA sequence of TaCPK2 was used in a BLAST search of the scaffolds of the A. tauschii draft genome sequence (Jia et al., 2013; Ling et al., 2013). DNA fragments 1500 bp upstream of the start codon and part of N-terminal variable region were amplified. By comparing the two coding sequences, two promoters belonging to TaCPK2-A and TaCPK2-D were distinguished. cis-Acting regulatory elements prediction was performed at the PLACE online service (http://www.dna.affrc.go.jp/PLACE/signalscan.html; Lescot et al., 2002).

Results

Isolation and analysis of TaCPK2 homoeologues

Wheat is an allohexaploid plant and the presence of multiple allelles of a gene (homoeoalles) provides the potential for functional divergence. To study whether all three homoeologous genes were expressed or in what kind of expression patterns, this study set out to isolate cDNA sequences of these genes. First, cDNAs were obtained from the diploid wheat species. Using a previously reported sequence as a template for primer design (AY704444, Li et al., 2008), TaCPK2 cDNA sequences were obtained from the diploid wheat species T. urartu (AA, UR206), Ae. speltoides (SS, putative BB progenitor, Y206), and Ae. tauscchii (DD, Y2282). Then, three cDNAs were isolated from the hexaploid Chinese Spring and were distinguished according to their polymorphisms with diploid cDNA sequences. The three cDNAs were named TaCPK2-A, TaCPK2-B, and TaCPK2-D from A, B, and D subgenomes respectively. The three homoeologous genes contained open reading frames of 1677, 1680, and 1671 bp that encoded 558, 559, and 556 amino acids, respectively. The three proteins were highly similar, with 96.9–98.6% identity (Supplementary Tables S1 and S2). The protein of OsCPK13 was first named OsCDPK7 before all rice CPK genes were systematically named (Asano et al., 2005). As shown in Fig. 1, compared with their rice orthologue OsCPK13, most divergent amino acids of wheat CPK2 proteins were located at the N-terminal domain of the protein, whereas key amino acids LGQQQFGT (red), K (teal), and D (pink) in the kinase domain and four EF hands (key amino acids D, D, S, E and D, D, D, E) were conserved, indicating that all three homoeologous genes should encode functional CPK proteins.

Chromosomal locations of TaCPK2 genes

Based on the single-nucleotide polymorphisms at the N-terminal variable region, this study designed gene-specific primers (Fig. 1 and Supplementary Table S5). The chromosome assignments of the TaCPK2-A, TaCPK2-B, and TaCPK2-D genes were determined using the Chinese Spring nulli-tetrasomic lines. The results showed that primers specific
to the *TaCPK2-A* gene did not amplify any product for N2AT2B and N2AT2D. Neither *TaCPK2-B* nor *TaCPK2-D* specific primers were able to obtain products from N2BT2A/N2BT2D and N2DT2A/N2DT2B lines, respectively. Therefore, *TaCPK2-A*, *TaCPK2-B*, and *TaCPK2-D* were mapped to chromosomes 2A, 2B, and 2D, respectively (Fig. 2).

**Fig. 1.** Multiple sequence alignment of CPK2 proteins. Wheat proteins are prefixed with Ta and rice with Os. Identical residues are shaded. Blue arrows indicate the kinase domain; red indicates ATP binding site; teal indicates K ATP binding site; pink indicates D proton acceptor site; black lines indicate EF hand domains. D–D–S–E and D–D–D–E are conserved amino acids in EF hands.

**TaCPK2 promoter sequences and expression patterns analysis**

Taking advantage of recently published A (*T. urartu*) and D (*Ae. tauschii*) draft genome sequences (Jia et al., 2013; Ling et al., 2013), the present study isolated two fragments corresponding to 1.5-kb upstream of the start codon of *TaCPK2-A*.
and TaCPK2-D in Chinese Spring. However, TaCPK2-B promoter sequences could not be isolated using the same sets of primers (Supplementary Table S5), indicating that it might be further diverged. cis-Element prediction using the program PLACE showed two defence-response WBOXATNPR1 elements at the promoter region of TaCPK2-A (Fig. 3A and Supplementary Table S3). In contrast, two cold-response LTRECOREATCOR15 (LTR) elements were predicted on the TaCPK2-D promoter. Because it has been shown that the rice orthologue OsCPK13 also responds to cold treatment (Saijo et al., 2000), this study then performed a similar prediction of the OsCPK13 promoter and found one cold-response element on it (Fig. 3A). These data suggest that the A and D promoters may have experienced significant divergence and may play different roles in the hexaploid wheat. To confirm this, qRT-PCR with gene-specific primers showed that TaCPK2-A responded to Bgt treatment only while the expression of TaCPK2-D responded weakly to Bgt treatment, but strongly to cold (Fig. 3B), suggesting that the divergence of these two promoters may lead to subfunctionalization of the two genes.

TaCPK2-A is required for powdery mildew resistance in wheat

As shown by its expression patterns, TaCPK2-A seems to be the major homo-allele involved in powdery mildew resistance in wheat, so this allele was knocked down using VIGS techniques. To make sure that only TaCPK2-A transcripts were targeted, a 190-bp fragment across the 5'-untranscribed region and part of the 5'-coding region of TaCPK2-A was selected to develop VIGS constructs because these regions were the most divergent for CPK genes (Fig. 4A). As shown in Fig. 4B, downregulation of TaCPK2-A by VIGS in leaves of PmA, a resistant wheat line, promoted the compatible interaction between Bgt and PmA, indicating that TaCPK2-A is indeed required for powdery mildew resistance. qRT-PCR showed that TaCPK2-A was significantly repressed in VIGS plants (Fig. 4C). To make sure that there was no ‘off target’ silencing, the expression levels of TaCPK2-B, TaCPK2-D, and a third CPK gene, TaCPK1, were determined. The results showed that TaCPK2-B and TaCPK2-D were slightly down-regulated while TaCPK1 was slightly upregulated; but none of these was significant, indicating that the VIGS assay was of high specificity (Fig. 4C). To further confirm that down-regulation of TaCPK2-A indeed enhanced Bgt penetration and growth, leaves spread with Bgt spores were microscopically examined. The results showed that there were more Bgt hyphae on BSMV:TaCPK2-A leaves than the BSMV:GFP control, indicating that the TaCPK2-A VIGS plants were more susceptible to powdery mildew infection (Fig. 4D). Statistic analysis showed a significant difference between powdery mildew penetration efficiencies of VIGS plants and controls (19.01±6.13 versus 0%; Table 1). Together, these data suggest that maintaining an overall high level of TaCPK2-A transcripts is essential for powdery mildew resistance in wheat.

TaCPK2-A overexpression enhanced bacterial blight resistance in rice

Phylogenetic analysis and sequence similarity has shown that the most possible orthologue of TaCPK2 in rice is OsCPK13 (Saijo et al., 2000; Li et al., 2008). In rice, overexpression of the full-length OsCPK13 gene enhances resistance to cold and salt stresses (Saijo et al., 2000). However, ectopic expression of a truncated OsCPK13 version in sorghum (Sorghum bicolor) causes accumulation of pathogenesis-related proteins and upregulation of defence-related genes, such as alanine aminotransferase and urorophyrinogen III decarboxylase (Mall et al., 2011). Therefore, the question arises as to whether overexpressing TaCPK2 in rice would increase its resistance to diseases as well. This study generated transgenic rice plants harbouring TaCPK2-A constructs driven by the maize ubiquitin promoter. The results showed that overexpressing TaCPK2-A in Kuiku 131, a Xoo-susceptible japonica rice cultivar, enhanced its resistance to bacterial blight for both seedling and booting stage plants (Fig. 5A). As shown in Fig. 5B, the lesion areas on Xoo-inoculated leaves were reduced to 6.78, 5.06, and 4.32% for transgenic plants lines 1, 2, and 3, respectively, when compared with the control, which is correlated with TaCPK2-A transgene expression levels (Fig. 5C). In addition, the populations of Xoo in the TaCPK2-A transgenic plants (lines 1, 2, and 3) were decreased more than 10-fold compared with the control plants at 15 d after Xoo inoculation (Supplementary Fig. S1). To further confirm that only TaCPK2-A transcripts was overexpressed and the rice orthologue was not affected, this study determined the expression levels of OsCPK13 and found that it was indeed not affected in TaCPK2-A transgenic plants (Fig. 5C). Together, these data indicate that the enhanced Xoo resistance was indeed caused by the increased expression of TaCPK2-A.

Activation of the transcription factor WRKY45 by TaCPK2-A

In rice, a pair of WRKY genes, OsWRKY45-1 and OsWRKY45-2, isolated from japonica and indica subspecies
respectively, play important roles in bacterial blight resistance (Shimono et al., 2007, 2012; Tao et al., 2009) by regulating SA- and JA-signalling genes (Tao et al., 2009). By isolating the \textit{WRKY45} DNA sequence, the current study confirmed that the japonica cultivar Kuiku 131 indeed contained the \textit{OsWRKY45-1} gene.

To ascertain that defence-responsive genes were inducible by \textit{TaCPK2-A}, this study analysed the expression of \textit{OsWRKY45-1} as well as 10 downstream genes in the transgenic rice plants, most of which were SA- or JA-signalling genes, including \textit{PAL1} (phenylalanine ammonia lyase 1; X16099) which is involved in SA synthesis by the phenylpropanoid pathway and \textit{ICSI}...
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(isochorismate synthase 1; AK120689) and PAD4 (phytoalexin-deficient 4; CX118864) which are putatively involved in SA synthesis in rice by the isochorismate pathway (Qiu et al., 2007). Additional genes were PR1a (acidic pathogenesis-related protein 1; AJ278436), NH1 (Arabidopsis NPR1 homologue 1; AY9123983), and OsWRKY13 (EF143611) which are associated with activation of the SA-dependent pathway and LOX (lipoxygenase; D14000) and AOS2 (allene oxide synthase 2; AY062258) for JA biosynthesis as well as PR1b (U89895) and PR10/PBZ1 (ribonuclease; D38170) which have been shown to function in both JA- and SA-dependent pathways (Qiu et al., 2007; Yuan et al., 2007). In Xoo-susceptible Kuiku 131, this study did not detect the upregulation of OsWRKY45-1 in inoculated leaves (Fig. 6A). Contrastingly, the analysis in Kuiku 131 transgenic plants showed that, compared with the wild type, overexpression of TaCPK2-A activated the expression of WRKY45-1, LOX, AOS2, PR1a, and PR1b (P < 0.05) upon Xoo infection (Fig. 6A). The expression of SA-related genes such as WRKY13, PAL, ICS1, PAD4, NH1, and PR10, however, were not significantly affected (Supplementary Fig. S2). These results suggest that TaCPK2-A may play an important role in regulating OsWRKY45-1- and JA-signalling pathway-related genes.

By contrast, in wheat leaves where TaCPK2-A was repressed by VIGS, the homologue (Ta-05961) of rice OsWRKY45-1 was significantly downregulated (P < 0.05) when compared with the control BSMV:GFP plants. Correspondingly, JA marker gene homologues including LOX (Ta-2498165446) and AOS2 (Ta-134687) for JA biosynthesis were downregulated, together with two canonical pathogenesis-related genes, PRIa (Ta-01596) and PRIb (Ta-141027) (Fig. 6B and Supplementary Table S4), suggesting a similar regulatory mechanism for TaCPK2-A in wheat. Thus, the result on WRKY45 here is in line with that of Bahrini et al. (2011), where overexpression of TaWRKY45 conferred disease resistance to multiple fungal diseases in transgenic wheat plants, reinforcing the idea that TaCPK2-A plays a role in fungal resistance in wheat via its regulation on TaWRKY45. Together, the current data indicate conserved mechanisms mediated by TaCPK2-A for powdery mildew (fungal) resistance in wheat and bacterial blight resistance in rice.

Discussion

Wheat is one of the most important staple crops of mankind, in which biotic and abiotic stresses are major factors affecting its growth and productivity. CPKs sense the calcium concentration changes in plant cells and play important roles in stress responses (Boudsocq and Sheen, 2013).

Subfunctionalization of TaCPK2 genes

Wheat is a hexaploid with three homoeologous genomes. Divergence of homoeologue functions in wheat has been reported such as LEAFY HULL STERILE1 (WLSH1) MADS-box genes and the domestication gene Q (Shitsukawa et al., 2007; Zhang et al., 2011). WLSH1 MADS-box genes showed genetic and epigenetic alterations. The A genome
**Table 1. Blumeria graminis f. sp. tritici penetration efficiency in wheat VIGS plants**

Numbers indicate the mean of three independent experiments; Beijing837, Bgt susceptible control; Mock, PmA leaves inoculated with GKP buffer; BSMV:GFP, PmA leaves infected with GFP BSMV construct, empty vector-like control; BSMV:TaCPK2-A, PmA leaves infected with TaCPK2-A BSMV construct; at least 15 VIGS plants were tested for each vector.

| Plant                  | Number of cells | Penetration efficiency (%) |
|------------------------|-----------------|---------------------------|
|                        | Attacked        | With elongated second hyphae |               |
| Mock (PmA)             | 100 ± 4.58      | 0 ± 0                     | 0             |
| BSMV:GFP              | 102.67 ± 4.93   | 0 ± 0                     | 0             |
| BSMV:TaCPK2-A         | 106.76 ± 14.53  | 20.29 ± 6.31              | 19.01 ± 6.13*|
| Beijing837 (negative control) | 100.33 ± 6.43 | 48 ± 5.29                 | 47.83 ± 4.32  |

*P < 0.01, Student’s t-test.

**WLHS1** homologue (**WLHS1-A**) had a structural alteration that contained a large DNA insertion in place of the K domain sequence. Yeast two-hybrid assay and transgenic analysis indicated that the **WLHS1-A** protein had no apparent function. Gene structures of the B and D genome homoeologues, **WLHS1-B** and **WLHS1-D**, were intact, although **WLHS1-B** was predominantly silenced by cytosine methylation. Consequently, of the three **WLHS1** homologues, only **WLHS1-D** was functional in the hexaploid wheat (Shitsukawa et al., 2007). In the case of the **Q** gene, the scenario for homoeoolle co-regulation was even more complicated. Combined phenotypic and expression analysis indicated that, whereas **5AQ** plays a major role in conferring domestication-related traits, **5Dq** contributes directly and **5Bq** indirectly to suppression of the speltoid phenotypes (a much longer and spear-shaped spike compared with the square-shaped spike of Chinese Spring). The evolution of the **Q/q** loci in polyploid wheat resulted in the hyperfunctionalization of **5AQ**, pseudogenization of **5Bq**, and subfunctionalization of **5Dq**, all contributing to the domestication traits (Zhang et al., 2011).

**TaCPK2** homoeologues may also experience such processes. This study shows that key functional domains of **TaCPK2** homoeologous proteins are highly similar. They are also conserved with the rice orthologous gene protein OsCPK13. Interestingly, promoters of **TaCPK2-A** and **TaCPK2-D** appeared to be diverged because they were predicted to confer different **cis**-elements. **TaCPK2-A** promoter contained defence-related elements where **TaCPK2-D** contained elements for cold response, similar to that of rice OsCPK13 which has been shown to be involved in abiotic responses including cold (Saijo et al., 2000; Wan et al., 2007). Accordingly, **TaCPK2-A** was experimentally confirmed to respond to Bgt infection whereas **TaCPK2-D** responded to cold. The fact that **TaCPK2-D** still weakly responded to Bgt indicates either the presence of additional unknown **cis**-elements or that its promoter is in the transient of divergence. Finally, it will be interesting if the **TaCPK2-B** promoter can be cloned and subjected to similar analysis.

**Fig. 5.** Overexpression of **TaCPK2-A** in rice enhances bacterial blight resistance. (A) **TaCPK2-A**-overexpressing plants showing enhanced resistance to the Philippines race P6 of *Xanthomonas oryzae* pv. *oryzae* at both the seedling and booting stages. Leaves of transgenic rice plants (lines 1, 2, 3) and the control wild-type Kuku 131 (CK) were collected 15 days after P6 inoculation. (B) Measurement of lesion areas on leaves of booting stage transgenic plants (lines 1, 2, 3) and CK. (C) Expression analysis of **TaCPK2-A** in transgenic rice plants as shown in (B); OsCPK13 was used as a non-‘off target’ control. *P < 0.05, Student’s t-test.

**Regulation on WRKY45 may underlie dual disease-resistance capability of TaCPK2-A**

Two rice **WRKY** genes, **OsWRKY45-1** and **OsWRKY45-2**, play important roles in both fungal and bacterial resistance (Shimoto et al., 2007, 2012; Tao et al., 2009). Similarly, overexpression of wheat **WRKY45** gene confers disease resistance to multiple fungi in transgenic wheat plants (Bahrini et al., 2011). In rice, **OsWRKY45-1** may play a role in regulating **SA**- and **JA**-signalling genes such as **PAL1**, **PAD4**, **PR1a**, **NH1**, **LOX**, and **PR1b**, while **OsWRKY45-2** regulated a
**Fig. 6.** TaCPK2-A regulates WRKY45 and defence-response genes. (A) Expression patterns of OsWRKY45-1, JA-signalling pathway, and pathogenesis genes from Tao *et al.* (2009) in TaCPK2-A-overexpressing transgenic rice plants inoculated with Xoo using quantitative real-time-PCR (qRT-PCR). Expression levels for each gene were relative to that in the wild-type Kuiku 131 (CK) before Xoo inoculation (0h) as a control. **LOX**, lipoxygenase; **AOS2**, allene oxide synthase; **PR1a**, acidic pathogenesis-related protein 1; **PR1b**, basic pathogenesis-related protein 1. (B) Expression patterns of homologues for rice OsWRKY45-1, JA-signalling pathway, and pathogenesis genes in wheat VIGS plants. For qRT-PCR, PmA/Beijing837 BC5F3 leaf samples were collected after 14 days of BSMV inoculation. Expression levels of corresponding genes in BSMV:TaCPK2-A plants were relative to that in BSMV:GFP control plants. Wheat homologues were the best matches in PlantGDB (http://www.plantgdb.org/cgi-bin/blast/PlantGDBblast). Ta, PUT-163b-Triticum_aestivum. **P** < 0.01, *P* < 0.05, Student’s t-test. Three independent biological replicates were performed.
set of partly overlapping genes (PAL1, ICS1, PAD4, PR1a, NH1, OsWRKY13, LOX, AOS2, and PR1b; Tao et al., 2009).

The current study shows that TaCPK2-A is able to regulate OsWRKY45-1, LOX, AOS2, PR1a, and PR1b expression both in transgenic rice and VIGS wheat plants. This demonstrates that TaCPK2-A is likely to participate in disease resistance by modulating the expression of OsWRKY45-1 and its associated genes. In fact, several transcription factors can be directly phosphorylated by CPKs, such as ABF4 (abscisic acid-responsive element-binding factor 4), RSG (repression of shoot growth), and HsfB2a (heat shock factor B2a) that have been characterized in vivo as CPK substrates for abscisic acid- (Choi et al., 2005), gibberellin- (Ishida et al., 2008; Ito et al., 2010), and herbivore-induced signalling (Kanchiswamy et al., 2010). Whether TaCPK2-A can phosphorylate WRKY45, directly or indirectly, or use additional transcription factors as direct substrate are interesting topics for further investigation.

Application of CPK2 genes for crop engineering

This study shows that the powdery mildew resistance ability of TaCPK2 is transferable to rice, where it appears to enhance bacterial blight resistance. This is in contrast to a recent work in which the transfer of rice OsCPK13 (TaCPK2 orthologue), which is supposed to be associated with cold tolerance, into sorghum instead causes disease-resistance-like reactions such as the lesion mimic phenotype and upregulation of a number of pathogenesis-related genes (Mall et al., 2011). Similar conflicting results have been reported for OsWRKY45-1. Shimono et al. (2012) showed that overexpression of a Nipponbare-derived OsWRKY45-1 cDNA, driven by maize ubiquitin promoter, results in very strong Xoo resistance while overexpression of OsWRKY45-1 genomic sequence under the control of the same promoter slightly increases the growth of X. oryzae when compared with non-transgenic plants (Tao et al., 2009). Because the protein sequences of wheat TaCPK2 and rice OsCPK13 genes are quite conserved, it is deduced that such a change in functions should contribute to the expression levels of the transgenes, i.e. the extent of signalling strength mediated by CPK2. The dosage of signalling molecules may determine the status of additional circuit to be connected in the highly integrated signalling network which may mask the initial effects in a feedback manner. Other possibilities may also exist, such as host–plant specificity. In other words, overexpressing the same CPK gene in different species may have different effects. In addition, disease resistance has not been tested in rice overexpressing OsCPK13. Finally, the different versions of genes used (full-length OsCPK13 in rice versus truncated OsCPK13 in sorghum) may also influence the effect of the proteins. In light of the involvement of a number of signalling molecules – Ca2+, JA, and SA – in compatible and incompatible interactions of plants and their pathogens, it is imaginable that the regulatory pathways of CPK genes should be complicated. Further dissection of CPK-signalling pathways should help to understand their complexity and hence their better application for biotic and abiotic improvement of wheat.

Supplementary material

Supplementary data are available at JXB online.

Supplementary Fig. S1. Xoo growth curves in TaCPK2-A transgenic rice plants.

Supplementary Fig. S2. TaCPK2-A regulations on additional defence-response genes in transgenic rice plants.

Supplementary Table S1. Similarity matrix of TaCPK2 and OsCPK13 genes.

Supplementary Table S2. Similarity matrix of TaCPK2 and OsCPK13 proteins.

Supplementary Table S3. cis-Elements on CPK2 gene promoters as predicted in PLACE.

Supplementary Table S4. Sequence similarity of SA- or JA-signalling pathway marker genes in rice and wheat.

Supplementary Table S5. Primers used in this study.

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