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RESEARCH PAPER

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

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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, Ca^{2+} influx, hormone biosynthesis, oxidative burst, and transcriptional reprogramming (Boudsocq and Sheen, 2013). Among them, Ca^{2+} plays an important role as a secondary messenger which can be sensed by calcium-dependent protein kinases (CDPKs, or lately

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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 (Freymark *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 *NtCPK2* 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, neofunctionalization, 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 subfunctionalization 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 BC5F3 (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 amphiploid derived from a cross between *Triticum durum* (AABB) accession DR147 and *Ae. tauschii* (Szyroki *et al.*, 2001) accession Ae39 (Zhou *et al.*, 2005). The line used for this study (PmA) is a BC5F3 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 RNeasy plant extraction Mini Kit (Tian-wei-shi-dai, China) according to the manufacturer's instructions. First-strand 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 constructs as described (Holzberg *et al.*, 2002). A 190-bp fragment of *TaCPK2-A* was amplified from the plasmid pTaCPK2-A with the forward primer 5′-*GCTAGC*TGTCCTTTGATGGGCAACGCA-3′ and the reverse primer 5′-*GCTAGC*CCGCGTGGCCGTCCGTCTT-3′. The underlined bases are *Nhe*I 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 tripartite 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). Wheat leaves (3 cm) were aligned on the surface of 0.5% agarose with 50 mg l^{-1} 2-[(4- chlorophenenyl) methyl]-1H-benzimidazol and sprayed with *Bgt* spores (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 g 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 homoeoalleles 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 pC:ubi: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 alleles 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. tauschii* (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* derived 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 LGQGQFGT (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

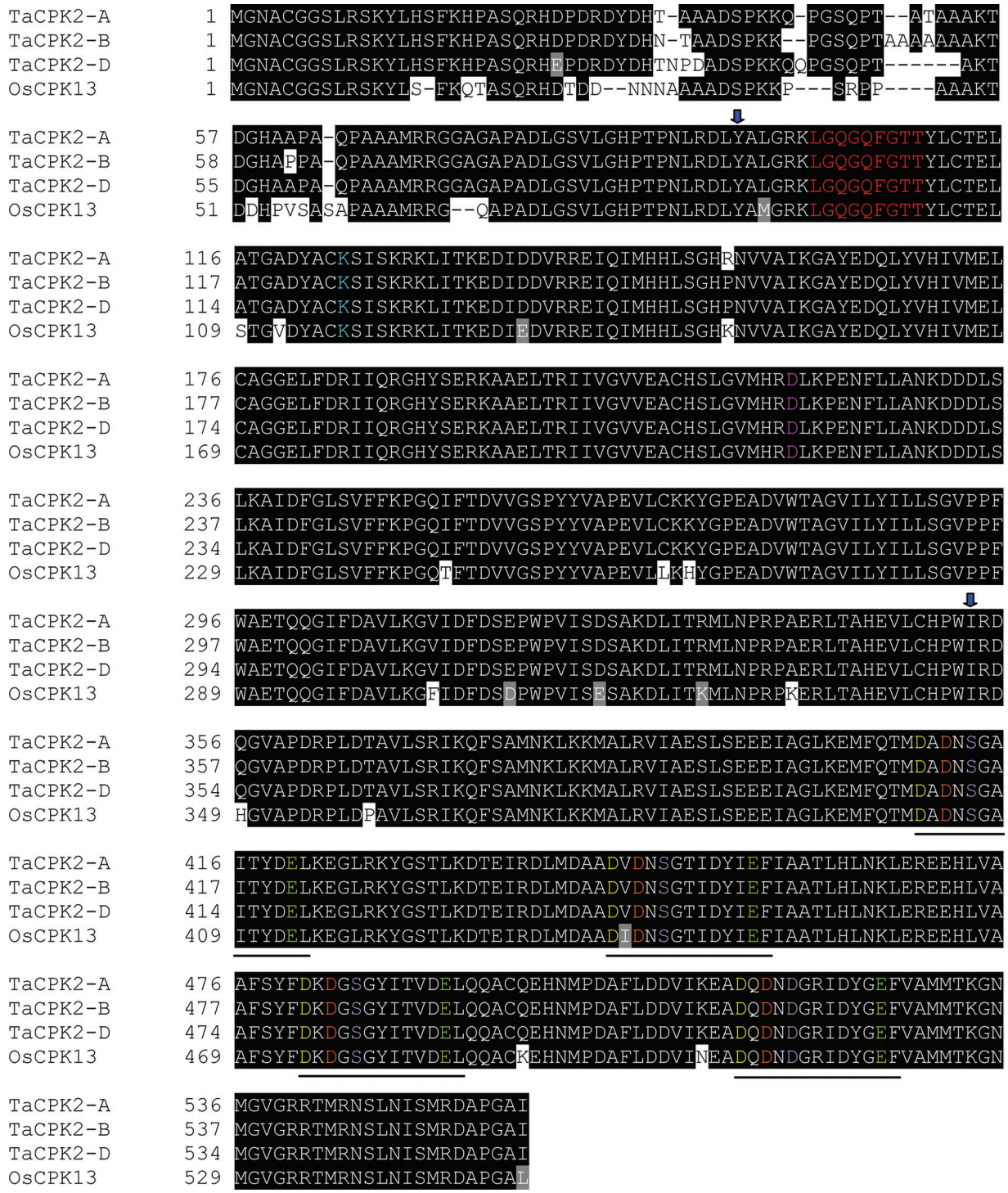

Fig. 1. Multiple sequence alignment of CPK2 proteins. Wheat proteins are prefixed with Ta and rice with Os. Identical residues are shaded. Blue arrows indicate the kinase domain; red indicates ATP binding site; teal indicates K ATP binding site; pink indicates D proton acceptor site; black lines indicate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

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).

TaCPK2 promoter sequences and expression patterns analysis

Taking advantage of recently published A (*T. uratu*) 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*

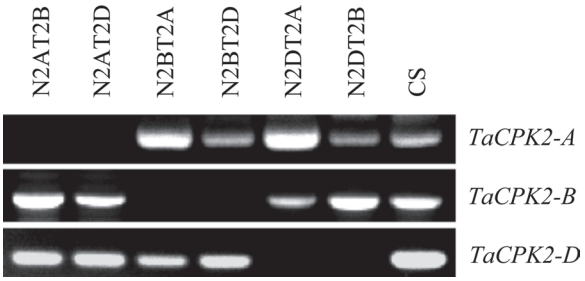

Fig. 2. Chromosomal locations of *TaCPK2* genes. Three pairs of genome-specific primers were used (Supplementary Table S5). A nulli-tetrasomic series of wheat cultivar Chinese Spring (CS) was used for chromosome assignment. Each line of the series lacks a given pair of homoeologous A, B, or D genome chromosomes (nullisomic condition) that are replaced by the corresponding homoeologous chromosome pair (tetrasomic condition).

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 homoeo-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 downregulated 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 downregulation 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 uroporphyrinogen 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

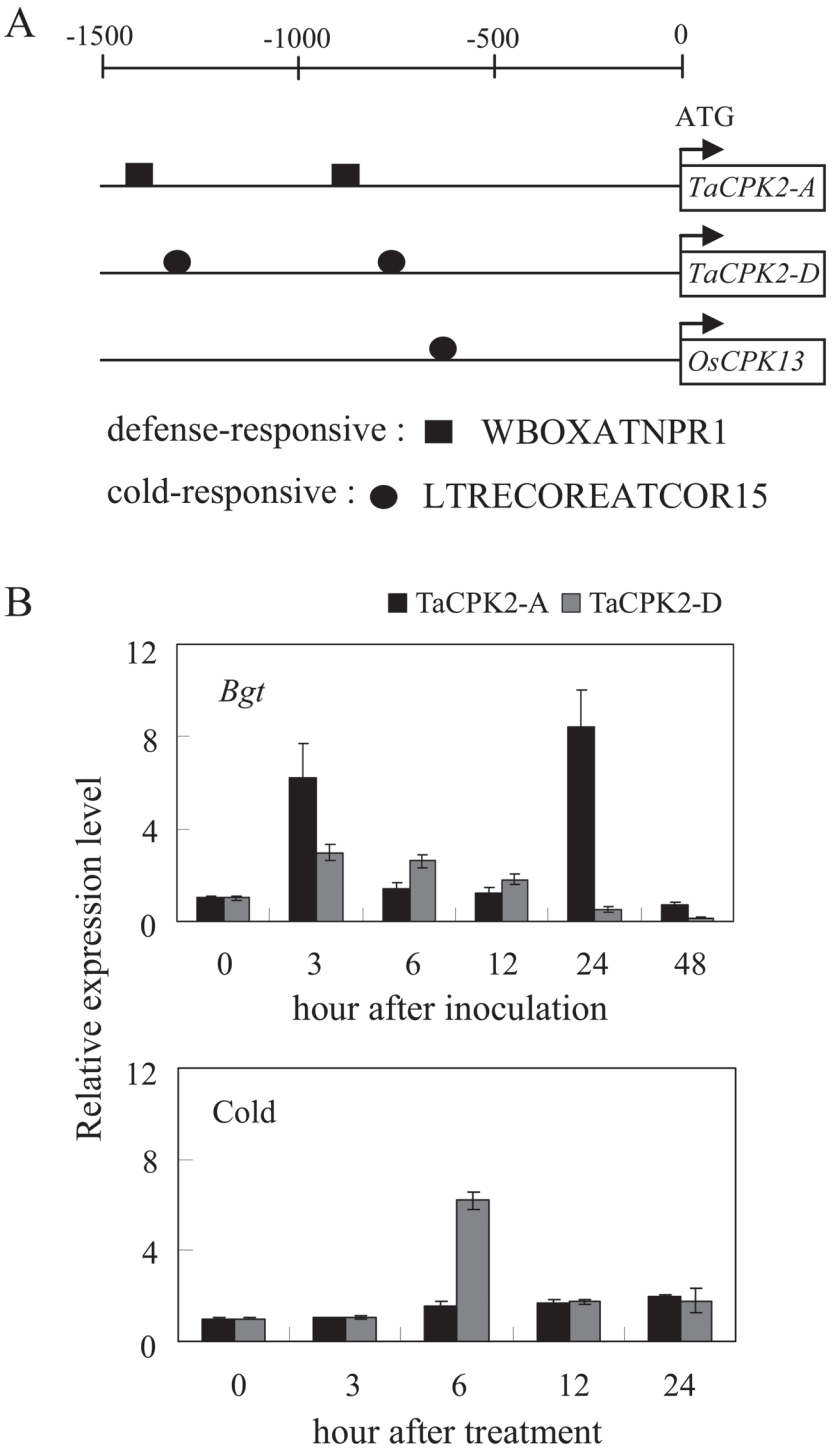

Fig. 3. Characterization of wheat A and D subgenome promoters of *TaCPK2* genes. (A) Presence of defence- and cold-response *cis*-elements on *TaCPK2-A*, *TaCPK2-D*, and rice *OsCPK13* promoters. (B) Expression patterns of *TaCPK2-A* and *TaCPK2-D* genes as detected by quantitative reala-time PCR using homoeoallele-specific primers. PmA used for PCR amplification is a powdery mildew-resistant line.

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 *WRKY45* DNA sequence, the current study confirmed that the japonica cultivar Kuiku 131 indeed contained the *OsWRKY45-1* gene.

To ascertain that defence-responsive genes were inducible by *TaCPK2-A*, this study analysed the expression of *OsWRKY45-1* as well as 10 downstream genes in the transgenic rice plants, most of which were SA- or JA-signalling genes, including *PAL1* (phenylalanine ammonia lyase 1; X16099) which is involved in SA synthesis by the phenylpropanoid pathway and *ICS1*

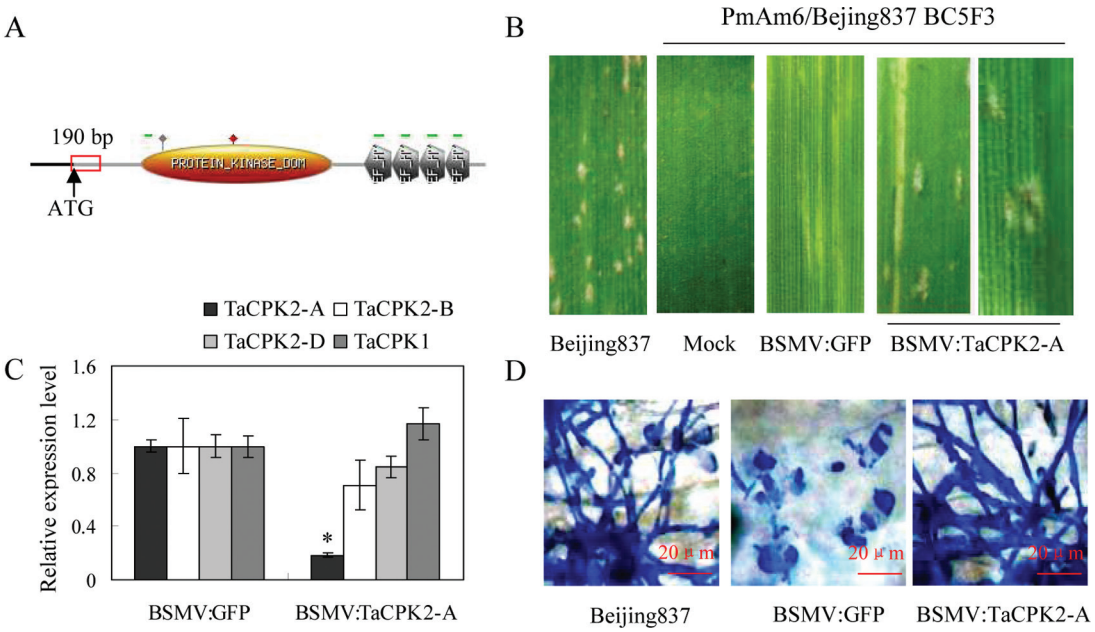

Fig. 4. Virus-induced gene silencing (VIGS) assay of *TaCPK2-A*. (A) Genomic structure of *TaCPK2-A* gene predicted by ScanProsite (http://prosite.expasy.org/scanprosite/); red box indicates the DNA fragment used for VIGS construct development. (B) *Bgt* inoculation and growth on leaves of PmAm6/Beijing837 BC5F3 *TaCPK2-A* VIGS plants and their controls for 8 days after inoculation; Bejing837, *Bgt* susceptible control; Mock, GKP buffer; BSMV:GFP, non-target control; BSMV:TaCPK2-A, *TaCPK2-A* VIGS construct; leaves from at least 15 plants were tested for each vector. (C) Significant downregulation of *TaCPK2-A* expression in PmAm6/Beijing837 BC5F3 plants infected with BSMV:TaCPK2-A VIGS constructs as detected by quantitative real-time PCR. $^*P < 0.05$, Student's t-test. (D) Microscopic observation of elongated secondary hyphae on leaves of *Bgt*-susceptible control Beijing837, non-target VIGS control BSMV:GFP, and *TaCPK2-A* VIGS construct BSMV:TaCPK2-A after 5 days of *Bgt* inoculation. Bars, 20 μm.

(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, *PR1a* (Ta-01596) and *PR1b* (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). *WLHS1* 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; Bejing837, *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 homoeologue (*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* homoeologues, only *WLHS1-D* was functional in the hexaploid wheat (Shitsukawa *et al.*, 2007). In the case of the *Q* gene, the scenario for homoeoallele 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).

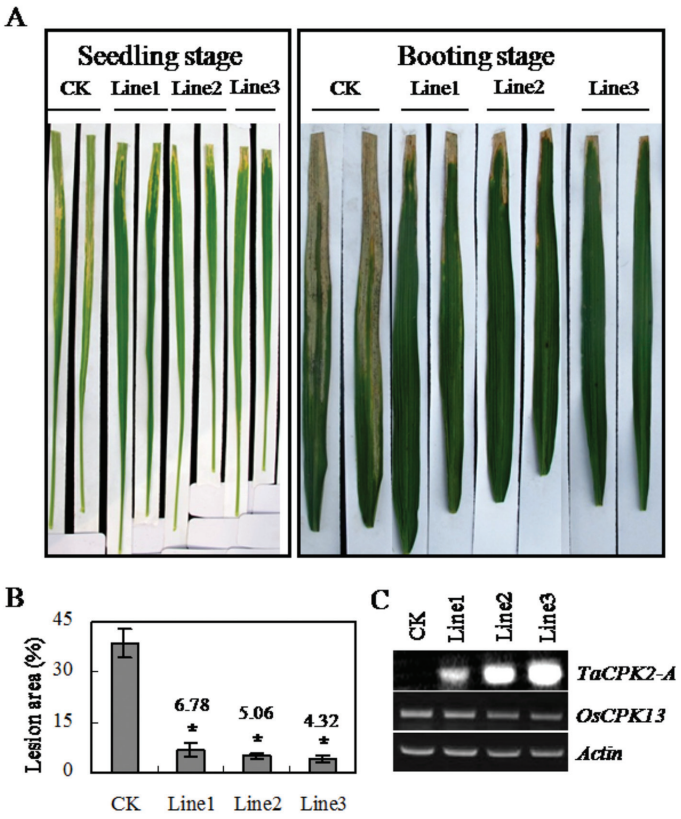

Fig. 5. Overexpression of *TaCPK2-A* in rice enhances bacterial blight resistance. (A) *TaCPK2-A*-overexpressing plants showing enhanced resistance to the Philippines race P6 of *Xanthomonas oryzae* pv. *oryzae* at both the seedling and booting stages. Leaves of transgenic rice plants (lines 1, 2, 3) and the control wild-type Kuiku 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.

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.

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 (Shimono *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

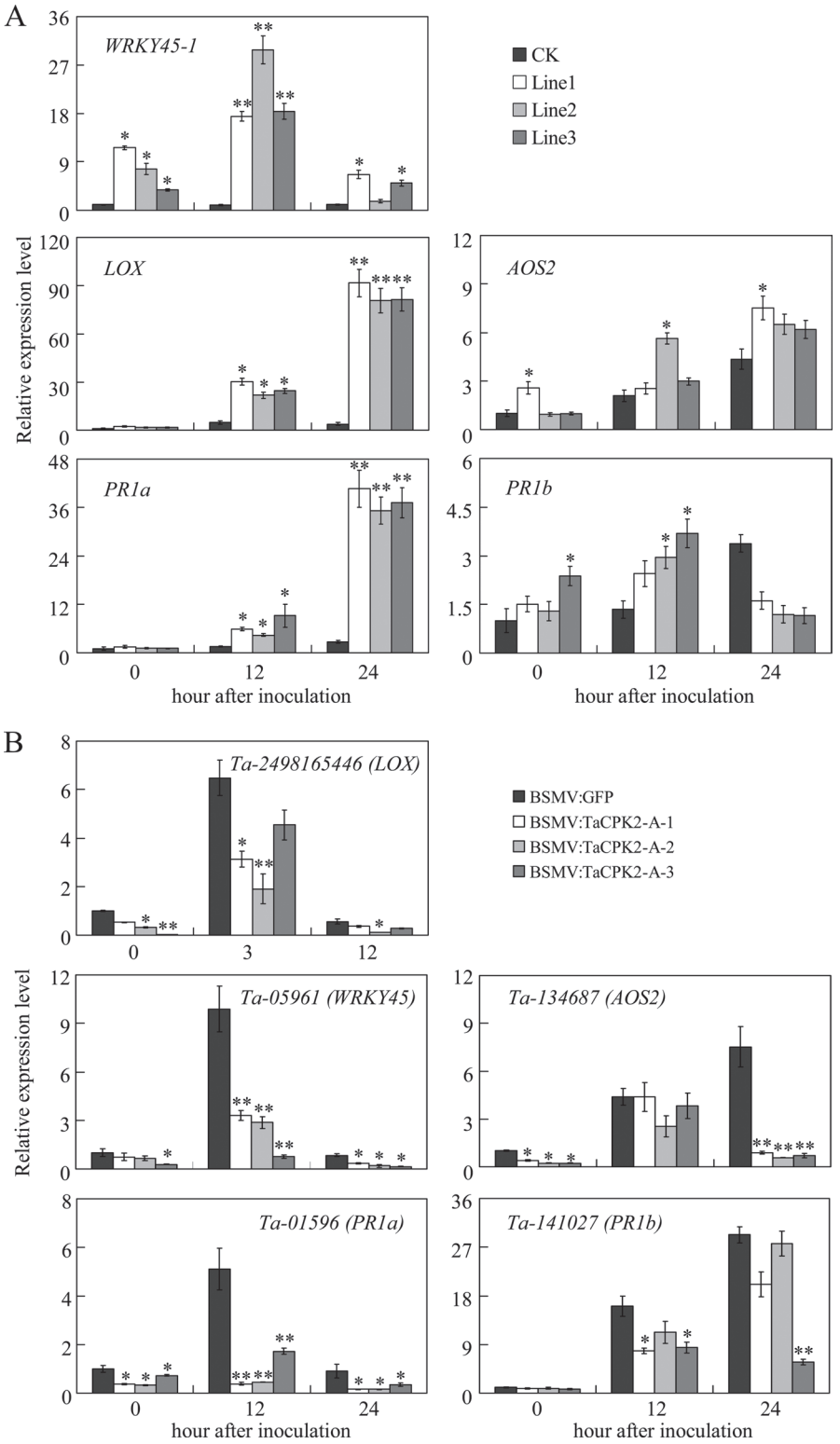

Fig. 6. *TaCPK2-A* regulates *WRKY45* and defence-response genes. (A) Expression patterns of *OsWRKY45-1*, JA-signalling pathway, and pathogenesis genes from Tao *et al.* (2009) in *TaCPK2-A*-overexpressing transgenic rice plants inoculated with *Xoo* using quantitative real-time-PCR (qRT-PCR). Expression levels for each gene were relative to that in the wild-type Kuiku 131 (CK) before *Xoo* inoculation (0 h) 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 increase the growth of *X. oryzae* when compared with non-transgenic plants (Tao *et al.*, 2009). Because the protein sequences of wheat *TaCPK2* and rice Os*CPK13* 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 – Ca^{2+}, 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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