Antagonistic Control of Powdery Mildew Host Cell Entry by Barley Calcium-Dependent Protein Kinases (CDPKs)

Gerald Freymark, Tobias Diehl, Marco Miklis, Tina Romeis, and Ralph Panstruga

Max-Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, D-50829 Köln, Germany

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Calcium-dependent protein kinases (CDPKs) are known to play pivotal roles in intracellular signaling during abiotic and biotic stress responses. To unravel potential functions of CDPKs in the course of barley (*Hordeum vulgare*)–powdery mildew (*Blumeria graminis*) interactions, we systematically analyzed the *HvCDPK* gene family. We found that, according to the existence of respective expressed sequence tags, at least nine paralogs are expressed in the barley leaf epidermis, the sole target tissue of powdery mildew fungi. We exemplarily selected two *HvCDPKs* with known full-length coding sequence for functional analysis. Transient expression of a putative constitutive active variant of one of these (*HvCDPK4*) in *Nicotiana benthamiana* triggered kinase-dependent mesophyll cell death in tobacco leaves. In a barley *mlo* mutant genotype, a constitutive active variant of the second paralog, *HvCDPK3*, partially compromised the highly effective resistance to *B. graminis* f. sp. *hordei*. A similar break of *mlo* resistance was seen upon expression of the junction domain of *HvCDPK4*, supposed to act as a dominant inhibitor of CDPK activity. Expression of a constitutive active *HvCDPK3* or *HvCDPK4* form also compromised penetration resistance to the inappropriate wheat powdery mildew fungus. Collectively, our data provide evidence for antagonistic roles of individual CDPK paralogs in the control of host cell entry during the early phase of powdery mildew pathogenesis.

Additional keywords: haustorium, nonhost resistance, phosphorylation, phylogenetic analysis.

Host cell entry defines an early and essential step during pathogenesis of many plant-pathogenic microbes, including biotrophic powdery mildew fungi. Powdery mildews are Ascomycete ectoparasites which exclusively exhibit hyphal growth and formation of sporangiophores on the outer surface of their respective epidermal host cells. Fungal sporelings attempt to breach the cell walls of plant cells briefly after germination

and endeavor to establish a specialized structure, the haustorium, on the interior of the respective host cells. Haustoria are thought to contribute to nutrient uptake and molecular communication with the host organism (e.g., for reprogramming of the host's metabolism to the benefit of the fungal intruder) (Panstruga 2003). However, even in compatible host–powdery mildew interactions, a certain percentage of attempted host cell entries fail, most likely due to basal plant defense mechanisms that suffice to limit fungal ingress. Extensive molecular studies on the interaction of barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) with their powdery mildew pathogens (*Blumeria graminis* f. sp. *hordei* and *B. graminis* f. sp. *tritici*, respectively) recently have uncovered several host genes that supposedly contribute positively or negatively to this basal defense at the host cell periphery (Panstruga 2004). Thus, these plant genes (and the respective encoded proteins) decisively determine the penetration success of powdery mildew sporelings. Pivotal host components known to be crucial for the outcome of the early stage of barley–powdery mildew interactions comprise the plant-specific heptahelical MLO protein (Büschges et al. 1997), the ROR2 t-SNARE (Collins et al. 2003), the small GTP-binding protein RACB (Schultheiss et al. 2002), and the BAX INHIBITOR 1 cell death regulator (Hückelhoven et al. 2003). Lack of functional MLO (as in recessively inherited loss-of-function *mlo* mutants) prevents successful entry of powdery mildew sporelings into host epidermal cells of monocot and dicot plant species (Büschges et al. 1997; Consonni et al. 2006). It has been suggested previously that the fungal pathogen exploits the presence of MLO proteins for defense suppression (e.g., by modulating a vesicle protein-associated and SNARE protein-dependent defense pathway at the cell periphery) (Panstruga 2005; Schulze-Lefert 2004). Consistent with this hypothesis, pharmacological as well as genetic evidence suggests that an intact actin cytoskeleton is required to prevent successful invasion by *B. graminis* f. sp. *hordei* in barley *mlo* genotypes (Miklis et al. 2007). The actin cytoskeleton appears likewise essential for penetration resistance of barley against inappropriate powdery mildew species, including the closely related wheat pathogen *B. graminis* f. sp. *tritici* (Kobayashi et al. 1997; Miklis et al. 2007).

Calcium-dependent protein kinases (CDPKs) represent a class of serine/threonine kinases that are unique to plants and some protists. In higher plants, CDPKs are encoded by medium-sized gene families comprising, for example, 29 and 34 family members in the monocot and dicot reference species *Oryza sativa* and *Arabidopsis thaliana*, respectively (Asano et al. 2005; Harmon et al. 2001; Harper and Harmon 2005). CDPKs share a common molecular structure, consisting of an N-terminal variable region, a prototypical kinase domain, an autoinhibitory junction domain, and a C-terminal calmodulin-like region bearing four calcium-binding EF-hands. A schematic model

G. Freymark and T. Diehl contributed equally to this work.

Corresponding author: R. Panstruga; Telephone: +49 (0)221 5062-316; Fax: +49 (0)221 5062-353; E-mail: panstrug@mpiz-koeln.mpg.de

Current address of G. Freymark: Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Strasse, D-35043 Marburg, Germany.

Current address of T. Romeis: Department of Plant Biochemistry, Institute for Biology, Free University of Berlin, Königin-Luise-Strasse 12-16, D-14195 Berlin, Germany.

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for the activation of CDPKs based on biochemical data obtained with recombinant enzymes and on in vivo studies after biotic and abiotic stress treatments for *NtCDPK2* is shown in Figure 1 (Böhmer et al. 2006; Harmon et al. 2001; Romeis et al. 2001). In the resting state, the autoinhibitory junction domain prevents substrate phosphorylation by blockage of the reactive kinase center. Upon activation by a calcium signal that is perceived by Ca^{2+} binding to the C-terminal EF-hands, CDPKs undergo a conformational change that leads to binding of the calmodulin-like domain to the junction domain concomitant with retraction of the junction domain from the kinase domain and exposure of the reactive center for substrate phosphorylation (Fig. 1).

Based on biochemical or transcriptional activation, CDPK signaling has been implicated previously in a range of abiotic and biotic stress responses, including light, cold, salinity, or wounding, as well as symbiotic plant-microbe interactions and plant defense (Böhmer et al. 2006; Ludwig et al. 2004). However, only a few examples are known where a specific biological function in plant defense or plant microbe interaction could be allocated to a single CDPK paralog. In tobacco, virus-induced gene silencing of *NtCDPK2* caused an attenuated and delayed hypersensitive response (HR) after elicitation of plants carrying the *Cf-9* disease resistance gene from tomato with the *Avr9* elicitor derived from the fungal pathogen *Cladosporium fulvum*, and similar results were obtained for the *Avr4/Cf-4*-conditioned gene-for-gene interaction (Romeis et al. 2001). In accordance with these data, in CDPK gain-of-function experiments, the ectopic expression of a truncated, constitutive active variant of *NtCDPK2* lacking its regulatory autoinhibitory and calcium-binding domains resulted in the induction of stereotypical plant defense responses, including production of reactive oxygen species (ROS), phytohormone signaling, activation

of pathogenesis-related genes, and an SGT1-dependent hypersensitive cell death (Ludwig et al. 2005). Using an RNA interference-based screen for gene function, *MtCDPK1* was identified in *Medicago truncatula*, resulting in plants with impaired root hair development (Ivashuta et al. 2005). In this report, loss-of-function of *MtCDPK1* by gene silencing was associated with enhanced production of ROS and increased expression of cell wall synthesis- and defense-related genes. Also, plants were less efficient in rhizobial and mycorrhizal colonization with *Sinorhizobium meliloti* and *Glomus versiforme*, respectively. These data suggest that specific CDPK paralogs function as key regulatory components in different signaling pathways that modulate cellular responses to control the plant response in symbiotic and pathogenic interactions.

In barley, CDPK-dependent signal transduction has hardly been characterized to date and the biological functions of specific barley CDPK paralogs are largely unknown. Only one family member, *HvCDPK1*, has been implicated recently in the gibberellic acid response of the barley aleurone through regulation of vacuolar function (McCubbin et al. 2004). Here, we address whether CDPKs are involved in barley antifungal defenses during host cell entry of powdery mildew pathogens. Owing to the lack of defined barley mutant lines in single *HvCDPK* genes, we conducted a combined gain- and loss-of-function approach in which either truncated CDPK variants lacking their regulatory junction and calcium-binding domains (constitutive active) or the autoinhibitory junction domain only (dominant negative) were employed. Respective variants of selected epidermally transcribed CDPKs were transiently expressed in *Nicotiana benthamiana* and in single barley leaf epidermal cells, and the effects on cell death and penetration resistance to appropriate and nonappropriate powdery mildew isolates were assessed. In conclusion, our findings provide evidence for an at least partially antagonistic role of CDPK paralogs in the control of host cell entry during the early phase of powdery mildew pathogenesis.

RESULTS

Identification of barley CDPK paralogs expressed in the leaf epidermis.

We searched the barley HarvEST database (version 1.51, assembly 31, released 9 June 2005) for *H. vulgare* expressed sequence tags (EST) encoding putative calcium-dependent protein kinases using the terms “CDPK” or “calcium-dependent protein kinase” for the “search by best BLAST hit keyword” function. Additionally, we performed a complementary TBLASTN search against barley EST deposited at the National Center for Biotechnology Information (NCBI) using the below-mentioned full-size *HvCDPK3* amino acid sequence as query. We focused on EST clones represented in the so-called barley HO cDNA library, an epidermis-specific cDNA library representing enrichment of transcripts of genes that are expressed in the target tissue of ectoparasitic powdery mildew fungi (Zierold et al. 2005). In the HarvEST database, we identified a total of 11 HO cDNA clones derived from seven uni-gene sets as bona fide candidates for epidermally expressed barley CDPKs (Table 1). Eight additional HO cDNA clones not represented in the HarvEST depository were found upon the TBLASTN search against the NCBI database, extending the number of epidermally expressed unigenes to nine (Table 1). Several other EST derived from distinct barley cDNA libraries encode additional CDPK members (*not shown*). Owing to the absence of matching cDNA clones in the HO cDNA library, it is unclear whether these paralogs are expressed in the barley leaf epidermis; therefore, we did not consider the respective unigenes in the present study.

Fig. 1. Domain architecture and model for regulation of plant-specific calcium-dependent protein kinases (CDPKs). **A**, Schematic drawing of the conserved CDPK structure. CDPKs consist of an N-terminal variable domain (V), which in some isoforms contains a myristoylation site (asterisk), C-terminally followed by a protein kinase domain (K), a short autoinhibitory junction domain (J), and a calmodulin-like domain (C) typically comprising four calcium-binding EF-hands. **B**, Based on in vitro and in vivo biochemical data, a model for CDPK activation has been proposed (Böhmer et al. 2006; Harper and Harmon 2005). In the resting state, CDPK protein kinase activity is blocked through intramolecular binding of the enzymes' intrinsic junction domain. Upon stimulation, calcium (Ca^{2+}) binding to the calmodulin-like domain causes conformational rearrangements resulting in the disengagement of the junction domain out of the active center, thereby releasing the autoinhibition and enabling the enzyme to phosphorylate its substrates. Please note that for simplicity only two of the likely four Ca^{2+} ions binding to the calmodulin-like domain are depicted.

Four of the nine identified contigs (HarvEST contigs no. 6387, *HvCDPK2*; 5769, *HvCDPK3*; 4541, *HvCDPK4*; and 6879, *HvCDPK7*) (Table 1) likely comprise complete open reading frames and, thus, potentially encode full-size barley CDPKs. We subjected the predicted amino acid sequences encoded by the four full-size contigs to analysis by the web-based pattern recognition tool “PlantsP.” We found that all four amino acid sequences revealed the entire set of motifs characteristic for a prototypical CDPK; namely, both an N-terminally located serine/threonine kinase domain and four C-terminally positioned calcium-binding EF-hands consisting of a calmodulin-like structure. Additionally, all four contain the consensus motif of an N-terminal myristoylation site (Table 1), indicating a likely association of the four CDPKs with lipid bilayers. Presence of an N-terminal myristoylation motif is a conserved feature of many though not all plant CDPKs; for example, 29 of 34 *Arabidopsis* CDPKs contain this signature. In summary, our in silico analysis identified four *H. vulgare* EST contigs likely representing full-size CDPK family members expressed in the barley leaf epidermis.

Phylogenetic analysis of epidermally expressed barley CDPKs.

We analyzed the phylogenetic relationships of full-size *HvCDPK* sequences by phylogenetic tree construction based on the PHYLIP software package (Felsenstein 1989) (discussed below). This analysis included all previously described *Arabidopsis* (34 *AtCPKs*), rice (29 *OsCPKs*) (Asano et al. 2005), and tobacco (*NtCDPK1-NtCDPK3*, *NtCPK4*, and *NtCPK5*) paralogs, plus the four full-length barley family members (*HvCDPK2*, *HvCDPK3*, *HvCDPK4*, and *HvCDPK7*) (Table 1) According to the classification of CDPK phylogenetic clades proposed by Harmon and associates (2001), *HvCDPK2* and *HvCDPK4* belong to clade I, whereas *HvCDPK3* resides in group II and *HvCDPK7* in clade III. (Fig. 2). Thus, the full-size epidermally expressed barley CDPK paralogs comprise

representatives of the three largest phylogenetic CDPK clades, suggesting diversification of CDPK functions in the barley leaf epidermis.

A constitutive active variant of *HvCDPK4* causes cell death in *N. benthamiana*.

Owing to the biotic and abiotic stress-responsiveness of CDPK members residing in phylogenetic clades I and II (Romeis et al., 2001) (T. Romeis, U. Dubiella, and S. Franz *unpublished data*), we selected full-size *HvCDPK3* and *HvCDPK4* for subsequent functional analysis. Translational fusions VK(3)-YFP and VK(4)-CFP were generated between the coding sequences of the VK variants lacking the autoinhibitory and calcium-binding domains of *HvCDPK3* and *HvCDPK4* and the 5' end of genes encoding fluorescent proteins (yellow fluorescent protein [YFP] or cyan fluorescent protein [CFP], respectively). Likewise, a fusion between the junction domain of *HvCDPK4* and YFP, (J[4]-YFP) was created. The fluorescent protein moieties served as an internal marker to monitor expression levels in planta by epifluorescence microscopy following plant transformation. Expression of the constructs was driven by the constitutive *Cauliflower mosaic virus* (CaMV) 35S promoter. As a control for the requirement of protein kinase activity, mutant variants harboring kinase activity inactivating single amino acid exchanges D209A (VK(3)_(D/A)) and D218A (VK(4)_(D/A)) were included in the experiments. The different *HvCDPK3* and *HvCDPK4* variants were transiently expressed in *N. benthamiana* leaves via infiltration of *Agrobacteria* carrying the respective binary vectors (Ludwig et al. 2005). At 2 to 3 days postinfiltration, the development of chlorosis and necrosis were assessed macroscopically and also visualized by leaf autofluorescence. Furthermore, cell death symptoms were analyzed after trypan-blue staining (details below). The expression of VK(4)-CFP induced severe chlorosis and necrosis (Fig. 3). This progressing cell death development was dependent on CDPK kinase

Table 1. Characteristics of barley unigene sets encoding predicted Calcium-dependent protein kinases (CDPK) polypeptides expressed in the leaf epidermis

HarvEST ^b	NCBI ^c	Barley 1 name ^d	cDNA clones ^e	Clones in set ^f	Gene designation	Domains in amino acid sequence ^a			
						V	K	J	C
6387	16130	06447	25/41	HO15J19, HO28I19	<i>HvCDPK2</i>	★			■ ■ ■ ■
5769	5775	05679	29/28	HO09C22, HO14H02	<i>HvCDPK3</i>	★			■ ■ ■ ■
4541	12358	04589	39/45	HO03G19, HO08J12, HO08K09, HO13E02, HO20D22	<i>HvCDPK4</i>	★			■ ■ ■ ■
11087	19431	11154	9/12	HO10N20	<i>HvCDPK5</i>				■ ■ ■ ■
10659	8121	11168	10/16	HO25I09, HO33F18	<i>HvCDPK6</i>				■ ■ ■ ■
6879	1517	07135	18/30	HO04C18, HO30J03	<i>HvCDPK7</i>	★			■ ■ ■ ■
18523	21794	16998	4/3	HO09F20	<i>HvCDPK8</i>				■ ■
36770	7572	17096	1/14	HO15F24	<i>HvCDPK9</i>				■ ■ ■ ■
13706	7572	17096	6/14	HO27A09					■ ■ ■ ■
16446	14972	15997	5/6	HO25J05	<i>HvCDPK10</i>				■ ■ ■ ■
18368	21623	18339	4/4	HO31E10	<i>HvCDPK11</i>	★			

^a Domains in corresponding amino acid sequence are depicted as follows. CDPKs consist of an N-terminal variable domain (V), which in some isoforms contains a myristoylation site (asterisk), C-terminally followed by a protein kinase domain (K), a short autoinhibitory junction domain (J), and a calmodulin-like domain (C) typically comprising four calcium-binding EF-hands. ★ = myristoylation site and ■ = EF-hand according to PlantsP prediction.

^b HarvEST unigene number.

^c National Center for Biotechnology Information (NCBI) unigene number.

^d Barley 1 GeneChip probe set name.

^e Number of cDNA clones in HarvEST/NCBI unigene set.

^f Clones of HO cDNA library present in unigene set. HO cDNA library, cDNA library derived from pathogen-challenged barley leaf epidermal tissue (Zierold et al. 2005).

activity because neither VK(4)_(D/A)-CFP, an inactive kinase form that carries a point mutation in the ATP-binding site, nor VKJ(4)-YFP, in which the junction domain constitutively blocks the kinase active center, were able to trigger these symptoms.

Accordingly, co-expression of the junction peptide J(4)-YFP with VK(4)-CFP partially compromised VK(4)-dependent cell death (Fig. 3). This identifies the isolated junction domain of HvCDPK4 as an inhibitory peptide that is able to impede CDPK

Fig. 2. Phylogenetic relationship of selected plant calcium-dependent protein kinases (CDPKs). The unrooted phylogenetic tree was generated using the PHYLIP software package based on the full-length amino acid sequences of HvCDPK2, HvCDPK3, HvCDPK4, and HvCDPK7 (shown in blue) and all previously described *Arabidopsis* (shown in black), rice (shown in red), and tobacco (shown in green) CDPK isoforms. Bootstrap support based on 100 replicates is indicated as color-coded circles at tree branches (for legend, see left bottom corner). Forks without circles have bootstrap support below 70. The scale (left bottom corner) indicates the number of amino acid substitutions per site. Major phylogenetic groups as defined by Harmon and associates (2001) are indicated by shading.

kinase activity in trans. The expression of neither VK(3)-YFP, VK(3)_(D/A)-YFP, nor J(4)-CFP caused similar severe, progressing cell death symptoms (Fig. 3), indicating isoform-specificity with regard to the cell-death-promoting function of VK(4)-CFP. Taken together, these data reveal *HvCDPK4* as a regulatory component of cell death induction similar to what has been described for *NtCDPK2* from tobacco (Ludwig et al. 2005), and suggest that *HvCDPK4* may be involved in environmental stress or plant defense signaling processes.

Transient expression of *HvCDPK* domains partially compromises *mlo*-based penetration resistance.

Next, we analyzed the functional contribution of *HvCDPK3* and *HvCDPK4* in the context of barley–powdery mildew interactions. Transient ballistic gene expression in single barley leaf epidermal cells represents a well-established assay to assess gene function in this plant–microbe interaction (Panstruga 2004). We engineered vectors carrying the above-mentioned *HvCDPK3* and *HvCDPK4* domains under the transcriptional control of the strong constitutive maize ubiquitin promoter. Coding sequences of CDPK domains were C-terminally fused in frame with fluorescent tags (YFP or CFP, respectively) to readily monitor transgene expression in transformed epidermal cells. We chose a highly resistant *mlo* genotype (*mlo-3* null mutant) as plant source for transient expression in this set of experiments because the nearly full immunity of this mutant to the compatible powdery mildew fungus, *B. graminis* f. sp. *hordei*, allows the detection of even subtle effects on the disease resistance phenotype. Before studying plant–microbe interactions, transgene expression was monitored via epifluorescence microscopy following particle bombardment. This indicated for each of the employed constructs clearly detectable fluorescence conveyed by the translational fusion with the fluorophore tag (data not shown), indicating that each gene was expressed at considerable levels.

Transient expression of pUbi-VK(3)-YFP resulted in a reproducible and statistically highly significant ($P < 0.01$ in Student's *t* test) increase in *B. graminis* f. sp. *hordei* host cell entry as compared with the pUbi-β-glucuronidase (GUS)-negative control (9.8 ± 8.4 versus $1.6 \pm 2.1\%$) (Fig. 4A). This increase was dependent on an active kinase function of *HvCDPK3*, because the single amino acid variant D209A (pUbi-VK(3)_(D/A)-YFP; discussed above) did not exhibit this effect ($0.7 \pm 1.9\%$). Likewise, neither expression of the pUbi-VK(4)-CFP construct nor of the plasmid encoding the respective inactive kinase variant (pUbi-VK(4)_(D/A)-YFP) resulted in enhanced host cell entry (0.7 ± 0.9 and $0.5 \pm 0.8\%$). Notably, however, transient expression of the junction domain (J) of *HvCDPK4*, supposed to exert a dominant negative effect on multiple CDPKs, also gave rise to reproducible and statistically highly significant ($P < 0.01$ in Student's *t* test) enhanced host cell penetration ($6.9 \pm 4.6\%$; Fig. 4A). Transient expression pUbi-GUS only ($1.6 \pm 2.1\%$) or pUbi-GUS plus pUbi-Mlo ($82.9 \pm 11.3\%$) served as negative and positive control, respectively, in this set of experiments. In sum, our results indicate that both the constitutive activation of a particular *HvCDPK* paralog (*HvCDPK3*) as well as dominant negative inhibition of one or more *HvCDPK* isoforms via the expression of the junction domain of *HvCDPK4* suffices to partially compromise *mlo* resistance in barley.

Transient expression of constitutive active *HvCDPK3* partially compromises penetration resistance to the inappropriate wheat powdery mildew fungus.

We next assessed the function of *HvCDPK3* and *HvCDPK4* in an inappropriate plant–microbe interaction between barley and the wheat powdery mildew *B. graminis* f. sp. *tritici*. The set of CDPK constructs was expressed transiently in single barley leaf epidermal cells of an *Mlo* genotype and challenged with conidia of the nonadapted fungal pathogen. Generally,

Fig. 3. Transient expression in *Nicotiana benthamiana* reveals a cell death-inducing function of constitutive active *HvCDPK4*. Chimeric genes encoding various *HvCDPK3* or *HvCDPK4* domains fused to a fluorescent tag (cyan fluorescent protein [CFP] or yellow fluorescent protein [YFP]) were transiently expressed in *N. benthamiana* leaves. At 2 to 3 days after *Agrobacterium* infiltration, cell death symptoms were assessed in leaf segments either as macroscopically visible necrotic lesions (upper panels), as autofluorescence upon UV excitation (middle panels), or after staining of leaf disks with trypan blue (lower panels). Adjustments in brightness, contrast, and color quality for leaf panels, autofluorescence panels, and trypan blue staining disks have been performed to visually optimize the graphic.

most penetration attempts by *B. graminis* f. sp. *tritici* on barley cells fail, and only rarely do fungal sporelings succeed in host cell entry and haustorium formation (Elliott et al. 2002). This is reflected by the low level of successful penetration events upon expression of the pUbi-GUS negative control ($1.7 \pm 1.3\%$) (Fig. 4B). Consistent with the results from the experiment in the *mlo* genotype (discussed above) (Fig. 4A), expression of VK(3)-YFP resulted in a highly significant increase in host cell entry ($27.3 \pm 13.6\%$; $P < 0.001$ in Student's *t* test) (Fig. 4B). This level of successful penetration also was significantly different from the previously reported (Elliott et al. 2002) and here verified enhanced entry rate upon overexpression of barley Mlo ($11.2 \pm 9.0\%$; $P < 0.05$ in Student's *t* test) (Fig. 4B). As in the barley *mlo*-*B. graminis* f. sp. *hordei* interaction, this effect was dependent on a functional *HvCDPK3*

kinase domain because expression of the kinase-inactive variant (plasmid pUbi-VK(3)_(D/A)-YFP) did not provoke any increased cell entry ($1.8 \pm 2.5\%$) (Fig. 4B). Unlike in the compatible barley-*B. graminis* f. sp. *hordei* interaction, expression of VK(4)-YFP also resulted in increased entry rates ($8.4 \pm 8.2\%$) (Fig. 4B). This effect was kinase-dependent, too, because expression of the inactive VK(4)_(D/A)-YFP mutant variant did not reveal this effect ($2.1 \pm 2.5\%$) (Fig. 4B). Additionally, also in contrast to the compatible barley-*B. graminis* f. sp. *hordei* interaction, expression of the junction domain of *HvCDPK4* (construct pUbi-J(4)-CFP) did not alter *B. graminis* f. sp. *tritici* entry rates ($3.3 \pm 3.4\%$) (Fig. 4B). Taken together, expression of constitutive active *HvCDPK3* and *HvCDPK4* results in enhanced fungal entry rates in this otherwise incompatible plant-microbe encounter.

Fig. 4. Transient expression of constitutive active *HvCDPK3* in barley epidermal cells partially compromises *mlo*-based penetration resistance and enables enhanced host cell entry by the inappropriate wheat powdery mildew fungus. Leaf segments of **A**, a barley *mlo* mutant genotype (BCI *mlo*-3) or **B**, barley wild-type cv. Golden Promise (*Mlo* genotype) were either bombarded with β -glucuronidase (GUS) reporter plasmid or co-bombarded with a GUS reporter plasmid plus a construct expressing either full-size Mlo or chimeric genes encoding *HvCDPK* domains fused to a fluorescent tag (cyan fluorescent protein [CFP] or yellow fluorescent protein [YFP]). Leaves then were inoculated with either **A**, *Blumeria graminis* f. sp. *hordei* isolate K1 or **B**, *B. graminis* f. sp. *tritici* isolate JIW2 and stained for GUS activity and fungal structures as described in Materials and Methods. Host cell entry was evaluated microscopically by the presence of mature haustoria or haustorium initials in GUS-stained epidermal cells attacked by fungal sporelings. Results represent mean $\pm$ standard deviation of 3 to 10 experiments per construct. Asterisks indicate $P < 0.01$, the plus sign indicates $P < 0.05$ (Student's *t* test) compared with the bombardments with a GUS-encoding plasmid only, and a hash indicates $P < 0.05$ (Student's *t* test) compared with the co-bombardments with GUS- and *Mlo*-encoding plasmids.

DISCUSSION

Here, we have shown evidence for a contribution of CDPK paralogs to the control of host cell entry in barley-powdery mildew interactions. This claim relies on enhanced fungal penetration rates upon transient expression of either constitutive active CDPK variants or a supposedly dominant negative-acting CDPK junction domain (Fig. 4). The increase in fungal host cell entry was observed both in the context of the highly resistant *mlo* genotype attacked by otherwise virulent *B. graminis* f. sp. *hordei* (Fig. 4A) as well as in the interaction between wild-type barley (*Mlo* genotype) and the inappropriate wheat powdery mildew pathogen, *B. graminis* f. sp. *tritici* (Fig. 4B). Successful host cell wall penetration was evidenced by the presence of either mature or developing haustoria (haustorium initials) inside transformed (GUS-positive) barley leaf epidermal cells; the frequent occurrence of the latter scenario (data not shown) may indicate that host cell entry commonly was either incomplete or delayed upon expression of the various CDPK variants. In case of the constitutive active CDPK variants, the observed effect was dependent on enzymatically active kinase domains because expression of mutant variants bearing single amino acid replacements in the active site of the kinase domain did not lead to enhanced entry rates in either plant-fungus interaction (Fig. 4). Likewise, at least in the interaction of the barley *mlo* genotype with *B. graminis* f. sp. *hordei*, we observed isoform-specificity because only constitutive active *HvCDPK3*, but not *HvCDPK4*, promoted fungal entry (Fig. 4A). Similarly, expression of the supposedly dominant negative-acting junction domain of *HvCDPK4* affected entry rates only in the context of the *mlo*-*B. graminis* f. sp. *hordei* but not the *Mlo*-*B. graminis* f. sp. *tritici* interaction (Fig. 4B). Taken together, the transient ectopic expression of respective CDPK variants caused altered efficiency in pathogen host cell entry which was restricted, in part, to a particular barley-powdery mildew interaction which was dependent on an intact protein kinase domain of the expressed enzymes, and which was specific to the expressed *HvCDPK3* or *HvCDPK4* isoform. Likewise, constitutive active *HvCDPK4*, but not *HvCDPK3*, induced spreading cell death in a kinase-dependent manner upon heterologous expression in *N. benthamiana* (Fig. 3). Such isoform specificity is in accordance with previous results obtained with tobacco CDPKs, where an activation of distinct plant defense responses, including an HR-like cell death response, was triggered by NtCDPK2-VK but not by closely related NtCDPK3-VK, a member of the same subgroup, sharing 94% amino acid identity (Ludwig et al. 2005). Furthermore, although neither reverse genetics nor biochemical data of members of the *Arabidopsis* CDPK family showing a function in plant-microbe interaction has been published yet, only a few CDPK members are able to trigger a similar cell death response upon ectopic expression in

tobacco. Remarkably, the two CDPK isoforms which are most closely related to HvCDPK4 belong to these enzymes (A.-C. Cazalé, A. Seal, C.-P. Witte, and T. Romeis, *unpublished*).

Interestingly, transient expression of constitutive active HvCDPK4 triggered a cell death response in *N. benthamiana* (Fig. 3) whereas we did not observe any indication of cell death upon transient expression of this variant in individual barley leaf epidermal cells. The latter is evidenced by the fact that a significant number of transformed cells supported the accommodation of *B. graminis* f. sp. *tritici* haustoria (Fig. 4B). Also, the number of pUbi-VK(4)-CFP + pUbi-GUS transformed cells showing positive GUS staining was comparable with the transformations with the other constructs (except pUbi-VK(3)-YFP, for which we consistently observed a lower number of GUS-stained cells) (data not shown). *N. benthamiana* possibly lacks additional regulatory proteins that prevent execution of cell death upon activation of HvCDPK4. Alternatively, the cell-death-promoting function of constitutive active HvCDPK4 may become effective only in mesophyll but not in epidermal cells.

Tobacco *NtCDPK2* and *NtCDPK3* previously have been implicated with abiotic and biotic stress responses and, in particular, with the execution of hypersensitive cell death in the course of isolate-specific gene-for-gene-type resistance reactions (Romeis et al. 2000, 2001). By contrast, here we show that epidermally expressed barley CDPKs may add to the establishment of a compatible interaction between the fungal powdery mildew pathogen and the plant host. This appears to be a conundrum in the light of the common belief that cytoplasmic calcium signaling plays a pivotal role in the activation of plant defense responses (Lecourieux et al. 2006). Previously, however, it has been shown that calcium-dependent association of the ubiquitous calcium sensor calmodulin with a specific binding domain in the cytoplasmic C-terminus of the heptahelical MLO promotes susceptibility to the barley powdery mildew fungus (Kim et al. 2002). Elevated calcium levels upon microbial attack may not only advantage this protein-protein interaction but, in addition, possibly stimulate CDPK activities which, in turn, also might either directly or indirectly support pathogen entry. Based on the presumed function of CDPKs in the phosphorylation of actin-depolymerizing factors (Allwood et al. 2001), a class of enzymes that catalyze the assembly or disassembly of actin microfilaments, one may hypothesize that interference with CDPK activities may obstruct the pathogen-triggered reorganization of the actin cytoskeleton toward biotic stress sites (Lipka and Panstruga 2005). Because actin cytoskeleton function is crucial for the execution of *mlo*-mediated and nonhost resistance in barley to powdery mildews (Kobayashi et al. 1997; Miklis et al. 2007), this scenario could satisfactorily explain the enhanced host cell entry rates observed in this study.

In addition to plant-pathogen interactions, calcium signaling is also pivotal during the onset of symbiotic plant-microbe interactions, and it has been shown that, in addition to CDPKs, another class of calcium-regulated protein kinases, CCaMKs, is involved (Mitra et al. 2004). This indicates that, in plants, different classes of calcium-regulated protein kinases are recruited for the generation and sensing of specific calcium signals and signatures at different states during symbiotic and pathogenic interactions. One of the urgent future goals is to unravel when and how these various types of calcium signatures occurring in the course of plant-microbe interactions are deciphered and translated in an appropriate cellular response (e.g., the activation of signal transducers such as CDPKs).

The junction domain of CDPKs is supposed to exert an intramolecular autoinhibitory function by preventing kinase activation and, consequently, substrate phosphorylation in the absence of an appropriate calcium stimulus. Consistent with this idea, transient co-expression of the junction domain of

NtCDPK2 largely compromised *NtVK(2)*-triggered cell death (Ludwig et al. 2005). Our experiments provide evidence that the inhibitory function of the junction domain also is effective to block activities of endogenous CDPKs when the junction domain is provided in trans (Fig. 4). This opens up the possibility to exploit expression of the junction domain as a general tool to interfere with CDPK functions in planta. Interference with CDPK functions via ectopic expression of the regulatory junction domain might be particularly useful in plant species for which reverse genetic tools are not yet available (e.g., barley). Additionally, this tool promises to be useful to overcome experimental difficulties associated with the presumptive functional redundancy of CDPK family members. At present, it is unclear how many CDPK paralogs would be affected by expression of a given junction domain. Based on the high overall sequence identity of CDPK junction domains, we consider it likely that at least multiple sequence-related family members belonging to the same phylogenetic group will be targeted. Thus, it currently is difficult to conclude inhibition of which barley CDPK paralogs might be responsible for the enhanced host cell entry rate observed upon transient expression of the HvCDPK4 junction domain.

In monocot as well as in dicot species, MLO proteins constitute key regulators of host cell entry by powdery mildew fungi (Büschges et al. 1997; Consonni et al. 2006). Presence of particular MLO paralogs is a prerequisite for successful fungal pathogenesis; absence of these MLOs, such as in *mlo* mutants, results in highly effective resistance. To date, in addition to barley loss-of-function alleles of two genes that are required for *mlo* resistance (*Ror1* and *Ror2*) (Freialdenhoven et al. 1996) and interference with actin cytoskeleton function (Miklis et al. 2007), overexpression of the antagonist of a pro-apoptotic protein BAX, barley BAX inhibitor 1, has been reported to partially compromise *mlo* resistance (Hückelhoven et al. 2003). Given the functional relation of both BAX inhibitor 1 and CDPKs to the regulation of cell death and powdery mildew host cell entry, it is tempting to speculate that BAX inhibitor 1 and the CDPKs identified in this study may act in a common pathway.

Though the exact molecular mechanism of *mlo*-mediated resistance remains elusive to date, there appears to be essential mechanistic overlap with so-called nonhost resistance to non-appropriate pathogens (Humphry et al. 2006). Accordingly, genetic loss- and gain-of-function experiments in *Mlo* wild-type and *mlo* mutant genotypes have been found to affect infection phenotypes of both adapted as well as nonadapted powdery mildews (Consonni et al. 2006; Eichmann et al. 2004; Elliott et al. 2002; Hückelhoven et al. 2003; Peterhänsel et al. 1997). The findings of this study, indicating a function for CDPKs in the context of *mlo* and nonhost resistance, support this notion and suggest a crucial role for phosphorylation and dephosphorylation events during antifungal defense at the cell periphery. Despite the proposed mechanistic similarities between *mlo*-mediated and nonhost resistance, there are apparent differences with respect to CDPK isoform-specificity in the two plant-powdery mildew interactions (discussed above) (Fig. 4). These differences may relate to either the different plant genotypes (*mlo* mutant versus *Mlo* wild type) or diverse fungal species (*B. graminis* f. sp. *hordei* versus *B. graminis* f. sp. *tritici*) used in the various sets of experiments. The function of constitutive active HvCDPK4, for example, either may require a functional MLO protein or might become evident only upon *B. graminis* f. sp. *tritici* challenge. The fact that both expression of a constitutive active CDPK variant and expression of a supposedly dominant negative version enhanced *B. graminis* f. sp. *hordei* host cell entry in the *mlo* genotype indicates that individual CDPKs likely have antagonistic functions in antifungal

Table 2. Oligonucleotides used in this study^a

Primer designation	Sequence
HvCDPK3 (V) F	5'- <u>CACC</u> ATG GGC CAG TGT TGC AGC-3'
HvCDPK3 (K) R	5'-TTC TCC TTC CCT GAG CCA TGG-3'
HvCDPK3 (D209A) F	5'-CAT GGG AGT GAT GCA CCG TGC ACT TAA GCC-3'
HvCDPK3 (D209A) R	5'-GGC TTA AGT GCA CGG TGC ATC ACT CCC ATG-3'
HvCDPK4 (V) F	5'- <u>CACC</u> ATG GGC AAC GCA TGC GGC GGT TCC CTT AG-3'
HvCDPK4 (K) R	5'-AAC TCC ATG ATC ACG AAT CCA TGG ATG GCA TAG-3'
HvCDPK4 (J) F	5'- <u>CACC</u> ATG GCT CCT GAT CGT CCT CTT G-3'
HvCDPK4 (J) R	5'-TAT TAC CCG CAA AGC CAT C-3'
HvCDPK4 (D218A) F	5'-CTC CTT AGG GGT CAT GCA CCG GGC ACT CAA-3'
HvCDPK4 (D218A) R	5'-TTG AGT GCC CGG TGC ATG ACC CCT AAG GAG-3'

^a pENTR-D/Topo cloning sequences are underlined, D to A mutations are highlighted in bold, and stretches in italics indicate silent introduced *MseI* (*HvCDPK3*) or *Bsu36I* (*HvCDPK4*) restriction sites.

defense. Identification of target proteins phosphorylated by the various CDPKs during barley-*B. graminis* interactions will be instrumental in deciphering the exact roles of the various CDPK paralogs in the control of host cell entry by phytopathogenic powdery mildew fungi.

MATERIALS AND METHODS

Plant and fungal material.

N. benthamiana, barley cv. Golden Promise, and BCI (back-cross Ingrid) *mlo-3* were cultivated at 22°C (16 h of light and 8 h of darkness). Powdery isolates *B. graminis* f. sp. *hordei* K1 and *B. graminis* f. sp. *tritici* JIW2 were propagated on susceptible barley and wheat cultivars, respectively.

Reverse-transcription PCR and cloning.

The truncated variant of *HvCDPK3* (VK[3]) was amplified by reverse-transcription polymerase chain reaction (PCR) with oligonucleotide primers shown in Table 2. RNA extracted from 8-day-old barley leaves (*H. vulgare*, cv. Golden Promise) inoculated for 1 day with *B. graminis* f. sp. *hordei* served as a template. Domains of *HvCDPK4* (VK[4], VKJ[4], and J[4]) were amplified by standard PCR with primers shown in Table 2 using EST clones HO08J12 (GenBank accession numbers CD055122 and CK568006) (Zierold et al. 2005) or HI11N11 (GenBank accession numbers CB859862 and BU998682) as template. All CDPK constructs were cloned directionally into the GATEWAY-compatible pENTR/D-Topo vector (Invitrogen, Carlsbad, CA, U.S.A.). Inserts were transferred into the binary destination vectors pXCSG-CFP and pXCSG-YFP (Feys et al. 2005) via GATEWAY-LR reactions for expression in *N. benthamiana* as well as into pUbi-GW-CFP and pUbi-GW-YFP (vectors bearing a maize ubiquitin promoter, a GATEWAY cassette, and either the *CFP* or *YFP* coding sequence) for expression in barley. Amino acid substitutions in VK(3) (D209A) and in VK(4) (D218A) were introduced by PCR-based, site-specific mutagenesis (details of oligonucleotides used are given in Table 2). All constructs were verified by the ADIS DNA sequencing core facility at the Max-Planck Institute for Plant Breeding Research (Cologne, Germany) on Abi Prism 377, 3100, and 3730 with BigDye-terminator v3.1 chemistry.

Phylogenetic analysis.

For phylogenetic analysis of CDPK proteins, the Phylip 3.66 software package was used (Felsenstein 1989). A distance ma-

trix was calculated by PROTDIST from a multiple sequence alignment (generated by CLUSTALW) and then transformed into a tree using the neighbor-joining method (NEIGHBOR). The resulting phylogenetic tree was visualized by TREEVIEW. For bootstrap support, SEQBOOT, PROTDIST, NEIGHBOR, and CONSENSE (100 replicates each) algorithms were applied sequentially. All programs were run with standard parameters. The full-size CDPK amino acid sequences and CLUSTALW alignment used for generation of the phylogenetic tree are provided in Supplement 1.

Agrobacterium-mediated transient expression in *N. benthamiana*.

Binary vector derivatives were electrotransformed into *Agrobacterium tumefaciens* GV3101::pMP90 RK and fusion proteins were transiently expressed in 4- to 6-week-old *N. benthamiana* plants mediated by *A. tumefaciens* as described (Ludwig et al. 2005). Plants were grown for 2 to 3 days in a growth chamber with a cycle of 16 h of high light (300 µE) and 8 h of darkness at 26°C and 50% relative humidity. For Trypan Blue staining experiments, leaf disks were boiled for 10 min in 1:1 Trypan Blue solution (33% in lactophenol) with ethanol and subsequently destained in a chloral hydrate solution at 2.5 g/ml.

Particle bombardment-mediated transient expression in *H. vulgare*.

Transient expression in single leaf epidermal cells upon ballistic transformation of barley leaves was performed essentially as described (Elliott et al. 2005). Briefly, detached barley leaves (genotype either back-cross line Ingrid *mlo-5* or Golden Promise *Mlo*) were either bombarded with a GUS-expressing reporter construct alone (pUbi-GUS) or co-bombarded with pUbi-GUS plus a plasmid expressing the respective test gene. Approximately 4 h after transformation, specimens were inoculated with either *B. graminis* f. sp. *hordei* or *B. graminis* f. sp. *tritici* conidiospores and cells stained for GUS activity at 48 h postinoculation. Epiphytic fungal structures were visualized by Coomassie Brilliant Blue staining and fungal penetration success evaluated by the presence or absence of haustoria in attacked epidermal cells.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

HarvEST barley EST database: harvest.ucr.edu
 PlantSP Pattern recognition tool: plantsp.genomics.purdue.edu
 PHYLIP phylogenetic analysis: evolution.gs.washington.edu/phylip.html
 CLUSTALW multiple sequence alignment: www.ebi.ac.uk/clustalw
 TREEVIEW phylogenetic tree visualization: taxonomy.zoology.gla.ac.uk/rod/treeview.html