

Calcium-dependent protein kinases play an essential role in a plant defence response

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Calcium-dependent protein kinases (CDPKs) comprise a large family of serine/threonine kinases in plants and protozoans. We isolated two related CDPK cDNAs (*NtCDPK2* and *NtCDPK3*) from *Nicotiana tabacum*. These CDPK transcripts are elevated after race-specific defence elicitation and hypo-osmotic stress. Transiently expressed myc-epitope-tagged *NtCDPK2* in *Nicotiana benthamiana* and *N.tabacum* leaves showed a rapid transient interconversion to an activated form after elicitation and hypo-osmotic stress. The *Avr9* race-specific elicitor caused a more pronounced and sustained response. This transition is due to phosphorylation of the CDPK. Immuno-complex kinase assays with epitope-tagged *NtCDPK2* showed that stress-induced phosphorylation and interconversion of *NtCDPK2* correlates with an increase in enzymatic activity. The function of *NtCDPK2* in plant defence was investigated by employing virus-induced gene silencing (VIGS) in *N.benthamiana*. CDPK-silenced plants showed a reduced and delayed hypersensitive response after race-specific elicitation in a gene-for-gene interaction, and lacked an accompanying wilting phenotype. Silencing correlated with loss of CDPK mRNA, whereas mRNA accumulation of mitogen-activated protein kinase WIPK remained unaltered.

Keywords: CDPK/hypo-osmotic stress/plant defence response/tobacco/VIGS

Introduction

Plants are constantly exposed to changes in their environment and have developed mechanisms to cope with biotic and abiotic stress. Among the earliest cellular responses to such stress stimuli are changes in the cytoplasmic calcium concentration, and a specific calcium signature is often established (McAinsh and Hetherington, 1998). Calcium-dependent protein kinases (CDPKs) may function as a potential sensor that decodes and translates the elevation of calcium concentration into enhanced protein kinase

activity and subsequent downstream signalling events (Harmon *et al.*, 2000).

CDPKs are calcium-binding serine/threonine protein kinases. In contrast to calmodulin-dependent kinases, in CDPKs the catalytic kinase domain in the N-terminal half of the protein is directly tethered via an autoinhibitory junction domain to a regulatory calmodulin-like domain, which usually contains four functional EF hands for calcium binding (Satterlee and Sussman, 1998). CDPKs have a highly conserved structure. Isoform-specific differences are mainly restricted to the N-terminal variable domain, which in many CDPKs also includes a fatty acylation site (Hrabak, 2000). Myristoylation and palmitoylation at these sites have been shown for CpCPK1 from zucchini (Ellard-Ivey *et al.*, 1999) and OSCP2 from rice (Martin and Busconi, 2000), and are necessary for targeting to the membrane.

So far, CDPKs have not been identified in yeast and animal systems. Protein kinase C and calmodulin-dependent kinases are well characterized as major mammalian calcium-dependent signalling molecules, and it has been proposed that CDPKs play the same role in plants (Roberts and Harmon, 1992). CDPKs comprise a large gene family (34 members in *Arabidopsis*; Harmon *et al.*, 2001). This suggests that individual isoforms have different functions and participate in multiple distinct signalling pathways. However, downstream CDPK-regulated processes remain largely unknown. The challenge that currently faces the CDPK field is not only to allocate defined biological functions to specific CDPK isoforms, but also to integrate CDPK signalling with other signal networks, for example, mitogen-activated protein (MAP) kinase cascades, whose complexity is also beginning to emerge.

Increasing evidence has been provided for CDPKs being involved in environmental stress signalling. CDPK transcript elevation was reported after exposure of *Arabidopsis* to cold, salt and drought (Urao *et al.*, 1994; Tähtiharju *et al.*, 1997), and remarkably, overexpression of rice *OsCDPK7* yielded cold and salt/drought-tolerant rice plants (Saijo *et al.*, 2000). Also, exposure to non-specific elicitors and mechanical wounding were reported to cause an increase of *NtCDPK1* transcript in tobacco (Yoon *et al.*, 1999). In a more physiological context, CDPK enzymatic activity has been correlated with osmotic stress (Takahashi *et al.*, 1997) and elicitation (Allwood *et al.*, 1999). However, in only one system so far, by choosing leaf protoplasts as an experimental approach, could the activity of a specific CDPK isoform from *Arabidopsis*, AtCPK10, be linked with the induction of environmental stress-related promoters after abscisic acid treatment (Sheen, 1996).

The tomato Cf-9 disease resistance gene confers responsiveness in transgenic tobacco to the *Avr9* race-specific elicitor (Hammond-Kosack *et al.*, 1998). We have

recently identified a 68/70 kDa CDPK from tobacco that becomes biochemically activated in response to Avr9 elicitation in Cf-9 tobacco (Romeis *et al.*, 2000). As demonstrated by in-gel kinase assays and western blot analysis, elicitation caused a phosphorylation-dependent transition from a non-elicited enzyme form into an elicited form, which differed in its electrophoretic mobility. This allowed us to characterize the enzyme in a defence-related biological system after applying an *in vivo* stimulus. These data suggested that the 68/70 kDa CDPK functions in a signalling pathway to recruit the plant defence.

In this study, we report the cloning of two tobacco CDPK cDNAs, *NtCDPK2* and *NtCDPK3*, which belong to the same subfamily. The cDNAs were isolated from tobacco cell suspension cultures expressing *Cf-9* as transgene, which were elicited with Avr9. *NtCDPK2* protein was biochemically characterized after expression of a triple-myc-tagged version in *Nicotiana benthamiana* and *Nicotiana tabacum* using an *Agrobacterium*-mediated transient assay. This allowed us to study the *NtCDPK2*-myc enzyme in response to external stress stimulation, irrespective of the presence of other endogenous CDPKs. *NtCDPK2*-myc showed a rapid and transient interconversion between two enzyme forms after osmotic stress and race-specific elicitation stimuli, and the activated form showed elevated protein kinase activity.

To address the function of *NtCDPK2* and *NtCDPK3* in plant defence we employed virus-induced gene silencing (VIGS) in *N. benthamiana* as a reverse genetics approach (Baulcombe, 1999). VIGS, based on a cosuppression mechanism, was predicted to silence closely related genes. This allowed us to circumvent the problem of gene redundancy in tobacco by potentially silencing the entire *NtCDPK2* subfamily. The CDPK-silenced plants were compromised in the Cf-9/Avr9- and Cf-4/Avr4-mediated activation of HR and lacked the concomitant characteristic wilting phenotype.

Our data show that: (i) a CDPK isoform, *NtCDPK2*, participates in signalling pathways triggered by different external biotic and abiotic stress stimuli; (ii) the phosphorylation-dependent transition between two enzyme forms is correlated with higher enzyme activity. (iii) *NtCDPK2* and/or closely related subfamily members are required in the defence-related signal cascade to induce HR triggered by a gene-for-gene interaction.

Results

Isolation of two cDNA clones, *NtCDPK2* and *NtCDPK3*, encoding CDPKs

The activity of protein kinases is frequently regulated by post-translational modifications such as phosphorylation. Changes in protein kinase activity can be accompanied by the transcriptional induction of the corresponding gene. This has been reported for MAP kinases from different plant species in response to cold, drought and wounding, but also after non-specific or race-specific elicitation (for review see Meskiene and Hirt, 2000; Zhang and Klessig, 2000). Biochemical activation followed by transcriptional induction of tobacco WIPK was also observed when Cf-9 tobacco cells or plants were treated with the corresponding race-specific elicitor Avr9 (Romeis *et al.*, 1999). Because a 68/70 kDa CDPK enzyme became phosphorylated and

activated in response to a Cf-9/Avr9 elicitation event, we set out to isolate CDPK genes coding for proteins of approximately that size, whose expression was induced in the same experimental system (Durrant *et al.*, 2000). Two full-length cDNAs, designated *NtCDPK2* and *NtCDPK3*, were isolated using an RT-PCR approach with degenerate primers on total RNA from elicited Cf-9 tobacco cells. *NtCDPK2* and *NtCDPK3* are 88 and 94% identical at nucleotide and amino acid level, respectively. The nucleotide sequence data will appear in DDBJ/EMBL/GenBank under accession Nos AJ344154 and AJ344155. The genes encode proteins of 581 and 578 amino acids with a predicted molecular mass of 64.73 and 64.72 kDa, respectively, which contain a myristoylation motif at the N-terminus. The closest homologs to *NtCDPK2* are CpCPK1 from zucchini (90% amino acid identity; DDBJ/EMBL/GenBank accession No. U90262; Ellard-Ivey *et al.*, 1999) and AtCPK2 from *Arabidopsis* (87% identity; DDBJ/EMBL/GenBank accession No. U38133; Hrabak *et al.*, 1996) (Figure 1). Therefore, both cDNAs from tobacco are members of the same subgroup (Harmon *et al.*, 2000).

A genomic Southern blot of *Bam*HI and *Eco*RV-digested genomic DNA hybridized with the full-length cDNA of *NtCDPK2* yielded four cross-reacting bands in *N. tabacum* (two in *Nicotiana sylvestris*) (data not shown). Because the *NtCDPK2* probe also recognized *NtCDPK3*,

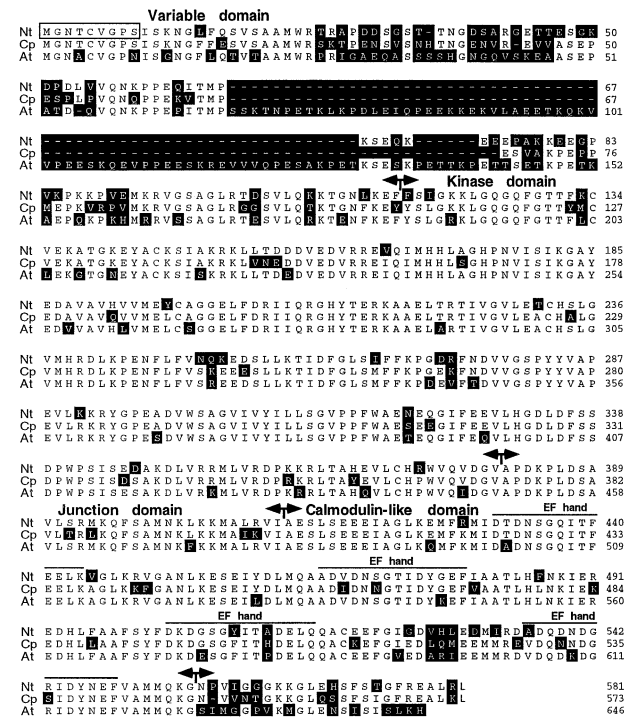


Fig. 1. Alignment of the predicted amino acid sequence of tobacco *NtCDPK2* with orthologous sequences. *NtCDPK2* (Nt) was aligned with CpCPK1 from zucchini (Cp; Ellard-Ivey *et al.*, 1999) and AtCPK1 from *Arabidopsis* (At; Harper *et al.*, 1993). Amino acids differing from the *NtCDPK2* sequence are shaded in black, gaps maximizing the alignment are shown by dashes. The boundaries between the variable, kinase, junction and calmodulin-like domain, as well as the positions of the four EF hands, are shown by arrows and bars. The N-terminal consensus motif for eukaryotic fatty acylation (Nimchuk *et al.*, 2000) is boxed, and numbers at the right indicate amino acid residues.

this indicates that *NtCDPK2* and *NtCDPK3* belong to a small gene subfamily of four members. A gene-specific probe comprising ~300 bp from the 5'- and 3'-UTRs of *NtCDPK3* hybridized with two bands in *N.tabacum* (one in *N.sylvestris*) (data not shown). *Nicotiana tabacum* is an allotetraploid species that is believed to have arisen from hybridization of the diploid species *N.sylvestris* and *Nicotiana tomentosiformis* (Lee *et al.*, 1988). The *NtCDPK2* and *NtCDPK3* alleles may therefore originate from different parental genomes.

Expression patterns of *NtCDPK2* and *NtCDPK3*

Transcript levels were analyzed in Cf-9 tobacco cell cultures and plants over a time course of 4 h after elicitation with IF(Avr9⁺) or IF(Avr9⁻), or after hypo-osmotic stress applied by diluting cells with water or by infiltrating water into leaves. RNA blot analysis with probes from the coding region of *NtCDPK2* or *NtCDPK3* resulted in cross hybridization between the two isoforms, whereas probes from the 5'- and 3'-UTRs were not sensitive enough to detect a signal (data not shown). Therefore, RT-PCR with gene-specific primers was conducted, using *WIPK* and *actin* as controls for an induced and a constitutively expressed gene, respectively (Figure 2).

In cell cultures, *WIPK* transcript was induced 30–60 min after dilution or elicitation with IF(Avr9⁺) but not after treatment with IF(Avr9⁻) (Figure 2). A similar pattern of transcript accumulation was observed for *NtCDPK3*, which was induced after hypo-osmotic stress and elicitation, except that the response was slightly delayed (maximal induction at 60–120 min; Figure 2). In contrast, no significant changes in *NtCDPK2* transcript accumulation could be observed, irrespective of the inducing stimulus. The transcript appeared to be constitutively expressed, perhaps due to the shaking of the cultures in the dark. The transcript analysis in Figure 2 represents one of three independent experiments showing identical expression patterns.

In plants, an increase in *NtCDPK2*, *NtCDPK3* and *WIPK* transcript levels could be detected after injecting IF(Avr9⁺), IF(Avr9⁻) or just water. This is caused by the mechanical stress of the infiltration procedure (flooding stimulus). Only for *WIPK* a stronger mRNA increase occurred through the contribution of both stimuli (flooding and specific elicitation), which confirms previous data obtained with RNA blot analysis (Romeis *et al.*, 1999).

Transient expression of *NtCDPK2-myc* in *N.tabacum* and *N.benthamiana*

To characterize *NtCDPK2* protein in the context of various stress stimuli a transient transformation system was employed. A tagged version of *NtCDPK2* was generated that includes a triple c-myc epitope at its C-terminus. *Agrobacterium tumefaciens* GV3101 carrying the construct under the control of the constitutive cauliflower mosaic virus 35S promoter (Table I) was infiltrated into tobacco leaves. Western analysis conducted 2 days after infiltration showed the presence of a protein of ~75 kDa

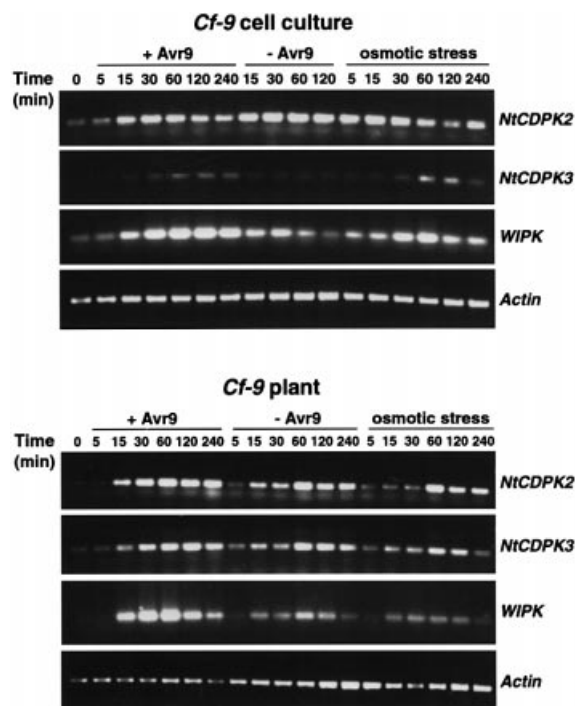


Fig. 2. Expression patterns of *NtCDPK2* and *NtCDPK3* after elicitation and hypo-osmotic stress. Cf-9 tobacco cell cultures and plants were treated with IF(Avr9⁺) or IF(Avr9⁻), which contains (+Avr9) or does not contain (-Avr9) the Avr9 peptide. Osmotic stress was applied by adding 2 vols of water to cells or by infiltrating water into leaves. At the time points indicated leaf samples were harvested and total RNA was isolated and used for RT-PCR as described in Materials and methods, applying 22, 24 and 22 amplification cycles with specific primers for the *NtCDPK2*, *NtCDPK3* and *WIPK* gene, respectively (gene indicated on the right). Equal cDNA amounts were controlled by amplification of constitutively expressed *actin* gene (24 cycles).

Table I. *Agrobacterium tumefaciens* GV3101 strains

Name	Purpose	Description of binary vector	Literature
NtCDPK2-myc	transient expression	35S <i>NtCDPK2</i> full-length gene fused to C-terminal triple c-myc tag in pBIN19	this study
NtCDPK2 ₁₋₃₈₀ myc	transient expression	35S truncated <i>NtCDPK2</i> , coding for variable and kinase domain only, fused to triple c-myc in pBIN19	this study
PVX-NtCDPK _{CLD}	silencing	417 bp of the calmodulin-like domain of <i>NtCDPK2</i> inserted into PVX genome behind coat protein promoter in pGR106	this study
PVX-GFP	silencing	GFP in pGR106	Jones <i>et al.</i> (1999)
4/456/Avr4	HR	35S <i>Cf-4</i> , 35S <i>Avr4</i>	Thomas <i>et al.</i> (2000)
4/456/Avr9	HR (control)	35S <i>Cf-4</i> , 35S <i>Avr9</i>	Thomas <i>et al.</i> (2000)
9/456/Avr9	HR	35S <i>Cf-9</i> , 35S <i>Avr9</i>	Thomas <i>et al.</i> (2000)

(Figure 3). No myc-tagged protein was detectable in the transformed *Agrobacterium* strain itself, 1 day after infiltration (data not shown), or in leaves infiltrated with the control vector (Figure 3A). As predicted by the putative myristoylation site, 90% of NtCDPK2-myc was localized in the membrane fraction (data not shown). In *N.benthamiana* and *N.tabacum* (Cf-9 tobacco), infiltration of water 2 days after *Agrobacterium* infiltration caused a rapid and transient interconversion of the expressed kinase from a faster migrating form (~75 kDa) to a slower migrating form (~77 kDa). In parallel, a decrease in the western signal intensity could be observed, suggesting that in addition to kinase interconversion, CDPK protein turnover was induced. Because in these experiments NtCDPK2-myc was expressed under the control of the 35S promoter, the activation of overexpressed enzyme may have preferentially induced a degradation pathway. When lower expression levels were achieved, no such decrease could be observed (see Figure 5B). The kinetics of the transition into the 77 kDa form was identical in both cultivars with the maximal shift peaking ~7.5 min after the inducing stimulus (Figure 3A). Such shift in electrophoretic mobility was previously correlated with an Avr9/Cf-9-mediated elicitation event in tobacco cell cultures, and was proposed to reflect a transition between a non-elicited, less active and an elicited, more active enzyme form (Romeis *et al.*, 2000).

To investigate the response of NtCDPK2-myc to a gene-for-gene-specific elicitation event, Cf-9 tobacco leaves that transiently expressed NtCDPK2-myc protein were infiltrated with IF(Avr9⁺) or IF(Avr9⁻). Again, CDPK interconversion from the non-elicited to elicited form was detectable (Figure 3B). In the IF(Avr9⁺) series this transition was maintained for 60 min before returning to basal level (Figure 3B, upper panel). When IF(Avr9⁻) was injected (lower panel), only a transient shift at 7.5 min, reminiscent of the hypo-osmotic stress series from Figure 3A, could be observed. These experiments were repeated at least three times with similar results. Thus, NtCDPK2-myc responded to two different external stimuli by adopting an identical mechanism but with altered duration.

Kinase activity of immunoprecipitated NtCDPK2-myc

We next studied whether the stimulus-induced transition of NtCDPK2-myc into the elicited form correlated with an increase in enzymatic activity. NtCDPK2-myc was transiently expressed in *N.benthamiana*, and samples were prepared before and after exposure to a stress stimulus. Both the non-elicited and elicited form of NtCDPK2-myc could be immunoprecipitated from crude solubilized membrane extracts with anti-c-myc antibodies. No myc-tagged protein was observed in the control without specific antibody (Figure 4A).

When immunoprecipitated NtCDPK2-myc that had been purified before or after the flooding stimulus was incubated with [γ -³³P]ATP, both CDPK forms became phosphorylated (Figure 4B). The reaction was calcium dependent and did not occur in the presence of 5 mM EGTA. Remarkably, even after prolonged reaction time, no change in the distribution of signal intensity between the non-elicited (75 kDa) and elicited (77 kDa) form took

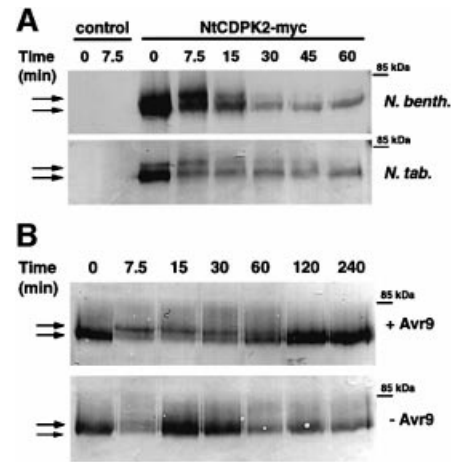


Fig. 3. Transiently expressed NtCDPK2-myc shows an elicitation- and osmotic stress-induced transition between two enzyme forms in *N.tabacum* (Cf-9 tobacco) and *N.benthamiana*. In a transient expression assay, *A.tumefaciens*, carrying the modified NtCDPK2 gene with an in-frame triple-myc tag at the 3' end under the control of a constitutive promoter (NtCDPK2-myc), or the empty vector (control) was infiltrated in leaves of *N.benthamiana* or *N.tabacum*. After 2 days, the injected area was exposed to hypo-osmotic stress by infiltrating water (A). Alternatively, in Cf-9 tobacco, the *Agrobacterium*-infiltrated area was elicited by injecting IF(Avr9⁺) or IF(Avr9⁻), which contains (+Avr9) or does not contain (-Avr9) the Avr9 peptide (B). At the time points indicated, samples were harvested and total solubilized membrane extracts were prepared. Proteins were separated on an SDS gel, transferred onto nitrocellulose, and the blots were subjected to immunodetection using an anti-c-myc antibody. Equal protein amount was confirmed by Ponceau S staining prior to immunodetection.

place. This suggests that both CDPK forms were able to autophosphorylate in a calcium-dependent manner. However, such *in vitro* autophosphorylation of isolated, immobilized NtCDPK2-myc alone could not mimic the stress-induced *in vivo* transition between the non-elicited and elicited CDPK form.

To address whether NtCDPK2-myc in its elicited form is more active, we conducted immunocomplex kinase assays with syntide-2 as artificial substrate. Cf-9 tobacco, transiently expressing NtCDPK2-myc, was elicited with IF(Avr9⁺) or IF(Avr9⁻), samples were harvested at different time points, and solubilized membrane fractions were analyzed for NtCDPK2-myc expression (Figure 4C, upper panel) or NtCDPK2-myc kinase activity in immunoprecipitates (lower panel). In parallel with the elicitor-induced transition into the elicited CDPK form, a 200-fold increase in kinase activity could be observed. No phosphorylation activity was detected in the absence of antibody or when the enzyme existed in the non-elicited form [for example, when returned to basal level 60 min after injection with control IF(Avr9⁻)].

In addition, immunocomplex kinase assays were conducted with transiently expressed NtCDPK2-myc isolated after the flooding stimulus. As shown in Figure 4D, immunoprecipitated NtCDPK2-myc efficiently phosphorylated syntide-2 in the presence of calcium (histogram, bar 1), but not in the presence of EGTA (bar 4) or when no antibody was used (bar 5). Incubation of the immobilized enzyme with a non-specific bacteriophage lambda phosphatase resulted in an interconversion back into the non-elicited form (Figure 4D, upper panel). At the

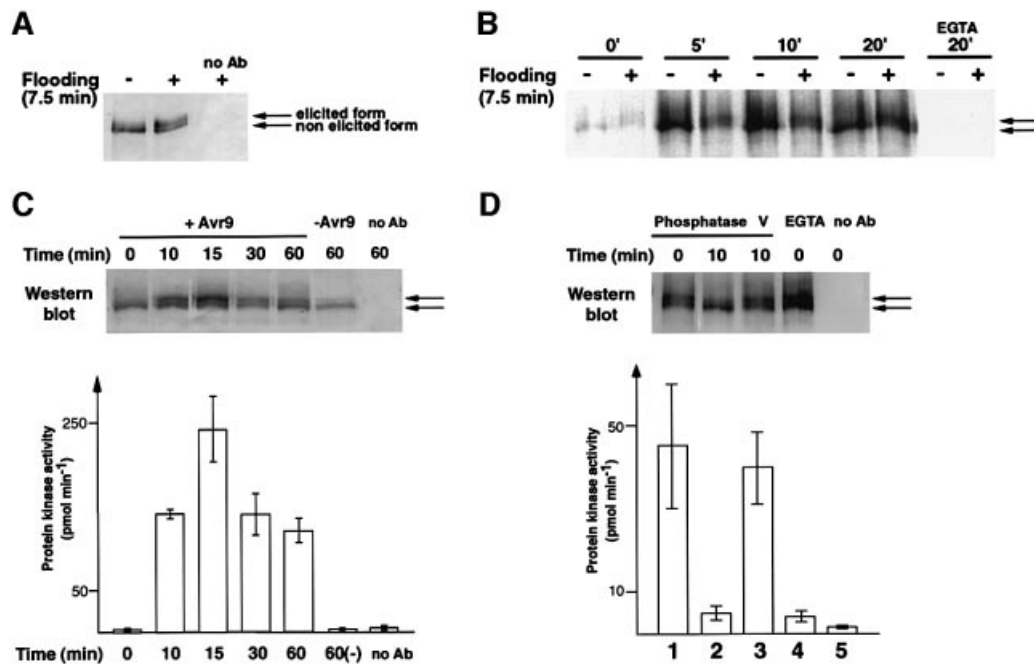


Fig. 4. Enzymatic activities of immunoprecipitated NtCDPK2-myc. (A) Leaf discs of *N.benthamiana* that transiently expressed NtCDPK2-myc were harvested before (–) or after (+) infiltration of water. Solubilized membrane extracts were subjected to immunoprecipitation with monoclonal anti-c-myc antibodies, and precipitated proteins were analyzed by western blotting and immunodetected using a polyclonal c-myc antiserum. (B) Immunoprecipitates of NtCDPK2-myc before and after the flooding stimulus were prepared as in (A), and analyzed for CDPK autophosphorylation by incubation with [γ - 33 P]ATP (92 kBq) for the time indicated in the presence of 1 mM calcium or 5 mM EGTA (last data pair). Samples were separated on an SDS gel and analyzed by autoradiography. The two arrows indicate the non-elicited and elicited enzyme forms. (C) Leaf discs of Cf-9 tobacco that transiently expressed NtCDPK2-myc were harvested over a time course after elicitation with IF(Avr $^{9+}$) or IF(Avr $^{9-}$) (–). Solubilized membrane extracts were analyzed by western blotting (upper panel) and subjected to immunocomplex kinase assays with 100 μ g/ml syntide-2 as substrate in the presence of 50 μ M [γ - 33 P]ATP (92 kBq) and 1 mM calcium for 5 min at 30°C (lower panel). Supernatants were spotted on phosphocellulose paper and subjected to scintillation counting (phosphorylation of syntide-2; histogram). (D) NtCDPK2-myc was immunoprecipitated from *N.benthamiana* leaf extracts after a flooding stimulus as described in (A). Aliquots containing the immobilized enzyme were incubated with non-specific λ phosphatase for 10 min where indicated, or with phosphatase in the presence of 50 mM NaF and 10 mM Na $_3$ VO $_4$ (lane 3). After separating the phosphatase by washing, immunocomplex kinase assays were conducted as described in (C) with syntide-2 as substrate in the presence of 50 μ M [γ - 33 P]ATP (92 kBq), 1 mM calcium (lanes 1–3 and 5) or 5 mM EGTA (lane 4). Supernatants were analyzed by western blotting (upper panel) as well as spotted on phosphocellulose paper and subjected to scintillation counting (lower panel).

same time, the corresponding immunocomplex kinase activity decreased \sim 10-fold (bar 2). In the presence of phosphatase inhibitors, *in vitro* transition and loss of kinase activity were compromised (lane and bar 3).

The immunocomplex kinase assay experiments were repeated three times with similar results in terms of induction pattern and kinetics. However, the kinase activity values were dependent on the amount of immunoprecipitated CDPK enzyme in each experimental series and were therefore influenced by varying expression levels in different tobacco plants. Our data indicate that the *in vivo* phosphorylation and interconversion of NtCDPK2-myc from the non-elicited into the elicited form is accompanied by a sustained (10- to 200-fold) increase in enzymatic activity.

VIGS of the NtCDPK2 subfamily

To examine the biological function of *NtCDPK2* we employed the recently established VIGS as a gene knockout system (Baulcombe, 1999; Burton *et al.*, 2000; R.Lu and D.C.Baulcombe, personal communication). VIGS is a rapid and transient method using potato virus X vectors that carry 300–500 bp elements from the exon of plant host genes. The silencing mechanism is based on cosuppression between transgene and

endogenous gene, likely involving the formation of double-stranded RNA, and should also target close homologs of the gene of interest (\sim 90% sequence identity). Because *NtCDPK2* and *NtCDPK3* are very closely related this technique should allow us to silence both genes.

VIGS of the *NtCDPK2* gene was conducted in *N.benthamiana*. Southern analysis of genomic DNA from *N.benthamiana* revealed two cross hybridizing signals with *NtCDPK2* full-length cDNA as probe (data not shown). The silencing construct was generated by inserting a 417 bp cDNA fragment, which comprised most of the calmodulin-like domain of *NtCDPK2*, into the viral vector. This fragment showed 98% identity on nucleotide level to *NtCDPK3*, and 96% identity to the corresponding *NtCDPK2* orthologous gene from *N.benthamiana* (DDBJ/EMBL/GenBank accession No. AJ344156; data not shown). Three weeks after the inoculation of seedlings with *A.tumefaciens* PVX-NtCDPK_{CLD} or PVX-GFP (Table I), plants were analyzed for CDPK and WIPK transcripts before and 1 h after a flooding stimulus (Figure 5A). mRNA levels were determined by RT-PCR using gene-specific primers that amplify a region outside the calmodulin-like domain (Burton *et al.*, 2000). Stress-induced mRNA accumulation of *N.benthamiana* orthologs to *NtCDPK2* and *NtCDPK3* could be detected in non-

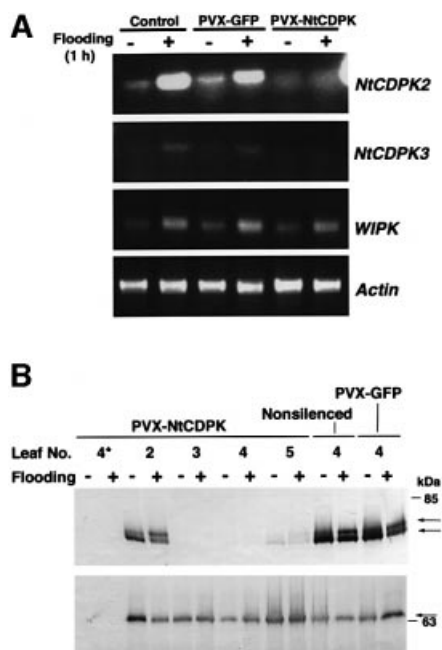


Fig. 5. VIGS of *NtCDPK2* in *N.benthamiana*. Seedlings were inoculated with *A.tumefaciens* PVX-*NtCDPK_{CLD}*, containing the silencing construct, in which a 417 bp fragment of the calmodulin-like domain of the *NtCDPK2* gene was integrated into the PVX genome, or an unrelated control insert (Jones *et al.*, 1999). After 3 weeks the silencing was accomplished, and if not otherwise stated, the fourth leaf above the inoculated one was then used for further experiments. (A) To determine the degree of silencing and analyze the transcript levels, the silenced fourth leaf of PVX-treated or the equivalent leaf of an untreated plant was exposed to hypo-osmotic stress by infiltrating water. Before (–) and 1 h after the flooding stimulus (+), samples were harvested and analyzed by RT-PCR for the expression of *N.benthamiana* homologs of *NtCDPK2*, *NtCDPK3* and *WIPK*, as described in the legend to Figure 2. *Actin* cDNA was amplified as an internal control for equal cDNA amounts. (B) Transient expression of *NtCDPK2*-myc and its truncated version in CDPK-silenced plants. Different leaves of plants previously inoculated with PVX-*NtCDPK_{CLD}* or leaf No. 4 of an untreated or PVX-GFP-inoculated control plant were infiltrated with *A.tumefaciens* *NtCDPK2*-myc (Table I) as described in the legend to Figure 3 (upper panel) or with *NtCDPK2_{1–380}*-myc lacking the junction and calmodulin-like domain (lower panel; Harper *et al.*, 1994). After two more days, the *Agrobacterium*-infiltrated area was subjected to hypo-osmotic stress by infiltrating water for 7.5 min. Leaf samples before (–) and after (+) the flooding stimulus were harvested, and extracts were prepared and analyzed by western blotting as described in the legend to Figure 3. The arrows at the right indicate *NtCDPK2*-myc and its truncated version (lower panel), respectively. Positions of the molecular mass markers are given at the right.

silenced (control) and PVX-GFP-treated plants, but were absent in CDPK-silenced plants. In contrast, the flooding-induced increase of *WIPK* mRNA was unaltered in all three plants. The *actin* gene was amplified as control for a constitutively expressed gene.

The degree of VIGS varies with the distance from the leaf used for the onset of silencing, and was reported to be highest on the third to fifth leaf above the originally inoculated one (R.Lu and D.C.Baulcombe, personal communication). Different leaves of silenced and control plants were analyzed for their ability to transiently express *NtCDPK2*-myc or a truncated version lacking the junction and calmodulin-like domain (Figure 5B), combined with the previously described flooding stimulus (see Figure 3).

If effective silencing were established, expression of full-length *NtCDPK2*-myc, which shares the 417 bp fragment with the PVX-delivered silencing construct, should be prevented. Two days after infiltration of *A.tumefaciens* *NtCDPK2*-myc and *NtCDPK2_{1–380}*-myc (Table I), *NtCDPK2*-myc was expressed in non-silenced and PVX-GFP control plants, and the flooding-induced shift between the non-elicited and elicited CDPK form could be observed (Figure 5B, upper panel). Western blot analysis of CDPK-silenced plants revealed some *NtCDPK2*-myc expression on leaf No. 2, no signal on leaf No. 3 and No. 4, and again a weak signal on leaf No. 5. Also no band could be seen when the vector control was used (first two lanes, leaf No. 4 marked by an asterisk). In contrast, a truncated *NtCDPK2*-myc construct, which lacked the domain used for the onset of silencing, was uniformly expressed on leaves in silenced and control plants (Figure 5B, lower panel).

Compared with untreated plants, the PVX-GFP- and PVX-CDPK-infected plants were ~10 and 5% shorter in stature, and PVX-infected leaves showed a slight mottling caused by the virus.

Next we studied the effect of CDPK silencing on defence-related responses. Our biochemical data using western blot and/or in-gel kinase assays demonstrated that elicitation of Cf-9 and Cf-4 tobacco cells with the corresponding Avr9 and Avr4 elicitor resulted in a gene-for-gene-specific CDPK interconversion and activation (Romeis *et al.*, 2000; data not shown). Here we determined the HR-inducing activity in the respective gene-for-gene interactions using an *Agrobacterium*-mediated transient assay in which the resistance gene (*Cf-4* or *Cf-9*) and the corresponding avirulence gene (*Avr4* or *Avr9*) were co-expressed (Thomas *et al.*, 2000). The necrotic reaction induced by *Cf-4/Avr4* or *Cf-9/Avr9* could be detected after 2 or 5 days in the upper leaf half of the non-silenced (Figure 6, lower panels) and PVX-control-treated plants (Figure 6, middle panels). In the CDPK-silenced plants, necrotic symptoms in the *Cf-4/Avr4* and *Cf-9/Avr9* combinations were significantly reduced and delayed, and developed only around the infiltration sites (Figure 6, upper panels). No HR-inducing activity was found when the inactive combination *Cf-4/Avr9* was infiltrated (Figure 6, lower leaf halves). This suggests that *NtCDPK2* alone or together with subfamily isoforms is required in the signalling cascade to activate HR. In addition, in CDPK-silenced plants the characteristic leaf wilting phenotype was absent (Figure 6, side and front views). This leaf flopping, which happens within a short period of time just before onset of necrosis in the *Cf-4/Avr4*-treated leaf half, could be observed in non-silenced and PVX control-infected plants. These HR and wilting phenotypes were repeated in several independent experiments over a long period of time. However, when the silencing was incomplete, so that a (less pronounced) increase in CDPK transcripts was still detectable by RT-PCR, gene-for-gene-dependent HR and leaf wilting were no longer affected.

Discussion

A challenge in plant signalling is to understand how cellular responses to environmental stimuli are activated

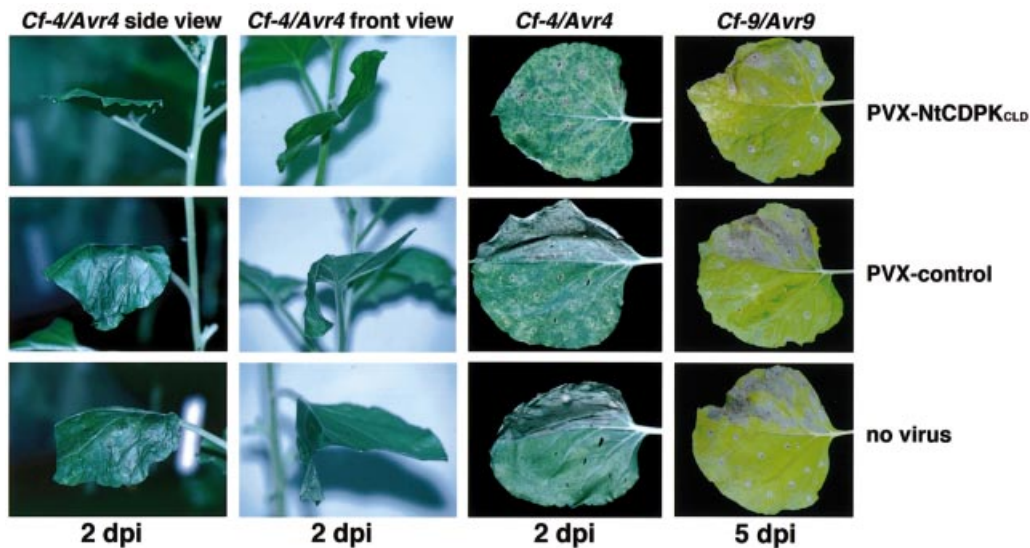


Fig. 6. CDPK-silenced leaves were compromised in gene-for-gene-dependent HR induction. For the onset of silencing, *N.benthamiana* seedlings were inoculated with *A.tumefaciens* PVX-NtCDPK_{CLD} (upper row), PVX-GFP (control; middle row) or remained untreated (lower row), as described in the legend to Figure 5. After 3 weeks the fifth leaf (first column) or the fourth leaf (second to fourth column) above the primary infected one was infiltrated with *A.tumefaciens* 4/456/Avr4 or 9/456/Avr9, as indicated, carrying either *Cf-4/Avr4* or *Cf-9/Avr9* gene-for-gene combinations on one leaf half (upper half in third and fourth column) or with strain 4/456/Avr9 as control carrying *Cf4/Avr9* on the other leaf half (lower half in third and fourth column). Photos of necrosis and wilting phenotype were taken as indicated.

and whether unique, overlapping or redundant signalling pathways are used (Bowler and Fluhr, 2000). Different signalling inputs converge at a single component (scenario 1); conversely, one stimulus can trigger components that are located in different signal cascades (scenario 2). Substantial progress has been made in characterizing the large CDPK gene family from various plant species and it has been shown that CDPKs are multifunctional (Harmon *et al.*, 2000). The future challenge is to address which specific isoforms are integrated in which cellular processes in which cell type and when.

***NtCDPK2* and *NtCDPK3* function in multiple signalling pathways**

Recently, a 68/70 kDa membrane-associated CDPK was identified by its characteristic shift in electrophoretic mobility in in-gel kinase assay and western blot analysis when Cf-9 tobacco cells were treated with the Avr9 elicitor (Romeis *et al.*, 2000), or Cf-4 tobacco cells with Avr4 (A.Ludwig, S.Rivas, J.D.G.Jones and T.Romeis, unpublished). The same shift, although less sustained, could be observed after diluting tobacco cells with 2 vols of water (data not shown). In this paper we have isolated two genes, *NtCDPK2* and *NtCDPK3*, and we conclude that *NtCDPK2* codes for that previously characterized 68/70 kDa Avr9/Cf-9-dependent CDPK. Transiently expressed NtCDPK2-myc protein is membrane associated, as predicted by its N-terminal myristoylation motif; it migrates at the correct size (allowing for the increase in molecular mass for the triple-myc tag); and it responds to hypo-osmotic stress and elicitation with reversible interconversion between two enzyme forms. Furthermore, although both genes were transcriptionally activated in response to stress stimuli in Cf-9 cells and plants, more *NtCDPK2* than *NtCDPK3* message accumulated after

stimulation. In particular in Cf-9 cells, *NtCDPK2* was constitutively highly expressed so that no further mRNA induction could be triggered by stress stimuli. This suggests that the major part of CDPK signalling in Cf-9 cells was contributed by *NtCDPK2*.

How can one enzyme be involved in different signalling pathways? Transiently expressed NtCDPK2-myc protein responds to osmotic stress and specific elicitation with a shift from a non-elicited (75 kDa) to an elicited enzyme form (77 kDa). Whereas after gene-for-gene-specific elicitation this transition was complete and sustained for ~60 min, flooding or treatment with control IF(Avr9⁻) caused a shorter response, peaking at 7.5 min, and only a portion of the enzyme available participated in the shift. This let us conclude that the enzyme is regulated by altering the extent and duration of the post-translational modification depending on the inducing stimulus.

Protein activation is followed by an increase in transcript level, and maximal mRNA accumulation was observed 60–120 min after hypo-osmotic stress and elicitation. Interestingly, this time course correlates with the CDPK shifting back into its non-elicited form after the Cf-9/Avr9 elicitation event in tobacco cells, as reported previously. Because CDPK activation is accomplished by phosphorylation, CDPK interconversion back to basal level and thus inactivation may be catalyzed by a protein phosphatase. Alternatively, the elicited form could be degraded by proteolysis, and reappearing CDPK in its non-elicited form is attributable to newly synthesized enzyme due to enhanced transcriptional activity. The latter hypothesis is supported by the fact that NtCDPK2 and NtCDPK3 contain a PEST sequence, which is a conserved protein degradation motif, in the N-terminal variable domain (Rechsteiner and Rogers, 1996). Protein degradation also became evident in addition to the mobility shift,

when transiently expressed NtCDPK2-myc was challenged by elicitor or flooding (Figure 3). What was described as scenario 1—one isoform participates in different signal cascades—has thus been demonstrated for *NtCDPK2* and its derived tagged gene product.

Enzymatic activity of NtCDPK2-myc protein

The fact that NtCDPK2 and NtCDPK3 share 94% amino acid sequence and have an identical N- and C-terminus hampered the design of isoform-discriminating anti-peptide antibodies. To investigate the activation and characteristics of the elicited and non-elicited CDPK without the interference of kinase activities caused by other protein kinases or endogenous CDPKs, tagged NtCDPK2-myc was transiently expressed. Enzymatic properties of the isolated CDPK isoform could then be analyzed in immunoprecipitates reflecting samples taken before or after *in vivo* stimulation in the biological system. In immunocomplex kinase assays (Figure 4C and D) the elicited form of isolated NtCDPK2-myc showed a 10- to 200-fold higher calcium-dependent enzymatic activity towards syntide-2 due to osmotic stress and elicitation, respectively, than the non-elicited form. This confirms previous studies in which a CDPK 'shift down' experiment after phosphatase treatment was shown using crude solubilized membrane extracts from elicited cells (Romeis *et al.*, 2000). Whether NtCDPK2-myc interconversion and activation requires identical intramolecular phosphorylation sites for both stimuli or whether different sites and phosphorylation kinetics are involved will be subject to future studies.

Both forms of purified NtCDPK2-myc were able to autophosphorylate *in vitro* (Figure 3B); however, CDPK autophosphorylation appears not to be sufficient for the observed shift between the two enzyme forms *in vivo*, suggesting that different and/or additional phosphorylation sites exist. This let us conclude that upon elicitation or abiotic stress, an upstream kinase is responsible for the phosphorylation and activation of NtCDPK2-myc in the biological system. This is consistent with the fact that the elicitation-induced *in vivo* transition between the two CDPK forms in Cf-9 cells was not compromised in the presence of inhibitors that would block CDPK activity (Romeis *et al.*, 2000). Whether this kinase is also responsive to multiple stress stimuli or whether branched cascades exist in which more than one enzyme is involved in CDPK activation remains to be shown. The latter scenario is reminiscent of MAP kinase signalling where in a stimulus-specific manner, upstream members of kinase cascades such as MAPKK or MAPKKK become recruited and are responsible for MAP kinase activation (Kiegerl *et al.*, 2000; Kovtun *et al.*, 2000; Liu *et al.*, 2000; for review see Meskiene and Hirt, 2000; Bent, 2001). By investigating CDPKs in transient assays, we therefore established an excellent system that will facilitate studies of the enzymes' *in vivo* activation mechanism, the identification of the phosphorylation acceptor sites and the upstream kinase.

Function of NtCDPK2 orthologs and homologs

NtCDPK2 and *NtCDPK3* belong to a small gene subfamily of four members in the allotetraploid *N.tabacum* genome. NtCDPK2-myc protein activation and *NtCDPK2*

transcript levels suggest that this subfamily functions in triggering cellular responses to extracellular stimuli. Is this task restricted to one enzyme, one subfamily or, in line with signalling scenario 2, are homologs from other CDPK subfamilies recruited?

Elicitation of Cf-9 cells with Avr9 resulted in the activation of a 68/70 kDa CDPK, likely to be attributable to NtCDPK2. However, it can not be excluded that NtCDPK3 and the other subfamily members also contributed to the observed signals on western blot and in-gel kinase activities. Activation of a 55 kDa CDPK in response to non-specific elicitation of soybean cell cultures has been suggested (Allwood *et al.*, 1999).

NtCDPK2 is orthologous to *AtCPK1* and *AtCPK2* from *Arabidopsis* and *CpCPK1* from zucchini and falls into the same group of the CDPK superfamily (Harmon *et al.*, 2000). *AtCPK1* is one of the best investigated CDPK isoforms, and detailed biochemical analysis of its enzyme mechanism is available (Harper *et al.*, 1993, 1994; Huang *et al.*, 1996; Vitart *et al.*, 2000). *In vitro* studies with recombinant AtCPK1 revealed that the enzyme interacted with 14-3-3 proteins (Camoni *et al.*, 1998), was able to activate a tonoplast chloride channel *in vitro* (Pei *et al.*, 1996), and could phosphorylate and inactivate an endoplasmic reticulum-localized, calmodulin-stimulated calcium pump (Hwang *et al.*, 2000). *CpCPK1* was reported to be highly expressed in hypocotyls of dark grown zucchini seedlings, and CpCPK1 protein could be myristoylated *in vitro* (Ellard-Ivey *et al.*, 1999). However, *in vivo* function has so far not been elucidated for any of these orthologs.

In response to drought and salt, CDPK transcript accumulation was shown for *AtCPK10* and *AtCPK11* in *Arabidopsis* (Urao *et al.*, 1994). The transient expression of *AtCPK10* in an active, calcium-independent form in protoplasts activated a cold, dark and osmotic stress response pathway, and mimicked the response to abscisic acid (Sheen, 1996). A salt/drought- and cold-induced increase in CDPK transcript was also reported for *OsCDPK7* from rice, and overexpression of the gene resulted in plants more tolerant to these environmental stresses (Saijo *et al.*, 2000). These CDPK isoforms fall into different side branches of the CDPK superfamily (Harmon *et al.*, 2000). So far, only one other tobacco CDPK homolog has been identified, *NtCDPK1*, which also classifies into a distant subgroup compared with *NtCDPK2* and *NtCDPK3*. Transcript accumulation of *NtCDPK1* and/or closely related subfamily members was induced by wounding and salt treatment of leaves, and by exposure to fungal elicitor or chitosan of BY2 tobacco cells (Yoon *et al.*, 1999). Therefore, one stimulus can activate more than one CDPK of more than one subfamily. How CDPK signalling contributed by different isoforms is integrated remains largely unknown. More data are needed on intracellular location, and cell- or tissue-specific expression patterns of CDPK isoforms.

Crosstalk between CDPKs and other signalling pathways?

The post-translational activation of a protein kinase followed by an increase in the transcript level of the corresponding gene, shown here for NtCDPK2, is known for one subclass of MAP kinases, which includes *WIPK*

from tobacco, *SAMK* from alfalfa, *AtMPK3* from *Arabidopsis* and *ERMK* from parsley (for review see Meskiene and Hirt, 2000; Zhang and Klessig, 2000; Romeis, 2001). Interestingly, these orthologous enzymes are multifunctional and participate in more than one signal cascade. The likely complexity of the MAP kinase signalling is further increased by the existence of SIPK, SIMK and AtMPK6 orthologs and/or by the AtMPK4 class of MAP kinases that also respond to multiple external stimuli (Ichimura *et al.*, 2000a,b). To study whether the CDPK and MAP kinase pathways are interconnected in an Avr9/Cf-9 interaction we previously applied pharmacological inhibitors. Data with W7, a rather non-specific inhibitor of calcium-dependent enzymes, suggested that in the Avr9/Cf-9 response a calcium-dependent enzyme is located upstream of the MAP kinase activation, which might or might not be a CDPK (Romeis *et al.*, 2000).

In this paper we used VIGS as a reverse genetics approach to silence signalling components that become transiently activated, which allowed us to address that question much more precisely. RT-PCR revealed that the flooding-induced increase in CDPK transcript was absent in the CDPK-silenced leaves, and therefore cross-silencing of both isoforms was obtained in *N.benthamiana*. On the other hand, the 417 bp fragment of the calmodulin-like domain of NtCDPK2 was <56% identical to calmodulin at the nucleotide level. This excludes the possibility that the observed effects are due to calmodulin cosuppression. In CDPK-silenced leaves both flooding-induced activation of WIPK and SIPK in-gel protein kinase activity towards myelin basic protein and flooding-induced accumulation of WIPK mRNA were unaffected (data not shown). Thus, NtCDPK2 subfamily members appear not to be involved in the activation of WIPK, and these signalling components, despite similar stress-induced kinase activities and transcript levels, function in distinct pathways.

Function of NtCDPK2 in the plant defence response

To dissect the biological function of CDPKs, loss-of-function genetic analysis is essential, and *Arabidopsis* mutant lines that carry T-DNA insertions in CDPK isoforms have already been identified (Krysan *et al.*, 1996). However, NtCDPK2 belongs to a gene subfamily in tobacco, and in employing the more conventional gene knock-out methods based on T-DNA or transposon insertions, a loss-of-function might have been compensated by functional homologs, for example, NtCDPK3. VIGS is based on cosuppression, and as confirmed by RT-PCR, closely related subfamily members are silenced.

By using VIGS we could show that plants silenced with the calmodulin-like domain of NtCDPK2 were compromised in generating a Cf-9/Avr9- and Cf-4/Avr4-dependent, defence-related HR, and also lacked an accompanying wilting phenotype. This indicates that NtCDPK2 and NtCDPK3 play a key role in HR induction. Early signal processes that activate an HR in response to a gene-for-gene interaction include changes in ion fluxes and protein kinase activation. An important role for reactive oxygen species such as H₂O₂ and NO has also been demonstrated (for review see Yang *et al.*, 1997; Richberg *et al.*, 1998; Scheel, 1998; Heath, 2000; Nürnberger and Scheel, 2001). However, the biological target(s) that become phosphoryl-

ated by NtCDPK2 are unknown. Although several potential pathogen-related CDPK targets have been discussed in the literature, including H⁺-ATPase (Schaller and Oecking, 1999), ion channels (see below) or NADPH-oxidase (Blumwald *et al.*, 1998; Romeis *et al.*, 2000), it is unclear at which step CDPK signalling feeds into the HR pathway. The leaf wilting phenotype, which precedes the onset of a macroscopic visible HR and is absent in CDPK-silenced leaves, suggests that NtCDPK2 may be involved in controlling the early changes in ion fluxes. Supraoptimal stomata opening allowing transpirational water loss had been observed in the Cf-9/Avr9 interaction and the loss of turgor and subsequent collapse of epidermal and mesophyll tissue may account for the developing gray necrosis (Hammond-Kosack *et al.*, 1996). In guard cells, calcium has been shown to be a messenger that affects both stomatal opening and stomatal closure, and the outcome very much depends on the specific calcium signature as well as on the availability of the calcium-sensing enzymes (Wang and Wu, 1999). Interestingly, recombinant AtCPK1, a NtCDPK2 ortholog, was shown to activate a guard cell tonoplast anion channel *in vitro*, and calcium-dependent activation of that channel might be required for stomatal opening (Pei *et al.*, 1996).

In investigating biochemical properties of CDPK isoforms in transient expression assays, studying their biological function with VIGS, and in combining both, we present novel approaches for the research in CDPK signalling that will facilitate future studies towards activation mechanism, the identification of upstream regulatory components, as well as downstream phosphorylation targets in a homologous biological system.

Materials and methods

Tobacco cell culture and plant treatments

Suspension cultures from *N.tabacum* cv Petite Havana and the derived Cf-9 line were subcultured at 2-weekly intervals and prepared for experiments as described previously (Romeis *et al.*, 2000). For elicitation, cells were challenged with 75 µl of intercellular fluid originating from transgenic tobacco that produces the Avr9 peptide apoplastically [IF(Avr9⁺)] or with control intercellular fluid [IF(Avr9⁻)] (Hammond-Kosack *et al.*, 1998). To apply osmotic stress, cells were diluted with 40 ml (twice the culture volume) of water. At the times indicated, cells were harvested by filtration, immediately frozen in liquid nitrogen, and stored at -70°C. *Nicotiana tabacum* cv Petite Havana, Cf-9 tobacco and *N.benthamiana* plants were grown in environmentally controlled growth and containment cabinets under a 16 h photoperiod at 22°C and an 8 h dark period at 18°C. Elicitation with IF (Cf-9 tobacco) was conducted on 6-week-old plants, just before the flower bud started to emerge, by infiltrating IF with a syringe in tiny cuts at the lower leaf side and thereby flooding the apoplastic space. The response to osmotic stress (without elicitation component) was investigated after the infiltration of water.

Isolation of CDPK cDNAs

Total RNA was extracted from Cf-9 tobacco cell suspension cultures treated with IF(Avr9⁺) for 30 min as described (Romeis *et al.*, 1999) and used for reverse transcription with degenerate primers A07 (5'-CCITAYTAYRTIGCICIGARGT-3') and WD73 (5'-CCYTTYTK-CATCATIGCIA CRAAYTC-3'), which bind within conserved regions of the kinase and calcium-binding domain following the protocol from Klimyuk *et al.* (1993). PCR fragments of ~750 bp were cloned into pGEMT (Promega) and sequenced. A 750 bp PCR fragment representing the majority of the clones obtained was then used as probe to screen a λZAP cDNA library established from elicited tobacco cells (Durrant *et al.*, 2000). Seven independent clones were found to encode two different CDPK isoforms, NtCDPK2 and NtCDPK3. The longest clones encompassing the entire reading frame were selected for further study.

DNA constructs and seedling infection for VIGS

A 417 bp cDNA fragment encompassing most of the calmodulin-like domain of *NtCDPK2* was amplified with phosphorylated primers CK1 (5'-GAAGAAATTGCTGGTCTG-3') and CK2 (5'-CTTTTCATCATGGCGACGAAC-3'), subcloned into pPCR-Script Amp SK(+) (Stratagene), and ligated as a *Clal*-*NotI* fragment into vector pGR106. The fragment is inserted downstream of the duplicated coat protein promoter in the PVX genome, which by itself is integrated into the pGreen binary vector derivative (Baulcombe *et al.*, 1995; Takken *et al.*, 2000; R.Lu and D.C.Baulcombe, personal communication). The construct contained the insert in sense orientation and was designated PVX-*NtCDPK_{CLD}*. PVX-GFP, a near full-length cDNA coding for the green fluorescent protein in the same vector, was used as PVX control. Both binary plasmids were transformed into strain GV3101, harboring transformation helper plasmid pSup (R.Lu and D.C.Baulcombe, personal communication).

For infection, second leaves of 2- to 3-week-old *N.benthamiana* seedlings were wounded with a tooth-pick that was streaked over an agar plate with the respective *A.tumefaciens* GV3101 strain (Table I). After an additional 3–4 weeks the fourth and fifth leaves above the primarily punched leaf of each plant were analyzed for CDPK transcript level and transient expression of *NtCDPK2-myc*.

To analyze for defence-related phenotypes, the silenced fourth and fifth leaves were infiltrated with *A.tumefaciens* 4/456/*Avr4* or 9/456/*Avr9* (expressing transiently *Cf-4* and *Avr4* or *Cf-9* and *Avr9*; gene-for-gene interaction) on one leaf half, and 4/456/*Avr9* (*Cf-4* and *Avr9*; control) on the other leaf half (Thomas *et al.*, 2000). In non-silenced plants, the hypersensitive cell death reaction can be observed after 2 and 5 days in the *Cf-4/Avr4* and *Cf-9/Avr9* combination, respectively (Wulff *et al.*, 2001).

Transcript levels and RT-PCR

Total RNA from leaf material or cultured cells was isolated with the RNA-Isolator (Genosys). Twenty micrograms of RNA were treated with 5 U of RNase-free DNase and 5 U of RNase inhibitor (both Amersham Pharmacia Biotechnology) for 30 min at 37°C, purified by phenol/chloroform extraction and dissolved in RNase-free water. Two micrograms of DNase-treated RNA were reverse transcribed for 90 min at 42°C in a 20 µl reaction volume containing 1 U of ExpandTM Reverse Transcriptase (Roche), 250 µM each dNTP, 30 µM oligo (dT)₃₀M primer, 20 U of RNase inhibitor and 10 mM dithiothreitol (DTT). One microliter of the RT reaction was used for PCR in a 20 µl volume with 1 U of Taq DNA-polymerase (Gibco-BRL), 100 µM each dNTP and 100 ng of each forward (CK11: 5'-ATGGGGAACTGTGTGGACC-3') and reverse primer (*NtCDPK2*-specific CK30-2: 5'-CTTAGGCTTACCGTCCCTCTC-3'; *NtCDPK3*-specific CK30-3: 5'-TTGGGCTTTTTGGTTGTTCTTT-3'). PCR conditions were the following: 3 min, 94°C (first cycle); 30 s, 94°C; 30 s, 50°C; 1.5 min, 72°C (22–26 cycles); 10 min, 72°C (last cycle). PCR products were separated on a 1% agarose gel and visualized after EtBr staining. As control for equal cDNA amount in each reaction, a PCR was performed with primers for actin AC1 (5'-ATGGCAGACGGTAGGATATTCA-3') and AC2 (5'-GCCTTGCAATCCACATCTGTTG-3').

Transient expression of *NtCDPK2* in *N.benthamiana* and *N.tabacum*

To generate a tagged version of *NtCDPK2*, the full-length cDNA and a truncated version coding for the variable and kinase domain were amplified by PCR with primers CK-V-*Nco*-F (5'-AATCCATGGGGAACTTGTGTGGACCA-3') and CK-CLD-*Bam*-R (5'-AATTGATCCAAGTCTAGAGCCTCTCTAA-3') or CK-K-*Bam*-R (5'-AATGGATCCCCACCATCAACTTGAACCC-3'), which introduced *Nco*I and *Bam*HI restriction sites at the 5' and 3' ends. The respective 1746 (full-length) or 1140 bp (truncated) fragment was inserted together with a 123 bp *Bam*HI-*Xba*I fragment coding for an in-frame C-terminal triple c-myc tag (Piedras *et al.*, 2000) into the *Nco*I and *Xba*I sites of the vector SLJ4D4 (Jones *et al.*, 1992). The *Eco*RI-*Hind*III fragments were then cloned into pBIN19 to yield plasmids pSLJ13791 and pSLJ13801 and the plasmids were electroporated into *A.tumefaciens* GV3101 (Table I). Overnight cultures were harvested by centrifugation, cells were resuspended in 10 mM MgCl₂, 10 mM MES pH 5.6 and 150 µM acetosyringone to an OD of 0.5, incubated for 2–5 h at room temperature, and infiltrated into leaves of 6-week-old *N.tabacum* (*Cf-9* tobacco) or *N.benthamiana* plants. Two days after *Agrobacterium* infiltration, leaf discs used for experiments were harvested, immediately frozen in liquid nitrogen and stored at -70°C.

Preparation of protein extracts

Leaf discs (2 cm diameter, 0.5 g wet weight) were ground in liquid nitrogen, thawed in 500 µl of extraction buffer [50 mM Tris-HCl pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 10 mM NaF, 10 mM Na₃VO₄, 25 mM β-glycerophosphate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 2 µg/ml antipain, 2 µg/ml aprotinin and 2 µg/ml leupeptin], and centrifuged at 100 000 g for 25 min at 6°C in a TL100 ultracentrifuge (Beckman). The pellet was resuspended in 250 µl of solubilization buffer (20 mM Tris-HCl pH 7.5, 1 mM MgCl₂, 1 mM DTT, 5 mM NaF, 1 mM Na₃VO₄, 10 mM β-glycerophosphate, 1 mM AEBSF, 2 µg/ml antipain, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 1% Triton X-100) and incubated for 30 min at 6°C with end-over-end rotating. After centrifugation (as above), the crude solubilized membrane extracts were analyzed directly or frozen in liquid nitrogen and stored at -70°C. The protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce) with bovine serum albumin as a standard.

Immunoblotting

Solubilized crude extracts (10 µg of total protein per lane) or immunoprecipitates (see below) were separated on a 10.5% SDS gel, and the proteins were transferred onto nitrocellulose (Amersham) by wet electroblotting (Mini-Protean II system; Bio-Rad). Equal loading of protein was confirmed by Ponceau S staining of the membrane, and the blots were subsequently blocked in TBST buffer (25 mM Tris-HCl pH 7.5, 100 mM NaCl and 0.1% Tween-20) with 5% fat-free milk powder for 30 min at room temperature. For immunodetection, blots were probed with a monoclonal (9E10 epitope; crude extracts) or polyclonal (A14 epitope; immunoprecipitates) anti-c-myc antiserum (both from Santa Cruz) in a 1:3000 dilution in TBST at 6°C overnight. Alkaline phosphatase-conjugated goat anti-mouse (crude extracts) or goat anti-rabbit (immunoprecipitates) IgG (1:3000 dilution; Sigma) was used as secondary antibodies, and the reaction was visualized by hydrolysis of tetrazolium-5-bromo-4-chloro-3-indolyl phosphate as substrate (Sigma).

Immunocomplex protein kinase assay

To determine kinase activity of transiently expressed *NtCDPK2-myc*, 150 µg of solubilized crude membrane extracts were incubated with 300 µl of solubilization buffer (lacking the detergent and DTT) and 0.25 µg of monoclonal anti-c-myc antibody with end-over-end rotation for 90 min at 6°C. After addition of 50 µl of protein G and a further incubation for 45 min, samples were harvested by centrifugation and washed with solubilization buffer containing 0.3% Triton X-100 (first and second wash), 0.3% Triton X-100 and 1 M NaCl (third wash). Subsequently, samples were aliquoted (5 µl of beads each), washed with kinase buffer and resuspended in 5 µl of kinase buffer (40 mM HEPES pH 7.4, 10 mM MgCl₂, 2 mM DTT, 0.1 mM EGTA).

For *in vitro* interconversion experiments, reactions with *NtCDPK2-myc* immunoprecipitates/beads were washed once with phosphatase buffer (NEB) and resuspended in 100 µl of the same buffer containing 2 U of non-specific λ phosphatase (NEB) in the absence or presence (control) of 50 mM NaF and 10 mM Na₃VO₄. After 10 min at 37°C the reaction was stopped by addition of NaF and Na₃VO₄, and the beads were washed twice to separate from residual (although inhibited) phosphatase and resuspended in 5 µl of kinase buffer.

The kinase reaction was started by addition of 25 µl of reaction mix to yield a final concentration of 10 mM MgCl₂, 100 µg/ml syntide-2, 0.925 MBq (2.5 µCi) [³³P]ATP (92 TBq/mmol; Amersham Pharmacia Biotech), 50 µM ATP, and either 1 mM CaCl₂ or 5 mM EGTA. After incubation for 5 min at 30°C, 20 µl of supernatant were spotted on P81 phosphocellulose paper squares and the incorporation of phosphate was determined as described (Romeis *et al.*, 2000). A 5 µl aliquot of each sample was analyzed in parallel by SDS-PAGE and immunoblotting.

Acknowledgements

We are grateful to Olivier Voinnet, Jack Peart, Rui Lu, Abdelhahid Bendahmane and David Baulcombe for supplying vectors and for advice and discussions concerning gene silencing. We thank Owen Rowland and Wendy Durrant for the cDNA library and Colwyn Thomas for the *A.tumefaciens* GV3101 strains 4/456/*Avr4*, 9/456/*Avr9* and 4/456/*Avr9*. We would also like to thank Alice Harmon (Gainesville, FL) for very stimulating discussions and critical reading of the manuscript. Matthew Smoker, Sara Perkins and Mike Hill are gratefully acknowledged for the propagation of cell cultures and the care and maintenance of plants. This work was supported by the Gatsby Charitable Foundation and the Crosstalk in Signalling in Plants project (EC Grant No. HRPN/CT-2000-00093).

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Received April 4, 2001; revised August 21, 2001;
accepted August 28, 2001