StCDPK5 confers resistance to late blight pathogen but increases susceptibility to early blight pathogen in potato via reactive oxygen species burst

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Summary
• Potato (Solanum tuberosum) calcium-dependent protein kinase (StCDPK5) has been shown to phosphorylate the N-terminal region of plasma membrane RBOH (respiratory burst oxidase homolog) proteins, and participate in StRBOHB-mediated reactive oxygen species (ROS) burst. The constitutively active form, StCDPK5VK, provides a useful tool for gain-of-function analysis of RBOH in defense responses.
• StCDPK5- and StCDPK5VK-green fluorescent protein fusion proteins were predominantly targeted to the plasma membrane, and conditional expression of StCDPK5VK activated StRBOHA-D. The interaction was confirmed by bimolecular fluorescence complementation assay. We generated transgenic potato plants containing StCDPK5VK under the control of a pathogen-inducible promoter to investigate the role of ROS burst on defense responses to blight pathogens.
• Virulent isolates of the late blight pathogen Phytophthora infestans and the early blight pathogen Alternaria solani induced hypersensitive response-like cell death accompanied by ROS production at the infection sites of transgenic plants. Transgenic plants showed resistance to the near-obligate hemibiotrophic pathogen P. infestans and, by contrast, increased susceptibility to the necrotrophic pathogen A. solani.
• These results indicate that RBOH-dependent ROS contribute to basal defense against near-obligate pathogens, but have a negative role in resistance or have a positive role in expansion of disease lesions caused by necrotrophic pathogens.

Introduction
Rapid production of reactive oxygen species (ROS) has been implicated in plant innate immunity (Doke, 1983). The NADPH oxidase, RBOH (respiratory burst oxidase homolog), has a central role in ROS burst in plants. RBOH participates in ROS-mediated diverse signaling networks, such as defense responses, plant development and cell elongation (Torres & Dangl, 2005). AtRBOHD and AtRBOHF function in ROS production during pathogen signals (Torres et al., 2002) and ABA-induced stomatal closure in guard cells (Kwak et al., 2003). Virus-induced gene silencing of NbRBOHA and NbRBOHB in Nicotiana benthamiana reduces ROS production and resistance to Phytophthora infestans (Yoshioka et al., 2003; Asai et al., 2008). Sagi et al. (2004) showed that using an antisense technique that loss-of-function of tomato RBOHs reduces ROS production in leaves and induces morphological abnormality. In root hair development, ROS production by AtRBOHC/RHD2 controls cell expansion through the activation of Ca2+ channels (Foreman et al., 2003).

RBOH proteins localize on the plasma membrane (Kobayashi et al., 2006), and an N-terminal cytosolic extension, which includes two Ca2+-binding EF-hand motifs, participates in the activation. Sagi & Fluhr (2001) showed by using denaturing gel assay that Ca2+ directly activates an RBOH-like enzyme in tomato and tobacco plasma membranes. Rac GTPase is also implicated in the regulation of RBOH by means of the N-terminal extension. Wong et al. (2007) showed there was direct interaction between Rac and the N-terminal of RBOH and that the interaction may activate NADPH oxidase activity in plants. They also suggested that cytosolic Ca2+ concentration might modulate NADPH oxidase activity by regulating the interaction between Rac and RBOH.

Transient influx of Ca2+ into the cytoplasm after perception of pathogen signals is an early element of the signaling pathway triggering ROS burst and hypersensitive response (HR) cell death (Blume et al., 2000; Grant et al., 2000; Lecourieux et al., 2006). Calcium-dependent protein kinases (CDPKs) are
serine/threonine protein kinases that include a Ca\(^{2+}\)-binding calmodulin-like domain and are the best-characterized calcium sensors in plants. CDPKs are encoded by a large multigenic family with possible redundancy or diversity, or both, in their functions (Harmon et al., 2000; Freymark et al., 2007). CDPK families are divided into four groups according to sequence homology-based clustering (Asano et al., 2005). Accumulating evidence indicates that CDPKs regulate many aspects of plant growth and development (Bachmann et al., 1996; Yoon et al., 2006), hormonal responses (McCubbin et al., 2004; Choi et al., 2005; Ludwig et al., 2005; Mori et al., 2006; Ishida et al., 2008; Gargantini et al., 2009; Kamiyoshihara et al., 2010), adaptation to biotic and abiotic stresses (Saijo et al., 2000; Chico et al., 2002; Iwashita et al., 2005) and plant immunity (Romei et al., 2001; Freymark et al., 2007; Coca & San Segundo, 2010; Asano et al., 2012). CDPKs may function upstream of ROS production (Ludwig et al., 2004; Kobayashi et al., 2007). Ecotopic expression of Arabidopsis AK1 (ArCPK1) increases NADPH oxidase activity and ROS production in tomato protoplasts (Xing et al., 2001). Tobacco CDPK2 (NtCDPK2) is activated biochemically and transcriptionally after gene-for-gene interaction and abiotic stress and then by ROS production (Romei et al., 2000). Arabidopsis group I CDPKs of CPK4, CPK5, CPK6, and CPK11 regulate redundantly bacterial elicitor flg22-induced ROS burst and pathogen defense (Boudsocq et al., 2010). Functional analysis using artificially activated CDPK has shown that CDPK consists of four domains: an N-terminal variable domain (V), a serine/threonine protein kinase domain (K), an autoinhibitory junction domain, and a C-terminal calmodulin-like domain including four EF-hand motifs (Harper et al., 2004). A junction domain between the kinase and calmodulin-like domains is a pseudosubstrate in the absence of Ca\(^{2+}\) and keeps low activity (Harmon et al., 1994). A truncated VK form, with the junction and calmodulin-like domains removed, shows constitutive activity in the absence of Ca\(^{2+}\) (Harper et al., 1994). Transient expression of NtCDPK2VK stimulates ROS production and HR cell death by abiotic hypo-osmotic and wound stresses; NtCDPK2VK increases the concentration of jasmonic acid, 12-oxo-phytodienoic acid and ethylene under abiotic stress stimuli and has a cross-talk with mitogen-activated protein kinases (MAPKs) in ethylene signaling (Ludwig et al., 2005).

We isolated StCDPK5 of group I as an StRBOHB kinase from potato (Kobayashi et al., 2007). StCDPK5 phosphorylates Ser-82 and Ser-97 in the N terminus of StRBOHB calcium dependently. Coexpression of the constitutively active form, StCDPK5VK, with StRBOHB in N. benthamiana stimulates ROS production, and immunoblot analysis using anti-pSer82 antibody showed that phosphorylation of Ser-82 can be detected in leaves (Kobayashi et al., 2007). These data suggest that StCDPK5VK activates StRBOHB-dependent ROS signaling by phosphorylation of the N-terminal of StRBOHB. StCDPK5VK is a useful tool for gain-of-function analysis of NADPH oxidase in defense responses during plant–pathogen interactions, because overexpression of the RBOH gene does not induce ROS production (Torres et al., 2004). Substitution of phosphorylated serine (S) by aspartic acid (D) of AtRBOHD does not produce a gain-of-function mutant (Nühse et al., 2007). We failed to produce a constitutively active mutant of StRBOH by double mutation of S82D/S97D in the N-terminal (M. Kobayashi, H. Yoshioka, unpublished results), even though double mutation of S82A/S97A lost ROS productivity by StCDPK5VK (Kobayashi et al., 2007).

Late blight, caused by the notorious oomycete pathogen P. infestans, is a devastating disease of potato and tomato. During the 1840s it caused the Irish potato famine and over one million fatalities. Although P. infestans is often considered a hemibiotrophic pathogen (i.e. pathogens that live partly as biotrophs and are partly often associated with later stages of infection as necrotrophs or saprophytes; Agrios, 2005). The means by which it is a near-obligate hemibiotrophic pathogen under natural and agricultural conditions suggest that it is better considered as a potential biotrophic pathogen (Fry, 2008).

In this study, we generated transgenic potato plants containing StCDPK5VK under the control of a pathogen-inducible promoter (Yamamizo et al., 2006). ROS production was induced by inoculation with virulent P. infestans and Alternaria solani at infection sites in transgenic plants. Transgenic plants indicated resistance to the near-obligate pathogen P. infestans after inoculation, but increased susceptibility to the necrotrophic pathogen A. solani. These results provide evidence that StCDPK5VK-mediated ROS production confers resistance to near-obligate pathogens, but increases susceptibility to necrotrophic pathogens.

Materials and Methods

Plant materials and growth conditions

Potato plants (Solanum tuberosum L.) and Nicotiana benthamiana L. plants were grown at 20 and 25°C, respectively, at 70% humidity under a 16 h : 8 h, light : dark photoperiod in biotron or environment-controlled growth cabinets. Fully expanded leaves of 1-month-old N. benthamiana plants and 2-month-old transgenic potato plants were used for the experiments. For GUS staining, 15-d-old transgenic potato plants were used.

Pathogen inoculation

Race 1.2.3.4 of P. infestans was maintained on susceptible potato tubers, and suspensions of Phytophthora zoospores were prepared as described by Yoshioka et al. (2003). For examination by reverse transcriptase (RT)-PCR, a spore suspension (2 × 10^4 zoospores ml\(^{-1}\)) was applied to attached leaves using a lens paper under 95% humidity at 20°C. For quantitative real-time PCR, a spore suspension (1 × 10^4 zoospores ml\(^{-1}\)) was sprayed on to the attached leaves using an airbrush. For microscopic observation, a spore suspension (1 × 10^4 zoospores ml\(^{-1}\)) was applied to the attached leaves using a lens paper.

Alternaria solani was grown on oatmeal agar for 5 d. Aerial mycelia on 5-d-old cultures were washed off by rubbing mycelial surfaces with cotton balls. The cultures were exposed to Black
Light Blue light at 25°C for 4 d to induce sporulation. The produced conidia were suspended in water and adjusted to a concentration of $5 \times 10^5$ spores ml$^{-1}$. For quantitative real-time PCR, 5 μl drops of suspended conidia were placed on the detached leaves. At the appropriate times, the inoculated regions of the tissue were harvested by cutting with a cork borer. Each sample for DNA isolation contained five leaf discs. For microscopic observation and RT-PCR, a spore suspension ($5 \times 10^5$ spores ml$^{-1}$) was sprayed on to the leaves using an airbrush.

**Agrobacterium tumefaciens-mediated transient expression (agroinfiltration) in *N. benthamiana***

A cDNA fragment of truncated (VK) variant of StCDPK5 was cloned into pGreen binary vector (Hellens et al., 2000) as described by Kobayashi et al. (2007). cDNA fragments of *StRBOHA*, *StRBOHB*, *StRBOHC* and *StRBOHD* were generated using PCR and cloned into the pGD binary vector (Goodin et al., 2002).

Binary plasmids were transformed into the *Agrobacterium* strain GV3101, which contains the transformation helper plasmid pSoup (Hellens et al., 2000), by electroporation and cultured overnight. The culture was diluted in 10-fold Luria-Bertani medium-kanamycin-rifampicin-tetracycline and cultured until transformed overnight. The culture was diluted in 10-fold Luria-Bertani medium-kanamycin-rifampicin-tetracycline and cultured until transformed overnight. The culture was diluted in 10-fold Luria-Bertani medium-kanamycin-rifampicin-tetracycline and cultured until transformed overnight.

**RNA isolation and RT-PCR**

Total RNA samples were prepared from potato leaves for RT-PCR. The following primer sets were used: *StCDPK5F* (5'-GTGCTTATGAGATCCTGAC-3') and *StCDPK5VR* (5'-AGCATATCCTGGAACATCGTATG-3'); *StCDPK5F* (5'-TTGTTATCGAAGCTGAGCTGAGTGCTG-GGG-3') and *StCDPK5R* (5'-CTTAGAGTGGATTTCTCTGGGACATAG-3'); and *StEF-1xF* (5'-TGCTGTAAACAGATGGATGCTACACCC-3') and *StEF-1xR* (5'-TCCCTTGTACCAGTGGTTGAGTACC-3').

**GUS staining**

Histochemical localization of GUS activity in situ was done using vacuum infiltration with a solution consisting of 50 mM sodium phosphate and 0.5 mg of 5-bromo-4-chloro-3-indolyl glucuronide ml$^{-1}$, and the mixture was incubated for 16 h at 37°C.

**ROS determination**

The relative intensity of ROS generation in *N. benthamiana* leaves was determined by counting photons from L-012-mediated chemiluminescence, as described by Kobayashi et al. (2007). To visualize H$_2$O$_2$ in potato leaves at the infection site of *P. infestans* or *A. solani*, potato leaves were infiltrated with 1 mg ml$^{-1}$ 3,3-diaminobenzidine (DAB) staining solution and inoculated with $1 \times 10^4$ *Phytophthora* zoospores ml$^{-1}$ using a lens paper. At 12 h after inoculation, the leaves were detached and fixed on the filter paper by immersion in a 3 : 1 solution of ethanol : acetic acid. For *A. solani*, $5 \times 10^3$ ml$^{-1}$ conidia water was sprayed on the detached leaves and the leaves were incubated at 95% humidity of 26°C. At 24 h after inoculation, the infected leaves were infiltrated with 1 mg ml$^{-1}$ DAB solution, and then 24 h later the DAB-treated leaves were fixed on to a filter paper by immersion in a 3 : 1 solution of ethanol : acetic acid.
Trypan blue staining

To visualize cell death and fungal hyphal structures in potato leaves infected with *P. infestans* or *A. solani*, infected leaves were transferred to a trypan blue stain solution (10 ml lactic acid, 10 ml glycerol, 10 g phenol, 10 ml H2O and 10 mg trypan blue) diluted in ethanol 1:1 and boiled for 1–2 min. The leaves were then destained overnight in 2.5 g ml⁻¹ chloral hydrate. Stained leaves were observed using a fluorescence microscope (Axio Imager, Carl Zeiss).

Extraction of microsomal proteins

Microsomal proteins from *N. benthamiana* leaves were prepared as described by Kobayashi *et al.* (2007). The leaves were ground with a pestle and mortar in extraction buffer (50 mM MOPS-KOH (pH 7.6), 0.5 M sorbitol, 20 mM 2-mercapto-ethanol, 5 mM EGTA, 5 mM EDTA, 0.1 M NaF, 1 mM Na3VO4, and 50 mM β-glycerophosphate) containing 0.1 mM AEBSF and 1 μM E-64, filtered through four layers of gauze, and centrifuged at 10 000 g for 15 min at 4°C. The supernatant was centrifuged at 150 000 g for 20 min at 4°C. The pellet was suspended in suspension buffer (20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 5 mM 2-mercaptoethanol, 10 mM NaF, 1 mM Na3VO4, 10 mM β-glycerophosphate, 0.1 mM AEBSF, and 1 μM E-64), and the suspension was used as microsomal proteins.

The protein concentration was determined using the Protein Assay Dye Reagent (Bio-Rad) with BSA as a standard.

Immunoblotting

Immunoblotting was done using SNAP i.d. according to the procedure of the manufacturer (Millipore). The microsomal protein extracts (15 μg) were separated on a 12% sodium dodecylsulfate (SDS)-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schunell, Keene, NH, USA) using a semi-dry blotter (ATTO, Tokyo, Japan) at 2 mA cm⁻². After blocking in Block Ace (milk proteins; Snow Brand Milk Products Co., Ltd, Tokyo, Japan), the membranes were incubated with monoclonal anti-FLAG antibody (M2, Sigma) or monoclonal anti-HA antibody (clone HA-7, Sigma) diluted with TBS-T (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 0.05% Tween 20) at room temperature for 10 min. After washing three times with TBS-T, the membranes were incubated with ECL™ anti-mouse Ig antibody (GE Healthcare, Buckinghamshire, UK) diluted with TBS-T at room temperature for 10 min. After washing three times with TBS-T, the antibody–antigen complex was detected using the ECL Western-Blotting detection kit (Amersham) and Light-Capture (ATTO).

Determination of pathogen biomass by quantitative real-time PCR

Necrotrophic pathogen biomass was determined using the modified method of Brouwer *et al.* (2003). Quantitative real-time PCR was used to determine the growth of *A. solani* on five potato leaf discs containing one drop of suspended conidia (5 × 10⁵ spores ml⁻¹). To amplify and detect the *A. solani*-specific DNA sequence, the following primers were used: *Tubulin-F* (5’-ACGACATCTGCACTGAGGACCCTC-3’) and *Tubulin-R* (5’-AACCATGGTGACGGCCAATTTCTCTC-3’). DNA was isolated using the DNeasy Plant Mini Kit (Qiagen). The tissues were mechanically disrupted in a Micro Smash (Tomy, Tokyo, Japan) by shaking the samples mixed with 2 mm glass beads, 5 mm zirconia beads, 400 μl lysis buffer and a drop of antiform emulsion for 120 s. DNA was extracted according to the manufacturer’s procedure. Quantitative real-time PCR was done with SYBR Premix Ex Taq™ (Takara, Otsu, Japan) by using a LightCycler 480 instrument (Roche Diagnostics Corp.). For PCR, samples were preheated at 95°C for 10 s. Then, 40 amplification cycles were run: 5 s at 95°C, 20 s at 60°C. Fluorescence (520 nm) was detected at the end of the elongation phase for each cycle. After the final amplification cycle, a melting curve was produced by heating to 95°C, cooling to 65°C and slowly heating to 95°C at 0.1°C s⁻¹ with continuous measurement of fluorescence at 520 nm.

To determine the growth of *P. infestans*, three inoculated potato leaves were used for quantitative real-time PCR according to the method described by Asai *et al.* (2008). To amplify and detect *P. infestans* - specific DNA sequences, the following primers were used: O8-3 (5’-GAAGGCATAGAAGGTAGA-3’) and O8-4 (5’-TAACCGGA-CCAAGTAGTAAA-3’) for *P. infestans* and *StEF-1x-F* (5’-GGTCTACCAACCTCGACTGTA-3’) and *StEF-1x-R* (5’-GGTTTGTCTGATGGCCTCTTG-3’) for plants.

Sequence data from this study are in the GenBank/DDBJ/EMBL data libraries under the following accession numbers: StCDPK5, AB279738; PVS3, AB022720; StRBOHA, AB050660; StRBOHB, AB050661; StRBOHC, AB198716; StRBOHD, AB198717; StEF-1x, AB061263 and Tubulin, Y17079.

Results

StCDPK5VK activates StRBOHA, StRBOHB, StRBOHC and StRBOHD in *N. benthamiana* leaves

We previously isolated *RBOH* cDNAs StRBOHA, StRBOHB, StRBOHC and StRBOHD from potato (Yoshioka *et al.*, 2001; Yamamizo *et al.*, 2006). In potato tubers, *StRBOHA* is constitutively expressed at low levels, and *StRBOHB* is induced by treatment with cell wall elicitor from *P. infestans* (Yoshioka *et al.*, 2001). In leaves, *StRBOHA*, *StRBOHB* and *StRBOHD* are expressed at low levels, but *StRBOHC* is markedly induced in response to *P. infestans* (Yamamizo *et al.*, 2006). StCDPK5 phosphorylates Ser-82 and Ser-97 in the N terminus of *StRBOH* calcium dependently, and it also phosphorylates *StRBOHA*, *StRBOHC* and *StRBOHD in vitro* because all *StRBOH* s contain consensus motifs for CDPK phosphorylation (Fig. 1a) (Huang & Huber, 2001; Huang *et al.*, 2001). From these results we expected that StCDPK5 would induce ROS burst mediated by these *StRBOH* s. To confirm the expectation,
StCDPK5VK, a constitutively active form, and StRBOHs were coexpressed in *N. benthamiana* leaves by agroinfiltration (Fig. 1b,c). Ectopic expression of StCDPK5VK alone and StRBOH alone slightly induced ROS production, but coexpression of StCDPK5VK and StRBOHs together synergistically induced ROS production. These results indicate that StCDPK5VK activates not only StRBOHB, but also StRBOHA, StRBOHC and StRBOHD. We confirmed the presence of transgene products in the microsomal membrane fractions of *N. benthamiana* leaves using anti-FLAG and anti-HA antibodies for StRBOHs and StCDPK5VK, respectively, by immunoblot analyses (Fig. 1d). The anti-FLAG antibody detected 110 kDa FLAG-StRBOHA, 100 kDa FLAG-StRBOHB, 106 kDa FLAG-StRBOHC and 99 kDa FLAG-StRBOHD. Although the intensity of the band for StRBOHA was faint compared with the other StRBOHs because of either inefficient transfer from SDS gel to membrane or instability of the protein, the activity of StRBOHB and C was much higher than that of StRBOHA and D, suggesting that the activation level by StCDPK5 may be dependent on the number of phosphorylation sites in the N-terminal region of each StRBOH (Fig. 1a).

**StCDPK5 interacts with StRBOHC on the plasma membrane**

To determine the subcellular localization of StCDPK5 *in vivo*, StCDPK5 and StCDPK5VK fusion constructs with GFP were generated and transiently expressed under the control of the 35S *CaMV* promoter in *N. benthamiana* leaves (Fig. 2a). Control green fluorescent signals were observed in the cytoplasm, plasma membrane and nuclei 2 d after agroinfiltration. However, the green fluorescent signals of StCDPK5-GFP and StCDPK5VK-GFP were predominantly observed in the plasma membrane and agreed with the feature of StCDPK5 in which the glycine residue at the second position and the cysteine residue at the fifth position are predicted myristoylation and palmitoylation sites for membrane association, respectively (Kobayashi et al., 2007).

To investigate if StCDPK5/StCDPK5VK and StRBOHC interact in plant cells, a BiFC assay was done. The C- and N-terminal halves of YFP were fused to either StCDPK5/StCDPK5VK or StRBOHC and were transiently coexpressed in onion epidermal cells (Fig. 2b). The reconstituted YFP fluorescence was detected on the plasma membrane as indicated by FM4-64 staining, indicating interactions between StCDPK5 and StRBOHC on the plasma membrane.

**A pathogen-inducible promoter is activated in response to *P. infestans* and *A. solani***

Zoospores of *P. infestans* encyst and initiate penetration from appressoria into an epidermal cell to form an infection vesicle within 2 h after inoculation. In susceptible plants, secondary hyphae with feeding structures known as haustoria expand from the infection vesicle to neighboring cells through the intercellular space. In resistant plants, HR cell death is induced soon after...
penetration and the pathogen is enclosed within neighboring dead host cells or within the penetrated epidermal cell, depending on the genotypes of the interacting plant and pathogen. HR lesions are visible macroscopically as brownish-black spots (Kamoun et al., 1999). However, A. solani, a typical necrotrophic pathogen, can successfully infect most major potato cultivars. Spores of the fungal pathogen penetrate tissues from appressoria-like structures, or directly through the epidermis or stomata. 12–24 h after inoculation, and develop infection hyphae across neighboring cells, including mesophyll cells, 48 h after infection. We observed this infection behavior after 24 h inoculation because germination of the spores did not synchronize under our laboratory conditions.

To assess the function of StCDPK5VK-mediated ROS production in the defense response, we developed transgenic potato plants containing StCDPK5VK. RBOH-mediated ROS production is related to hormonal response and development, such as ABA-induced stomatal closure and cell expansion in root hair formation (Foreman et al., 2003; Kwak et al., 2003). To avoid improper ROS production by StCDPK5VK other than defense signaling, StCDPK5VK was controlled by the potato vesitradiene synthase 3 (PVS3) promoter, which is a pathogen-inducible promoter (Yamamizo et al., 2006). Complementary DNAs encoding PVS1-4, which are sesquiterpene cyclases that catalyze farnesyl diphosphate into vesitradiene, a precursor of the phytoalexins lubimin and rishitin, have been isolated. Infection of P. infestans with potato tubers causes transient increases in the transcript abundance of PVS during compatible and incompatible interactions (Yoshioka et al., 1999). Only PVS3 is markedly induced by pathogen infection in potato leaves.

![Fig. 2 Subcellular localization of StCDPK5-GFP or StCDPK5VK-GFP and interaction of StCDPK5 and StRBOHC in plants. (a) Nicotiana benthamiana leaves were observed 2 d after infiltration of Agrobacterium-containing GFP, StCDPK5-GFP or StCDPK5VK-GFP. DAPI was used for nucleus staining. (b) Bimolecular fluorescence complementation (BiFC) visualization of StCDPK5 (St5)–StRBOHC (StC) and StCDPK5VK (StVK)–StRBOHC (StC) interactions. Onion epidermal cells were cotransformed by particle bombardment. FM4-64 was used for plasma membrane staining. Merged images with differential interference contrast (DIC) are shown in the right panels. Bars, 50 μm. GFP, green fluorescent protein; YFP, yellow fluorescent protein.](image-url)
(Yamamizo et al., 2006). Heterologous expression analysis in *N. benthamiana* indicated that the *PVS3* promoter is activated by defense-related MAPKs, salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK) (Yamamizo et al., 2006). The *PVS3*:GUS construct (Fig. 3a) was transformed into potato plants. Drops of zoospore were inoculated onto the indicated leaf points of *PVS3*:GUS transgenic potato plants as positive controls for the GUS staining (Fig. 3b). GUS activity was not detected in shoot apical meristem (Fig. 3c) and in roots (Fig. 3d), but it was detected at the infection site of virulent *P. infestans* 12 h after inoculation. Cystospores and appressoria were seen under the microscope (Fig. 3e). The *PVS3*...
Transgenic potato plants containing PVS3:StCDPK5VK indicate resistance to virulent P. infestans

We constructed the PVS3:StCDPK5VK gene to generate transgenic potato plants (Fig. 4a). We finally obtained only two transformants (line 7 and line 8) of 50 regenerated plants. The others were all escaped plants lacking a transgene. Transcript accumulation of StCDPK5VK in the transgenic plants was analyzed using RT-PCR. StCDPK5VK expression in line 7 was more strongly induced by inoculation of virulent P. infestans (Fig. 4b) or A. solani (Fig. 4c) than in line 8, whereas expression levels of endogenous StCDPK5 did not change in both lines. Transgenic leaves and tubers showed normal growth and development (Fig. 4d). Leaves were inoculated with virulent P. infestans, and H2O2 production was stained by DAB solution. DAB precipitates resulting from oxidation by H2O2 were detected only in transgenic plants (Fig. 4e) and were markedly produced in line 7 leaves. Microscopic analyses showed accumulation of DAB precipitates in cells at the penetration sites 12 h after inoculation in both lines (Fig. 4e, lower panels).

PVS3:StCDPK5VK plants were tested for resistance to virulent P. infestans. Eight days after inoculation, nontransgenic potato plants indicated typical disease symptoms and inoculated leaves were completely blighted. By contrast, transgenic plants showed high resistance (line 7) or delayed development of symptoms (line 8) (Fig. 5a). These phenotypes were correlated with the expression profiles of StCDPK5VK (Fig. 4b,c). Inoculated tissues were stained by trypan blue and observed under a microscope at 3 d after inoculation with virulent P. infestans (Fig. 5b). In leaves of wild-type plants, appressoria were observed on epidermal cells and infection hyphae spread around intercellular spaces (Fig. 5b). In transgenic plants, HR-like cell death was observed and trypan blue staining was seen around the attacked cells. Massive HR-like cell death was also observed as browning in the penetrated cells in line 7.

Determination of P. infestans biomass by quantitative real-time PCR showed that line 7 and line 8 displayed strong resistance until 3 d after inoculation (Fig. 5c). However, 8 d after inoculation, line 7 showed higher resistance than line 8 (Fig. 5a). These results indicate that PVS3:StCDPK5VK increases resistance to P. infestans.

PVS3:StCDPK5VK plants induce ROS production but indicate increased susceptibility to necrotrophic pathogen A. solani

We also investigated if PVS3:StCDPK5VK potato plants confer resistance to early blight pathogen A. solani. Intriguingly, inoculated leaves in line 7 and line 8 plants indicated more severe disease symptoms than wild-type leaves 3 d after inoculation (Fig. 6a). H2O2 was stained by DAB solution 2 d after inoculation and observed under a microscope (Fig. 6b). There was modest ROS production at the penetration sites in wild-type leaves. By contrast, massive ROS production was detected at the penetration sites in transgenic plants (Fig. 6b). Trypan blue staining of the leaves showed strong staining in transgenic plants compared with wild-type plants, confirming that severe cell death was induced in the transgenic plants infected with A. solani (Fig. 6c). Determination of A. solani biomass by quantitative real-time PCR showed more intensive spreading of fungal hyphae in line 7 than in line 8 (Fig. 6d). These results suggest that PVS3:StCDPK5VK increases susceptibility to necrotrophic pathogen.

Discussion

The importance of RBOH-dependent ROS production has been shown using rboh mutants or knockdown techniques (Torres et al., 2002; Yoshioka et al., 2003; Sagi et al., 2004; Asai et al., 2008). By contrast, gain-of-function analyses of RBOH have not been done, because ectopic expression of RBOH did not induce ROS production (Fig. 1). We previously showed that RBOH requires NADPH produced by glucose-6-phosphate dehydrogenase, derivatives of riboflavin as cofactors, and StCDPK5 as an activator (Kobayashi et al., 2007; Asai et al., 2010, 2011). In this study, we generated transgenic potato plants containing constitutively active StCDPK5VK and investigated the roles of RBOH-dependent ROS production in resistance to near-obligate and necrotrophic pathogens.

Production of ROS was observed by coexpression of StCDPK5VK with SrRBOHA, SrRBOHB, SrRBOHC or SrRBOHD (Fig. 1), indicating that StCDPK5 activates these SrRBOHs. Predicted amino acid sequences implied that these four SrRBOHs have phosphorylation motifs (Fig. 1a), and the recombinant N-terminal peptides were shown to be phosphorylated by StCDPK5 in vitro (Kobayashi et al., 2007). Phospho-proteomics analyses of elicitor signaling in Arabidopsis indicate that the N-terminal region of AtRBOHD is phosphorylated in vivo when treated with flg22 or xylanase, and mutagenesis in some of the phosphorylation sites decreases flg22-triggered ROS burst (Benschop et al., 2007; Nühse et al., 2007). AtRBOHD is synergistically activated by Ca2+ binding and phosphorylation in a heterologous expression system using a mammalian cell line (Ogasawara et al., 2008). This evidence suggests that N-terminal phosphorylation is a conserved process in RBOH activation. Mammalian NADPH oxidase 5, which carries an N-terminal extension including EF-hand motifs, appears also to be regulated by calcium/calmodulin-dependent kinase II-mediated direct phosphorylation (Pandey et al., 2011).

In this study, we showed that transgenic potato plants containing PVS3:StCDPK5VK induce ROS burst when infected by virulent P. infestans, and confer resistance to the pathogen. However, the plants were more susceptible than wild-type plants to infection by necrotrophic pathogen A. solani. Fig. 7 summarizes a proposed mechanism of ROS burst in transgenic plants in response to virulent pathogens. Pathogen attack induces Ca2+ influx into the cytoplasm and pre-existing StCDPK5 activity to some extent.
Fig. 4 Transgenic potato (Solanum tuberosum) plants containing the PVS3:StCDPK5VK gene participate in reactive oxygen species (ROS) production after infection by virulent Phytophthora infestans. (a) Scheme of the construct of PVS3:StCDPK5VK. The plasmid PVS3 promoter up to −2648 bp was transcriptionally fused to the StCDPK5VK gene. The sequence of the restriction enzyme (SpeI) is underlined. Transcript accumulation of StCDPK5VK (StVK) and endogenous StCDPK5 (St5) in response to virulent P. infestans (b) or Alternaria solani (c), and constitutively expressed EF-1α were monitored using reverse transcriptase (RT)-PCR in line 7 and line 8. (d) Transgenic plants and tubers developed normally. (e) Wild type (WT) and transgenic leaves of line 7 and line 8 were inoculated with virulent P. infestans and H2O2 accumulation was visualized by 3,3-diaminobenzidine (DAB) staining 12 h after the inoculation. Bar, 50 μm.
Following activation of the MAPK cascade, the StRBOHC gene is up-regulated in potato leaves (Yamamizo et al., 2006), and StCDPK5VK is simultaneously induced by the MAPK-responsive PVS3 promoter. StCDPK5VK might intensely phosphorylate and activate StRBOHC, which leads to ROS production. The ROS burst could provoke high resistance to near-obligate or biotrophic pathogens, but high susceptibility to necrotrophic pathogens. This suggests that StCDPK5-mediated ROS production has a pivotal role in defense signaling, and that necrotrophic pathogens hijack the defense-related cell death signaling to invade host cells by host-selective toxins as a pathogenicity factor (Gilchrist, 1998; Glazebrook, 2005; Kliebenstein & Rowe, 2008). Arabidopsis mutants with delayed or reduced cell death response are generally more resistant to the necrotrophic
pathogen *Botrytis cinerea* that causes gray mold disease, but mutants in which cell death is accelerated are more susceptible (van Baarlen et al., 2007). Growth of *B. cinerea* and the necrotrophic fungal pathogen *Sclerotinia sclerotiorum* is suppressed in the HR-deficient Arabidopsis mutant *dnd1* and is increased by pretreatment with glucose and glucose oxidase (GO), which generate H$_2$O$_2$ (Govrin & Levine, 2000). Arabidopsis mutants lacking DELLAs increase RBOH-dependent ROS accumulation in biotic and abiotic responses and show substantially strong disease symptoms caused by *B. cinerea* and *Alternaria brassicicola*, and resistance to the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Achard et al., 2008; Navarro et al., 2008). The role of ROS burst in pathogenesis has been investigated by virus-induced gene silencing in *N. benthamiana*. *NbRBOH* silencing has a strong effect on resistance to the potential pathogen *P. infestans* (Yoshioka et al., 2003), but reduces the disease lesion of *B. cinerea* (Asai & Yoshioka, 2009). ROS seem to have a negative role in resistance or a positive role in expansion of disease lesions during *B. cinerea* and *N. benthamiana* interaction. Taking these results together, phenotypes of *PVS3:StCDPK5VK* plants seem to reflect roles of ROS produced by RBOH in infected cells.

**Fig. 6** Transgenic potato (*Solanum tuberosum*) plants containing *PVS3:StCDPK5VK* show increased susceptibility to *Alternaria solani*. (a) Three days after inoculation of wild type (WT) and transgenic leaves of line 7 and line 8 with *A. solani*. (b) H$_2$O$_2$ accumulation visualized by 3,3-diaminobenzidine (DAB) was observed under a microscope at 2 d after inoculation. Arrows indicate penetration sites of *A. solani*. (c) Cell death and hyphae visualized by trypan blue observed under a microscope at 3 d after inoculation. Black arrows indicate internal hyphae of *A. solani*. Bars, 50 µm. (d) Determination of *A. solani* biomass by quantitative real-time PCR with *A. solani*-specific primers using DNA isolated from inoculated leaves. Data are means ± SD from three experiments. Data were analysed by Student’s *t*-test: *, *P* < 0.05 vs wild-type leaves.
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state of StCDPK5 with insufficient increase in Ca²⁺. However, direct phosphogen–plant interactions, StRBOHC is not activated because of the inactive PVS3 MAPKs, the endogenous directly modulate resistance and susceptibility to pathogens as indicated by unknown defense-related components in the plasma membrane and susceptibility to necrotrophic pathogens. StCDPK5VK might phosphorylate ROS burst confers resistance to biotrophic pathogens, but increases susceptibility to necrotrophic pathogens. StCDPK5VK might phosphorylate unknown defense-related components in the plasma membrane and directly modulate resistance and susceptibility to pathogens as indicated by dotted arrows.

Hypersensitive response-like cell death was observed in cells expressing StCDPK5VK in response to P. infestans, which is surprising because Agrobacterium-mediated transient expression of StCDPK5VK did not induce visible cell death in N. benthamiana leaves (data not shown). These observations suggest that ROS activate cell death in cooperation with other signals induced by virulent P. infestans infection. One of the best-characterized signals as a counterpart of ROS in cell death is nitric oxide (NO), which is generated in plant–pathogen interactions by putative NO synthase (NOS) or nitrate reductase (Delledonne et al., 1998; Yamamoto-Katou et al., 2006). NO and ROS together, but not individually, are required to induce cell death, and balanced production of NO and H₂O₂ seems to be important to induce cell death (Delledonne et al., 2001). The constitutively active form of MAPKK, MEK²DD, activates SIPK, which induces both NO and RBOH-dependent ROS production in N. benthamiana (Asai et al., 2008). Transgenic potato plants expressing StMEK²DD, controlled by the same pathogen-inducible promoter as for StCDPK5VK, are resistant to both P. infestans and A. solani (Yamamizo et al., 2006). Leaf areas infiltrated with the NOS inhibitor N⁶-nitro-L-Arg-methyl ester (L-NAME) show marked expanding disease lesions of B. cinerea (Asai & Yoshioka, 2009). StCDPK5VK did not induce NO production (data not shown), agreeing with PVS3:StCDPK5VK plants that show high susceptibility to necrotrophic pathogens. These results support the idea that NO contributes to disease resistance against A. solani and B. cinerea. Simultaneously, the synergistic work of NO with ROS might induce cell death, so providing an advantage for these necrotrophic pathogens. However, they seem unlikely to overcome robust resistance induced by MEK²DD-mediated NO burst.

Transgenic potato plants expressing fungal GO increase concentrations of H₂O₂ and show resistance to a broad range of plant pathogens, including A. solani and P. infestans (Wu et al., 1995, 1997). These conflicting responses with PVS3:StCDPK5VK plants may be attributed to temporal and quantitative differences in ROS generation. In this study, PVS3:StCDPK5VK plants induced ROS production only in pathogen interactions, but GO-transgenic plants have constitutively higher amounts of ROS and increased defense-related gene expression, salicylic acid accumulation, and lignin content (Wu et al., 1997). Pathogen infection often causes immediate phase I ROS burst, and then a massive phase II burst. A protein synthesis inhibitor only abolishes the phase II burst in potato leaves and tubers (Chai & Doke, 1987; Yoshioka et al., 2001), suggesting that StCDPK5VK-induced ROS production in transgenic potato increases the phase II burst. A recent study indicated that the phase I burst triggered by pathogen-associated molecular patterns (PAMPs) is negatively regulated by MAPKs (Segonzac et al., 2011). This rapid burst seems unlikely to depend on RBOH expression, because the first ROS burst started < 5 min after elicitation, which would presumably allow insufficient time for de novo transcription and translation of the RBOH enzyme. Therefore, a MAPK-dependent ROS burst may participate in the massive phase II burst triggered by effectors secreted by pathogens and cognate R protein of the host plant, as reviewed by Yoshioka et al. (2011). We think the increased massive second ROS burst may cause cell death in pathogen-attacked cells expressing StCDPK5VK. Thus, several arguments exist with regard to the regulation and role of ROS during plant and pathogen interactions. However, our study of the gain-of-function analysis of NADPH oxidase suggests that ROS may have a positive effect on disease resistance to biotrophic pathogens and a negative effect on disease resistance to necrotrophic pathogens.

Despite the fact that CDPKs are expected to be multifunctional with many target substrates by in vitro kinase reactions, significant differences in substrate specificity for recombinant CDPKs were shown by assays using various substrates (Kanchiswamy et al., 2010; Curran et al., 2011), suggesting mechanisms by which an individual CDPK specifically recognizes its substrate. The variable N-terminal domain of tobacco NtCDPK1 has an essential role in the specific recognition of the substrate (Ito et al., 2010). Specific subcellular locations often provide the potential for isoform-specific differences in regulating various cellular functions. The defense-related MAPKs translocate between the cytosol and nucleus (Lee et al., 2004; Ishihama et al., 2011) and potential substrates include transcription factors (Katou et al., 2005; Popescu et al., 2009; Ishihama et al., 2011), suggesting that the substrates phosphorylated by MAPKs regulate many defense-related genes. In Arabidopsis, CDPKs comprise a gene family with 34 members (Cheng et al., 2002). There is evidence of CDPKs in multiple subcellular localizations, including the plasma membrane, endoplasmic reticulum.
membrane, endosperm storage vesicles, actin cytoskeletal system, mitochondria, peroxisomes, cytoplasm and nucleus (Lu & Hrabak, 2002; Dammann et al., 2003; Wurzinger et al., 2011). Arabidopsis transcriptome analyses have shown that AtCPK5, which is homologous to StCDPK5, and StMEK2 ortholog At-MKK4 could act either synergistically or independently (Boudsocq et al., 2010). The defense-related MAPKs, SIPK and WIPK, in N. benthamiana exist in the cytoplasm and nuclei (Ishihama et al., 2011), and StCDPK5 is predominantly on the plasma membrane. Different modes of action are supported by the different subcellular localization of the involved kinases (Mehlmer et al., 2010; Wurzinger et al., 2011). However, CDPKs and MAPKs are somehow regulated in a coordinated manner by pathogen attacks. Activation of defense-related MAPKs partially depends on the Ca^{2+} influx (Segonzac et al., 2011).

StCDPK5 contains predicted myristoylation and palmitoylation sites for membrane association (Kobayashi et al., 2007). Mutations of the myristoylation site of Arabidopsis CDPKs impair membrane attachment and their function (Benetka et al., 2008). In this study we showed that GFP-tagged StCDPK5 proteins are predominantly on the plasma membrane by using a transient expression assay of epidermal cells of N. benthamiana leaves. The BiFC assay showed an interaction of StRBOHC and StCDPK5 on the plasma membrane (Fig. 2). These observations suggest that StCDPK5 targets plasma membrane proteins, and might phosphorylate StRBOHC (Fig. 7, gray arrows). However, we cannot rule out the possibility that StCDPK5VK phosphorylates unknown defense-related components in the plasma membrane and directly modulates resistance and susceptibility to pathogens (Fig. 7, dotted arrows).

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