The Tomato Calcium Sensor Cbl10 and Its Interacting Protein Kinase Cipk6 Define a Signaling Pathway in Plant Immunity

Fernando de la Torre,a Emilio Gutiérrez-Beltrán,a Yolanda Pareja-Jaime,a Suma Chakravarthy,b Gregory B. Martin,b,c,d and Olga del Pozoa,1

a Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas/Universidad de Sevilla, 41092 Seville, Spain
b Boyce Thompson Institute for Plant Research, Ithaca, New York 14853
c Department of Plant Pathology and Plant-Microbe Biology, Cornell University, Ithaca, New York 14853
d Genomics and Biotechnology Section, Department of Biological Sciences, King Abdulaziz University, Jeddah 21589, Saudi Arabia

Ca2+ signaling is an early and necessary event in plant immunity. The tomato (Solanum lycopersicum) kinase Pto triggers localized programmed cell death (PCD) upon recognition of Pseudomonas syringae effectors AvrPto or AvrPtoB. In a virus-induced gene silencing screen in Nicotiana benthamiana, we independently identified two components of a Ca2+ -signaling system, Cbl10 (for calcineurin B-like protein) and Cipk6 (for calcineurin B-like interacting protein kinase), as their silencing inhibited Pto/AvrPtoB-elicited PCD. N. benthamiana Cbl10 and Cipk6 are also required for PCD triggered by other plant resistance genes and virus, oomycete, and nematode effectors and for host susceptibility to two P. syringae pathogens. Tomato Cipk6 interacts with Cbl10 and its in vitro kinase activity is enhanced in the presence of Cbl10 and Ca2+, suggesting that tomato Cbl10 and Cipk6 constitute a Ca2+ -regulated signaling module. Overexpression of tomato Cipk6 in N. benthamiana leaves causes accumulation of reactive oxygen species (ROS), which requires the respiratory burst homolog RbohB. Tomato Cbl10 and Cipk6 interact with RbohB at the plasma membrane. Finally, Cbl10 and Cipk6 contribute to ROS generated during effector-triggered immunity in the interaction of P. syringae pv tomato DC3000 and N. benthamiana. We identify a role for the Cbl/Cipk signaling module in PCD, establishing a mechanistic link between Ca2+ and ROS signaling in plant immunity.

INTRODUCTION

Plants respond to pathogen infection by activating a two-part immune response, including pattern-triggered immunity (PTI) and effector-triggered immunity (ETI; Jones and Dangl, 2006). PTI results from the recognition of microbe- or pathogen-associated molecular patterns (PAMPs), whereas ETI occurs when cytoplasmic resistance (R) proteins detect specific pathogen effectors. ETI is more robust than PTI and is often accompanied by a localized programmed cell death (PCD) event known as the hypersensitive response (HR), which is believed to limit pathogen establishment and spread by killing both the pathogen and host cell (Greenberg, 1997).

Both PTI and ETI share early signaling events, including changes in protein phosphorylation status, cytosolic Ca2+ elevation, production of reactive oxygen species (ROS) in the oxidative burst, and activation mitogen-activated protein kinase (MAPK) cascades that lead to common downstream defense responses differing mainly in their kinetics and magnitude (Boiler and Felix, 2009; Tsuda and Katagiri, 2010). Protein phosphorylation and Ca2+ and ROS signaling were described as sequential events (Blume et al., 2000; Grant et al., 2000; Lecourieux et al., 2002; Garcia-Brugger et al., 2006) that influence each other differently depending on the pathosystem. For instance, in Nicotiana benthamiana plants treated with flagellin, Ca2+ influx was required for the ROS burst, which negatively regulated Ca2+ influx (Segonzac et al., 2011), whereas in the cryptogeous/tobacco cells system, ROS burst stimulated extracellular Ca2+ influx to the cytosol (Lecourieux et al., 2002).

The main enzymatic source for the oxidative bursts in Arabidopsis thaliana, the membrane-bound NADPH oxidases (also known as respiratory burst homolog [RBOH] proteins), are synergistically regulated by Ca2+ and phosphorylation (Torres, 2010). RBOHs possess an N-terminal cytoplasmic regulatory domain, which contains Ca2+-binding EF hands and phosphorylation sites by Ca2+-dependent protein kinases (CDPKs or CPKs) that are necessary for RBOH function (Kobayashi et al., 2007; Ogasawara et al., 2008; Dubiella et al., 2013). Thus, a complex spatio-temporal Ca2+/ROS crosstalk exists in early immune signaling. To date, it is still unclear what are the signaling components (proteins and second messengers) that link initial PAMP or effector recognition with Ca2+, ROS, and MAPK signaling and how these signaling events influence each other. A current challenge is to understand how the cytoplasmic Ca2+ increase is interpreted and turned into a functional immune response (Segonzac et al., 2011).

The bacterial pathogen Pseudomonas syringae pv tomato (Pst) causes bacterial speck disease in tomato (Solanum lycopersicum),...
which affects tomato fruit quality and yield resulting in important economic losses (Pedley and Martin, 2003). Pto, a tomato Ser/Thr kinase, recognizes Pst effectors AvrPto and AvrPtoB and together with Prf, a protein containing a nucleotide binding site and a region of leucine rich repeats elicit an ETI resistance response against Pst, which includes activation of a complex array of signaling cascades and involves PCD (Mucyn et al., 2006; Oh and Martin, 2011). AvrPto and AvrPtoB are delivered into the host cell through a type III secretion system where they suppress PTI and promote plant disease susceptibility (Anderson et al., 2006; Xiao et al., 2007; Shan et al., 2008; Hann et al., 2010). Several components (~25) of the Pto-mediated signaling pathway have been identified in tomato, tobacco (Nicotiana tabacum), and N. benthamiana using different loss-of-function approaches (Oh and Martin, 2011). Still, relevant aspects of early signaling after Pto/Prf activation remain to be characterized, like the participation of Ca2+ and ROS as second messengers.

A virus-induced gene silencing (VIGS) screen using randomly selected cDNA fragments from N. benthamiana identified several genes that caused an alteration in Pto/AvrPto-induced PCD upon silencing. Among the candidates identified, MAPKKKx, a positive regulator of PCD, was further characterized and found to regulate both ETI and disease-associated PCD (del Pozo et al., 2004). Here, we report the identification and characterization of two components of a Ca2+-signaling module also identified in this VIGS screen: a calcium sensor (calcineurin B-like protein [CBL]) and its putative target, a calcineurin B-like interacting protein kinase (CIPK). In the general context of R protein/effectector silencing, few proteins that sense and relay Ca2+ signals have been described, among them the CDPKs/CIPKs, which have been shown to play a role in defense activation in different pathosystems (Boudsocq et al., 2010; Kobayashi et al., 2012; Dubiela et al., 2013). Ca2+-related downstream signaling events include salicylic acid synthesis, nitric oxide generation, and transcriptional gene activation (Ma et al., 2008; Du et al., 2009; Boudsocq et al., 2010; Ma et al., 2012). In Pto-mediated ETI, APR134, a tomato Calmodulin-like protein, has been the only Ca2+-signaling component characterized in this pathway (Chiasson et al., 2005).

Calcium plays an important role as a universal second messenger in adaptation of cells to environmental changes. After pathogen perception, apoplastic Ca2+ enters the cytoplasm presumably via cyclic nucleotide-gated channels and Glu receptor-like channels (Ma et al., 2009; Kwaataa et al., 2011; Tapken et al., 2013). Specificity is achieved by the stimulus-specific spatial and temporal Ca2+ influx pattern (calcium signature) and by the local presence of Ca2+ binding proteins or sensors (CBLs, CaMs, Calmodulin-like proteins, and CDPKs), which bind Ca2+ with high affinity through EF hands, a helix-loop-helix structural motif (Harper et al., 2004). Ca2+ binding changes EF-hand protein conformation, resulting in activity changes in the sensor itself or modulating the function of downstream target proteins (sensor relays), transforming Ca2+ changes into cellular responses.

CBLs bind Ca2+ through four EF hands and relay the signal by interacting specifically with a C-terminal regulatory domain (NAF or FISL) of CIPKs, Ser/Thr protein kinases belonging to Snf1-RELATED KINASE3 family (Suc nonfermenting 1-related kinases, group 3; SnRK3) also known as PKS (protein kinase related to SOS2) regulating their kinase activity (Ishitani et al., 2000; Albrecht et al., 2001; Gong et al., 2002; Hrabak et al., 2003). Thus, it is assumed that CBL/CIPKs modules relay Ca2+ signals into target protein(s) phosphorylation. To date, few CIPK phosphorylation targets have been described and most of them are membrane proteins (Quintero et al., 2002; Li et al., 2006; Ho et al., 2009). Database analysis identified 10 CBLs and at least 26 CIPKs in Arabidopsis (www.arabidopsis.org). Because a specific CBL can interact with several CIPKs and a specific CIPK with several CBLs, CBL/CIPK module combinatorial possibilities are vast, thus conferring an extraordinary diversity and versatility of Ca2+ responsiveness to this signaling system, and their roles are only beginning to emerge. Most CBLs and CIPKs described so far are involved in different aspects of abiotic stress signaling and plant nutrient acquisition (Quintero et al., 2002; Kim et al., 2007; Quan et al., 2007; Ho et al., 2009; Weinl and Kudla, 2009). Their involvement in biotic stress signaling has been speculated about (Kudla et al., 2010), but their role in defense responses remains unclear.

We report the identification and characterization of two tomato and N. benthamiana participants, Cbl10 and Cipk6, in PCD events associated with ETI mediated by Pto/AvrPto and by different R proteins and effectors from oomycetes, fungus, and virus, thus constituting a convergent signaling node in ETI. We demonstrate that tomato Cbl10 and Cipk6 interact in planta and that Cipk6 kinase activity is positively regulated in the presence of Ca2+ and Cbl10, indicating that Cbl10/Cipk6 constitutes a functional signaling module regulated by Ca2+. In an attempt to explore Cipk6 downstream signaling connections, we found that Cipk6 overexpression led to ROS generation, which was dependent on Cipk6 kinase activity and required respiratory burst homolog NbRbohB. Importantly, both Cbl10 and Cipk6 interact with RbohB and the complexes localize at the plasma membrane. Finally, we demonstrate that N. benthamiana Cbl10 and Cipk6 contribute to the ROS peak generated during ETI in N. benthamiana in response to Pst infection. Altogether, we present Cbl10/Cipk6 as participants in ETI PCD, likely integrating Ca2+ and ROS signaling events via phosphorylation.

RESULTS

N. benthamiana Cbl10 and Cipk6 Play a Role in Pto-Mediated PCD

In a VIGS screen of randomly selected gene fragments from N. benthamiana directed at identifying components of Pto-mediated PCD, several genes were identified (del Pozo et al., 2004). N. benthamiana plants silenced with Potato virus X (PVX)-based constructs cNbME25F4 or cNbME30H4 showed compromised Pto-mediated PCD in all of the events examined (for cNbME25F4, four out of four events showed complete PCD inhibition, whereas for cNbME30H4, two out of four events showed partial and two no PCD).

A BLAST search with the cNbME25F4 and cNbME30H4 CIPKs in the tomato EST databases (www.sgn.cornell.edu and www.tigr.org) identified two tomato contigs representing partial sequences for each clone (SGN-U229233 and SGN-E250261 for cNbME25F4;
SGN-U566705 and SGN-E339676 for cNbME30H4 sharing 95.6 and 92.3% identity, respectively, at the protein level with the N. benthamiana identified clones. A potato (Solanum tuberosum) contig, SGN-U271168, spanning the complete coding sequence for St-Cipk6 was later identified (see Supplemental Figure 1 online). BLAST performed with both sequences in the Arabidopsis database (www.tair.org) established that cNbME25F4 was a partial clone of the N. benthamiana ortholog for At-CBL10 (also known as SOS3-LIKE Ca2+ BINDING PROTEIN8 [SCaBP8], a CBL protein (Kim et al., 2007; Quan et al., 2007), and that cNbME30H4 corresponded to a partial N. benthamiana ortholog clone for At-CIPK6, a CIPK, a target for CBLs (Shi et al., 1999; Tripathi et al., 2009).

Tomato cDNA clones having the complete open reading frame were obtained, and we refer to them as Sl-Cbl10 and Sl-Cipk6 (see Supplemental Figure 1 online). Because both N. benthamiana and tomato displayed a high degree identity at the nucleotide level (91.2 and 92.4%, with several stretches of more than 21 to 24 nucleotides with 100% identity), either Sl or Nb constructs were used for effective silencing in both species, and in silencing experiments we refer to them generally as Cbl10 and Cipk6.

Protein sequences were derived and aligned for all 10 CBLs present in Arabidopsis, along with Sl-Cbl10, Nb-Cbl10, and a putative rice (Oryza sativa) Os-CBL10 protein (GenBank DQ201203), and the alignment used to build a phylogenetic tree (Figure 1A; see Supplemental Data Set 1 and Supplemental Figure 2 online). As expected, cNbME25F4 appears to be the ortholog of At-CBL10 (Figure 1A). Sl-Cbl10 has four EF hands and an extended N-terminal hydrophobic domain characteristic of At-CBL10 necessary for membrane association, which is also present in the putative rice ortholog Os-CBL10 but absent in other CBLs (Quan et al., 2007). Ser-241 residue in At-CBL10, phosphorylated by SOS2, is also conserved in Sl-Cbl10 (Ser-237) (Figure 1C; see Supplemental Figure 2 online) (Lin et al., 2009).

The closest sequence for cNbME30H4 in the Arabidopsis database was At-CIPK6. Protein sequences of putative At-CIPK6 orthologs from tomato, chickpea (Cicer arietinum; GenBank accession number EU492906), and rice (Q6Z9F4) were aligned, together with a representation of other CIPKs with assigned functions (see Supplemental Data Set 2), and a phylogenetic tree was developed (Figure 1B; see Supplemental Figure 3 online). Indeed, cNbME30H4 and its putative tomato ortholog were located in the same clade as At-CIPK6, Ca-CIPK6, and Os-CIPK6 and therefore were designated as Nb-Cipk6 and Sl-Cipk6, respectively. Sl-Cipk6 has a typical SnRK3 structure, displaying an N-terminal catalytic domain and a C-terminal regulatory domain separated by a junction region (Hrabak et al., 2003). The regulatory domain contains the NAF/FISL region, which binds CBLs and

---

**Figure 1.** Tomato and N. benthamiana Cbl10 and Cipk6 Phylogenetic Analysis and Protein Structure.

(A) and (B) Phylogenetic trees of CBL amino acid sequences from tomato, Sl-Cbl10; N. benthamiana, Nb-Cbl10; rice, Os-CBL10; Arabidopsis CBLs; and Calcineurin B protein from Drosophila melagonaster used as the outgroup (A) as well as CIPK6 amino acid sequences from tomato, Sl-Cipk6; N. benthamiana, Nb-Cipk6; chickpea, Ca-CIPK6; Arabidopsis, At-CIPK6; rice, Os-CIPK6; Arabidopsis CIPKs, including At-CIPK8, At-CIPK24 (SOS2), At-CIPK3, At-CIPK9, and At-CIPK23; tomato Sl-Cipk11 and Sl-Cipk14 (B). At-SnRK2 was used as the outgroup. Phylogenetic trees were done using the neighbor-joining method (ClustalW program; Thompson et al., 1994) and MEGA 4 software. The scale represents amino acid substitutions, and numbers on the tree represent bootstrap scores.

(C) Schematic structure of tomato Cbl10 protein representing conserved Ca2+ binding EF-hands, N-terminal hydrophobic domain, and putative phosphorylatable Ser-237 residue.

(D) Schematic structure of tomato Cipk6 protein representing N-terminal catalytic, junction, and C-terminal regulatory domains, protein phosphatase interaction, NAF/FISL, and activation loop domains. Residues mutagenized for Cipk6 characterization Lys-43 to Met (K43M) and Thr-172 to Asp (T172D) are marked.
physically overlaps with a protein phosphatase interaction binding domain (Liu et al., 2000; Albrecht et al., 2001; Ohta et al., 2003). In the catalytic domain, the conserved ATP binding Lys (Lys-43), and within the activation loop, a putative trans-phosphorylatable Thr residue (Thr-172) was observed (Figure 1D).

To confirm and further characterize the phenotype associated with the loss of function of Cbl10 and Cipk6 in plant immunity, we cloned cDNA fragments from tomato (SlME25F4 and SlME30H4, respectively) corresponding to those originally identified as N. benthamiana VIGS clones into a tobacco rattle virus vector (TRV; Liu et al., 2002), which causes a more uniform and persistent silencing phenotype than PVX. In order to substantiate the specificity of their silencing phenotypes, we generated additional nonoverlapping silencing constructs in TRV for Cbl10 (Sl-Cbl10-5') and Cipk6 (Sl-Cipk6-Sand Sl-Cipk6-3') (see Supplemental Figure 1 online) and used them for VIGS. PCD was monitored visually and classified according to its extent (Figure 2A). Similar loss of PCD phenotypes were observed using either the originally identified clones or each of the alternative silencing constructs for either N. benthamiana Cbl10 or Cipk6 gene, indicating that the phenotype is due to specific silencing of Cipk6 or Cbl10 (Figure 2B). Full Pto/AvrPto-mediated PCD developed in TRV control plants in 80% of the cases, whereas it ranged between 15 and 25% in TRV-Sl-Cipk6-5', TRV-Sl-Cipk6-3', or TRV-Nb-Cipk6-silenced N. benthamiana leaves and between 15 and 25% in TRV-Sl-Cbl10-5' or TRV-Nb-Cbl10-silenced N. benthamiana leaves. The degree of silencing was assessed by RT-PCR, which showed that N. benthamiana Cbl10 and Cipk6 transcript abundances were reduced ~90% compared with TRV control plants (see Supplemental Table 1 online).

Silencing specificity was determined by checking possible changes in transcript abundances of the closest genes found in the databases (see Supplemental Table 1 online). The tomato genome sequence was then released and BLAST analysis identified a sequence, SGN-US3600, which shared three stretches of 23, 26, and 25 nucleotides 100% identical to Cipk6 located toward the 3' end of the putative gene sequence. Two of them overlapped with the TRV-Sl-Cipk6-3' silencing construct, whereas none overlapped with TRV-Sl-Cipk6-5' or TRV-Nb-Cipk6 silencing constructs. RT-PCR performed with specific oligonucleotides for SGN-US3600 and cDNA from infected or noninfected tomato and N. benthamiana leaves did not amplify any band, suggesting that this putative gene is not transcribed in leaves (see Supplemental Table 1 online). From these experiments, we conclude that Cbl10 and Cipk6 participate in Pto/AvrPto-triggered PCD.

**N. benthamiana** Cbl10 and Cipk6 Contribute to PCD Triggered by Different Elicitors

Next, we asked whether Cbl10 and Cipk6 expression was also required in other R/Avr- or elicitor-triggered PCD. For that purpose, we used *Agrobacterium tumefaciens*–mediated transient transformation of leaves (agroinfiltration) to express NPP-1 (for necrosis-inducing Phytophthora protein1; Fellibrich et al., 2002), potato Gpa2/RBP-1 from potato cyst nematode (Sacco et al., 2009), and potato Rx2/coat protein (CP) from PVX (Bendahmane et al., 2000) in N. benthamiana leaves silenced for Cbl10, Cipk6, and TRV control-infected leaves. PCD triggered by NPP-1, Gpa2/
RBP-1, and Rx2/CP was substantially suppressed in TRV-Cipk6- and TRV-Cbl10–silenced plants compared with TRV control plants (Figure 2C). These results demonstrate that Cbl10 and Cipk6 are required by different R genes/effectors in N. benthamiana, thereby constituting a convergent signaling node in plant immunity shared by different pathogen-responsive pathways.

Tomato Cbl10 and Cipk6 Transcript Abundances Increase upon Pst Infection

Cipk6 and Cbl10 were both identified through VIGS in a leaf PCD assay in N. benthamiana. Therefore, we wished to confirm their expression in tomato leaves and other tissues. Tomato Cbl10 and Cipk6 were moderately expressed in all the tissues analyzed with the exception of stems, where both transcripts were in low abundance, especially Cipk6 (see Supplemental Figure 4A online). Cbl10 and Cipk6 transcripts were also detectable in roots. Cbl10 and Cipk6 expression in roots and stems contrasts with their low abundance reported in roots for Arabidopsis SCABP8/CBL10 and chickpea CIPK6 and their high level of expression detected in stems for both Arabidopsis orthologs CIPK6 and CBL10 (Quan et al., 2007; Kim et al., 2007; Tripathi et al., 2009). These differences might underlie divergent functions for CBLs and CIPKs orthologs in different plant species.

We next examined whether transcript abundance of tomato Cbl10 and Cipk6 changed during ETI and susceptible responses of tomato to Pst DC3000 (which expresses AvrPto and AvrPtoB) (see Supplemental Figures 4B and 4C online). Both responses in tomato are accompanied by PCD, associated with the HR or with bacterial speck lesion formation, respectively, and are
Figure 4. Tomato Cipk6 Is an Active Kinase and Is Regulated by Cbl10 and Ca^{2+}.

(A) Tomato Cipk6 schematic diagram showing kinase and regulatory domains and mutant derivatives. NAF, CBL interaction domain; AD, activation domain. Mutagenized residues and deleted domains are marked. WT, full-length Cipk6. K43M and T172D, single amino acid substitution of Lys-43 to Met and Thr-172 to Asp, respectively. △NAF (NAF/FISL amino acids 302 to 321) and △Cterm (amino acids 302 to 432) deletions. (T172D)/△NAF and (T172D)/△Cterm combine both mutations within the same protein.

(B) For the phosphorylation assays, Cipk6 and mutant versions were incubated with 1 µg of MBP in the presence [γ-32P]ATP and kinase buffer, electrophoresed on SDS-polyacrylamide gel, and autoradiographed. a.u., arbitrary units.

(C) For the autophosphorylation assays, proteins were incubated with [γ-32P]ATP, and samples were processed as in (B).

(D) Equimolecular levels of protein corresponding to Sl-Cipk6 and mutant versions were confirmed by immunoblot using anti-cMyc antibodies.

(E) Cipk6 kinase activity is regulated by Cbl10 in a Ca^{2+}-dependent manner. MBP substrate was incubated in the presence of [γ-32P]ATP and Ca^{2+} or EGTA in kinase buffer with Cipk6 and with or without GST-Cbl10.
separated in the timing of their appearance (~16 to 18 h for HR
and 3 to 4 d for specks). For ETI, resistant tomato plants ex-
pressing Pto (RG-PtoR) were infiltrated with a high titer of
Pst DC3000 (1 × 10^8 colony-forming units [cfu/mL]), which gener-
ated visible PCD 16 h later, and Cipk6 and Cbl10 mRNA accumu-
lation was determined 0, 4, 8, and 16 h after. Cipk6 transcript
abundance sharply increased 4 h after Pst DC3000 inoculation
and decreased 8 h after. By contrast, Cbl10 transcript accumu-
dation was only slightly increased 8 h after inoculation and de-
creased thereafter.

For the disease interaction, susceptible tomato plants (RG-PtoS)
were inoculated with Pst DC3000 (5 × 10^5 cfu/mL), and tissue was
collected 0, 1, 2, 3, and 4 d later. Specks were first noticeable 3 d
after infection (DAI). Both Cipk6 and Cbl10 transcript accumula-
tion increased during the infection process, albeit showing different
patterns. Cipk6 transcript increase could be detected 1 DAI and
continued steadily until it reached its peak accumulation at 3 DAI.
By contrast, Cbl10 transcript accumulation doubled at 3 DAI and
increased at 4 DAI (see Supplemental Figure 4C online).

Tomato Cipk6 and Cbl10 Are Required for Pto/AvrPto ETI
Triggered by Pst DC3000

Next, we examined Cipk6 and Cbl10 function in tomato host re-
spones upon Pst DC3000 infection. To test their role in ETI, we used
VIGS to silence Cbl10 and Cipk6 in resistant tomato plants (RG-
PtoR). Tomato leaves were syringe infiltrated with a PCD-inducing
titer of Pst DC3000 (0.5 × 10^7 cfu/mL), along with TRV-Prf and TRV
control-inoculated plants, and PCD was visualized 18 h after in-
filtration. Infiltrated areas were excised and stained with trypan blue.
PCD did not develop in TRV-Cbl10, TRV-Cipk6 (Figure 3A), or TRV-
Prf plants (data not shown; see del Pozo et al., 2004), as trypan blue
staining was greatly reduced in comparison to TRV control plants,
which showed confluent PCD with uniform trypan blue staining
covering the entire infiltrated area. These results were supported
by a conductivity assay, which measures ion leakage in leaves
undergoing PCD (Figure 3B). No significant differences were found
in bacterial populations 18 h after infiltration of the RG-PtoR leaves
when comparing TRV control plants with TRV-Cbl10 and TRV-
Cipk6 plants (Figure 3C).

N. benthamiana and Tomato Cipk6 and Cbl10 Also
Participate in Disease Lesion Formation Caused by
Two P. syringae Strains

P. syringae is a hemibiotrophic pathogen that suppresses PCD
early during infection, but later in the infection process, induces
cell death leading to disease lesions that may contribute to

Figure 5. Tomato Cipk6 Expression Results in ROS Generation: Cbl10
and Cipk6 Contribute to ROS Production in Response to Pst DC3000.

(A) Luminol-enhanced chemiluminescent assay in leaf discs agro-
expressing Sl-Cipk6, Sl-Cipk6 mutant versions, or control (empty vector)
in pER8 XVE vector after 17β-estradiol treatment. ROS was quantified as
relative light units (RLU) 2 to 3 h after. Data presented are means of 8/10
measures of independent discs. The experiment was repeated five times
with similar results. Asterisks indicate a significant increase compared
with control (Student’s t test; P < 0.05). WT, the wild type.

(B) ROS mediated by Sl-Cipk6(T172D) or Sl-Cipk6(T172D)ΔCterm was
dramatically reduced in TRV-Nb-RbohB plants. Experiment was repeated
twice with similar results, with eight biological replicates and three
technical replicates. Single asterisks, significant increase compared with
control (empty vector-agroinfiltrated). Double asterisks, significant de-
crease compared with TRV control plants (Student’s t test; P < 0.01).

(C) ROS is greatly diminished in TRV-Cbl10 and TRV-Cipk6 and almost
undetectable in TRV-Nb-RbohB Nb plants compared with TRV control
plants after Pst DC3000 (2.5 × 10^8 cfu/mL) inoculation. ROS were
measured for 7 h as in (A). Pst DC3000 ΔhrcC was employed as a control
for the ETI (2.5 × 10^8 cfu/mL). The experiment was repeated three times
with similar results.
Lesion-associated cell death is considered a PCD that shares molecular mechanisms with ETI PCD, differing mainly in the timing and number of cells undergoing this response (Tao et al., 2003; del Pozo et al., 2004). We hypothesized that *N. benthamiana* Cipk6 and Cbl10 might also play a role in disease-associated PCD as was observed previously for MAPKKKα (del Pozo et al., 2004). We analyzed the disease symptoms caused by $5 \times 10^5$ cfu/mL *P. syringae* pv *tabaci* infection in TRV-Cipk6, TRV-Cbl10, TRV-Cipk6/TRV-Cbl10–silenced, and TRV control *N. benthamiana* leaves (see Supplemental Figure 5 online). Four days after *P. syringae* pv *tabaci* infection, fully collapsed tissue due to successful disease progression was observed in TRV control plants (see Supplemental Figure 5A online). However, no disease-associated PCD was apparent in 50 to 60% of the infiltrated areas in single or cosilenced plants for Cipk6 and/or Cbl10 (see Supplemental Figures 5A and 5B online). Bacterial growth was reduced in TRV-Cbl10, TRV-Cipk6, and to the same extent in TRV-Cipk6/TRV-Cbl10 plants (see Supplemental Figure 5C online). These observations were substantiated by a conductivity assay (see Supplemental Figure 5D online).

To characterize Cipk6/Cbl10 role in disease-associated PCD in tomato caused by *Pst* DC3000, we silenced Cipk6 and Cbl10 individually or together in susceptible tomato plants (RG-prf3) and inoculated the resulting plants with $2.5 \times 10^4$ cfu/mL of *Pst* DC3000. Five days later, mild disease symptoms, consisting of few and weak specks, could be observed in TRV-Cipk6 and TRV-Cbl10 plants, whereas in TRV control plants, numerous well-defined speck lesions were present (Figure 3D). Bacterial growth was reduced in TRV-Cipk6, TRV-Cbl10, and TRV-Cipk6/TRV-Cbl10 RG-prf3 plants compared with the TRV control (Figure 3E). TRV-Cipk6/TRV-Cbl10 plants showed similar reduction in bacterial specks and growth as single silenced TRV-Cipk6 or TRV-Cbl10, suggesting that Cipk6 and Cbl10 genetically function in the same pathway. These results were observed in five independent experiments. Thus, both *N. benthamiana* and tomato Cbl10 and Cipk6 are involved in PCD signaling that occurs during both ETI and disease and contributes to disease susceptibility to two *P. syringae* pathovars.

**Tomato Cipk6 Kinase Activity Is Enhanced by SlCbl10 and Ca$^{2+}$**

Despite the central role attributed to Ca$^{2+}$ signaling in plant immunity, little is known about how plants decode Ca$^{2+}$ signals into cellular responses leading to plant immunity. Cbl10/Cipk6 participation in immunity-associated PCD prompted us to test if tomato Cipk6 could participate as a Ca$^{2+}$ relay through phosphorylation events. We set up to test and characterize Cipk6 kinase activity (Figure 4). As a negative control, a kinase inactive version was generated, where the conserved catalytic Lys within the kinase domain was substituted by Met (Sl-Cipk6[K43M]) (Liu et al., 2000). We also generated different Cipk6 versions mimicking previously described mutations in other CIPKs that conferred enhanced kinase activity (Gong et al., 2002). Initial yeast two-hybrid analysis followed by structural information has demonstrated that the N-terminal kinase domain and the C-terminal regulatory domain of SOS2 (*Arabidopsis* CIPK24) interact intramolecularly through the NAF/FISL domain, inhibiting kinase pathogen dissemination. Lesion-associated cell death is considered a PCD that shares molecular mechanisms with ETI PCD, differing mainly in the timing and number of cells undergoing this response (Tao et al., 2003; del Pozo et al., 2004). We hypothesized that *N. benthamiana* Cipk6 and Cbl10 might also play a role in disease-associated PCD as was observed previously for MAPKKKα (del Pozo et al., 2004). We analyzed the disease symptoms caused by $5 \times 10^5$ cfu/mL *P. syringae* pv *tabaci* infection in TRV-Cipk6, TRV-Cbl10, TRV-Cipk6/TRV-Cbl10–silenced, and TRV control *N. benthamiana* leaves (see Supplemental Figure 5 online). Four days after *P. syringae* pv *tabaci* infection, fully collapsed tissue due to successful disease progression was observed in TRV control plants (see Supplemental Figure 5A online). However, no disease-associated PCD was apparent in 50 to 60% of the infiltrated areas in single or cosilenced plants for Cipk6 and/or Cbl10 (see Supplemental Figures 5A and 5B online). Bacterial growth was reduced in TRV-Cbl10, TRV-Cipk6, and to the same extent in TRV-Cipk6/TRV-Cbl10 plants (see Supplemental Figure 5C online). These observations were substantiated by a conductivity assay (see Supplemental Figure 5D online).

To characterize Cipk6/Cbl10 role in disease-associated PCD in tomato caused by *Pst* DC3000, we silenced Cipk6 and Cbl10 individually or together in susceptible tomato plants (RG-prf3) and inoculated the resulting plants with $2.5 \times 10^4$ cfu/mL of *Pst* DC3000. Five days later, mild disease symptoms, consisting of few and weak specks, could be observed in TRV-Cipk6 and TRV-Cbl10 plants, whereas in TRV control plants, numerous well-defined speck lesions were present (Figure 3D). Bacterial growth was reduced in TRV-Cipk6, TRV-Cbl10, and TRV-Cipk6/TRV-Cbl10 RG-prf3 plants compared with the TRV control (Figure 3E). TRV-Cipk6/TRV-Cbl10 plants showed similar reduction in bacterial specks and growth as single silenced TRV-Cipk6 or TRV-Cbl10, suggesting that Cipk6 and Cbl10 genetically function in the same pathway. These results were observed in five independent experiments. Thus, both *N. benthamiana* and tomato Cbl10 and Cipk6 are involved in PCD signaling that occurs during both ETI and disease and contributes to disease susceptibility to two *P. syringae* pathovars.

**Tomato Cipk6 Kinase Activity Is Enhanced by SlCbl10 and Ca$^{2+}$**

Despite the central role attributed to Ca$^{2+}$ signaling in plant immunity, little is known about how plants decode Ca$^{2+}$ signals into cellular responses leading to plant immunity. Cbl10/Cipk6 participation in immunity-associated PCD prompted us to test if tomato Cipk6 could participate as a Ca$^{2+}$ relay through phosphorylation events. We set up to test and characterize Cipk6 kinase activity (Figure 4). As a negative control, a kinase inactive version was generated, where the conserved catalytic Lys within the kinase domain was substituted by Met (Sl-Cipk6[K43M]) (Liu et al., 2000). We also generated different Cipk6 versions mimicking previously described mutations in other CIPKs that conferred enhanced kinase activity (Gong et al., 2002). Initial yeast two-hybrid analysis followed by structural information has demonstrated that the N-terminal kinase domain and the C-terminal regulatory domain of SOS2 (*Arabidopsis* CIPK24) interact intramolecularly through the NAF/FISL domain, inhibiting kinase
activity possibly by blocking substrate access to the catalytic site (Albrecht et al., 2001; Sánchez-Barrena et al., 2007; Akaboshi et al., 2008). We generated a deletion mutant version that lacked the regulatory domain (SI-Cipk6ΔCterm) (Figure 4A). A SI-Cipk6ΔNAF version, lacking the NAF/FISL binding site was previously generated (see above). It has been also described that substitution of a conserved Thr within the activation loop to Asp, mimics phosphorylation (and possibly activation) by unknown upstream kinases (Guo et al., 2001; Gong et al., 2002). We generated, through site-directed mutagenesis, a version with this substitution, SI-Cipk6(T172D), and obtained double mutants by combining this point mutation with deletion mutants described above (Figure 4A).

To determine whether tomato Cipk6 is an active kinase, we cloned Cipk6 and mutant versions into the pY/L436 tandem affinity purification (TAP) vector (Rubio et al., 2005), agroinfiltrated them into N. benthamiana leaves, and purified the tagged proteins. Cipk6 and mutant versions were observed at the expected molecular masses, and an in vitro kinase assay was performed with the recombinant proteins to determine their kinase activity in the presence of $\gamma^{32}$P-ATP and myelin basic protein (MBP) as a substrate as well as their autophosphorylation activity (Figures 4B and 4D). Wild type SI-Cipk6 was observed to have a weak transphosphorylation and autophosphorylation activity, whereas SI-Cipk6(K43M) (kinase inactive) had no activity (Figures 4B and 4C). Altogether, the Cipk6 variants (SI-Cipk6[T172D], SI-Cipk6ΔNAF, and SI-Cipk6ΔCterm) showed noticeably increased kinase and autophosphorylation activity, with Sl-Cipk6(T172D) displaying the highest kinase activity (approximately threefold above the wild type).

Interestingly, Cipk6 variants resulting from the addition of the single substitution, T172D, to the deletion mutants (SI-Cipk6[T172D]ΔNAF and SI-Cipk6[T172D]ΔCterm) increased both kinase activity and most noticeably autophosphorylation activity with SI-Cipk6(T172D)ΔCterm displaying highest autophosphorylation activity compared with single mutant versions. Kinase and autophosphorylation assays were performed in parallel with identical sample loadings, along with an immunoblot to assess that similar amount of protein was used (Figure 4D). Thus, it appears that the highest kinase activity is achieved through transactivation and highest autophosphorylation through release of the catalytic domain of the inhibitory effect of the regulatory domain.

To test whether tomato Cipk6 kinase activity was modulated by Cbl10 and Ca$^{2+}$, in vitro transphosphorylation assays were performed using MBP as substrate. Cipk6 kinase activity increased significantly ($\approx$16×) in the presence of both Cbl10 and Ca$^{2+}$, therefore demonstrating that Cipk6 kinase activity is regulated by Cbl10 in a Ca$^{2+}$-dependent manner (Figure 4E). No Cbl10 phosphorylation by Cipk6 could be observed in the conditions assayed (Figure 4E). Arabidopsis CBL10 was found previously to be phosphorylated by SOS2 (CIPK24) (Lin et al., 2009).

**Tomato Cipk6 Kinase Activity Is Associated with ROS Production**

An increase in cytoplasmic Ca$^{2+}$ is among the earliest responses upon pathogen recognition and blocking this influx with inhibitors demonstrated that Ca$^{2+}$-derived signals are necessary for downstream signaling processes, including ROS production and PCD (Blume et al., 2000; Grant et al., 2000; Segonzac et al., 2011). Therefore, given the participation of the Cbl10/Cipk6 module in ETI-associated PCD, we set out to investigate whether tomato Cipk6 kinase activity could also affect ROS production.

Tomato Cipk6 and its mutant versions were agroinfiltrated using an estradiol-inducible system in N. benthamiana leaves, and production of ROS was quantified in the presence of luminol in a luminometer (Figure 5A). ROS were detected in samples expressing SI-Cipk6 and increased more than two- and fourfold in samples expressing enhanced kinase activity versions SI-Cipk6(T172D) and SI-Cipk6(T172D)/ΔCterm, respectively, whereas ROS was much reduced after expression of the kinase-inactive version SI-Cipk6(K43M), indicating that SI-Cipk6 kinase activity is necessary for ROS generation. The highest ROS accumulation was detected in SI-Cipk6(T172D)/ΔCterm-expressing samples, thus correlating with highest kinase and autophosphorylation activity (Figures 4B and 4C) likely as a result of combining both de-repression and mimicking-transactivation of kinase activity. Cipk6 and all the mutant versions proteins were expressed similarly (Figure 5A).

Membrane-bound RBOHs play a key role as ROS-producing enzymes in plants. N. benthamiana RbohB silenced plants were susceptible to an avirulent pathogen and showed reduced HR (Yoshioka et al., 2003). RbohB was also required for ROS accumulation during PTI in response to PAMPs (Segonzac et al., 2011). In order to determine if RbohB was required for ROS generation mediated by Cipk6, we expressed SI-Cipk6(T172D) and SI-Cipk6(T172D)/ΔCterm and measured ROS as described in Figure 5A in N. benthamiana plants silenced for RbohB and nonsilenced control plants (Figure 5B). Silencing RbohB completely abolished SI-Cipk6(T172D)– and SI-Cipk6(T172D)/ΔCterm–mediated ROS generation. ROS were detected in samples expressing SI-Cipk6(T172D) and SI-Cipk6(T172D)/ΔCterm in TRV control plants. This result clearly indicates that N. benthamiana RbohB is required for tomato Cipk6-mediated ROS generation.

We also determined if tomato Cbl10 and Cipk6 contributed to ROS generation during the response of N. benthamiana to Pst DC3000 infection. Using the in vivo luminol-based assay, TRV control plants infected with Pst DC3000 showed two ROS peaks approximately at 2.5 and 6 h after infection (Figure 5C). The early ROS burst is associated with PTI, whereas the later one corresponds to ETI, since TRV control plants infected with Pst DC3000 hrcC (deficient in the type III secretion system and therefore eliciting PTI but not ETI) showed the first but not the second peak. Both ROS peaks were substantially reduced in TRV-Cipk6 or TRV-Cbl10 but not completely abrogated, as observed in TRV-RbohB N. benthamiana plants (Figure 5C). Collectively, these data demonstrate that Cbl10 and Cipk6 contribute to ROS generation during the immune response to Pst DC3000 in N. benthamiana.

**Cipk6 and Cbl10 Interact in Vivo and Constitute a Signaling Module**

The fact that plants cosilenced for Cipk6/Cbl10 did not show an additive loss of PCD or reduced bacterial growth compared with plants silenced individually for these genes suggested that
their proteins might act together as a PCD-inducing signaling module. To test if these two proteins interact physically, we first performed a yeast two-hybrid assay. Yeast two-hybrid analysis and in vitro binding assays have demonstrated that CBLs often bind a number of CIPKs with different affinity (Albrecht et al., 2001; Batistic and Kudla, 2009). We found that tomato Cbl10 interacted with Cipk6, whereas, as expected, Sl-Cipk6ΔNAF, an altered protein lacking the NAF/FISL domain necessary for CBL binding, did not interact (Figure 6A). Cbl10 did not interact with Cipk11 or Cipk14, tomato CIPK proteins with high amino acid similarity to Cipk6, therefore suggesting that the Cipk6/Cbl10 interaction has a high level of specificity (Figure 6A). Cbl10, Cipk6, Cipk6ΔNAF, Cipk11, and Cipk14 were expressed in yeast (see Supplemental Figure 6 online).

To further examine the Cipk6 and Cbl10 interaction and to test if the presence of Ca²⁺ influenced their binding, we attempted pull-down experiments with tomato Cipk6 and Cbl10 proteins isolated from Escherichia coli. We successfully expressed Cbl10 fused to glutathione S-transferase (GST), Sl-Cbl10-GST, and because Cipk6 protein production was limited, we produced Sl-Cipk6-MetS358-labeled protein in vitro. Pull-down experiments demonstrated direct interaction between Cipk6 and Cbl10, which was not dependent on the presence of Ca²⁺ in our in vitro assay (see Supplemental Figure 7 online). The interaction of Arabidopsis CIPK24 (SOS2) and CBL10 (SCaBP8) is also not affected by Ca²⁺ (Quan et al., 2007).

In order to examine the likelihood that tomato Cipk6 and Cbl10 constitute a signaling module in vivo and to further test the specificity of their interaction, we performed coimmunoprecipitation (co-IP) experiments. We transiently expressed by Ca²⁺-mediated responses, including salinity, drought, cold, and in response to abscisic acid. In this study, we report the characterization as positive regulators of PCD events in ETI mediated by different R genes/effectors from bacteria, oomycete, nematode, and virus and in susceptible responses to two pathogenic strains of P. syringae. We propose that tomato Cbl10 and Cipk6 act in a convergent regulatory node in ETI- and disease-associated PCD signaling in plant immunity.

Biochemical characterization indicated that tomato Cipk6 is an active kinase, whose activity is positively regulated by Cbl10 and Ca²⁺. Because Cbl10 and Cipk6 interact in planta, we conclude that Cbl10/Cipk6 constitute a signaling module regulated by Ca²⁺. In an attempt to investigate Cipk6 downstream signaling connections, we found that Cipk6 overexpression results in ROS generation in plants, which required Cipk6 kinase activity and NADPH oxidase RbohB. Both Cbl10 and Cipk6 interacted in vivo with RbohB at the plasma membrane, thus suggesting that RbohB is a target of Cbl10/Cipk6. Importantly, Cbl10 or Cipk6 loss-of-function analysis in N. benthamiana revealed their contribution to ROS bursts occurring during both PTI and ETI in response to Pst DC3000 infection, thus validating their physiological role in the plant immune response.

Our studies reveal a functional role for a CBL/CIPK module in ETI PCD and resistance and provide molecular insights into the mechanistic conversion of Ca²⁺ signaling into ROS signaling in plant immunity through still unknown phosphorylation events in plants. Based on these results, we propose a model where Ca²⁺ influx after pathogen perception might be detected by Cbl10, which interacts with Cipk6, thereby derepressing its kinase activity, which in turn phosphorylates RbohB, resulting in ROS production contributing to PCD and resistance responses.

Ca²⁺ seems not to be necessary for the Cbl10/Cipk6 interaction, at least in our in vitro assay. However, Cipk6 kinase activity increased notably in the presence of Cbl10 and Ca²⁺, demonstrating the relevance of Ca²⁺ regulation in the system. Similarly, SOS2/SOS3 and Arabidopsis CBL2/CIPK14 displayed Ca²⁺-independent interaction and Ca²⁺-dependent kinase activity (Halfter et al., 2000; Akaboshi et al., 2008). Among the single mutants, Sl-Cipk6(T172D) displayed the greatest kinase and autophosphorylation activity. Thr-172 is a phosphorylatable residue located in the activation loop and is conserved at the same position in all SnRK3s. Substitution of this residue to Asp, to mimic Thr phosphorylation, gives CIPKs enhanced kinase activity (Guo et al., 2001; Gong et al., 2002). Because this activated state was observed in several cases in which Ser/Thr was mutated to an Asp, it has been proposed that CIPKs could also be activated by a phosphorylation cascade (Harper et al., 2004).

Changes in the phosphorylation state of proteins are among the earliest events in plant immunity (Dietrich et al., 1990; Felix et al., 1991). In the future, identification of a kinase(s) that could regulate Cipk6 activity could fill in knowledge gaps in early immune signaling in plants between early phosphorylation events and Ca²⁺-mediated responses. One possible candidate is Pto.
However, the participation of *N. benthamiana* Cipk6 in diverse *R* gene/effector-elicited PCD events would argue for the involvement of a convergent kinase playing this role.

In plants, cell death induced by pathogen infection is associated with both disease resistance and susceptibility. In the tomato/Pst DC3000 interaction, both ETI and susceptibility are associated with PCD events, which seem to share some common regulators and physiological changes but their development occurs at different timing and involves different number of cells (del Pozo et al., 2004; López-Solanilla et al., 2004; Tao et al., 2003). In contrast with HR PCD, the molecular basis of disease-associated PCD that takes place during compatible interactions is largely unknown. In this work, we observed that tomato and *N. benthamiana* Cbl10 and Cipk6 positively regulate both immunity-associated PCD and disease-associated PCD and contribute to disease susceptibility in a fashion similar as described previously for MAPKKK_	ext{p} (del Pozo et al., 2004). This raises the possibility that Cbl10/Cipk6 and MAPKKK_	ext{p} act in the same pathway or that crosstalk between them might exist. We did not detect any interaction between MAPKKK_	ext{p} (full-length or kinase domain) and tomato Cipk6 (wild-type or constitutive active derivatives) by yeast two-hybrid assays (O. del Pozo, unpublished data). MAPKKK_	ext{p} overexpression results in PCD, whereas over-expression of tomato Cipk6 (wild-type or enhanced kinase activity versions) in the presence or absence of Cbl10 did not result in PCD (O. del Pozo, unpublished data). Possibly, an increase of cytosolic Ca\textsuperscript{2+} is required for full Cipk6 kinase activation. Alternatively, Cipk6 kinase activity and ROS generation might be necessary but not sufficient for PCD execution. In this regard, it is known that H\textsubscript{2}O\textsubscript{2} can diffuse and activate PCD; however, it must be accompanied by suppression of ROS detoxification mechanisms and by a balanced concentration of nitric oxide (Mittler et al., 1999; Delledonne et al., 2001). Because *C. elegans* and Cipk6 loss-of-function phenotypes are reminiscent of those previously described for MAPKKK_	ext{p} (del Pozo et al., 2004), it will be important to explore in the future possible crosstalk between Cipk6 and MAPKKK_	ext{p} or other MAPK cascades in PCD regulation. However, as we have stated previously for MAPKKK_	ext{p} (del Pozo et al., 2004), we cannot rule out that Cbl10/Cipk6 might regulate other susceptibility-related pathways in addition to controlling cell death. Perturbation of these pathways could also lead to inhibition of bacterial growth as there is no direct link between lesion formation and virulence or ETI PCD and increased resistance as shown in *Pst* DC3000 mutant strains that affect both forms of PCD (López-Solanilla et al., 2004; Gassmann, 2005).

We discovered evidence of a direct and positive connection between tomato Cipk6 kinase activity and ROS production in plants; more importantly, we provide genetic and physiological in vivo evidence that Cbl10 and Cipk6 contribute to ROS generated during both PTI and ETI peaks in the *N. benthamiana/Pst* DC3000 interaction. These results indicate that ROS generation in ETI and PTI might be mechanistically linked at the Cbl10/Cipk6 signaling node. Because Cbl10 and Cipk6 are interacting partners in yeast and in planta (using co-IP and both interact directly with RbohB in *N. benthamiana* plasma membrane (by BIFC), we propose that RbohB is a target of the Cbl10/Cipk6 module, thus providing a mechanistic link between Ca\textsuperscript{2+}-derived signals, phosphorylation, and ROS signaling during the plant immune response to bacterial pathogens.

Work performed in elicitor-treated suspension cultures indicated that phosphorylation and Ca\textsuperscript{2+} signaling are required for ROS generation (Blume et al., 2000; Grant et al., 2000; Kimura et al., 2012), whereas other studies demonstrated that ROS production was necessary for Ca\textsuperscript{2+} influx or played a positive feedback role (Van Breusegem et al., 2008). These studies revealed a specific and complex crosstalk between Ca\textsuperscript{2+} and ROS signaling in early immune responses in different plant–pathogen interactions. An oxidative burst is a hallmark in nearly all plant responses to pathogen attack and plays an important signaling role in PCD (Torres, 2010). Different enzymatic sources of ROS have been described (Daudi et al., 2012; Ishiga et al., 2012; Rojas et al., 2012); however, the membrane-bound RBOH complex seems to play a central role in generating apoplastic ROS in PTI and ETI and possibly in amplifying ROS generated in different cellular compartments (Torres, 2010). The role of specific RBOH enzymes in pathogen-elicited PCD and resistance is complex, and they play distinct roles depending on the pathogen and the plant species. For instance, *Arabidopsis* *rbohF* plants are more resistant to *Peronospora parasitica*, displaying enhanced HR, but are impaired in resistance to compatible bacteria, whereas *N. benthamiana* plants silenced for *RbohF* (*Arabidopsis* *RBOHF* ortholog) are more susceptible to *Phytophthora infestans* and present reduced HR, but appear not to contribute to bacterial resistance (Yoshioka et al., 2003; Segonzac et al., 2011; Chaouch et al., 2012). A role for *Arabidopsis* RBOHs in signaling has been proposed, as they regulate the spread of salicylic acid–mediated cell death and the synthesis of salicylic acid and defense-related metabolites (Torres et al., 2005; Chaouch et al., 2012). Recently, a model for CPK5 and RBOHD in a self-activating circuit that activates distal plant defense has been proposed (Dubiel et al., 2013). In the future, it will be interesting to determine if Cipk6 regulation of RbohB contributes to defense-related metabolite production and if it affects the outcome of defense responses regulation in both tomato and *N. benthamiana*.

Plant RBOH proteins are regulated by Ca\textsuperscript{2+} at different levels. They contain EF hands that can bind Ca\textsuperscript{2+} (Keller et al., 1998) and are phosphorylated during pathogen responses by CDPKs or CPKs (Kobayashi et al., 2007; Dubiel et al., 2013). Additional events of RBOH phosphorylation had been described in proteomic analysis performed in *Arabidopsis* cells after elicitor treatment (Benshop et al., 2007; Nühse et al., 2007). These results highlight the importance of Ca\textsuperscript{2+} and phosphoregulation of RBOHs in early immune signaling, and we propose that CIPKs are candidates for such phosphorylation events in plant responses to pathogen attack, in addition to CDPKs or CPKs.

Recent reports indicate that at least in vitro, *Arabidopsis* RBOH proteins might be also regulated by different Ca\textsuperscript{2+}-regulated kinases that play a role in abiotic stress responses (Sirichandra et al., 2009; Drerup et al., 2013). *Arabidopsis* CIPK6, CBL10, and orthologs have been shown to participate in different processes like salt stress tolerance, development, regulation of potassium channel AKT2 activity or modulation, or abscisic acid signaling (Kim et al., 2007; Quan et al., 2007; Tripathi et al., 2009; Held et al., 2011; Chen et al., 2012; Ren et al., 2013). We did not observe any abnormal growth or development in *N. benthamiana* or tomato plants.
silenced for either Cbl10, Cipk6, or both, growing under standard greenhouse conditions. Because N. benthamiana and tomato plants become fully silenced when they are over 1 month old, such early development-associated phenotypes might not have been apparent in our silenced plants. Interestingly, ROS are also generated during salt stress and during development (Borsani et al., 2001; Hernández et al., 2001), and RBOHs participate in initial phases of salt stress (Jiang et al., 2012). It will be interesting to determine whether Arabidopsis CIPK6 also mediates ROS production and its possible contribution to immunity, salt tolerance, or development.

Earlier, a connection between CIPKs and reactive oxygen signaling was suggested, as different SOS2-interacting proteins (NUCLEOSIDE DIPHOSPHATE KINASE2 and two catalases [CAT2 and CAT3]) involved in ROS signaling and decomposition, respectively, were identified (Verslues et al., 2007). SOS1, a Na+/H+ antiporter, which is a target of the SOS3/SOS2 complex, has been reported to be posttranscriptionally regulated by ROS and to interact with oxidative responses regulators (Katayar-Agarwal et al., 2006; Chung et al., 2008). In rice cultured cells treated with PAMPs, CIPK14/15 appear to mediate ROS production (Kurusu et al., 2010), and, more recently, Arabidopsis CIPK26 was shown to interact with RBOHF in plants and positively and negatively regulate its ROS-producing activity in human cultured cells (HEK293T) (Kimura et al., 2012; Drerup et al., 2013). In planta validation of such results in the context of a specific physiological response will consolidate these promising results.

CBIs and CIPKs have been described to interact specifically with protein phosphatases, the chaperone-like protein DNAJ and a 14-3-3 protein (Fuglsang et al., 2007; Yang et al., 2010). Understanding the regulation of the formation of the Cbi10/Cipk6 complex, identification of additional regulatory components, together with transcriptional regulation of their genes under developmental-, tissue-, and cell-specific and environmental or biotic stress conditions will be important for understanding how down-stream Cbi10/Cipk6 signaling specificity is achieved from upstream Ca2+-derived signals in abiotic and biotic stress signaling.

Our results and other recent reports that suggest the participation of rice CIPK14/15 in regulation of defense gene expression and phytoalexin production in rice cultured cells and the possible regulation of NPR1 by PKS5 (Arabidopsis CIPK24) phosphorylation (Kurusu et al., 2010; Xie et al., 2010) support a role for CBL/CIPK participation in different aspects of plant defense. Thus, CBL/CIPK complexes are important players in modulating outputs during biotic interactions. In the future, we will investigate the molecular basis of tomato Cbl10/Cipk6 regulation of RbohB ROS production. For that purpose, we will determine if RbohB is a Cipk6 phosphorylation substrate, identify the putative residues phosphorylated, and analyze the contribution of these putative phosphorylation events to ROS generation, PCD, and resistance in the plant immune responses to pathogenic bacteria through functional analysis. Alternatively, phosphorylation-independent regulation of RbohB by Cipk6 also could be plausible, in a similar fashion to SOS2 (CIPK24) regulation of the H+/Ca2+- antiporter CAX1 (Cheng et al., 2004). Thus, characterization of RbohB regulation by Cipk6 and identification and characterization of additional Cipk6 phosphorylation targets involved in plant immunity will be the next important steps to gain understanding about the cellular processes regulated by this key regulator of PCD.

METHODS

Plant Material and Bacterial Strains

Resistant tomato (Solanum lycopersicum) line Rio Grande-PtoR (RG-PtoR; Pto/Pto and Prf/Prf), susceptible line Rio Grande-PtoS (RG-PtoS; ptp/ptp and Prf/Prf), and Rio Grande-prf3 (RG-prf3; Pto/Prf and prf/prf) and Nicotiana benthamiana were used for VIGS. Agrobacterium tumefaciens strains GV2260 and GV3101 were used for VIGS in N. benthamiana and tomato, respectively, and C58C1 for Agrobacterium-mediated transient expression in N. benthamiana. Pseudomonas syringae pv tomato strain DC3000 and P. syringae pv tomato strain DC3000 ΔhrcC- (Pet DC3000 ΔhrcC-) were used in tomato and N. benthamiana infection analysis, and P. syringae pv tabaci strain 11528R was used for pathogen assays in N. benthamiana.

cDNA Cloning for Tomato Cipk6, Cbl10, and RbohB

Tomato Cipk6, Cbl10, and RbohB cDNAs containing the complete open reading frames were obtained by RT-PCR using as a template a tomato cDNA library obtained from Pet DC3000-infected leaves using Gateway-adapted oligonucleotides OPS-13/OPS-14 for Cipk6, OPS-56/OPS-64 for Cbl10, and OPS-637/OPS-638 for RbohB and cloned into the pDONR207 vector (Invitrogen; Supplemental Table 2).

Site-Directed Mutagenesis and Deletion Mutants

PCR-based site mutagenesis was performed to generate the substitutions K43M and T172D in Sl-Cipk6 using the QuickChange kit (Stratagene) using the following oligonucleotides: OPS-40/OPS-41 for Sl-Cipk6 (Cheng et al., 2004). Thus, characterization of RbohB, CIPK26 was shown previously to SOS2 (CIPK24) regulation of the H+/Ca2+ antiporter CAX1 (Cheng et al., 2004). Thus, characterization of RbohB regulation by Cipk6 and identification and characterization of additional Cipk6 phosphorylation targets involved in plant immunity will be the next important steps to gain understanding about the cellular processes regulated by this key regulator of PCD.

Electrolyte Leakage Assay and PCD Staining

Tryptan blue staining in tomato was performed as described by del Pozo et al. (2004). Ion leakage assays were performed with three discs per plant (1 cm diameter) floated in 3 mL of MilliQ water for 2 h at room temperature with gentle shaking, and conductivity was measured with an Acorn Con 8 meter (Oakton Instruments).

VIGS

PVX clones containing N. benthamiana partial cDNAs corresponding to Cipk6 (cNbME30H4) and Cbl10 (cNbME35F4) were identified previously (del Pozo et al., 2004). Tomato identical clones were amplified and transferred into the TRV silencing vector pYL215 (Liu et al., 2002) to obtain TRV-Cipk6 and TRV-Cbl10. TRV-Cipk6-5, a 403-bp Cipk6 fragment, Cipk6-3, a 463-bp fragment, and TRV-Cbl10-5, a 205-bp fragment, were PCR amplified using Gateway-adapted oligonucleotides OPS-15/OPS-16, OPS-19/OPS-20, and OPS-22/OPS-24, respectively. PCR products were gel purified, inserted into the TRV vector by the Gateway reaction (Invitrogen), sequenced, and transformed into Agrobacterium strain GV2260. TRV-Nb-RbohB silencing construct was kindly provided by Cecile Segonzac (Segonzac et al., 2011). For silencing in tomato and N. benthamiana, seedlings with two emerging leaflets and 14-d-old plants, respectively, were syringe infiltrated with cultures containing pYL215 derived constructs and mixed with pTRV1, both with an OD$_{600}$ of 0.075, according to procedures described by Liu et al. (2002).
**Agrobacterium-Mediated Transient Transformation Assay**

Agrobacterium-mediated transient expression of proteins in *N. benthamiana* leaves was performed as described by He et al. (2004). Agrobacterium GV2260 cultures carrying Pto, avrPto, Cf9, avr9, PtoY207D, and Bax were infiltrated at OD_{600} = 0.2; for cultures carrying RBP1 or Gpa2, OD_{600} = 0.1; and for cultures carrying NPP1, RX2, or CP, OD_{600} = 0.04 was used. OD_{600} was adjusted for each culture to synchronize PCD development in *GV2260* cultures carrying *Agrobacterium* (Rubio et al., 2005) (oligonucleotides OPS-189, OPS-190, and OPS-191). Yeast Two-Hybrid Assay

Tomato Cbl10 tagged with an HA epitope was cloned into pJG4-5 vector under control of the Gal-inducible promoter and tomato Cipk6 gene and derivatives (fused to the LexA DNA binding domain) into pEG202 vector and transformed in yeast strain EGY48 (ura3, his3, trp1, lexAPO-leu2) following the LiAc/PEG method (Yeast Protocols Handbook, Clontech). Two-hybrid tests were performed on indicator plates supplemented with 2% Gal and 1% raffinose to induce prey fusion protein expression. Activation of the chromosomal LEU2 reporter was tested by scoring growth on minimal medium lacking uracil, His, Trp, and Leu (Golemis and Khazak, 1997). Expression of fusion proteins was verified by immunoblot using monoclonal antibodies mouse anti-LexA SC-7544 (Santa Cruz Biotechnology) or rat anti-HA 3F10 (Roche Applied Science).

**Preparation and Purification of Tomato Cipk6 Recombinant Proteins**

Tomato Cipk6 and derivatives were PCR amplified with LIC-compatible primer OPS-189 (forward) and OPS-190 or OPS-191 (reverse) oligonucleotides for full-length or Cipk6ΔCterm, respectively, and cloned as described by Popescu et al. (2007 Supplemental Table 2). Six-week-old *N. benthamiana* leaves were infiltrated with a mixture 1:2 (v/v) with extraction buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.1% Triton X-100, and 1 mM DTT) at 4°C. Elution from the IgG beads was performed by incubation with 10 g of horseradish peroxidase. Expression was induced by adding 5 nM 17β-estradiol and 0.1% Silwet L-77. For ROS detection after bacterial infection, 2.5 × 10^8 cfu/mL *Pst* DC3000 or *Pst* DC3000 ΔhrcC was added to the wells, and ROS were measured in vivo as luminescence using a Varioscan Flash Multimode Reader (Thermo Scientific) every 30 s up to 8 h.

**BiFC Assay**

Tomato Cipk6 and Cbl10 were Gateway cloned into the pXCGW vector, using oligonucleotides OPS13/OPS63 and OPS66/OPS64, respectively; tomato *RbohB* was cloned into the pXNGW vector (OPS-637/OPS-638). For the BiFC assay, Agrobacterium C58C1 cultures (OD_{600} = 0.5) carrying pXCGW-SiCipk6 or pXCGW-Si-Cbl10, pXNGW-Nb-RbohB and tomato bushy stunt virus *p19*, were mixed at 1:1:1 v/v/v ratio, were infiltrated in *N. benthamiana* plants and kept in the greenhouse for 48 h. YFP recombination was visualized by confocal microscopy. FM64-64 was infiltrated (20 µM) and observed immediately by confocal microscopy. See Supplemental Table 2.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: cNvME2554F4 (JF974260), cNvME30H4 (JF974261), Nb-Cipk6 (EB449468), Si-Cbl3 (SGN-US58014), Si-Cbl4 (SGN-US58071), Si-Cbl10 (JF831199; Solyc08g065330), Si-Cipk3 (SGN-US580739), Si-Cipk6 (JF831200; Solyc12g010130), Si-Cipk11 (JF831201; Solyc08g024440), Si-Cipk14 (JF831202; Solyc10g085450), Si-RbohB (Solyc09g117980), Os-Cbl10 (DQ201204), Os-Cipk6 (Q629IF4), Ca-Cipk6 (ACC96114), and Dm-CalciB (NM_080002). For Arabidopsis, they are as follows: At-Cipk6 (AT4g30960), At-Cipk3 (AT4g24400), At-Cipk24 (AT5g35440), At-Cipk3 (AT2g26980), At-Cipk9 (AT1g01140), At-Cipk23 (AT1g30270), At-Snr2X (At1g09400), At-Cbl1 (AT4G17615), At-Cbl2 (AT5G55990), At-Cbl3 (AT4G26570), At-Cbl4 (AT5G24270), At-Cbl5 (AT4G16020), At-Cbl6 (AT4G16350), At-Cbl7 (AT4G23660), At-Cbl8 (AT1G64480), At-Cbl9 (AT5G47100), and At-Cbl10 (AT4g33000).
Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. ORF Cloning of Sl-Cbl10 and Sl-Cipk6 and Derivative VIGS Constructs.

Supplemental Figure 2. Cbl10 Protein Sequence Alignment.

Supplemental Figure 3. SlCipk6 Protein Sequence Alignment.

Supplemental Figure 4. Cipk6 and Cbl10 Genes Are Expressed in All Tissues Analyzed and Their Expression Is Induced after Pathogen Infection.

Supplemental Figure 5. Reduction of Disease Symptoms in N. benthamiana Plants Silenced for Cipk6 or Cbl10.

Supplemental Figure 6. Tomato Cipk and Cbl10 Proteins Were Expressed in Yeast.

Supplemental Figure 7. Cbl10/Cipk6 Interaction Is Not Calcium Dependent in in Vitro Pull-Down Assays.

Supplemental Table 1. TRV-Cbl10 and TRV-Cipk6 Constructs Specifically Target Their Corresponding mRNAs in Tomato.

Supplemental Table 2. Oligonucleotides Used in This Work.

Supplemental Data Set 1. Text File of Alignment Corresponding to Phylogenetic Analysis in Figure 1A.

Supplemental Data Set 2. Text File of Alignment Corresponding to Phylogenetic Analysis in Figure 1B.

ACKNOWLEDGMENTS

We thank Peter Moffett for providing Rx2/CP and Gpa2/RBP-1 constructs and the co-IP protocol, Cecile Segonzac for TRV-Nb-RbohB construct and helping with the ROS assay, and Mary Beth Mudgett for Gateway-compatible BiFC vectors. We thank Jose Manuel Pardo for critically reading the article and for suggesting fruitful experiments. This work was funded in part by the European Regional Development Fund through the Ministerio de Ciencia e Innovación (BIO2005-02136 and BIO2009-08648), Junta de Andalucía (PO7-CVI-03171), Marie Curie Programme through the International Reintegration grants (MIRG-CT-2005-031174) (O.D.); National Science Foundation IOS-0841807 and IOS-1025642 (G.B.M.). O.D. was supported in part by the Junta de Andalucía (Programa de Retorno de Investigadores), F.D. by a Juan de la Cierva contract (Ministerio de Ciencia e Innovación; Spain), and Y.P.-J. by Grant BIO2009-08648. E.G.-B. was a recipient of an Formación de Personal Investigador fellowship (Ministerio de Educación, Spain).

AUTHOR CONTRIBUTIONS

F.D. and O.D. designed experiments and analyzed the data. O.D. wrote most of the article, and F.D. and G.B.M. helped with the writing. F.D. performed most of experiments. O.D. performed initial VIGS assays. E.G.-B. performed yeast two-hybrid and RT-PCR analyses. Y.P.-J. performed tomato RbohB cloning and BiFC. S.C. performed in planta experiments using oomycete, viral, and nematode effectors. G.B.M. assisted with data analysis.

Received May 13, 2013; revised July 1, 2013; accepted July 12, 2013; published July 31, 2013.

REFERENCES


Held, K., Pascaud, F., Eckert, C., Gajdanowicz, P., Hashimoto, K.,


Feilbrich, G., Romanski, A., Varei, A., Blume, B., Brunner, F.,

Engelhardt, S., Felix, G., Kemmerling, B., Krzyzowski, M., and


Garcia-Brugger, A., Lamotte, O., Vandelle, E., Bourque, S.,


Held, K., Pascual, F., Eckert, C., Gajdanowicz, P., Hashimoto, K.,


Jiang, C., Befield, E.J., Mithani, A., Visscher, A., Ragoussis, J.,


Kimura, S., Kaya, H., Kawarasaki, T., Hiraoka, G., Senzaki, E.,


Kobayashi, M., Yoshioka, M., Asai, S., Nomura, H., Kuchimura, K.,


