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Minireview

Molecules and Cells

Molecular Mechanisms of Generation for Nitric Oxide and Reactive Oxygen Species, and Role of the Radical Burst in Plant Immunity

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Rapid production of nitric oxide (NO) and reactive oxygen species (ROS) has been implicated in the regulation of innate immunity in plants. A potato calcium-dependent protein kinase (StCDPK5) activates an NADPH oxidase StRBOHA to D by direct phosphorylation of N-terminal regions, and heterologous expression of StCDPK5 and StRBOHs in *Nicotiana benthamiana* results in oxidative burst. The transgenic potato plants that carry a constitutively active StCDPK5 driven by a pathogen-inducible promoter of the potato showed high resistance to late blight pathogen *Phytophthora infestans* accompanied by HR-like cell death and H_2O_2 accumulation in the attacked cells. In contrast, these plants showed high susceptibility to early blight necrotrophic pathogen *Alternaria solani,* suggesting that oxidative burst confers high resistance to biotrophic pathogen, but high susceptibility to necrotrophic pathogen. NO and ROS synergistically function in defense responses. Two MAPK cascades, MEK2-SIPK and cytokinesis-related MEK1-NTF6, are involved in the induction of *NbRBOHB* gene in *N. benthamiana*. On the other hand, NO burst is regulated by the MEK2-SIPK cascade. Conditional activation of SIPK in potato plants induces oxidative and NO bursts, and confers resistance to both biotrophic and necrotrophic pathogens, indicating the plants may have obtained during evolution the signaling pathway which regulates both NO and ROS production to adapt to wide-spectrum pathogens.

INTRODUCTION

Rapid production of nitric oxide (NO) and reactive oxygen species (ROS), called NO burst and oxidative burst, respectively, have been implicated in diverse physiological processes, such as resistance to biotic and abiotic stress, hormonal signaling and development in plants (Doke, 1983; Hong et al., 2008; Torres et al., 2006; Wendehenne et al., 2004). Recently, NO has attracted attention as a radical that participates in plant innate immunity. NO activates the mitogen-activated protein kinase (MAPK) cascade (Clarke et al., 2000) and increases the expression of defense genes, such as those coding for phenylalanine ammonia-lyase (PAL) and pathogenesis-related proteins (Durner et al., 1998). In animals, NO is produced by NO synthase (NOS). The sources of NO synthesis in plants include reduction in nitrite by nitrate reductase (NR), oxidation of arginine to citrulline by NOS. Although evidences for arginine-dependent NO synthesis in plants have accumulated, no gene or protein that has a sequence similar to known mammalian-type NOS has been found in plants (Butt et al., 2003; Garcia-Mata and Lamattina, 2003). Guo et al. (2003) identified a NOS-like enzyme from Arabidopsis (*Arabidopsis thaliana*) (AtNOS1) with a sequence similar to a protein that has been implicated in NO synthesis in the snail *Helix pomatia*. The AtNOS1 protein has no NOS activity (Zemojtel et al., 2006), and therefore the AtNOS1 was renamed AtNOA1 for NO ASSOCIATED1 (Crawford et al., 2006). Recent studies showed that AtNOA1 has circularly permuted GTPase activity (Moreau et al., 2008). The function of AtNOA1 remains to be resolved. However, the Arabidopsis mutant *noa1* is still useful for its phenotype, which shows reduced levels of NO in plant growth, fertility, hormonal signaling, salt tolerance and plant-pathogen responses (Guo et al., 2003; He et al., 2004; Kato et al., 2008; Zeidler et al., 2004; Zhao et al., 2007). Knocking out or down the *NOA1* provides a powerful tool to analyze the NO function.

The activation mechanism of NADPH oxidase has been well investigated for mammals. NADPH oxidases have Nox/Duox (NADPH oxidase/dual oxidase) as a catalytic subunit. In phagocytes, $gp91^{phox}$, known as Nox2, forms a multi-protein complex with $p22^{phox}$, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$ and Rac2 (Sumimoto, 2008). Nox organizer 1 (Noxo1) and Nox activator 1 (Noxa1) are homologs of $p47^{phox}$ and $p67^{phox}$, respectively, and are required for Nox1 activation (Takeya et al., 2003). Nox5, Duox1, and Duox2 have N-terminal extensions, including EF-hand motifs, and are likely regulated by Ca^{2+} directly (Bánfi et al., 2004; Dupuy et al., 1999). *Respiratory burst oxidase homolog* (*RBOH*) is a plant homolog of Nox5 in mammalian NADPH oxidase and found in several plant genomes, such as Arabidopsis, rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), and *Nicotiana benthamiana* (Amicucci et al., 1999; Groom et al., 1996; Keller et al.,

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1998; Simon-Plas et al., 2002; Torres et al., 1998; Yoshie et al., 2005; Yoshioka et al., 2001; 2003). Several lines of evidence indicate that RBOH has a pivotal role in ROS-mediated signaling, such as defense responses, plant development and cell elongation. *AtRBOHD* and *AtRBOHF* function in ROS production during pathogen signals (Torres et al., 2002) and abscisic acid-induced stomatal closure in guard cells (Kwak et al., 2003). Virus-induced gene silencing (VIGS) of *NbRBOHA* and *NbRBOHB* in *N. benthamiana* attenuates ROS production and resistance to *Phytophthora infestans* (Asai et al., 2008; Yoshioka et al., 2003). Loss-of-function of tomato *RBOHs* by an antisense technique reduces ROS production in leaves and induces morphological abnormality (Sagi et al., 2004). In root hair development, ROS production by *AtRBOHC/RHD2* controls cell expansion through the activation of Ca^{2+} channels (Foreman et al., 2003). Except for RBOH and Rac, the homologs of other subunits of phagocyte NADPH oxidase are not found by Arabidopsis genome sequencing (Arabidopsis Genome Initiative, 2000). RBOH protein has been shown to localize on the plasma membrane (Kobayashi et al., 2006) The N-terminal extension includes two Ca^{2+} binding EF-hand motifs and suggests participation of Ca^{2+} signaling in the activation process (Keller et al., 1998). Sagi and Fluhr (2001) showed that Ca^{2+} directly activates an RBOH-like enzyme in tomato and tobacco plasma membranes by using denaturing gel assay and then by regeneration. Rac GTPase is also implicated to regulate RBOH by means of N-terminal extension. Wong et al. (2007) indicated direct interaction between Rac and the N-terminal of RBOH and the interaction may activate NADPH oxidase activity in plants. They also suggested that cytosolic Ca^{2+} concentration might modulate NADPH oxidase activity by regulating the interaction between Rac and RBOH. Furthermore, it was reported that a calcium-dependent protein kinase (CDPK) activates NADPH oxidase by the direct phosphorylation of its N-terminal region (Kobayashi et al., 2007). The conditional expression of an active CDPK in transgenic potato plants induce oxidative burst and confers resistance to virulent *P. infestans*. However, the transgenic potato plants are more susceptible than wild-type plants to infection by necrotrophic pathogen *Alternaria solani*.

NO and ROS together, but not individually, are required to induce cell death (Delledonne et al., 1998), and balanced production of NO and H_2O_2 is important to induce hypersensitive response (HR) cell death (Delledonne et al., 2001). However, little is known how these molecules are coordinately regulated, and functions in plant immune responses. Recently, we found that MAPKs regulate production of NOA1-associated NO and NADPH oxidase-dependent ROS (Asai et al., 2008). In this review, we discuss the molecular mechanisms of the regulation of NO and ROS production and roles of radical bursts in resistance to pathogens.

MAPKs regulate NADPH oxidase genes

The timely recognition of invading microbes and the rapid induction of defense responses are essential for plant disease resistance. At least two recognition systems are used by plants (Jones and Dangl, 2006). Plant defenses are often initiated by a gene-for-gene interaction between a dominant plant resistance (R) gene and a pathogen avirulence (Avr) gene, which provides race-specific resistance that is easily overcome by pathogen mutations. Plants also use a much less specific recognition system that identifies pathogen-associated molecular patterns (PAMPs), such as flagellin (Zipfel et al., 2004) and EF-Tu (Zipfel et al., 2006), so-called general elicitors. Both animals and plants can recognize invariant PAMPs that are characteristic of pathogenic microorganisms by corresponding pattern recognition receptors.

The MAPK cascade is a major evolutionarily conserved signaling pathway used to transduce extracellular stimuli into intracellular responses among eukaryotes (MAPK Group, 2002; Pitzschke et al., 2009). In the MAPK signal transduction cascade, a MAPK is activated by a MAPK kinase (MAPKK), which itself is activated by a MAPKK kinase (MAPKKK). Many studies have extensively characterized plant MAPKs, including tobacco wound-induced protein kinase (WIPK) (Seo et al., 1999) and salicylic acid (SA)-induced protein kinase (SIPK) (Zhang and Klessig, 1997) and their orthologs in other plant species (Katou et al., 2005; Lee et al., 2004; Pedley and Martin, 2005). WIPK and SIPK participate either in *R* gene-dependent resistance to tobacco mosaic virus (TMV) (Jin et al., 2003), to *Cladosporium fulvum*-derived effector *Avr9* (Romeis et al., 1999) in a gene-for-gene-specific way or in PAMPs-responsive resistance in *Colletotrichum orbiculare-N. benthamiana* interactions (Tanaka et al., 2009). NtMEK2, a tobacco MAPKK, is upstream of both WIPK and SIPK (Yang et al., 2001). Expression of $NtMEK2^{DD}$, a constitutively active mutant of NtMEK2, induces HR-like cell death, defense gene expression, and generation of ROS, all of which are preceded by activation of endogenous WIPK and SIPK (Ren et al., 2002; Yang et al., 2001). NPK1-MEK1/NQK1-NTF6/NRK1 is a pivotal MAPK cascade in the regulation of cytokinesis (Sasabe et al., 2006). Like WIPK or SIPK, silencing *NbNPK1*, *NbMEK1* or *NbNTF6* attenuates *N*- and *Pto*-mediated resistance against TMV (Jin et al., 2002; Liu et al., 2004) and *Pseudomonas syringae AvrPto*, respectively (Ekengren et al., 2003). These studies indicated that MAPK cascades MEK2-WIPK/SIPK and NPK1-MEK1-NTF6 participate in disease resistance in plants.

The potato ortholog of tobacco NtMEK2, StMEK2 (formerly described as StMEK1) was also cloned (Katou et al., 2003). Transient expression of $StMEK2^{DD}$ induced HR-like cell death and ROS production in *N. benthamiana* leaves (Katou et al., 2003; 2005). We found that $StMEK2^{DD}$ induces accumulation of *NbRBOHB* mRNA (Yoshioka et al., 2003), suggesting that the transcriptional activation of *RBOH* is one of the functions of the MAPK cascade in ROS production. We also showed that silencing *NbRBOHA* and *NbRBOHB* in *N. benthamiana* plants leads to less ROS production and reduced resistance to *P. infestans* (Yoshioka et al., 2003). NTF4 is a tobacco MAPK that reveals high homology with SIPK (93.6% in identity) and a similar expression pattern and activation profile of SIPK (Ren et al., 2006). The conditional expression of *NTF4* induces autophosphorylation and HR-like cell death (Ren et al., 2006). It was shown that NbNTF4, like NbSIPK, also regulates the radical bursts (Asai et al., 2008). NTF4 may play a similar role to SIPK in the signaling of basal defense. INF1-induced expression of *NbRBOHB* was compromised in *SIPK*/*NTF4*/*NTF6*-silenced leaves, but not in *WIPK*-silenced leaves (Asai et al., 2008). Furthermore, conditional expression of $NbMEK1^{DD}$ induced expression of *NbRBOHB*. These results indicated that INF1 regulates NbRBOHB-dependent ROS generation through MEK2-SIPK/NTF4 and MEK1-NTF6 cascades. Fig. 1 summarizes mechanisms of radical burst regulated by MAPK cascades.

CDPK activates NADPH oxidase by direct phosphorylation of its N-terminal region

Transient influx of Ca^{2+} into cytoplasm after perception of pathogen signals constitutes an early element of signaling cascades triggering oxidative burst and HR cell death (Blume et al., 2000; Grant et al., 2000; Lecourieux et al., 2006). CDPKs are

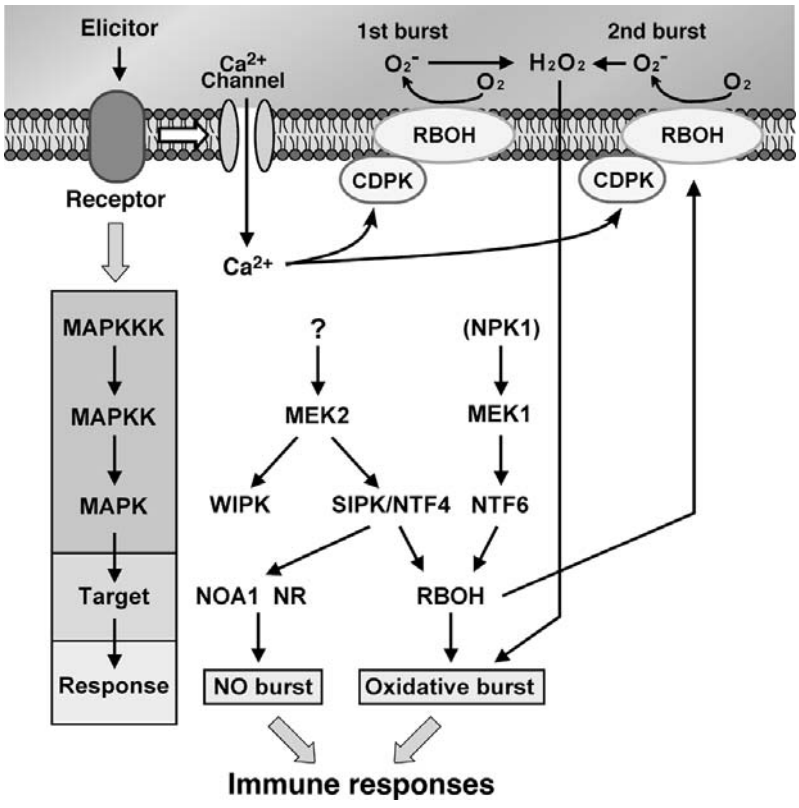

Fig. 1. Scheme of proposed model for signaling pathway leading to radical burst. Elicitor induces Ca^{2+} influx. Increase of intracellular Ca^{2+} concentration provokes Ca^{2+} binding to EF-hand motifs of CDPK and RBOH N-terminal region. Phosphorylation of RBOH by the CDPK results in the phase I (1st) and phase II (2nd) bursts. The elicitor induces NOA1- and NR-mediated NO burst by means of MEK2-SIPK/NTF4 cascade. MEK2-SIPK/NTF4 and (NPK1)-MEK1-NTF6 cascades up-regulate inducible form of *RBOH* gene. Question mark indicates unidentified MAPKKK.

Ser/Thr 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 multigene family with possible redundancy or diversity or both in their functions (Freymark et al., 2007; Harmon et al., 2001). Accumulating evidence indicates that CDPKs regulate many aspects of plant growth and development (Bachmann et al., 1996; Yoon et al., 2006), hormonal responses (Gargantini et al., 2009; McCubbin et al., 2004; Mori et al., 2006) and adaptation to biotic and abiotic stresses (Chico et al., 2002; Freymark et al., 2007; Ivashuta et al., 2005; Ludwig et al., 2005; Romeis et al., 2001; Saijo et al., 2000). Some *CDPK* genes are induced transcriptionally by pathogen signals (Chico et al., 2002; Murillo et al., 2001; Romeis et al., 2001; Yoon et al., 1999). CDPKs have been suggested to function upstream of ROS production (Kobayashi et al., 2007; Ludwig et al., 2004). Ectopic expression of Arabidopsis *AK1* (*AtCPK1*) increases NADPH oxidase activity and ROS production in tomato protoplasts (Xing et al., 2001). Functional analysis using artificially activated CDPK has shown that CDPK consists of four domains: an N-terminal variable domain (V), a Ser/Thr 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 pseudo-substrate in the absence of Ca^{2+} and keeps a low activity state (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). Ludwig et al. (2005) showed that transient expression of NtCDPK2VK stimulates ROS production and HR-like cell death by abiotic hypo-osmotic and wound stresses, and that NtCDPK2VK increases the level of jasmonic acid, 12-oxo-phyodienoic acid and ethylene under abiotic stress stimuli and has a cross-talk with MAPKs in ethylene signaling.

Pathogen infection or elicitor treatment causes immediate phase I oxidative burst, and then massive phase II oxidative burst. A protein synthesis inhibitor abolishes only phase II oxidative burst in potato leaves and tubers (Chai and Doke, 1987; Yoshioka et al., 2001). We previously isolated *StRBOHA* to *D* from potato plants (Yamamizo et al., 2006; Yoshioka et al., 2001). In potato tubers, *StRBOHA* is constitutively expressed at a low level, 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 a low level, but *StRBOHC* is markedly induced in response to *P. infestans* (Yamamizo et al., 2006). The promoter analysis of *StRBOHC* demonstrated that MEK2 regulates the gene activation at the transcriptional level (unpublished results). NADPH oxidase inhibitor diphenylene iodonium (DPI) blocked both bursts, whilst pretreatment of tuber with the protein synthesis inhibitor cycloheximide abolished only the second burst. These data suggest that StRBOHA and StRBOHB plus C contribute to phase I and phase II bursts, respectively (Yamamizo et al., 2006; Yoshioka et al., 2001). We found that both bursts are also inhibited by a protein kinase inhibitor or a calcium inhibitor (Miura et al., 1995). These findings led us to investigate the direct phosphorylation of the N-terminal region of the StRBOH protein by certain protein kinases for the activation of the enzymes. We identified Ser82 and Ser97 in the N-terminus of potato StRBOHB as potential phosphorylation sites by in-gel kinase assays using the mutated N-terminal proteins of StRBOHB (Kobayashi et al., 2007). Moreover, an anti-phosphopeptide (pSer82) antibody indicated that the Ser82 was phosphorylated by pathogen signals in plants. We cloned StCDPK5 by cDNA expression screening using the anti-pSer82 antibody and cells expressing an N-terminus of StRBOHB, and

mass spectrometry analyses showed that the CDPK phosphorylated only Ser82 and Ser97 in the N-terminus in a calcium-dependent manner. Ectopic expression of the constitutively active mutant of StCDPK5, StCDPK5VK, provoked ROS production in *N. benthamiana* leaves. The CDPK-mediated ROS production was disrupted by knockdown of *NbRBOHB* in tobacco. The loss-of-function was complemented by heterologous expression of wild-type potato StRBOHB, but not by a mutant (S82A/S97A). Furthermore, the heterologous expression of StCDPK5VK phosphorylated Ser82 of StRBOHB in tobacco. Comparing the differences in predicted amino acid sequences of the four StRBOHs, StRBOHA, StRBOHC and StRBOHD also have presumable phosphorylation motifs corresponding to those of StRBOHB, and the recombinant N-terminal peptides of the four StRBOHs are phosphorylated by StCDPK5 *in vitro* (Kobayashi et al., 2007). Phosphoproteomics analyses of elicitor signaling in Arabidopsis indicate that the N-terminal region of AtRBOHD is phosphorylated *in vivo* when treated with flg22 and xylanase, and mutagenesis in some of the phosphorylation sites decreases flg22-triggered oxidative bursts (Benschop et al., 2007; Nühse et al., 2007). AtRBOHD is synergistically activated by Ca^{2+} binding and phosphorylation in a heterologous expression system using a mammalian cell line (Ogasawara et al., 2008). AtRBOHC/RHD2, which is required for root hair elongation, activates ROS production through calcium-dependent protein phosphorylation (Takeda et al., 2008). These results suggest that N-terminal phosphorylation is a conserved process in RBOH activation. Mammalian NOX5, which carries an N-terminal extension including EF-hand motifs, is regulated by direct phosphorylation (Jagnandan et al., 2007; Serrander et al., 2007).

Furthermore, analyses by the BiFC (bimolecular fluorescence complementation) method indicated that StRBOHB and StCDPK5 interact on the plasma membrane and mutations of *N*-myristoylation and palmitoylaytion sites of the StCDPK5, which are responsible for localization on the membrane, eliminate these interactions (unpublished results). These lines of evidence suggest that the StCDPK5 induces phosphorylation of RBOHs and regulates the oxidative burst. Figure 1 indicates mechanisms of oxidative burst regulated by CDPK.

CDPK-triggered oxidative burst confers resistance to late blight but increases susceptibility to early blight pathogen in potato

The importance of RBOH-dependent ROS production has been shown by using *RBOH* mutants or knock-down techniques (Asai et al., 2008; Foreman et al., 2003; Kwak et al., 2003; Sagi et al., 2004; Simon-Plas et al., 2002; Torres et al., 2002; 2005; Yoshie et al., 2005; Yoshioka et al., 2003). However, gain-of-function analysis of NADPH oxidase has not been reported. StCDPK5 phosphorylates the N-terminus of StRBOHs calcium dependently, and induces oxidative burst. These data suggest that 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., 2005). Substitution of phosphorylated Ser by Asp of AtRBOHD does not produce a gain-of-function mutant (Nühse et al., 2007). We also failed to produce a constitutively active mutant of StRBOHB by double mutation of S82D/S97D in the N-terminal, even though double mutation of S82A/S97A lost ROS productivity by StCDPK5VK (Kobayashi et al., 2007). We generated transgenic potato plants containing constitutively active StCDPK5VK and investigated the role of RBOH-dependent ROS production in resistance to biotrophic and necrotrophic pathogens and defense response (Kobayashi et al., 2009).

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 part of their lives as biotrophs, and the other part, often associated with later stages of infection, as necrotrophs or saprophytes; Agrios, 2005), the means by which it is a near-obligate pathogen under natural and agricultural conditions suggest that it is better considered a potential biotrophic pathogen (Fry, 2008). We generated transgenic potato plants containing *StCDPK5VK* under control of a pathogen-inducible promoter *PVS3* (Yamamizo et al., 2006). ROS production was induced by inoculation with-virulent *P. infestans* and *A. solani* at the infection sites in the transgenic plants. Transgenic potato plants containing *PVS3:: StCDPK5VK* indicated resistance to biotrophic pathogen *P. infestans* after inoculation, but increased susceptibility to necrotrophic pathogen *A. solani*. These results provide evidence that StCDPK5VK- mediated ROS production confers resistance to biotrophic pathogens, but increases susceptibility to necrotrophic pathogens (Kobayashi et al., 2009). This suggests that StCDPK5-mediated ROS production has a pivotal role in defense signaling, and that necrotrophic pathogens hijacks the host cell death signaling to invade host cells together with host-specific toxins as a pathogenicity factor (Gilchrist, 1998; Glazebrook, 2005; Greenberg, 1997). Arabidopsis mutants with delayed or reduced cell death response are generally more resistant to 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 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_2O_2 (Govrin and Levine, 2000). Arabidopsis mutants lacking DELLAs increases RBOH-dependent ROS accumulation in biotic and abiotic responses and shows substantially strong disease symptoms caused by *B. cinerea* and *Alternaria brassicicola*, and resistance to hemibiotrophic bacterial pathogen *P. syringae* pv. *tomato* DC3000 (Achard et al., 2008; Navarro et al., 2008). Loss-of-function analyses using inhibitors and VIGS have investigated the role of the radical burst in pathogenesis. ROS function has a negative role in resistance or has a positive role in expansion of disease lesions during *B. cinerea* and *N. benthamiana* interaction (Asai and Yoshioka, 2009). Taking these results together, phenotypes of *PVS3:: StCDPK5VK* plants seem to reflect roles of ROS produced by RBOHs in infected cells.

HR-like cell death was observed by 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. This suggests that ROS activate cell death by 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 NO (Zaninotto et al., 2006). NO is generated in plant-pathogen interactions by putative NOS or NR (Delledonne et al., 1998; Yamamoto et al., 2003). NO and ROS together, but not individually, are required to induce cell death (Delledonne et al., 1998), and balanced production of NO and H_2O_2 is important to induce HR cell death (Delledonne et al., 2001). Catalase-deficient tobacco leaves, which accumulate sublethal levels of H_2O_2 under moderate high light condition, show cell death with

NO treatment but not leaves of wild-type plants (Zago et al., 2006). Genome-wide cDNA-amplified fragment length polymorphism analysis has shown that many genes are specifically induced by combined action of NO and H_2O_2 (Zago et al., 2006). Peroxynitrite ($ONOO^-$) generated from the reaction of NO and O_2^- is also related to plant-pathogen interactions. Urate, a natural peroxynitrite scavenger, reduces HR cell death caused by avirulent *P. syringae* in Arabidopsis (Alamillo and García-Olmedo, 2001). Taking these results together, we speculate that increased ROS production causes cell death by coordinated and balanced production of NO in response to virulent pathogens.

Studies have shown that the effects of the ROS and NO production on defense response appear to be diverse in plant-pathogen interactions. Arabidopsis lacking the NO ASSOCIATED1 (AtNOA1) mutant that reduces NO production shows an increase in susceptibility to virulent *P. syringae* (Zeidler et al., 2004), indicating that NO participates in basal defense during compatible hemi-biotrophic bacterial pathogen and Arabidopsis interaction. In *N. benthamiana*, *NbRBOHB* silencing has a strong effect on resistance to the potential pathogen *P. infestans* (Yoshioka et al., 2003) but not to *B. cinerea*, and *NbNOA1* silencing induces high susceptibility to *B. cinerea* but not to *P. infestans* (Asai and Yoshioka, 2009; Asai et al., 2008). We indicated that transgenic potato plants expressing *StCDPK5VK* fused to a pathogen-inducible promoter show high resistance to *P. infestans* but high susceptibility to the necrotrophic pathogen *A. solani*. StCDPK5VK induces production of ROS, but not of NO. The results support the conclusion that ROS may have a negative role in disease resistance to necrotrophic pathogens or a positive role in expansion of disease lesions. Transgenic potato plants expressing a constitutively active form of MAPKK, *StMEK2* (*StMEK2*^{DD}) controlled by the same pathogen-inducible promoter as for *StCDPK5VK*, are resistant to both *P. infestans* and *A. solani* (Yamamizo et al., 2006). MEK2^{DD} activates SIPK, which induces both RBOH-dependent ROS and NOA1-mediated NO production in *N. benthamiana* (Asai et al., 2008). These results support the idea that NO contributes to disease resistance against necrotrophic pathogens *A. solani* and *B. cinerea*, but synergistically works with ROS to induce cell death providing an advantage for these necrotrophic pathogens. NO may have diverse functions as a counterpart of ROS to contribute to cell death or resistance to a wide spectrum of pathogens, or both, leaving open the question how NO and ROS direct the cellular phenotype. One possible explanation for the diverse effect is that changes in redox balance correspond to a shift toward host cell death or defense response. ROS production does not always result in increased susceptibility, which depends on the timing and the intensity of ROS (Asselbergh et al., 2007). *B. cinerea* endo-polygalacturonase 1 (BcPG1), first known as a virulence factor (ten Have et al., 1998), elicits cell death and several defense responses, including production of NO and ROS and activation of MAPKs (Kars et al., 2005; Poinssot et al., 2003; Vandelle et al., 2006). However, mutants overproducing BcPG1 indicate decreased pathogenicity in grape and bean, triggering intense oxidative bursts (Kunz et al., 2006). Loss-of-function analysis indicates that ROS produced by AtRBOHD acts as a negative regulator of the runaway cell death in Arabidopsis *lsd1* mutant (Torres et al., 2005).

Transgenic potato plants expressing a fungal GO increase levels of H_2O_2 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 production. *PVS3::StCDPK5VK* plants induced ROS production only in pathogen interactions, but GO-transgenic plants have constitutively higher levels of ROS and increased defense-related gene expression, SA accumulation, and lignin content (Wu et al., 1997). Pathogen infection or elicitor treatment causes immediate phase I oxidative burst, and then massive phase II oxidative burst. A protein synthesis inhibitor abolishes only phase II oxidative burst in potato leaves and tubers (Chai and Doke, 1987; Yoshioka et al., 2001), suggesting that StCDPK5VK-induced ROS production in transgenic potato increases phase II oxidative burst. We think the increased massive second oxidative burst may cause cell death in pathogen-attacked cells expressing *StCDPK5VK*. Thus, several arguments exist about the role of ROS during plant and pathogen interactions. Gain-of-function analysis of NADPH oxidase at least shows that ROS has a positive effect on disease resistance to biotrophic pathogens and a negative effect on disease resistance to necrotrophic pathogens.

Conclusions and perspectives

ROS and NO are believed to play important roles independently or coordinately in plant innate immunity. ROS generated on the plasma membrane are released to the apoplast where they activate the Ca^{2+} channel to increase the level of cytosolic Ca^{2+} (Lecourieux et al., 2002). Ca^{2+} may function not only as an inducer of the oxidative burst, but also as a signaling molecule downstream of the oxidative burst and causes various cellular responses, including defense (Asai and Yoshioka, 2008). However, NO signaling includes various messenger molecules, such as cGMP, cADP ribose and Ca^{2+} (Durner et al., 1998; Romero-Puertas et al., 2004; Wendehenne et al., 2001), which both directly and indirectly modulate the expression of specific genes (Parani et al., 2004; Polverari et al., 2003). NO signaling pathways often include post-translational modification of target proteins, such as NO-dependent Cys *S*-nitrosylation that can modulate the activity and function of different proteins (Feechan et al., 2005; Lindermayr et al., 2005; Romero-Puertas et al., 2004; Sokolovski and Blatt, 2004). NO can also react with O_2^- to form the reactive molecule $ONOO^-$, which leads to formation of NO_2 and the effective oxidant hydroxyl radical. $ONOO^-$ is also responsible for Tyr nitration (Saito et al., 2006), which is the major toxic reactive nitrogen species in animal cells (Stamler et al., 1992). $ONOO^-$ is relevant to HR and defense gene expression (Alamillo and García-Olmedo, 2001). One study emphasized that the combination of NO and H_2O_2, but not $ONOO^-$, takes part in the induction of defense responses (Delledonne et al., 2001). Recent studies indicate that these redox changes regulate MAPK activity by attenuating a protein Tyr phospathase activity (Gupta and Luan, 2003) and the conformation of NPR1, a master regulator of SA-mediated defense genes (Tada et al., 2008). Thus, identification of sensor and effector proteins for radicals will be most important in dissecting the complexities of defense-related redox signaling.

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REFERENCES

Achard, P., Renou, J.-P., Berthomé, R., Harberd, N.P., and Genschik, P. (2008). Plant DELLAs restrain growth and promote survival of adversity by reducing the levels of reactive oxygen

species. Curr. Biol. *18*, 656-660.
Alamillo, J.M., and García-Olmedo, F. (2001). Effects of urate, a natural scavenger of peroxynitrite-mediated toxicity, in the response of *Arabidopsis thaliana* to the bacterial pathogen *Pseudomonas syringae*. Plant J. *25*, 529-540.
Amicucci, E., Gaschler, K., and Ward, J.M. (1999). NADPH oxidase genes from tomato (*Lycopersicon esculentum*) and curly-leaf pondweed (*Potamogeton crispus*). Plant Biol. *1*, 524-528.
Arabidopsis Genome Initiative (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature *408*, 796-815.
Asai, S., and Yoshioka, H. (2008). The role of radical burst via MAPK signaling in plant immunity. Plant Signal. Behavior *3,* 920-922.
Asai, S., and Yoshioka, H. (2009). Nitric oxide as a partner of reactive oxygen species participates in disease resistance to necrotrophic pathogen *Botrytis cinerea* in *Nicotiana benthamiana*. Mol. Plant-Microbe Interact. *22*, 619-629.
Asai, S., Ohta, K., and Yoshioka, H. (2008). MAPK signaling regulates nitric oxide and NADPH oxidase-dependent oxidative bursts in *Nicotiana benthamiana*. Plant Cell *20*, 1390-1406.
Asselbergh, B., Curvers, K., Franca, S.C., Audenaert, K., Vuylsteke, M., van Breusegem, F., and Hofte, M. (2007). Resistance to *Botrytis cinerea* in *sitiens*, an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis. Plant Physiol. *144*, 1863-1877.
Bachmann, M., Shiraishi, N., Campbell, W.H., Yoo, B.C., Harmon, A.C., and Huber, S.C. (1996). Identification of a Ser-543 as the major regulatory phosphorylation site in spinach leaf nitrate reductase. Plant Cell *8*, 505-517.
Bánfi, B., Tirone, F., Durussel, I., Knisz, J., Moskwa, P., Molnár, G.Z., Krause, K.-H., and Cox, J.A. (2004). Mechanism of Ca^{2+} activation of the NADPH oxidase 5 (NOX5). J. Biol. Chem. *279*, 18583-18591.
Benschop, J.J., Mohammed, S., O'Flaherty, M., Heck, A.J., Slijper, M., and Menke, F.L. (2007). Quantitative phosphoproteomics of early elicitor signaling in *Arabidopsis*. Mol. Cell. Proteomics *6*, 1192-1214.
Blume, B., Nürnberger, T., Nass, N., and Scheel, D. (2000). Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. Plant Cell *12*, 1425-1440.
Butt, Y.K.-C., Lum, J.H.-K., and Lo, S.C.-L. (2003). Proteomic identification of plant proteins probed by mammalian nitric oxide synthase antibodies. Planta *216*, 762-771.
Chai, H.B., and Doke, N. (1987). Activation of the potential of potato leaf tissue to react hypersensitively to *Phytophthora infestans* by cytospore germination fluid and the enhancement of this potential by calcium ions. Physiol. Mol. Plant Pathol. *30*, 27-37.
Chico, J.M., Raíces, M., Téllez-Iñón, M.T., and Ulloa, R.M. (2002). A calcium-dependent protein kinase is systemically induced upon wounding in tomato plants. Plant Physiol. *128*, 256-270.
Clarke, A., Desikan, R., Hurst, R.D., Hancock, J.T., and Neill, S.J. (2000). NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. Plant J. *24*, 667-677.
Crawford, N.M., Galli, M., Tischner, R., Heimer, Y.M., Okamoto, M., and Mack, A. (2006). Response to Zemojtel et al: Plant nitric oxide synthase: back to square one. Trends Plant Sci. *11*, 526-527.
Delledonne, M., Xia, Y., Dixon, R.A., and Lamb, C. (1998). Nitric oxide functions as a signal in plant disease resistance. Nature *394*, 585-588.
Delledonne, M., Zeier, J., Marocco, A., and Lamb, C. (2001). Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. Proc. Natl. Acad. Sci. USA *98*, 13454-13459.
Doke, N. (1983). Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. Physiol. Plant Pathol. *23*, 345-357.
Dupuy, C., Ohayon, R., Valent, A., Noël-Hudson, M.-S., Déme, D., and Virion, A. (1999). Purification of a novel flavoprotein involved in the thyroid NADPH oxidase: cloning of the porcine and human cDNAs. J. Biol. Chem. *274*, 37265-37269.
Durner, J., Wendehenne, D., and Klessig, D.F. (1998). Defense gene induction in tobacco by nitric oxide, cyclic GMP and cyclic ADP-ribose. Proc. Natl. Acad. Sci. USA *95*, 10328-10333.
Ekengren, S.K., Liu, Y., Schiff, M., Dinesh-Kumar, S.P., and Martin, G.B. (2003). Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato. Plant J. *36*, 905-917.
Feechan, A., Kwon, E., Yun, B.W., Wang, Y., Pallas, J.A., and Loake, G.J. (2005). A central role for *S*-nitrosothiols in plant disease resistance. Proc. Natl. Acad. Sci. USA *22*, 8054-8059.
Foreman, J., Demidchik, V., Bothwell, J.H.F., Mylona, P., Miedema, H., Torres, M.A., Linstead, P., Costa, S., Brownlee, C., Jones, J.D.G., et al. (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature *422*, 442-446.
Freymark, G., Diehl, T., Miklis, M., Romeis, T., and Panstruga, R. (2007). Antagonistic control of powdery mildew host cell entry by barley calcium-dependent protein kinases (CDPKs). Mol. Plant-Microbe Interact. *20*, 1213-1221.
Fry, W. (2008). *Phytophthora infestans*: the plant (and *R* gene) destroyer. Mol. Plant Pathol. *9*, 385-402.
Garcia-Mata, C., and Lamattina, L. (2003). Abscisic acid, nitric oxide and stomatal closure - is nitrate reductase one of the missing links? Trends Plant Sci. *8*, 20-26.
Gargantini, P.R., Giammaria, V., Grandellis, C., Feingold, S.E., Maldonado, S., and Ulloa, R.M. (2009). Genomic and functional characterization of StCDPK1. Plant Mol. Biol. *70*, 153-172.
Gilchrist, D.G. (1998). Programmed cell death in plant disease: The purpose and promise of cellular suicide. Annu. Rev. Phytopathol. *36*, 393-414.
Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. *43*, 205-227.
Govrin, E.M., and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Curr. Biol. *10*, 751-757.
Grant, M., Brown, I., Knight, M., Ainslie, A., and Mansfield, J. (2000). The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. Plant J. *23*, 441-450.
Greenberg, J.T. (1997). Programmed cell death in plant-pathogen interactions. Annu. Rev. Plant Physiol. Plant Mol. Biol. *48*, 525-545.
Groom, Q.J., Torres, M.A., Fordham-Skelton, A.P., Hammond-Kosack, K.E., Robinson, N.J., and Jones, J.D.G. (1996). *rbohA*, a rice homologue of the mammalian *gp91phox* respiratory burst oxidase gene. Plant J. *10*, 515-522.
Guo, F.-Q., Okamoto, M., and Crawford, N.M. (2003). Identification of a plant nitric oxide synthase gene involved in hormonal signaling. Science *302*, 100-103.
Gupta, R., and Luan, S. (2003). Redox control of protein tyrosine phosphatases and mitogen-activated protein kinases in plants. Plant Physiol. *132,* 1149-1152.
Harmon, A.C., Yoo, B.-C., and McCaffery, C. (1994). Pseudosubstrate inhibition of CDPK, a protein kinase with a calmodulin-like domain. Biochemistry *33*, 7278-7287.
Harper, J.F., Huang, J.-F., and Lloyd, S.J. (1994). Genetic identification of an autoinhibitor in CDPK, a protein kinase with a calmodulin-like domain. Biochemistry *33*, 7267-7277.
Harper, J.F., Breton, G., and Harmon, A. (2004). Decoding Ca^{2+} signals through plant protein kinases. Annu. Rev. Plant Physiol. Plant Mol. Biol. *55*, 263-288.
He, Y., Tang, R.-H., Hao, Y., Stevens, R.D., Cook, C.W., Ahn, S.M., Jing, L., Yang, Z., Chen, L., Guo, F., et al. (2004). Nitric oxide represses the *Arabidopsis* floral transition. Science *305*, 1968-1971.
Hong, J.K., Yun, B.-W., Kang, J.-G., Raja, M.U., Kwon, E., Sorhagen, K., Chu, C., Wang, Y., and Loake, G.J. (2008). Nitric oxide function and signalling in plant disease resistance. J. Exp. Bot. *59*, 147-154.
Ivashuta, S., Liu, J., Liu, J., Lohar, D.P., Haridas, S., Bucciarelli, B., VandenBosch, K.A., Vance, C.P., Harrison, M.J., and Gantt, J.S. (2005). RNA interference identifies a calcium-dependent protein kinase involved in *Medicago truncatula* root development. Plant Cell *17*, 2911-2921.
Jagnandan, D., Church, J.E., Bánfi, B., Stuehr, D.J., Marrero, M.B., and Fulton, D.J. (2007). Novel mechanism of activation of NADPH oxidase 5. calcium sensitization via phosphorylation. J. Biol. Chem. *282*, 6494-6507.
Jin, H., Axtell, M.J., Dahlbeck, D., Ekwenna, O., Zhang, S., Staskawicz, B., and Baker, B. (2002). NPK1, an MEKK1-like mi-

togen-activated protein kinase kinase kinase, regulates innate immunity and development in plants. Dev. Cell *3*, 291-297.
Jin, H., Liu, Y., Yang, K.-Y., Kim, C.Y., Baker, B., and Zhang, S. (2003). Function of a mitogen-activated protein kinase pathway in *N* gene mediated resistance in tobacco. Plant J. *33*, 719-731.
Jones, J.D., and Dangl, J.L. (2006). The plant immune system. Nature *444*, 323-329.
Kars, I., Krooshof, G.H., Wagemakers, L., Joosten, R., Benen, J.A., and van Kan, J.A. (2005). Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. Plant J. *43*, 213-225.
Kato, H., Asai, S., Yamamoto-Katou, A., Yoshioka, H., Doke, N., and Kawakita, K. (2008). Involvement of *NbNOA1* in NO production and defense responses in INF1-treated *Nicotiana benthamiana*. J. Gen. Plant Pathol. *74*, 15-23.
Katou, S., Yamamoto, A., Yoshioka, H., Kawakita, K., and Doke, N. (2003). Functional analysis of potato mitogen-activated protein kinase kinase, StMEK1. J. Gen. Plant Pathol. *69*, 161-168.
Katou, S., Yoshioka, H., Kawakita, K., Rowland, O., Jones, J.D.G., Mori, H., and Doke, N. (2005). Involvement of PPS3 phosphorylated by elicitor-responsive mitogen-activated protein kinases in the regulation of plant cell death. Plant Physiol. *139*, 1914-1926.
Keller, T., Damude, H.G., Werner, D., Doerner, P., Dixon, R.A., and Lamb, C. (1998). A plant homolog of the neutrophil NADPH oxidase gp91^{phox} subunit gene encodes a plasma membrane protein with Ca^{2+} binding motifs. Plant Cell *10*, 255-266.
Kobayashi, M., Kawakita, K., Maeshima, M., Doke, N., and Yoshioka, H. (2006). Subcellular localization of Strboh proteins and NADPH-dependent O_2^--generating activity in potato tuber tissues. J. Exp. Bot. *57*, 1373-1379.
Kobayashi, M., Ohura, I., Kawakita, K., Yokota, N., Fujiwara, M., Shimamoto, K., Doke, N., and Yoshioka, H. (2007). Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. Plant Cell *19*, 1065-1080.
Kobayashi, M., Yoshioka, M., Asai, S., Kuchimura, K., Mori, H., Doke N., and Yoshioka, H. (2009). Calcium-dependent protein kinase-induced oxidative burst confers resistance to late blight but increases susceptibility to early blight pathogen in potato. (in submission)
Kunz, C., Vandelle, E., Rolland, S., Poinssot, B., Bruel, C., Cimerman, A., Zotti, C., Moreau, E., Vedel, R., Pugin, A., et al. (2006). Characterization of a new, nonpathogenic mutant of *Botrytis cinerea* with impaired plant colonization capacity. New Phytol. *170*, 537-550.
Kwak, J.M., Mori, I.C., Pei, Z-.M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S., Jones, J.D.G., and Schroeder, J.I. (2003). NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*. EMBO J. *22*, 2623-2633.
Lecourieux, D., Mazars, C., Pauly, N., Ranjeva, R., and Pugin, A. (2002). Analysis and effects of cytosolic free calcium increases in response to elicitors in *Nicotiana plumbaginifolia* cells. Plant Cell *14*, 2627-2641.
Lecourieux, D., Raneva, R., and Pugin, A. (2006). Calcium in plant defence-signalling pathways. New Phytol. *171*, 249-269.
Lee, J., Rudd, J.J., Macioszek, V.K., and Scheel, D. (2004). Dynamic changes in the localization of MAPK cascade components controlling pathogenesis-related (*PR*) gene expression during innate immunity in parsley. J. Biol. Chem. *279*, 22440-22448.
Lindermayr, C., Saalbach, G., and Durner, J. (2005). Proteomic identification of *S*-nitrosylated proteins in *Arabidopsis*. Plant Physiol. *137*, 921-930.
Liu, Y., Schiff, M., and Dinesh-Kumar, S.P. (2004). Involvement of MEK1 MAPKK, NTF6 MAPK, WRKY/MYB transcription factors, *COI1* and *CTR1* in *N*-mediated resistance to tobacco mosaic virus. Plant J. *38*, 800-809.
Ludwig, A.A., Romeis, T., and Jones, J.D.G. (2004). CDPK-mediated signalling pathways: specificity and cross-talk. J. Exp. Bot. *55*, 181-188.
Ludwig, A.A., Saitoh, H., Felix, G., Freymark, G., Miersch, O., Wasternack, C., Boller, T., Jones, J.D.G., and Romeis, T. (2005). Ethylene-mediated cross-talk between calcium-dependent protein kinase and MAPK signaling controls stress responses in plants. Proc. Natl. Acad. Sci. USA *102*, 10736-10741.
MAPK Group (2002). Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends Plant Sci. *7*, 301-308.
McCubbin, A.G., Ritchie, S.M., Swanson, S.J., and Gilroy, S. (2004). The calcium-dependent protein kinase HvCDPK1 mediates the gibberellic acid response of the barley aleurone through regulation of vacuolar function. Plant J. *39*, 206-218.
Miura, Y., Yoshioka, H., and Doke, N. (1995). An autophotographic determination of the active oxygen generation in potato tuber discs during hypersensitive response to fungal infection or elicitor. Plant Sci. *105*, 45-52.
Moreau, M., Lee, G.I., Wang, Y., Crane, B.R., and Klessig, D.F. (2008). AtNOS/A1 is a functional *Arabidopsis thaliana* cGTPase and not a nitric oxide synthase. J. Biol. Chem. *283*, 32957-32967.
Mori, I.C., Murata, Y., Yang, Y., Munemasa, S., Wang, Y.F., Andreoli, S., Tiriac, H., Alonso, J.M., Harper, J.F., Ecker, J.R., et al. (2006). CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca^{2+}-permeable channels and stomatal closure. PLoS Biol. *4*, 1749-1761.
Murillo, I., Jaeck, E., Cordero, M.J., and San Segundo, B. (2001). Transcriptional activation of a maize calcium-dependent protein kinase gene in response to fungal elicitors and infection. Plant Mol. Biol. *45*, 145-158.
Navarro, L., Bari, R., Achard, P., Lisón, P., Nemri, A., Harberd, N.P., and Jones, J.D.G. (2008). DELLAs control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. Curr. Biol. *18*, 650-655.
Nühse, T.S., Bottrill, A.R., Jones, A.M., and Peck, S.C. (2007). Quantitative phosphoproteomic analysis of plasma membrane proteins reveals regulatory mechanisms of plant innate immune responses. Plant J. *51*, 931-940.
Ogasawara, Y., Kaya, H., Hiraoka, G., Yumoto, F., Kimura, S., Kadota, Y., Hishinuma, H., Senzaki, E., Yamagoe, S., Nagata, K., et al. (2008). Synergistic activation of the *Arabidopsis* NADPH oxidase AtrbohD by Ca^{2+} and phosphorylation. J. Biol. Chem. *283*, 8885-8892.
Pedley, K.F., and Martin, G.B. (2005). Role of mitogen-activated protein kinases in plant immunity. Curr. Opin. Plant Biol. *8*, 541-547.
Pitzschke, A., Djamei, A., Bitton, F., and Hirt, H. (2009). A major role of the MEKK1-MKK1/2-MPK4 pathway in ROS signalling. Mol. Plant *2*, 120-137.
Plant Pathology (2005). G.N., Agrios, ed., (Burlington, MA: Elsevier Academic Press).
Poinssot, B., Vandelle, E., Bentejac, M., Adrian, M., Levis, C., Brygoo, Y., Garin, J., Sicilia, F., Coutos-Thevenot, P., and Pugin, A. (2003). The endopolygalacturonase 1 from *Botrytis cinerea* activates grapevine defense reactions unrelated to its enzymatic activity. Mol. Plant-Microbe Interact. *16*, 553-564.
Polverari, A., Molesini, B., Pezzotti, M., Buonaurio, R., Marte, M., and Delledonne, M. (2003). Nitric oxide-mediated transcriptional changes in *Arabidopsis thaliana*. Mol. Plant-Microbe Interact. *16*, 1094-1105.
Ren, D., Yang, H., and Zhang, S. (2002). Cell death mediated by mitogen-activated protein kinase pathway is associated with the generation of hydrogen peroxide in *Arabidopsis*. J. Biol. Chem. *277*, 559-565.
Ren, D., Yang, K.-Y., Li, G., Liu, Y., and Zhang, S. (2006). Activation of Ntf4, a tobacco MAPK, during plant defense response and its involvement in hypersensitive response-like cell death. Plant Physiol. *141*, 1482-1493.
Romeis, T., Piedras, P., Zhang, S., Klessig, D.F., Hirt, H., and Jones, J.D.G. (1999). Rapid Avr9- and Cf9-dependent activation of MAP kinases in tobacco cell cultures and leaves: convergence of resistance gene, elicitor, wound, and salicylate responses. Plant Cell *11*, 273-287.
Romeis, T., Ludwig., A.A., Martin, R., and Jones, J.D.G. (2001). Calcium-dependent protein kinases play an essential role in a plant defence response. EMBO J. *20*, 5556-5567.
Romero-Puertas, M.C., Perazzolli, M., Zago, E.D., and Delledonne, M. (2004). Nitric oxide signalling functions in plant-pathogen interactions. Cell. Microbiol. *9*, 795-803.
Sagi, M., and Fluhr, R. (2001). Superoxide production by plant homologues of the gp91^{phox} NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection. Plant Physiol. *126*, 1281-1290.
Sagi, M., Davydov, O., Orazova, S., Yesbergenova, Z., Ophir, R., Stratmann, J.W., and Fluhr, R. (2004). Plant respiratory burst oxidase homologs impinge on wound responsiveness and de-

velopment in *Lycopersicon esculentum*. Plant Cell *16*, 616-628.
Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K., and Izui, K. (2000). Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J. *23*, 319-327.
Saito, S., Yamamoto-Katou, A., Yoshioka, H., Doke, N., and Kawakita, K. (2006). Peroxynitrite generation and tyrosine nitration in defense responses in tobacco BY-2 cells. Plant Cell Physiol. *47,* 689-697.
Sasabe, M., Soyano, T., Takahashi, Y., Sonobe, S., Igarashi, H., Itoh T. J., Hidaka, M., and Machida, Y. (2006). Phosphorylation of NtMAP65-1 by a MAP kinase down-regulates its activity of microtubule bundling and stimulates progression of cytokinesis of tobacco cells. Genes Dev. *20*, 1004-1014.
Seo, S., Sano, H., and Ohashi, Y. (1999). Jasmonate-based wound signal transduction requires activation of WIPK, a tobacco mitogen- activated protein kinase. Plant Cell *11*, 289-298.
Serrander, L., Jaquet, V., Bedard, K., Plastre, O., Hartley, O., Arnaudeau, S., Demaurex, N., Schlegel, W., and Krause, K.H. (2007). NOX5 is expressed at the plasma membrane and generates superoxide in response to protein kinase C activation. Biochimie *89*, 1159-1167.
Simon-Plas, F., Elmayan, T., and Blein, J.-P. (2002). The plasma membrane oxidase NtrbohD is responsible for AOS production in elicited tobacco cells. Plant J. *31*, 137-147.
Sokolovski, S., and Blatt, M.R. (2004). Nitric oxide block of outward-rectifying K^+ channels indicates direct control by protein nitrosylation in guard cells. Plant Physiol. *136*, 4275-4284.
Stamler, J.S., Singel, D.J., and Loscalzo, J. (1992). Biochemistry of nitric oxide and its redox-activated forms. Science *258*, 1898-1902.
Sumimoto, H. (2008). Structure, regulation and evolution of Nox-family NADPH oxidases that produce reactive oxygen species. FEBS J. *275*, 3249-3277.
Tada, Y., Spoel, S.H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., Zuo, J., and Dong, X. (2008). Plant immunity requires conformational changes of NPR1 via S-nitrosylation and thioredoxins. Science *321,* 952-956.
Takeda, S., Gapper, C., Kaya, H., Bell, E., Kuchitsu, K., and Dolan, L. (2008). Local positive feedback regulation determines cell shape in root hair cells. Science *319*, 1241-1244.
Takeya, R., Ueno, N., Kami, K., Taura, M., Kohjima, M., Izaki, T., Nunoi, H., and Sumimoto, H. (2003). Novel human homologues of $p47^{phox}$ and $p67^{phox}$ participate in activation of superoxide-producing NADPH oxidases. J. Biol. Chem. *278*, 25234-25246.
Tanaka, S., Ishihama, N., Yoshioka, H., Huser, A., O'Connell, R., Tsuji, G., Tsuge, S., and Kubo, Y. (2009). The *Colletotrichum orbiculare ssd1* mutant enhances *Nicotiana benthamiana* basal resistance by activating a mitogen-activated protein kinase pathway. Plant Cell *21*, 2517-2526.
ten Have, A., Mulder, W., Visser, J., and van Kan, J.A. (1998). The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. Mol. Plant-Microbe Interact. *11*, 1009-1016.
Torres, M.A., Onouchi, H., Hamada, S., Machida, C., Hammond-Kosack, K.E., and Jones, J.D.G. (1998). Six *Arabidopsis thaliana* homologues of the human respiratory burst oxidase (*$gp91^{phox}$*). Plant J. *14*, 365-370.
Torres, M.A., Dangl, J.L., and Jones, J.D.G. (2002). *Arabidopsis* $gp91^{phox}$ homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc. Natl. Acad. Sci. USA *99*, 517-522.
Torres, M.A., Jones, J.D.G., and Dangl, J.L. (2005). Pathogen-induced, NADPH oxidase-derived reactive oxygen intermediates suppress spread of cell death in *Arabidopsis thaliana*. Nat. Genet. *37*, 1130-1134.
Torres, M.A., Jones J.D.G., and Dangl, J.L. (2006). Reactive oxygen species signaling in response to pathogens. Plant Physiol. *141*, 373-378.
van Baarlen, P., Woltering, E.J., Staats, M., and van Kan, J.A.L. (2007). Histochemical and genetic analysis of host and non-host interactions of Arabidopsis with three *Botrytis* species: an important role for cell death control. Mol. Plant Pathol. *8*, 41-54.
Vandelle, E., Poinssot, B., Wendehenne, D., Bentejac, M., and Pugin, A. (2006). Integrated signaling network involving calcium, nitric oxide, and active oxygen species but not mitogen-activated protein kinases in BcPG1-elicited grapevine defenses. Mol. Plant-Microbe Interact. *19*, 429-440.
Wendehenne, D., Pugin, A., Klessig, D.F., and Durner, J. (2001). Nitric oxide: Comparative synthesis and signaling in animal and plant cells. Trends Plant Sci. *6*, 177-183.
Wendehenne, D., Durner, J., and Klessig, D.F. (2004). Nitric oxide: a new player in plant signalling and defence responses. Curr. Opin. Plant Biol. *7*, 449-455.
Wong, H.L., Pinontoan, R., Hayashi, K., Tabata, R., Yaeno, T., Hasegawa, K., Kojima, C., Yoshioka, H., Iba, K., Kawasaki, T., et al. (2007). Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. Plant Cell *19*, 4022-4034.
Wu, G., Shortt, B.J., Lawrence, E.B., Levine, E.B., Fitzsimmons, K.C., and Shah, D.M. (1995). Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. Plant Cell *7*, 1357-1368.
Wu, G., Shortt, B.J., Lawrence, E.B., León, J., Fitzsimmons, K.C., Levine, E.B., Raskin, I., and Shah, D.M. (1997). Activation of host defense mechanisms by elevated production of H_2O_2 in transgenic plants. Plant Physiol. *115*, 427-435.
Xing, T., Wang, X.J., Malik, K., and Miki, B.L. (2001). Ectopic expression of an *Arabidopsis* calmodulin-like domain protein kinase-enhanced NADPH oxidase activity and oxidative burst in tomato protoplasts. Mol. Plant-Microbe Interact. *14*, 1261-1264.
Yamamizo, C., Kuchimura, K., Kobayashi, A., Katou, S,. Kawakita, K., Jones, J.D.G., Doke, N., and Yoshioka, H. (2006). Rewiring mitogen-activated protein kinase cascade by positive feedback confers potato blight resistance. Plant Physiol. *140*, 681-692.
Yamamoto, A., Katou, S., Yoshioka, H., Doke, N., and Kawakita, K. (2003). Nitrate reductase, a nitric oxide-producing enzyme: induction by pathogen signals. J. Gen. Plant Pathol. *69*, 218-229.
Yang, K.-Y., Liu, Y., and Zhang, S. (2001). Activation of a mitogen-activated protein kinase pathway is involved in disease resistance in tobacco. Proc. Natl. Acad. Sci. USA *98*, 741-746.
Yoon, G.M., Cho, H.S., Ha, H.J., Liu, J.R., and Lee, H.S.P. (1999). Characterization of NtCDPK1, a calcium-dependent protein kinase gene in *Nicotiana tabacum*, and the activity of its encoded protein. Plant Mol. Biol. *39*, 991-1001.
Yoon, G.M., Dowd, P.E., Gilroy, S., and McCubbina, A.G. (2006). Calcium-dependent protein kinase isoforms in Petunia have distinct functions in pollen tube growth, including regulating polarity. Plant Cell *18*, 867-878.
Yoshie, Y., Goto, K., Takai, R., Iwano, M., Takayama, S., Isogai, A., and Che, F.-S. (2005). Function of the rice $gp91^{phox}$ homologs *OsrbohA* and *OsrbohE* genes in ROS-dependent plant immune responses. Plant Biotech. *22*, 127-135.
Yoshioka, H., Sugie, K., Park, H.-J., Maeda, H., Tsuda, N., Kawakita, K., and Doke, N. (2001). Induction of plant gp91 *phox* homolog by fungal cell wall, arachidonic acid, and salicylic acid in potato. Mol. Plant-Microbe Interact. *14*, 725-736.
Yoshioka, H., Numata, N., Nakajima, K., Katou, S., Kawakita, K., Rowland, O., Jones, J.D.G., and Doke, N. (2003). *Nicotiana benthamiana* $gp91^{phox}$ homologs *NbrbohA* and *NbrbohB* participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. Plant Cell *15*, 706-718.
Zago, E., Morsa, S., Dat, J.F., Alard, P., Ferrarini, A., Inzé, D., Delledonne, M., and Van Breusegem, F. (2006). Nitric oxide- and hydrogen peroxide-responsive gene regulation during cell death induction in tobacco. Plant Physiol. *141*, 404-411.
Zaninotto, F., La Camera, S., Polverari, A., and Delledonne, M. (2006). Cross talk between reactive nitrogen and oxygen species during the hypersensitive disease resistance response. Plant Physiol. *141*, 379-383.
Zeidler, D., Zahringer, U., Gerber, I., Dubery, I., Hartung, T., Bors, W., Hutzler, P., and Durner, J. (2004). Innate immunity in *Arabidopsis thaliana*: Lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. Proc. Natl. Acad. Sci. USA *101*, 15811-15816.
Zemojtel, T., Frohlich, A., Palmieri, M.C., Kolanczyk, M., Mikula, I., Wyrwicz, L.S., Wanker, E.E., Mundlos, S., Vingron, M., Martasek, P., et al. (2006). Plant nitric oxide synthase: A never-ending story? Trends Plant Sci. *11*, 524-525.
Zhang, S., and Klessig, D.F. (1997). Salicylic acid activates a 48 kD MAP kinase in tobacco. Plant Cell *9*, 809-824.
Zhao, M.-G., Tian, Q.-Y., and Zhang, W.-H. (2007). Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in Arabidopsis. Plant Physiol. *144*, 206-217.
Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D.,

Felix, G., and Boller, T. (2004). Bacterial disease resistance in *Arabidopsis* through flagellin perception. Nature *428*, 764-767.
Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. Cell *125*, 749-760.