NADPH Oxidase-Mediated Reactive Oxygen Species Production: Subcellular Localization and Reassessment of Its Role in Plant Defense

Jeannine Lherminier,¹ Taline Elmayan,¹ Jérôme Fromentin,¹ Khadija Tantaoui Elaraoui,¹ Simona Vesa,¹ Johanne Morel,¹ Jean-Louis Verrier,² Bernard Cailleteau,² Jean-Pierre Blein,¹ and Françoise Simon-Plas¹

¹UMR PME INRA 1088/CNRS 5184/Université de Bourgogne, 17 Rue Sully, BP 86510 21065, Dijon-cedex, France; ²ALTADIS-Institut du Tabac 769 route de Sainte-Alvère, 24100 Bergerac, France

Submitted 2 October 2008. Accepted 24 February 2009.

Chemiluminescence detection of reactive oxygen species (ROS) triggered in tobacco BY-2 cells by the fungal elicitor cryptogin was previously demonstrated to be abolished in cells transformed with an antisense construct of the plasma membrane NADPH oxidase, *NtrbohD*. Here, using electron microscopy, it has been confirmed that the first hydrogen peroxide production occurring a few minutes after challenge of tobacco cells with cryptogin is plasma membrane located and *NtrbohD* mediated. Furthermore, the presence of *NtrbohD* in detergent-resistant membrane fractions could be associated with the presence of *NtrbohD*-mediated hydrogen peroxide patches along the plasma membrane. Comparison of the subcellular localization of ROS in wild-type tobacco and in plants transformed with antisense constructs of *NtrbohD* revealed that this enzyme is also responsible for the hydrogen peroxide production occurring at the plasma membrane after infiltration of tobacco leaves with cryptogin. Finally, the reactivity of wild-type and transformed plants to the elicitor and their resistance against the pathogenic oomycete *Phytophthora parasitica* were examined. *NtrbohD*-mediated hydrogen peroxide production does not seem determinant for either hypersensitive response development or the establishment of acquired resistance but it is most likely involved in the signaling pathways associated with the protection of the plant cell.

Plants are exposed to a large number of microorganisms, although only a relatively small proportion of them are able to cause diseases. Prevention of penetration by pathogens during plant infection is generally dependent on an accurate time course of the pathogen perception by host cells and activation of a signaling cascade triggering a network of coordinated responses. The rapid production of reactive oxygen species (ROS), called the oxidative burst, is one of the earliest events detected during incompatible interactions between plants and pathogens. The generation of ROS has been considered for a long time to contribute to several disease resistance strategies

(antimicrobial activity, cross-linking of cell wall proteins, induction of defense related genes, and so on) (Bolwell 1999; Bolwell and Wojtaszek 1997; Lamb and Dixon 1997; Wojtaszek 1997). More recently, the complex role of ROS in the regulation of cell death accompanying the hypersensitive response (HR) has been underlined (Torres et al. 2005). Because of the key role played by these ROS in the orchestration of plant defense (Sagi and Fluhr 2006; Torres et al. 2006), the mechanisms of their biosynthesis have been extensively studied using whole plants or cell suspension cultures treated with various elicitor molecules.

Cell-wall-located enzymes have been proved to be responsible for apoplastic ROS production during biotic stress (Apel and Hirt 2004; Mittler et al. 2004). The cell-wall-associated oxalate oxidase, also known as germin, releases H₂O₂ and CO₂ from oxalic acid (Wojtaszek 1997). This enzyme was reported to be involved in apoplastic hydrogen peroxide accumulation during interactions between different cereals species and fungi (Lane 2002). Moreover, its overexpression in sunflower or oilseed rape induced an increase in H₂O₂ levels following pathogen inoculation compared with wild-type plants (Dong et al. 2008; Hu et al. 2003). The generation of ROS by cell-wall-located peroxidases during plant defense processes has also been demonstrated; for instance, during the HR triggered in cotton by the bacterium *Xanthomonas campestris* pv. *malvacearum* (Martinez et al. 1998) and on lettuce by the bacterium *Pseudomonas syringae* pv. *phaseolicola* (Bestwick et al. 1997). Several studies have also shown that amine oxidase-like enzymes may contribute to defense responses occurring in the apoplast following biotic stress, mainly through H₂O₂ production. Amine oxidases catalyze the oxidative deamination of polyamines (i.e., putrescine, spermine, and spermidine) using FAD as a cofactor (Cona et al. 2006a). Such enzymes, located in the apoplast, have been proven to be a source of ROS production; for instance, in *Zea mays* treated with the protein phosphatase inhibitor cantharidin (Cona et al. 2006b) and in tobacco cv. Bright Yellow 2 (BY-2) cells challenged with a fungal elicitor (Yoda et al. 2006).

However, studies performed on different plant species have demonstrated that plasma membrane (PM)-located respiratory burst oxidase homologue (rbob) enzymes, similar to the respiratory burst oxidase of mammalian neutrophil cells, are an essential ROS-producing system during the early stages of plant-microbe interactions (Amicucci et al. 1999; Desikan et al. 1996; Groom et al. 1996; Keller et al. 1998; Simon-Plas et al. 2002; Torres et al. 1998; Yoshioka et al. 2003). The mammalian NADPH oxidase is a proteic complex composed of a

Corresponding author: Françoise Simon-Plas; Telephone: +33 3 80 69 32 75; Fax: +33 3 80 69 37 53; E mail: simon@dijon.inra.fr

Current address of T. Elmayan: Laboratoire de Biologie Cellulaire, INRA Route de Saint-Cyr, 78026 Versailles-cedex, France.

*The e-Xtra logo stands for “electronic extra” and indicates that five supplemental figures are published online.

membrane-bound heteroflavocytochrome and cytosolic regulatory proteins (Babior 2004; Cross and Segal 2004). The flavocytochrome is formed of two subunits, p22^{phox} and a transmembrane protein, gp91^{phox}, which contain the NADPH binding site and the electron transport chain necessary for the reduction of molecular oxygen into superoxide anion. The plant oxidase has so far been described as a single protein which exhibits some characteristic features of gp91^{phox} (i.e., the number and position of the putative membrane spanning helices, part of the C-terminus domain containing the motifs responsible for the binding of FAD and NADPH cofactors, and, in particular, four histidines postulated to bind the two hemes) (Davis et al. 1998), but it also possesses a hydrophilic N-terminal extension with a Ca²⁺-binding motif, typical of plant oxidases (Amicucci et al. 1999; Keller et al. 1998; Torres et al. 1998).

The *Arabidopsis* genome contains 10 *Atrboh* paralogues exhibiting different patterns of expression throughout the plant and variable sensitivity to environmental factors. For instance, *AtrbohD* and *F* have been identified as main isoforms involved in responses to biotic stress, and *AtrbohC* as an actor of root hair development (Sagi and Fluhr 2006). ROS accumulation in the *Arabidopsis thaliana* double mutant *rbohDrbohF* is greatly decreased during incompatible interactions with an avirulent strain of the bacteria *P. syringae* or with the oomycete *Peronospora parasitica* (Torres et al. 2002). *Nicotiana benthamiana* plants silenced for *rbohA* and *rbohB* isoforms (orthologs of *Arabidopsis* isoforms D and F, respectively), using the virus-induced gene silencing (VIGS) approach, also show a reduced level of H₂O₂ accumulation in leaves 24 h after infection with the oomycete *Phytophthora infestans* (Yoshioka et al. 2003). However, this downregulation of *rboh* genes expression leads to contrasted effects on pathogen development and HR. For instance, *Ntrboh*-silenced plants are susceptible to a normally avirulent *P. infestans* strain and exhibit a reduced HR (Yoshioka et al. 2003), whereas the *A. thaliana* mutant *rbohF* is more resistant to a weakly virulent strain of *Peronospora parasitica* and expresses enhanced HR (Torres et al. 2002). Moreover, additional work indicates that ROS produced by *Atrboh* proteins stimulated by exogenous ROS can suppress cell death in cells surrounding sites of NADPH oxidase activation (Torres et al. 2005). Thus, although it seems clear that *rboh* proteins are required for pathogen-induced ROS production, the signaling functions of ROS in general and those related to disease resistance and HR in particular are still far from being fully understood. A precise picture of the timing and localization at the cellular level of ROS production is necessary to gain new insights into the physiological roles of these proteins.

Elicitins, a family of low molecular weight proteins secreted by many species of the oomycete *Phytophthora*, induce a hypersensitive-like response and acquired resistance in tobacco (Ricci 1997). In order to understand the biochemical processes triggered by elicitors, the effects of one of them, cryptogein, on tobacco cell suspensions have been studied for several years. Early events following cryptogein treatment include fixation of a sterol molecule (Osman et al. 2001; Vauthrin et al. 1999); binding of the elicitor to a high-affinity site located on the PM (Wendehenne et al. 1995); alkalinization of the extracellular medium (Blein et al. 1991); efflux of potassium, chloride, and nitrate (Blein et al. 1991; Wendehenne et al. 2002); fast influx of calcium (Lecourieux et al. 2002); mitogen-activated protein kinase activation (Lebrun-Garcia et al. 1998; Zhang et al. 1998); nitric oxide (NO) production (Gould et al. 2003; Lamotte et al. 2004); and development of an oxidative burst (Rusterucci et al. 1996; Simon-Plas et al. 1997). We previously identified a tobacco homologue of gp91^{phox}, *NtrbohD*, located on the PM. Downregulation of this protein by an

antisense strategy abolished ROS accumulation upon challenge of tobacco BY-2 cells with cryptogein (Simon-Plas et al. 2002). Very recently, Ashtamker and associates (2007) reported a complex spatial compartmentalization of ROS accumulation after cryptogein treatment of the same cell type. This interesting work, however, did not unambiguously link ROS accumulation to identified producing enzymes, and the use of optical microscopy limited the accuracy of the spatial localization of these ROS.

The aim of this article is to connect different aspects of the study of ROS production, which have been generally studied independently. We first used electron microscopy to define precisely the subcellular localization of H₂O₂ in wild-type tobacco BY-2 cells treated with cryptogein. By comparing results obtained with wild-type cells and cells inactivated for *NtrbohD*, we determined the contribution of this enzyme to the observed ROS accumulation. We then applied this technique to leaves of wild-type tobacco and plants transformed with antisense constructs of *NtrbohD* treated with cryptogein. Finally, the response of wild-type and transformed plants to the elicitor and their resistance against the pathogen oomycete *Phytophthora parasitica* were evaluated. Results obtained shed a new light on the role of ROS produced by *rboh* proteins in response to biotic stress.

RESULTS

Subcellular location of H₂O₂ accumulation in BY-2 or gp3 cells after treatment with cryptogein.

Production of H₂O₂ was investigated using chemiluminescence and, as previously observed (Simon-Plas et al. 2002), wild-type BY-2 cells exhibited a transitory accumulation of H₂O₂ detectable 5 min after elicitation with cryptogein, with a maximum oxidative burst at 15 min (Supplementary Fig. S1). Thus, we focused our studies on the period corresponding to this first wave of ROS accumulation (from 0 to 30 min) after addition of cryptogein. Gp3 cells, transformed with antisense constructs of the PM NADPH oxidase *NtrbohD*, did not show any ROS accumulation upon addition of the elicitor, as previously reported (Simon-Plas et al. 2002).

A transmission electron microscopy (TEM) study, using the cerium chloride technique which leads to the formation of perhydroxide precipitates in presence of H₂O₂, was performed to localize H₂O₂ in BY-2 cells and precisely measure the kinetics of its accumulation. This method has been widely used in TEM studies in animal (Briggs et al. 1975; Miura et al. 2003; Soares et al. 1994; Warren et al. 1989) and plant cells and tissues (Bestwick et al. 1997, 1998; Boka et al. 2007; Brown et al. 1998; Czaninski et al. 1993; Fester and Hause 2005; Grant et al. 2000; Lee et al. 2005; Melillo et al. 2006; Olmos et al. 2003; Pellinen et al. 1999; Romero-Puertas et al. 2004; Rubio et al. 2004; Soyulu et al. 2005).

In control BY-2 and gp3 cells, only sparse precipitates were observed in some mitochondria (Fig. 1A). Approximately 20% of BY-2 and 30% of gp3 cells exhibited a weak cerium staining of the tonoplast (Fig. 1B). Although unexplained, such an observation has also been reported using cerium chloride to localize hydrogen peroxide in lettuce (Bestwick et al. 1997). This pattern of cerium staining was not modified during the time course of the experiment in either control or elicited cells.

In contrast, 75% of elicited BY-2 cells, sampled 5 min after the addition of cryptogein, exhibited small and discrete patches (100 nm) of cerium deposits on the PM (Fig. 1B). Fifteen minutes after elicitation, the same proportion of cells exhibited densely packed precipitates along the PM (Fig. 1C). Thirty minutes after cryptogein addition, discrete precipitates were still observed in zones of the PM, but only in 20% of

Fig. 1. Electron microscope localization of H_2O_2 accumulation triggered by cryptogein in **A** through **D**, *Nicotiana tabacum* cv. Bright Yellow 2 (BY-2) cells and **E** and **F**, gp3 cells after staining by CeCl_3 . CeCl_3 precipitates with H_2O_2 at reaction sites, forming electron-dense cerium perhydroxides that are visualized as black spots. **A**, Experiment on nonelicited BY-2 cells: plasma membrane is free of cerium precipitates (arrowhead). Weak precipitates are observed in mitochondria (arrow). **B**, Aliquots of BY-2 cells sampled 5 min after the addition of 50 nM cryptogein. Patches (100 nm) of cerium perhydroxides are located along the plasma membrane (arrows). Tonoplasmic staining can be visualized on this photograph (small arrow). **C**, Aliquots of BY-2 cells sampled 15 min after the addition of cryptogein. Abundant cerium precipitates are visualized on plasma membrane. **D**, Aliquots of BY-2 cells sampled 30 min after the addition of cryptogein: discrete stained patches can be still observed on the plasma membrane (arrows). **E**, Aliquots of gp3 cells sampled 5 min after the addition of 50 nM cryptogein. No staining is observed on the plasma membrane (arrowhead). **F**, Aliquots of gp3 cells sampled 30 min after the addition of the elicitor. No staining is observed on the plasma membrane (arrowhead). CW = cell wall; pm = plasma membrane; mit = mitochondria; tn = tonoplast. Bars = 0.2 μm .

BY-2 cells (Fig. 1D). The nucleus of elicited BY-2 cells was not stained during the time course of the experiment. Elicited gp3 cells transformed with antisense constructs of *NtrbohD* did not show any precipitates along the PM at any time of the experiment (0 to 30 min after cryptogein addition) (Fig. 1E and F).

The presence of *NtrbohD* on detergent-resistant membrane (DRM) fractions of tobacco PM has already been evidenced (Mongrand et al. 2004). Here, PM was prepared from suspension cells treated for 5 min with cryptogein, and the different fractions collected from the sucrose gradient used to isolate DRM fractions were blotted with an antibody directed against *NtrbohD*: fractions 1 to 5 correspond to solubilized membranes and fraction 6 to the ring of DRM fractions. *NtrbohD* is present only in the DRM fraction (Fig. 2). This association of *NtrbohD* with specific domains of the PM is consistent with the cytochemical localization of H_2O_2 accumulation in discrete patches of the membrane.

Characterization of wild-type *N. tabacum* L. cv. Xanthi or tobacco plants transformed with an antisense construct of *NtrbohD*.

Cell suspensions were generated using leaves of either wild-type cv. Xanthi or independent lines of transgenic plants expressing an antisense construct of *NtrbohD* as a starting material to produce callus. Xanthi cells exhibited an intense accumulation of ROS during the first 90 min after cryptogein treatment, whereas two cell lines from transgenic plants ('gp1' and 'gp15') did not (Supplementary Fig. S2). In Xanthi cells, this was associated with a quick and strong accumulation of *NtrbohD* transcripts, whereas their level remained low and unaffected in the two transgenic cell lines. Subsequent experiments were focused on the homozygous gp15 plants, which had a monolocus insertion. The expression of the native *NtrbohD* gene and of the antisense transgene was investigated in wild-type and transgenic gp15 plants by Northern blot analysis (Fig. 3). In wild-type plants, the cDNA probe corresponding to part of the *NtrbohD* 3' coding and untranslated region, hybridized with a transcript of 3.8 kb as previously reported (Simon-Plas et al. 2002). This transcript was hardly detectable in mature leaves, and transiently induced during the first hour after water infiltration before returning to its basal level (Fig. 3). After infiltration of leaves with cryptogein, a similar induction occurred 30 min after treatment, and a second increase in transcript level in the infiltrated area with a maximum at 8 h is observed. In gp15 plants, the probe hybridized mainly with a

transcript of 3.3 kb (corresponding to the 2.63-kb fragment of the *NtrbohD* coding region introduced in antisense in the transgene construct, and to the 0.7 kb of the *rbcs* terminator region), even though a slight hybridization with a transcript of 3.8 kb, likely to correspond to a very low level of the native gene, was also observed. The abundance of these transcripts is not significantly modified by either water or cryptogein infiltration between 0 and 24 h of treatment (Fig. 3).

The 2,633-bp fragment used as antisense for the transformation covers the 234-bp consensus fragment of *NbrbohA* from *N. benthamiana* used to silence by VIGS the two members, A and B, of *Nbrboh* subfamily in *N. benthamiana* (Yoshioka et al. 2003). Thus, we assume that our antisense construct was able to silence tobacco *NtrbohD* and *F* in the transgenic plants, because *NtrbohD* and *F* share 98 and 96% identity with their orthologs *NbrbohB* and *A*, respectively (Yoshioka et al. 2003). In this study, *Ntrboh F* transcripts were not detectable by Northern blot using a specific probe either before or after treatment of wild-type tobacco leaves with cryptogein (data not shown). This result is in agreement with the very low constitutive level of *NbrbohA* expression observed by Yoshioka and associates (2003) and suggests no or a minor contribution of this isoform to ROS accumulation upon biotic stress.

In order to monitor ROS accumulation upon cryptogein treatment, epidermal peels from tobacco leaves from wild-type or gp15 plants were loaded with 0.5 μ M of the dichlorofluorescein diacetate (DCFH-DA) probe. This led, in all cases, to a transitory fluorescence of the chloroplasts, similar to that observed in the same conditions by Allan and Fluhr (1997). We waited for return to a basal state before starting the experiment. Nonelicited Xanthi epidermal and stomata guard cells did not exhibit any fluorescence 12 min after return to the basal state (Fig. 4A). In Xanthi epidermal peels treated with cryptogein, a strong fluorescence of guard cell nuclei was detectable throughout the experiment after elicitation. Approximately 11 to 12 min after treatment with the elicitor, 90% of guard cells exhibited nuclear fluorescent staining, among which 40% also had fluorescent spots at their periphery and a diffuse fluorescence in the cytosol in the vicinity of the PM (Fig. 4B). From 15 to 20 min after the addition of cryptogein, 75% of the nuclei were still fluorescent whereas fluorescence of the spots at the cell periphery and the diffuse fluorescence in the cytoplasm were still observed in 9 and 20% of the cells, respectively (Fig. 4C). Surprisingly, this burst of fluorescence

Fig. 2. Western blotting indicating that *NtrbohD* is associated with detergent-resistant membranes (DRMs). Plasma membranes extracted from *Nicotiana tabacum* cv. Bright Yellow 2 cells treated for 5 min with cryptogein were solubilized with Triton X-100 at low temperature and centrifuged on a sucrose gradient. The different fractions collected from the sucrose gradient used to isolate DRMs were blotted with an antibody directed against *NtrbohD*: fractions 1 to 5 correspond to solubilized membranes and fraction 6 to the ring of DRMs. An equal amount of proteins present in the fractions collected were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted with an anti-*NtrbohD* antibody.

Fig. 3. Expression of *NtrbohD* in wild-type and gp15 tobacco leaves upon cryptogein treatment. Mature leaves of either wild-type tobacco plants (cv. Xanthi) or tobacco plants transformed with the antisense construct of *NtrbohD* cDNA (gp15) were infiltrated with water (Control) or with 500 μ l of an aqueous solution (1 μ g/ml) of cryptogein (Cryptogein). Total RNAs (10 μ g) extracted at various times after treatment were analyzed by Northern blot and probed with a 32 P-labeled restriction fragment corresponding to the *NtrbohD* 3' coding region and untranslated region. The autoradiograph of the hybridized blot is shown above the corresponding gels stained with ethidium bromide.

both in the nucleus and at the periphery of the cell was more obviously visualized in only one guard cell of the stomatal complex. Unlike *Xanthi* epidermal cells, gp15 stomata cells did not exhibit any induced nuclear or peripheral fluorescence after addition of the elicitor at any time, as shown 16 min after treatment with cryptogein (Fig. 4D).

Subcellular location of H₂O₂ accumulation in tobacco leaves of *Xanthi* or transgenic gp15 plants infiltrated with cryptogein.

Following the observed kinetics of expression of *NtrbohD* in *Xanthi* or transgenic gp15 tobacco leaves infiltrated with cryptogein, a TEM study was performed to establish the subcellular localization of H₂O₂ in tissues of infiltrated leaves at 6, 8, and 10 h after cryptogein treatment. All images in Figures 5 and 6 are clearly representative of a very high proportion (>80%) of cells observed in the area infiltrated with the elicitor. Considering results obtained on epidermal peels with the fluorescent probe, particular attention was paid to stomatal guard cells. Epidermal cells of *Xanthi* leaves infiltrated with sterile water showed a very low background of cerium precipitates, corresponding to basal H₂O₂ metabolism (Fig. 5A). Infiltration of wild-type leaves with cryptogein resulted, 6 h after treatment, in strong black spots of precipitates on the PM (Fig. 5B). From there on, H₂O₂ seemed to diffuse into the cytosolic compartment, as seen 8 h after cryptogein infiltration (Fig. 5C). At this time, no precipitate was observed in either cell walls (Fig. 5B and C) or the nucleus of these reacting cells (Fig. 5B). By 10 h after infiltration of the elicitor, cerium pre-

cipitates were still visible along the PM and, at this time, some small precipitates were also pointed out in cell walls (Fig. 5D). In gp15 epidermal cells of leaves infiltrated with cryptogein, H₂O₂ was exclusively accumulated in cell walls without any staining on the PM or inside the cytoplasm 6, 8, and 10 h after infiltration (Fig. 5E through H).

Kinetics of H₂O₂ location was also studied in different leaf tissues: mesophyll, phloem, and xylem. In wild-type or gp15 plants infiltrated with water, no cerium staining was observed 6 to 10 h after treatment. In wild-type leaf tissues sampled 6 h after cryptogein infiltration, cell walls of mesophyll cells were free of precipitates, although black points indicating an accumulation of H₂O₂ were located on the PM and detected in the cytosol (Fig. 6A). The same H₂O₂ pattern occurred in vascular tissues, with the accumulation on the PM of companion cells in phloem bundles (Fig. 6B) and of parenchyma cells in xylem vessels (Fig. 6C) associated with a diffusion of the cerium precipitates into the cytosol of these cells. At this time, H₂O₂ was detected in gp15-transformed plant cells only in primary cell walls of mesophyll (Fig. 6D), phloem bundle (Fig. 6E), and xylem cells (Fig. 6F). By 10 h after leaf infiltration with cryptogein, H₂O₂ accumulation was observed in cell walls, and no longer on the PM or in the cytosol of wild-type mesophyll cells (Fig. 7A). This cell wall staining was still present in mesophyll cells of the gp15 mutant. However, at this time, severe ultrastructural damage affected spongy mesophyll cells of these gp15 plants, with a strong H₂O₂ accumulation in the chloroplasts (Fig. 7B). A quantification of these observations indicated that, 10 h after cryptogein treatment, among 54 *Xanthi* mesophyll cells, only 12% were dead whereas this proportion increased to 74% among the 77 gp15 mesophyll cells analyzed.

HR and resistance induced by cryptogein in wild-type and transgenic gp15 plants.

Wild-type or gp15 plants were treated with cryptogein either by infiltration of attached leaves or by deposit on a fresh section of cut stem. A well-described necrotic symptom (Ricci 1997) developed in a similar manner in wild-type or transgenic lines, whatever the way elicitor was applied. An HR only occurred in the infiltrated area and necrosis started to be visible after 20 h similarly in all plants (Supplementary Fig. S3).

When cryptogein was applied to stems of decapitated tobacco plants, necrotic spots typical of an HR developed on distant leaves 16 h after treatment. The proportion of leaves exhibiting necrosis on each plant ($61 \pm 2\%$ for *Xanthi* and $64 \pm 3\%$ for gp15), the kinetics of symptom appearance (steady state reached after 48 h), and the extent of lesions on each leaf were not significantly different between wild-type and gp15 plants.

Leaves of wild-type or gp15 plants were also infiltrated with zoospores of the oomycete *P. parasitica* able to cause the black shank disease of tobacco, 48 h after cryptogein treatment (or water for control plants) on the cut stem to evaluate the extent of resistance triggered by the elicitor (Ricci 1997). No significant difference was observed between wild-type and gp15 plants: development of the pathogen was reduced by approximately 60% in both lines 7 days after inoculation when plants were pretreated with cryptogein. A deposit of either water or cryptogein was applied on the cut stem of wild-type or gp15 tobacco plants. After 48 h, two medium-sized leaves of each plant were infiltrated with spores of the pathogen. After 7 days, the surface of colonized areas were measured. The ratio of mean colonized surface observed in cryptogein treated plants/water-treated plants with standard errors of mean was 0.41 ± 0.09 for *Xanthi* and 0.37 ± 0.11 for gp15. Three independent experiments, comprising eight plants of each line, were performed.

Fig. 4. Epidermal tissue loaded with the probe dichlorofluorescein diacetate and observed under a fluorescent microscope. Cryptogein is added during the time course of image acquisition. **A**, Stomata of control non-elicited wild-type cv. *Xanthi* epidermal tissue observed 12 min after the beginning of the kinetics. No fluorescence is observed. **B**, Stomata of cryptogein-elicited wild-type *Xanthi* epidermal tissues observed 11 min after the beginning of the kinetics of elicitation. Nuclei are fluorescent. Small fluorescent spots at the periphery of the cells and a diffuse fluorescence into the cytoplasm are detectable (arrows). **C**, Stomata of cryptogein-elicited wild-type *Xanthi* epidermal tissues observed 20 min after the beginning of the kinetics of elicitation. Note the fluorescence of the nucleus and the diffuse fluorescence in the cytoplasm. **D**, Stomata of cryptogein-elicited transformed gp15 epidermal tissues observed 16 min after the beginning of the kinetics of elicitation. Note the absence of fluorescence. Photographs presented in the picture and statistics indicated in the text are representative of three independent experiments. N = nucleus; Cy = cytoplasm. Bars = 6 μ m.

Fig. 5. Subcellular localization of H_2O_2 accumulation in wild-type Xanthi and transformed gp15 leaf guard cells with CeCl_3 staining and transmission electron microscopy. **A** through **D**, Kinetics of H_2O_2 accumulation in Xanthi-infiltrated leaves. **A**, Control leaf infiltrated with sterile water. **B**, Detection of H_2O_2 in leaf infiltrated with cryptogeyn for 6 h: black spots of cerium precipitates are visualized on the plasma membrane of epidermal guard cells (arrows). Cell walls are free of detectable H_2O_2 . **C**, At 8 h after cryptogeyn treatment: H_2O_2 accumulation is detected both on the plasma membrane (arrows) and in the cytosol (arrowheads) of the guard cells. **D**, At 10 h after cryptogeyn treatment, cerium perhydroxides precipitates were also visualized in the cell wall of guard cells (arrowheads). **E** through **H**, Kinetics of H_2O_2 accumulation in **E**, transformed gp15 control and **F** through **H**, cryptogeyn-infiltrated leaves. No cerium precipitate is visualized in control gp15 sections. At each time point (**F**, **G**, and **H** = 6, 8, and 10 h after infiltration, respectively), H_2O_2 is found to accumulate only in cell walls of the elicited stomata guard cells. CW, cell wall; N, nucleus; cy, cytosol; pm, plasma membrane. Bars: **A** through **E** = 0.5 μm and **F** through **H** = 1 μm .

Finally, careful observations of the development of the pathogen in water-treated plants indicated a similar progression of *P. parasitica*, characterized by the area of macroscopic lesions, in wild-type and gp15 plants 7 days after inoculation. Medium leaves of wild-type or gp15 tobacco plants were infiltrated with 100 μ l of a spore suspension as described below. The surface of the lesion was measured 7 days after infiltration and expressed as a percentage of the surface of initial infiltrated zone. For each experiment, eight plants were used, and two leaves were infiltrated on each plant. The progression of infection was $510 \pm 145\%$ for Xanthi and $440 \pm 173\%$ for gp15. The results are the mean of three independent experiments. Earlier measurements performed 3 days after inoculation did not reveal further differences between the lines. Moreover, the appearance of the lesion was identical in wild-type or gp15 plants as well as the development of the oomycete assessed by trypan blue staining and microscopic observations, using a high or a low amount of spores for the inoculation (Supplementary Fig. S4).

These results suggest that neither resistance to *P. parasitica* (basal or induced by cryptogein) nor HR triggered by the elicitor are impaired in gp15 plants.

DISCUSSION

Primary accumulation of hydrogen peroxide triggered on BY-2 cells is located on the PM.

The observation, based on cerium chloride detection, that H_2O_2 occurs as distinct and local patches on the PM of cryptogein-elicited BY-2 cells with a maximum 15 min after elicitation, is in agreement with the detection of H_2O_2 accumulation using chemiluminescence. This is also consistent with the detection of hydrogen peroxide accumulation along the PM of cultured cells of *Rubia tinctorum* challenged with a fungal elicitor (Boka et al. 2007). It is interesting to note that, using this specific method, H_2O_2 accumulation was not detected in either nuclei or any other organelles of BY-2 cells following cryptogein treatment. However, an increase of fluorescence

Fig. 6. Subcellular localization of H_2O_2 accumulation in **A** through **C**, wild-type Xanthi and **D** through **F**, transformed gp15 leaf tissues 6 h after cryptogein infiltration. **A**, Black cerium precipitates, indicating H_2O_2 accumulation, were visualized on the plasma membrane (arrow) and in cytosol (arrowheads) of the mesophyll cells of Xanthi-infiltrated leaves. **B**, In phloem bundle, H_2O_2 accumulation was visualized on plasma membrane (arrow) and cytosol (arrowhead) of companion cells. **C**, Same pattern of H_2O_2 accumulation occurred in parenchyma xylem cells. **A** through **C**, Note that primary cell walls are free of detectable H_2O_2 . **D** through **F**, H_2O_2 accumulation in transformed gp15-infiltrated leaves is then found only in primary cell walls and intercellular spaces in the different tissues. CW = cell wall; pm = plasma membrane; chl = chloroplasts; se = sieve element, Cc = companion cell, Xy = xylem; Xy Pc = xylem parenchyma cell, Lig = lignin; IS = intercellular space. Bars: A, B, and E = 0.5 μ m; C = 0.25 μ m; and D = 1 μ m.

was observed in the nuclei of either BY-2 (data not shown) or Xanthi epidermal cells (Fig. 4) after a few minutes of elicitation with cryptogein using DCFH-DA staining, which is in complete agreement with a recently published work (Ashtamker et al. 2007). This suggests that this nuclear fluorescence could originate from a ROS other than H_2O_2 , as proposed by Allan and Fluhr (1997), who observed that the DCF fluorescence triggered in the nuclei of epidermal cells elicited with cryptogein was insensitive to catalase.

Limitations to the interpretation of DCF fluorescence as a specific marker for intracellular H_2O_2 formation have already been described (Tarpey et al. 2004). Moreover, application of L-arginine to DCFH-loaded cells of tobacco epidermal peels resulted in a large increase in DCF fluorescence, inhibited by NO synthase inhibitor, demonstrating NO detection by this probe (Allan and Fluhr 1997). In our model, NO is synthesized during cryptogein elicitation with a timing similar to H_2O_2 , and visualized in the nuclear region, a few minutes after stimulation of epidermal tobacco peels with cryptogein (Foissner et al. 2000). Thus, the nucleus fluorescence registered in wild-type cells and epidermal guard cells could be due to the accumulation of peroxynitrite. Allan and Fluhr (1997) reported an insensitivity of cryptogein-induced DCF fluorescence to the NO synthase inhibitor NAME, but used at a concentration (0.5 mM) far below the one (10 mM) proved to be necessary to inhibit by 50% NO production induced by cryptogein with the specific probe DAF-2DA (Lamotte et al. 2004).

Altogether, these experiments indicate clearly that the primary production of hydrogen peroxide triggered in BY-2 cells by cryptogein is located on the PM, and that no intracellular accumulation could be observed within 30 min of treatment.

All hydrogen peroxide detected early after cryptogein treatment is produced via an NADPH oxidase located in particular domains of BY-2 cell PM.

Chemiluminescence experiments failed to detect ROS accumulation following treatment with cryptogein in BY-2 cells transformed with antisense constructs of the PM NADPH oxidase NtrbohD, indicating that this enzyme is the ROS-producing system in this model (Simon-Plas et al. 2002). Because these cells do not exhibit any cerium precipitates on the PM after elicitation, it can be assumed that the patches observed along the PM of elicited wild-type cells represent the accumulation of H_2O_2 generated by NtrbohD. Similar cerium precipitates on the PM were reported following treatment of BY-2 cells with cadmium concentrations leading to an oxidative

burst (Olmos et al. 2003), further demonstrated to be mediated by NtrbohD (Garnier et al. 2006).

Over the last 10 years, a new aspect of membrane biology, the existence of membrane domains, also called “lipid rafts”, has resulted from biological, biochemical, and biophysical studies. According to this concept, biological membranes should no longer be considered as homogeneous bilayers composed of lipids and proteins. Indeed, “discrete islands” with specific location and composition exist in cellular membranes, particularly in the PM. Such domains allow the segregation of active components in membranes and are involved in many cellular processes (Rajendran and Simons 2005). Characterization of membrane domains mainly relies on their property of insolubility to detergent at cold temperatures, leading to the isolation of DRM fractions. They have been extensively studied because they can help to explain numerous biochemical processes occurring in cell membranes, in terms of specificity, selectivity, and rapidity, that cannot be readily explained by other models. Recently, a consensus has emerged about the characteristics of these domains: both proteins and lipids contribute to the formation and stability of membrane domains that should be called “membrane rafts” (MR) and envisaged as small (10 to 200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein–protein and protein–lipid interactions (Pike 2006). This organizational hierarchy of lipid and protein constituents in PM mediates many life processes involving perception and early transduction of external stimuli, allowing the adaptation of an organism to cope with pathogen attack and adverse environmental conditions (Rajendran and Simons 2005).

Although the study of MR has mainly focused on animals and yeasts, contributions have recently come from the field of membrane domains in plants (Lefebvre et al. 2007; Mongrand et al. 2004; Morel et al. 2006). These have described the peculiar protein and lipid content of plant MR, characterized by a strong enrichment in sphingolipids together with free sterols and sterol conjugates. A proteomic study of BY-2 cell PM domains has led to the identification of 146 proteins, among which a high proportion are linked to signaling in response to biotic stress, supporting the hypothesis of the involvement of PM domains in plant defense responses (Morel et al. 2006).

Previous studies, using either immunology or mass spectrometry, revealed the presence of NtrbohD in DRM fractions of tobacco BY-2 cells (Mongrand et al. 2004; Morel et al.

Fig. 7. Subcellular localization of H_2O_2 accumulation in **A**, wild-type Xanthi and **B**, transformed gp15 mesophyll 10 h after cryptogein infiltration. **A**, H_2O_2 is found to accumulate mainly in primary cell walls (arrow) of wild-type Xanthi leaf mesophyll. **B**, In parallel, severe ultrastructural damages were regularly observed in mesophyll cells of gp15 leaves with H_2O_2 accumulating in chloroplasts (arrow). CW = cell wall; pm = plasma membrane; chl = chloroplast. Bars = 0.5 μ m.

2006). The Western-blotting experiment presented in this study indicates that, 5 min after cryptogeiin treatment, all the protein present on the PM was associated with the DRM fraction. This is one of the first correlations to be reported between the association of a protein with particular membrane domains of specific lipid and protein composition and the detection of its biological activity on distinct areas of the PM.

H₂O₂ production occurs on PM and cell wall via two different sources upon infiltration of tobacco leaves with cryptogeiin.

Very little has been described concerning the subcellular localization of ROS production during the set up of plant defense, because most of the studies related to the role of ROS in these processes have been conducted using diaminobenzidine (DAB) to evaluate the global level of ROS accumulation in a given plant or organ.

Among the few papers reporting the use of cerium chloride to detect hydrogen peroxide production in planta during biotic interactions, the first (Bestwick et al. 1997) described the accumulation of H₂O₂ in the wall of lettuce cells challenged with avirulent strains of *Pseudomonas syringae*.

Six hours after cryptogeiin infiltration of tobacco leaves, CeCl₃ staining of the PM membrane of epidermal cells indicated the presence of hydrogen peroxide, followed by a cytoplasmic diffusion of this compound (Fig. 5). Ten hours after cryptogeiin treatment, accumulation of hydrogen peroxide became detectable in the cell wall. These observations corroborate previous studies performed on different plant tissues using the same technique. Treatment of pea leaves with cadmium resulted in accumulation of cerium precipitates on the PM of transfer, mesophyll, and epidermal cells (Romero-Puertas et al. 2004). The use of different inhibitors suggested that the main source of this H₂O₂ could be an NADPH oxidase. Similarly, an 8-h exposure of birch plants to ozone led to the detection of cerium precipitates on the PM of mesophyll cells, followed by accumulation in the cell wall after 10 h (Pellinen et al. 1999). The use of inhibitors suggested that both NADPH oxidases and cell wall peroxidases could be involved in this ROS production.

Experiments performed on gp15 tobacco plants transformed with an antisense construct of *NtrbohD* displayed quite a different pattern of ROS accumulation upon cryptogeiin infiltration, because cerium precipitates could only be detected in cell walls 6 to 10 h after treatment.

The fact that inactivation of *NtrbohD* results in the disappearance of H₂O₂ accumulation on the PM confirms that this oxidase is, not only in cell suspensions but also in planta, involved in the initial oxidative burst triggered by cryptogeiin. Studies performed on *N. benthamiana* and *A. thaliana*, indicating that *NbrbohB* and *AtrbohD* (respective homologues of *NtrbohD*) are the isoforms essentially involved in early ROS production occurring during an incompatible interaction, are in agreement with this result. In *Arabidopsis*, the intensity of DAB staining was greatly reduced in leaves of the *AtrbohD* mutant challenged with an avirulent strain of *P. syringae* or with the pathogenic oomycete *Peronospora parasitica* compared with wild-type plants (Torres et al. 2002). In *N. benthamiana*, silencing of the *NbrbohB* gene led to a decrease of DAB staining in leaves infected with a pathogenic strain of *Phytophthora* (Yoshioka et al. 2003) and of chemiluminescence of ROS accumulation in leaves treated with INF1, an elicitor produced by *Phytophthora infestans* (Asai et al. 2008).

What seems particularly interesting is that cell wall accumulation of hydrogen peroxide occurring in gp15 plants cannot result from the activity of *NtrbohD*. Very interestingly, Yoda and associates (2006) have reported a cryptogeiin-induced cell-

wall-associated polyamine oxidase activity in BY-2 cells, which leads to hydrogen peroxide accumulation within this compartment via the degradation of polyamines. In this same study, cells treated with cryptogeiin in the presence of α -difluoromethyl-Orn (DFMO), an inhibitor of polyamine synthesis, were greatly affected in late H₂O₂ accumulation, whereas the early generation of superoxide anions was not significantly affected. These results suggest that two systems could be involved in ROS production induced by cryptogeiin: a NADPH oxidase for the early phase and a polyamine oxidase for the late phase. The results of cytochemical localization of hydrogen peroxide presented here concurred with the possibility that two distinct systems contribute in planta to H₂O₂ accumulation on the PM and in the cell wall.

Thus, the major outcome of the present observations is that the PM-located NADPH oxidase is not the only ROS-producing system induced in tobacco plants by cryptogeiin, and that it does not govern cell-wall-associated hydrogen peroxide accumulation.

Gp15 plants are still able to develop HR and resistance to *P. parasitica* upon cryptogeiin treatment.

The following question concerns the role specifically played by this PM-associated ROS production in HR development and response to pathogens.

No significant differences could be observed between wild-type and gp15 plants, concerning their susceptibility to *P. parasitica* and *Peronospora tabacina* (data not shown). Previous results indicated that *N. benthamiana* plants silenced for *NbrbohB* are more susceptible to *Phytophthora infestans* (Asai et al. 2008; Yoshioka et al. 2003); however, infection of first leaves of *A. thaliana* seedlings with a virulent strain of *Peronospora parasitica* led to a similar development of the oomycete (estimated by the number of sporangiophores 7 days after inoculation) in the wild-type and *atrbohD* mutants (Torres et al. 2002).

Both wild-type and gp15 plants treated with cryptogeiin either by infiltration or by deposit on a fresh section of cut stem exhibited the well-described necrotic symptoms upon elicitor treatment, indicating that ROS produced by *NtrbohD* are not directly responsible for the development of the HR. This confirms previous findings of groups working on other models, because *atrboh* mutants and, particularly, *atrbohD* ones showed no clear differences with wild-type plants upon trypan blue staining, indicative of dying cells following challenging with avirulent strains of *Pseudomonas syringae* (Torres et al. 2002). This conclusion is again quite consistent with the work of Yoda and associates (2006) who reported, using both pharmacological approaches and RNAi strategy, that a cell-wall-associated polyamine oxidase involved in the late H₂O₂ accumulation was also responsible for the cell death induced by the elicitor. In that study, an inhibition of this enzyme by DFMO led to a decrease of 80% of the cell death induced by cryptogeiin on BY-2 cells. A similar decrease of cell death upon cryptogeiin treatment was observed when transgenic lines inactivated for this polyamine oxidase were compared with wild-type BY-2 cells. All these experiments have been performed using cell suspension and their conclusions thus do not refer to HR but to cell death. However, previous work published by the same group (Yoda et al. 2003) clearly indicates that polyamines accumulate in apoplasts of tobacco leaves infected with *Tobacco mosaic virus* (TMV) and that, in presence of DFMO inhibiting their synthesis, the size of necrotic lesions induced by the virus became considerably reduced. The use of guazatin, an inhibitor of polyamine oxidase activity, also drastically reduced the formation of necrotic lesions upon TMV infection, supporting the idea that polyamine oxidase could play a critical role dur-

ing hypersensitive cell death (Yoda et al. 2003). Moreover, the accumulation of hydrogen peroxide revealed by DAB staining in necroses induced by infiltration of polyamines in tobacco leaves suggests that degradation of polyamines by polyamines oxidase could lead to H₂O₂ accumulation associated with HR. Thus, the hypothesis that cell-wall-associated H₂O₂ production observed in both wild-type and gp15 tobacco plants after cryptogeiin infiltration could result from such an activity should be considered.

Application of cryptogeiin on the stem of decapitated tobacco plants has been proven to trigger a durable nonspecific resistance against a wide range of pathogens, including the oomycete *Phytophthora parasitica* (Keller et al. 1996a), and it has been demonstrated that this resistance was mediated by salicylic acid (Keller et al. 1996b). If the role of NADPH-mediated ROS production has been studied in links to either HR development following infection with avirulent strains of bacteria or fungi or natural resistance to various pathogens (Soylu et al. 2005; Torres et al. 2002, 2005; Yoshioka et al. 2003), its function in the establishment of a systemic acquired resistance (SAR) has not been widely investigated with genetic tools so far. As indicated earlier, the protection induced by cryptogeiin toward infection caused by zoospores of *P. parasitica* is similar in wild-type and gp15 plants. These results definitely rule out the possibility that ROS produced via NtrbohD could act as the translocated signal that mediates SAR. Moreover, they provide the first evidence of a dissociation between NtrbohD-mediated ROS production and other signaling events triggered by cryptogeiin and leading to the set up of plant resistance. This is consistent with the similar induction observed in wild-type and gp15 plants of transcripts of genes considered as markers of SAR, such as PR1a and PR3, or of HR such as 9 lipoxygenase (Supplementary Fig. S5). These data corroborate previous findings indicating that SAR triggered by avirulent strains of *Pseudomonas* could occur on *ntrbohD* mutants (Torres et al. 2002) and are in perfect agreement with a very recent paper (Galletti et al. 2008) indicating that, whereas *AtrbohD* mutants of *Arabidopsis* did not exhibit the oligogalacturonide (OG)-induced oxidative burst observed in wild-type plants, they exhibited similar OG-induced defense gene expression and levels of resistance to *Botrytis cinerea*. Thus, the authors conclude that the elicitor-mediated oxidative burst elicited by OG in *A. thaliana* is dispensable for the activation of defense responses effective against *B. cinerea*.

A role for NtrbohD-mediated H₂O₂ production?

The severe ultrastructural damage observed in mesophyll cells of gp15 tobacco leaves 10 h after cryptogeiin infiltration did not occur in wild-type plants. Similarly, 50% of gp3 cells treated for 30 min with cryptogeiin exhibited morphological damage, mainly associated with tonoplast lysis and distortion of vacuoles, whereas the morphology of wild-type BY-2 cells was not affected by the same treatment. This would suggest a protective effect of ROS produced by NtrbohD toward the plant cell. This is completely in agreement with the work of Torres and associates (2005) indicating that, in *Arabidopsis* plants challenged with an avirulent strain of *Pseudomonas syringae*, ROS produced by *AtrbohD* might protect cells from death by regulating ROS metabolism and counterbalance the elevation of salicylic acid levels required to initiate defense responses. This adaptive role of ROS has already been noted and concerns both biotic and abiotic stresses (Foyer and Noctor 2005). For instance, following exposure of pea leaves to cadmium, there are indications that the PM oxidative burst is primarily composed of O₂⁻, which is subsequently transformed into H₂O₂ via Cu-Zn superoxide dismutases (SOD) (Romero-Puertas et al. 2004). These authors also observed that inhibi-

tion of these SOD, preventing hydrogen peroxide formation, was associated with severely damaged cells. The involvement of NtrbohD in this PM oxidative burst following treatment of tobacco BY-2 cells with cadmium has also been further demonstrated (Garnier et al. 2006). Finally, the putative function of an intracellular signal aimed at protecting the cell is quite consistent with the cytoplasmic diffusion observed following PM accumulation. A cytoplasmic diffusion of H₂O₂, via endocytic vesicles reported as sites of ROS production, has been described following salt stress of *Arabidopsis* roots and associated with a positive effect toward salt tolerance (Leshem et al. 2007).

Conclusion

The complex role of ROS produced by plants during biotic interactions has been extensively investigated. However, the numerous indications obtained originate from quite different models and methodologies. The results reported in this article, using a unique model and different approaches to address the question from a subcellular level (origin of ROS production) to the whole plant (role of ROS during biotic interaction), bring together some pieces of the puzzle. They also improve our understanding of the tobacco cryptogeiin model, confirming the involvement of different enzymes in response to a single elicitor, leading to ROS accumulation at different times in different subcellular compartments, each of them triggering a particular response. The major outcomes of this study are to i) confirm that the first hydrogen peroxide production occurring a few minutes after challenging tobacco cells with cryptogeiin is PM located and NtrbohD mediated; ii) correlate the presence of NtrbohD in DRM fractions and the presence of NtrbohD-mediated hydrogen peroxide as patches along the PM; iii) establish that NtrbohD is also responsible for hydrogen peroxide production occurring at PM after cryptogeiin treatment of tobacco leaves; iv) indicate that such a treatment also leads to cell-wall-associated hydrogen peroxide production, independent of the previous one (a detailed study of the putative correlation between these two ROS producing systems being an attractive challenge); and v) show that NtrbohD-mediated hydrogen peroxide production does not seem to be involved in either HR development or the set up of acquired resistance but, rather, in the signaling pathways associated with plant cell protection toward damage possibly encountered during these processes. Microarray profiling of transcript experiments in wild-type and transformed plants and cells upon challenge with the elicitor would be very informative to elucidate the targets of ROS produced by NtrbohD and how they lead to appropriate adjustments in plant gene expression, metabolism, and physiology.

MATERIALS AND METHODS

Plant materials.

Tobacco (*N. tabacum*) cv. BY-2 cells and gp3 cells lacking *NtrbohD* expression (Simon-Plas et al. 2002) were grown in Chandler's medium (Chandler et al. 1972) at 25°C under continuous light (200 µE/m²/s) on a rotary shaker (125 rpm). The suspensions were subcultured every 7 days at 1:20-ml dilution in Murashige and Skoog (1962) medium supplemented with sucrose at 0.3 g/liter, KH₂PO₄ at 200 mg/liter, inositol at 100 mg/liter, morpholineethanesulfonic acid (MES) at 2 g/liter, thiamine at 1 mg/liter, and 2,4-D at 0.2 mg/liter. All experiments were performed with 7-day-old BY-2 or gp3 cells harvested, filtered, and resuspended (1 g for 10 ml) in I2 buffer, a 2 mM MES buffer, pH 5.8, containing 175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, for a 3-h equilibration period on a rotary shaker (140 rpm).

Wild-type tobacco plants and gp15 tobacco transformed with antisense constructs of *NtrbohD* (*N. tabacum* L. cv. Xanthi)

were grown in a greenhouse for the duration of the experiments. Greenhouse conditions were 22 to 25°C (day) and 12 to 16°C (night) and 40 to 80% relative humidity with a 16-h photoperiod.

Antisense gene construction and plant transformation.

An *Sst*I/*Xho*I fragment of 2,633 bp from *NtrbohD* cDNA, truncated in the 5' coding region, was inserted antisense into pKYLX71–35S2, a plant transformation vector allowing inserts to be cloned between a 35S promoter with a duplicated enhancer and *rbcS* terminator region of 700 bp (Simon-Plas et al. 2002). This fragment covered the 234-bp consensus fragment of *NtrbohA* from *N. benthamiana* used by Yoshioka and associates (2003) to silence by VIGS the *Ntrboh* subfamily in *N. benthamiana*. The resulting plasmid, introduced by triparental matings into a disarmed strain of *Agrobacterium tumefaciens*, C58C1 (pMP90), was used to obtain transformed shoots from leaf fragments of tobacco as described by Horsch and associates (1986). Homozygous transgenic lines expressing the antisense construct of *NtrbohD* were obtained and characterized for further analysis.

Pathogens.

Phytophthora parasitica aggressive strain 329 was cultivated on malt-agar medium (1% wt/vol) at 25°C and transferred monthly to fresh medium. Zoospores of *P. parasitica* were obtained as described by Pettitt and associates (2002).

Chemical and fluorescent probes.

Cryptogein (from *P. cryptogea*) was purified according to Ricci and associates (1989) and kindly provided by M. Ponchet (INRA, Antibes, France). DCFH-DA (Molecular Probes, Invitrogen, Eugene, OR, U.S.A.) was kept as a 100 mM stock solution in dimethyl sulfoxide (Allan and Fluhr 1997) at –20°C.

Elicitor treatment of suspension cells and tobacco plants.

BY-2 or gp3 cells were elicited by the addition of cryptogein at the final concentration of 50 nM. H₂O₂ accumulation was monitored by chemiluminescence as previously described (Simon-Plas et al. 2002) and, in parallel, samplings were done for H₂O₂ accumulation visualization in TEM (see below).

Fully expanded leaves of wild-type Xanthi or gp15 tobacco plants were infiltrated with 500 µl of an aqueous solution of cryptogein (1 µg/ml) (Rusterucci et al. 1996). The necrotic activity was noted and samplings of tissues in the infiltrated area were performed for both RNA extraction and cytochemical detection of H₂O₂ in TEM study.

For defense induction assays, wild-type or gp15 tobacco plants were decapitated and each sectioned stem immediately received 50 µl of an aqueous solution of cryptogein (0.04 mg/ml) whereas control plants received sterile water after decapitation.

Zoospore inoculations.

Inoculations of *P. parasitica* were performed as follows: 100 µl of a suspension containing 50 or 200 zoospores was applied to the lower side of attached leaves. The leaf surface that turned brown (indicating fungal invasion) was measured 7 days after pathogen inoculation. Plants were kept in cages equipped with brumization to ensure high relative humidity. When indicated, treatment with elicitor was applied 2 days before inoculation.

RNA extraction and analyses.

Sampling was performed 6, 8, and 10 h after infiltration of tobacco leaves with cryptogein. Total RNA was extracted and

purified, followed by a CsCl gradient according to Sambrook and associates (1989). Samples of total RNA (10 µg) were analyzed by electrophoresis in 8% formaldehyde/1.5% agarose gels and transferred to Pall Biotyne B membranes according to the manufacturer's instructions. A fragment of 0.86 kb corresponding to part of the *NtrbohD* 3' coding and untranslated region was labeled to 108 cpm µg⁻¹ using random priming and ³²P-dCTP (Amersham Pharmacia Biotech, Orsay, France). Labeling, hybridization, and washes were performed as described by Sambrook and associates (1989).

Cell fractionation.

Cells were collected by filtration, frozen in liquid N₂, and homogenized by grinding in buffer (50 mM Tris-MES, pH 8.0; 500 mM sucrose; 20 mM EDTA; 10 mM dithiothreitol; 1 mM PMSF). The homogenate was centrifuged at 16,000 × g for 20 min. After centrifugation, supernatants were collected, filtered through two successive screens (63 and 38 µm), and centrifuged at 96,000 × g for 35 min. This microsomal fraction was purified by partitioning in an aqueous two-phase system to obtain the PM fraction (Larsson et al. 1994). PM were resuspended in a buffer containing 50 mM Tris-HCl, pH 7.4; 3 mM EDTA; and 1 mM 1,4-dithiothreitol and treated with 1% Triton X-100 (wt/vol) for 30 min on ice, with very gentle shaking every 10 min. Solubilized membranes were placed at the bottom of a centrifuge tube and mixed with 60% sucrose (wt/wt) to reach a final concentration of 48% (wt/wt) and overlaid with a discontinuous sucrose gradient (40, 35, 30, and 20% [wt/wt]). After centrifugation for 20 h at 100,000 × g, fractions of 1 ml were collected and proteins were precipitated by 20% trichloroacetic acid (wt/vol). The amount of proteins present in each fraction was determined according to Schaffner and Weissman (1973) using bovine serum albumin as a standard.

Western blotting experiments.

A polyclonal rabbit serum raised against amino acids 138 to 152 (CLNKRPIPTGRFDRNK) and 784 to 798 (IAKNKGKSGSASGGC) of the *NtrbohD* protein, coupled to KLH, was obtained from Eurogentec (Seraing, Belgium). Immunoglobulin G (IgG) was then purified according to Hardie and van Regenmortel (1977) and resuspended in distilled water at a concentration of 2 mg/ml. Samples of 15 µg of protein were diluted with one volume of Laemmli buffer 2× and loaded on an 8% sodium dodecyl sulfate polyacrylamide gel. After electrophoresis separation (1 h, 40 mA), protein fractions were electroblotted onto nitrocellulose membrane (20 min, 15 V, Trans-Blot SD, semi-dry transfer cell; BioRad, Hercules, CA, U.S.A.). Probing and detection of Western blots were performed as described in the ECL Western blotting detection kit (Amersham, Tokyo). Primary antibodies were used at a dilution of 1:1000 in TBS-Tween (2 mM Tris, 15 mM NaCl, and 0.05% Tween 20, pH 7.6). The horseradish peroxidase anti-rabbit IgG antibody (BioRad) was used at a 1:25,000 dilution in TBS-Tween. The revelation was performed using an exposition time of 2 min.

Detection of ROS accumulation in epidermal peels of tobacco leaves.

Epidermal peels were removed from the abaxial surface of fully expanded leaves from wild-type or gp15 mutant tobacco plants. Peels were then placed in a petri dish containing DCFH-DA (0.5 µM final) in 5 ml of loading buffer (10 mM Tris buffer, pH 7.2) for 5 min. To wash off excess fluorescent probe, peels were then rinsed twice for 5 min in the same buffer in the dark (Allan and Fluhr 1997). Peels were then transferred in 500 µl of this buffer in a 1,000-µl perfusion chamber (Electron Microscopy Sciences, Seattle, WA, U.S.A.)

sealed on a microscope slide surface. Cryptogein (50 nM) was added directly in the chamber. Pieces of cover glasses enabled gentle fixation of the peels for observation under the microscope. To avoid illumination of the same area of the specimen preparation, image recordings were performed each minute in different areas of the sample.

Observations were performed with a DMRB (Leica, Rueil-Malmaison, France) fluorescent microscope equipped with a filter combination I3 (488-nm excitation and 515-nm barrier filters) and coupled with a Hamamatsu Orca 100 camera driven by Visilog image analysis software (Noesis, Les Ulis, France). Images from the microscope were acquired during a 200-ms exposure time.

Subcellular location of H₂O₂ accumulation in suspension cells or tobacco leaves.

To localize in situ H₂O₂ in either tobacco BY-2 or gp3 cell suspensions or tobacco leaves of wild-type and transformed gp15 plants, the cytochemical method based on the generation of cerium perhydroxides developed by Bestwick and associates (1997) was used. BY-2 or gp3 cells suspended in I2 were sampled 5, 15, and 30 min after elicitation with 50 nM cryptogein. Nonelicited cells were similarly sampled. For plant assays, tissue pieces were excised from tobacco leaf infiltrated zones 6, 8, and 10 h after treatment with cryptogein. For controls, the same samplings were done on nonelicited tobacco leaves treated with water instead of cryptogein.

Cell suspensions or leaf tissues were preincubated in freshly prepared 5 mM CeCl₃ in 50 mM 3-(N-morpholino) propane sulfonic acid (MOPS) at pH 7.2 for 45 min at room temperature. Incubation of cells or tissues in MOPS buffer without CeCl₃ was performed as negative controls. Cells were not allowed to sediment and were regularly resuspended in the incubating solution. Leaf pieces were immersed in this incubating solution under vacuum to allow a rapid penetration of CeCl₃ into plant cells. After four washes in 50 mM MOPS buffer, cells or plant tissues were fixed in a mixture of 100 mM sodium cacodylate buffer (pH 7.2) containing 1.25% (vol/vol) glutaraldehyde and 1.25% (wt/vol) paraformaldehyde for 1 h at room temperature. After fixation, samples were washed several times in the same buffer over a period of 30 min. Cells were then pelleted by low-speed centrifugation (30 s, 6,000 rpm) and embedded in 2.5% (wt/vol) agarose. Agarose blocks containing cells or leaf pieces were then treated with 1% (wt/vol) osmium tetroxide in 50 mM sodium cacodylate buffer for 1 h at room temperature. Cells or tissues were then dehydrated through a graded ethanol series and propylene oxide, and embedded in Epon (Spi-Chem, Neyco, Paris) according to the standard procedure (Luft 1961). Ultrathin sections (80 nm) were cut on a Reichert Ultracut E ultramicrotome (Leica, Rueil-Malmaison, France). We have found that staining the specimens can often add confusion; therefore, no subsequent treatment of the sections was performed to enhance contrast.

Thin sections were examined with a Hitachi H7500 transmission electron microscope (Hitachi Scientific Instruments Co., Tokyo) operating at 80 kV and equipped with an AMT camera driven by AMT software (AMT, Danvers, MA, U.S.A.).

ACKNOWLEDGMENTS

We thank M. Ponchet and Benoit Industrie from INRA Sophia-Antipolis for providing purified cryptogein; F. Panabières and M.-L. Kuhn for their help with the *Phytophthora* strain; the Department SPE of INRA for constant support, in particular a postdoctoral grant for K. Tantaoui; the Conseil Régional de Bourgogne and the Association pour la Recherche sur les Nicotianées for financial support and especially Ph.D. and postdoctoral grants to J. Morel and S. Vesa, respectively; D. Wendehenne for helpful discussion; and V. Gianinazzi-Pearson for critical reading of the manuscript.

LITERATURE CITED

- Allan, A. C., and Fluhr, R. 1997. Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. *Plant Cell* 9:1559-1572.
- 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.
- Apel, K., and Hirt, H. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55:373-399.
- Asai, S., Ohta, K., and Yoshioka, H. 2008. MAPK signaling regulates nitric oxide and NADPH oxidase-dependant oxidative burst in *Nicotiana benthamiana*. *Plant Cell* 20:1390-1406.
- Ashtamker, C., Kiss, V., Sagi, M., Davydov, O., and Fluhr, R. 2007. Diverse subcellular locations of cryptogein-induced reactive oxygen species production in tobacco bright yellow-2 cells. *Plant Physiol.* 143:1817-1826.
- Babior, B.M. 2004. NADPH oxidase. *Curr. Opin. Immunol.* 16:42-47.
- Bestwick, C. S., Brown, I. R., Bennett, M. H. R., and Mansfield, J. W. 1997. Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv. *phaseolicola*. *Plant Cell* 9:209-221.
- Bestwick, C. S., Brown, I. R., and Mansfield, J. W. 1998. Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce. *Plant Physiol.* 118:1067-1078.
- Blein, J. P., Milat, M. L., and Ricci, P. 1991. Response of cultured tobacco cells to cryptogein, a proteinaceous elicitor from *Phytophthora cryptogea*. Possible plasmalemma involvement. *Plant Physiol.* 95:486-491.
- Boka, K., Orban, N., and Kristof, Z. 2007. Dynamics and localization of H₂O₂ production in elicited plant cells. *Protoplasma* 230:89-97.
- Bolwell, G. P. 1999. Role of active oxygen species and NO in plant defence responses. *Curr. Opin. Plant Biol.* 2:287-294.
- Bolwell, G. P., and Wojtaszek, P. 1997. Mechanisms for the generation of reactive oxygen species in plant defence—a broad perspective. *Physiol. Mol. Plant Pathol.* 51:347-366.
- Briggs, R. T., Dratch, D. B., Karnovsky, M. L., and Karnovsky, M. J. 1975. Localization of NADH oxidase on the surface of human polymorphonuclear leukocytes by a new cytochemical method. *J. Cell Biol.* 67:566-596.
- Brown, I., Trethowan, J., Kerry, M., Mansfield, J., and Bolwell, G. P. 1998. Localization of components of the oxidative cross-linking of glycoproteins and of callose synthesis in papillae formed during the interaction between non-pathogenic strains of *Xanthomonas campestris* and French bean mesophyll cells. *Plant J.* 15:333-343.
- Chandler, M. T., Tandeau de Marsac, N., and de Kouchkovsky, N. 1972. Photosynthetic growth of tobacco cells in liquid suspension. *Can. J. Bot.* 50:2265-2270.
- Cona, A., Rea, G., Angelini, R., Federico, R., and Tavladoraki, P. 2006a. Functions of amine oxidases in plant development and defence. *Trends Plant Sci.* 11:80-88.
- Cona, A., Rea, G., Botta, M., Corelli, F., Federico, R., and Angelini, R. 2006b. Flavin-containing polyamine oxidase is a hydrogen peroxide source in the oxidative response to the protein phosphatase inhibitor cantharidin in *Zea mays* L. *J. Exp. Bot.* 57:2277-2289.
- Cross, A. R., and Segal, A. W. 2004. The NADPH oxidase of professional phagocytes-prototype of the NOX electron transport chain system. *Biochim. Biophys. Acta* 1657:1-22.
- Czaniński, Y., Sachot, R. M., and Cateson, A. M. 1993. Cytochemical localization of hydrogen peroxide in lignifying cell walls. *Ann. Bot.* 72:547-550.
- Davis, A. R., Mascolo, P. L., Bunger, P. L., Sipes, K. M., and Quinn, M. T. 1998. Cloning and sequencing of the bovine flavocytochrome *b* subunit proteins, gp91-phox and p22-phox: Comparison with other known flavocytochrome *b* sequences. *J. Leukoc. Biol.* 64:114-123.
- Desikan, R., Hancock, J. T., Coffey, M. J., and Neill, S. J. 1996. Generation of active oxygen in elicited cells of *Arabidopsis thaliana* is mediated by a NADPH oxidase-like enzyme. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 382:213-217.
- Dong, X., Ji, R., Guo, X., Foster, S. J., Chen, H., Dong, C., Liu, Y., Hu, X., and Liu, S. 2008. Expressing a gene encoding wheat oxalate oxidase enhances resistance to *Sclerotinia sclerotiorum* in oilseed rape (*Brassica napus*). *Planta* 228:331-340.
- Fester, T., and Hause, G. 2005. Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. *Mycorrhiza* 15:373-379.
- Foissner, I., Wendehenne, D., Langebartels, C., and Durner, J. 2000. In vivo imaging of an elicitor-induced nitric oxide burst in tobacco. *Plant J.* 23:817-824.
- Foyer, C. H., and Noctor, G. 2005. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. *Plant Cell Environ.* 28:1056-1071.

- Galletti, R., Denoux, C., Gambetta, S., Dewdney, J., Ausubel, F. M., De Lorenzo, G., and Ferrari, S. 2008. The AtRbohD-mediated oxidative burst elicited by oligogalacturonides in *Arabidopsis* is dispensable for the activation of defense responses effective against *Botrytis cinerea*. *Plant Physiol.* 148:1695-1706.
- Garnier, L., Simon-Plas, F., Thuleau, P. A., Blein, J. P., Ranjeva, R., and Montillet, J. L. 2006. Cadmium affects tobacco cells by a series of three waves of reactive oxygen species that contribute to cytotoxicity. *Plant Cell Environ.* 29:1956-1969.
- Gould, K. S., Lamotte, O., Klinguer, A., Pugin, A., and Wendehenne, D. 2003. Nitric oxide production in tobacco leaf cells: A generalized stress response? *Plant Cell Environ.* 26:1851-1862.
- Grant, M., Brown, I., Adams, S., 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.
- Groom, Q. J., Torres, M. A., Fordham-Sketon, 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.
- Hardie, G., and van Regenmortel, M. H. 1977. Isolation of specific antibody under conditions of low ionic strength. *J. Immunol. Methods* 15:305-314.
- Horsch, R. B., Klee, H. J., Stachel, S., Winans, S. C., Nester, E. W., Rogers, S. G., and Fraley, R. T. 1986. Analysis of *Agrobacterium tumefaciens* virulence mutants in leaf disks. *Proc. Natl. Acad. Sci. U.S.A.* 83:2571-2575.
- Hu, X., Bidney, D. L., Yalpani, N., Duvick, J. P., Crasta, O., Folkerts, O., and Lu, G. H. 2003. Overexpression of a gene encoding hydrogen peroxide-generating oxalate oxidase evokes defense responses in sunflower. *Plant Physiol.* 133:170-181.
- Keller, H., Blein, J. P., Bonnet, P., and Ricci, P. 1996a. Physiological and molecular characteristics of elicitor-induced systemic acquired resistance in tobacco. *Plant Physiol.* 110:365-376.
- Keller, H., Bonnet, P., Galiana, E., Pruvot, L., Friedrich, L., Ryals, J., and Ricci, P. 1996b. Salicylic acid mediates elicitor-induced systemic acquired resistance, but not necrosis in tobacco. *Mol. Plant-Microbe Interact.* 9:696-703.
- 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.
- Lamb, C. J., and Dixon, R. A. 1997. The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:251-275.
- Lamotte, O., Gould, K., Lecourieux, D., Sequeira-Legrand, A., Lebrun-Garcia, A., Durner, J., Pugin, A., and Wendehenne, D. 2004. Analysis of nitric oxide signaling functions in tobacco cells challenged by the elicitor cryptogein. *Plant Physiol.* 135:516-529.
- Lane, B. G. 2002. Oxalate, germins, and higher-plant pathogens. *IUBMB Life* 53:67-75.
- Larsson, C., Sommarin, M., and Widell, S. 1994. Aqueous Two-Phase Systems: Isolation of Highly Purified Plant Plasma Membranes and Separation of Inside-Out and Right-Side-Out Vesicles. Academic Press Inc., San Diego, CA, U.S.A..
- Lebrun-Garcia, A., Ouaked, F., Chiltz, A., and Pugin, A. 1998. Activation of MAPK homologues by elicitors in tobacco cells. *Plant J.* 15:773-781.
- 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.
- Lee, M. Y., Shin, K. H., Kim, Y. K., Suh, J. Y., Gu, Y. Y., Kim, M. R., Hur, Y. S., Son, O., Kim, J. S., Song, E., Lee, M. S., Nam, K. H., Sung, M. K., Kim, H. J., Chun, J. Y., Park, M., Ahn, T. I., Hong, C. B., Lee, S. H., Park, H. J., Park, J. S., Verma, D. P. S., and Cheon, C. I. 2005. Induction of thioredoxin is required for nodule development to reduce reactive oxygen species levels in soybean roots. *Plant Physiol.* 139:1881-1889.
- Lefebvre, B., Furt, F., Hartmann, M. A., Michaelson, L. V., Carde, J. P., Sargueil-Boiron, F., Rossignol, M., Napier, J. A., Cullimore, J., Bessoule, J. J., and Mongrand, S. 2007. Characterization of lipid rafts from *Medicago truncatula* root plasma membranes: A proteomic study reveals the presence of a raft-associated redox system. *Plant Physiol.* 144:402-418.
- Leshem, Y., Seri, L., and Levine, A. 2007. Induction of phosphatidylinositol 3-kinase-mediated endocytosis by salt stress leads to intracellular production of reactive oxygen species and salt tolerance. *Plant J.* 51:185-197.
- Luft, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* 9:409-414.
- Martinez, C., Montillet, J. L., Bresson, E., Agnel, J. P., Dai, G. H., Daniel, J. F., Geiger, J. P., and Nicole, M. 1998. Apoplastic peroxidase generates superoxide anions in cells of cotton cotyledons undergoing the hypersensitive reaction to *Xanthomonas campestris* pv. *malvacearum* race 18. *Mol. Plant-Microbe Interact.* 11:1038-1047.
- Melillo, M. T., Leonetti, P., Bongiovanni, M., Castagnone-Sereni, P., and Bleve-Zacheo, T. 2006. Modulation of reactive oxygen species activities and H_2O_2 accumulation during compatible and incompatible tomato-root-knot nematode interactions. *New Phytol.* 170:501-512.
- Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. 2004. reactive oxygen gene network of plants. *Trends Plant Sci.* 9:490-498.
- Miura, H., Bosnjak, J. J., Ning, G., Saito, T., Miura, M., and Gutterman, D. D. 2003. Role for hydrogen peroxide in flow-induced dilation of human coronary arterioles. *Circ. Res.* 92:e31-40.
- Mongrand, S., Morel, J., Laroche, J., Claverol, S., Carde, J. P., Hartmann, M. A., Bonneau, M., Simon-Plas, F., Lessire, R., and Bessoule, J. J. 2004. Lipid rafts in higher plant cells: Purification and characterization of TX-100 insoluble microdomains from tobacco plasma membrane. *J. Biol. Chem.* 279:36277-36286.
- Morel, J., Claverol, S., Mongrand, S., Furt, F., Fromentin, J., Bessoule, J. J., Blein, J.-P., and Simon-Plas, F. 2006. Proteomics of plant detergent-resistant membranes. *Mol. Cell Proteomics* 5:1396-1411.
- Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15:221-251.
- Olmos, E., Martinez-Solano, J. R., Piqueras, A., and Hellin, E. 2003. Early steps in the oxidative burst induced by cadmium in cultured tobacco cells (BY-2 line). *J. Exp. Bot.* 54:291-301.
- Osman, H., Vauthrin, S., Mikes, V., Milat, M.-L., Panabières, F., Marais, A., Brunie, S., Maume, B., Ponchet, M., and Blein, J.-P. 2001. Mediation of elicitor activity on tobacco is assumed by elicitor-sterol complexes. *Mol. Biol. Cell* 12:2825-2834.
- Pellinen, R., Palva, T., and Kangasjarvi, J. 1999. Subcellular localization of ozone-induced hydrogen peroxide production in birch (*Betula pendula*) leaf cells. *Plant J.* 20:349-356.
- Pettitt, T. R., Wakeham, A. J., Wainwright, M. F., and White, J. G. 2002. Comparison of serological, culture, and bait methods for detection of *Pythium* and *Phytophthora* zoospores in water. *Plant Pathol.* 51:720-727.
- Pike, L. J. 2006. Rafts defined: A report on the Keystone Symposium on lipid rafts and cell function. *J. Lipid Res.* 47:1597-1598.
- Rajendran, L., and Simons, K. 2005. Lipid rafts and membrane dynamics. *J. Cell Sci.* 118:1099-1102.
- Ricci, P. 1997. Induction of the hypersensitive response and systemic acquired resistance by fungal proteins: The case of elicitors. Pages 53-75 in: *Plant-Microbe Interactions*. N. S. Jacobellis, A. Collmer, S. W. Hutcheson, J. W. Mansfield, C. E. Morris, J. Murillo, N. W. Schaad, D. E. Stead, G. Surico, and M. Ullrich, eds. Kluwer Academic Chapman and Halls, Dordrecht, The Netherlands.
- Ricci, P., Bonnet, P., Huet, J.-C., Sallantin, M., Beauvais-Cante, F., Bruneteau, M., Billard, V., Michel, G., and Pernollet, J.-C. 1989. Structure and activity of proteins from pathogenic fungi *Phytophthora* eliciting necrosis and acquired resistance in tobacco. *Eur. J. Biochem.* 183:555-563.
- Romero-Puertas, M. C., Rodriguez-Serrano, M., Corpus, F. J., Gomez, M., Del Rio, L. A., and Sandalio, L. M. 2004. Cadmium-induced subcellular accumulation of O_2^- and H_2O_2 in pea leaves. *Plant Cell Environ.* 27:1122-1134.
- Rubio, M. C., James, E. K., Clemente, M. R., Bucciarelli, B., Fedorova, M., Vance, C. P., and Becana, M. 2004. Localization of superoxide dismutases and hydrogen peroxide in legume root nodules. *Mol. Plant-Microbe Interact.* 17:1294-1305.
- Rusterucci, C., Stallaert, V., Milat, M. L., Pugin, A., Ricci, P., and Blein, J. P. 1996. Relationship between active oxygen species, lipid peroxidation, necrosis, and phytoalexin production induced by elicitors in *Nicotiana*. *Plant Physiol.* 111:885-891.
- Sagi, M., and Fluhr, R. 2006. Production of reactive oxygen species by plant NADPH oxidases. *Plant Physiol.* 141:336-340.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A..
- Schaffner, W., and Weissman, C. 1973. A rapid, sensitive, and specific method of the determination of protein in dilute solution. *Anal. Biochem.* 56:502-514.
- Simon-Plas, F., Rustérucci, C., Milat, M.-L., Humbert, C., Montillet, J.-L., and Blein, J.-P. 1997. Active oxygen species production in tobacco cells elicited by cryptogein. *Plant Cell Environ.* 20:1573-1579.
- 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.
- Soares, F. A., Shaughnessy, S. G., MacLarkey, W. R., and Orr, F. W. 1994. Quantification and morphologic demonstration of reactive oxygen species produced by Walker-256 tumor cells in vitro and during metastasis in vivo. *Lab. Invest.* 71:480-489.

- Soylu, S., Brown, I., and Mansfield, J. W. 2005. Cellular reactions in *Arabidopsis* following challenge by strains of *Pseudomonas syringae*: From basal resistance to compatibility. *Physiol. Mol. Plant Pathol.* 66:232-243.
- Tarpey, M. M., Wink, D. A., and Grisham, M. B. 2004. Methods for detection of reactive metabolites of oxygen and nitrogen: In vitro and in vivo considerations. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 286:R431-444.
- Torres, M. A., Onuchi, H., Hamada, S., Machida, G., Hammond-Kosak, K. E., Robinson, N. J., 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., Dangel, J., 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. U.S.A.* 99:517-522.
- Torres, M. A., Jones, J. D. G., and Dangel, 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 Dangel, J. L. 2006. Reactive oxygen species signaling in response to pathogens. *Plant Physiol.* 141:373-378.
- Vauthrin, S., Mikes, V., Milat, M. L., Ponchet, M., Maume, B., Osman, H., and Blein, J. P. 1999. Elicitins trap and transfer sterols from micelles, liposomes and plant plasma membranes. *Biochim. Biophys. Acta* 1419:335-342.
- Warren, J. S., Kunkel, R. G., Simon, R. H., Johnson, K. J., and Ward, P. A. 1989. Ultrastructural cytochemical analysis of oxygen radical-mediated immunoglobulin A immune complex induced lung injury in the rat. *Lab. Invest.* 60:651-658.
- Wendehenne, D., Binet, M. N., Blein, J. P., Ricci, P., and Pugin, A. 1995. Evidence for specific, high-affinity binding sites for a proteinaceous elicitor in tobacco plasma membrane. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 374:203-207.
- Wendehenne, D., Lamotte, O., Frachisse, J. M., Barbier-Brygoo, H., and Pugin, A. 2002. Nitrate efflux is an essential component of the cryptogein signaling pathway leading to defense responses and hypersensitive cell death in tobacco. *Plant Cell* 14:1937-1951.
- Wojtaszek, P. 1997. Oxidative burst: An early plant response to pathogen infection. *Biochem. J.* 322:681-692.
- Yoda, H., Yamaguchi, Y., and Sano, H. 2003. Induction of hypersensitive cell death by hydrogen peroxide produced through polyamine degradation in tobacco plants. *Plant Physiol.* 132:1973-1981.
- Yoda, H., Hiroi, Y., and Sano, H. 2006. Polyamine oxidase is one of the key elements for oxidative burst to induce programmed cell death in tobacco cultured cells. *Plant Physiol.* 142:193-206.
- 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 H₂O₂ accumulation and resistance to *Phytophthora infestans*. *Plant Cell* 15:706-718.
- Zhang, S., Du, H., and Klessig, D. F. 1998. Activation of the tobacco SIP kinase by both a cell wall-derived carbohydrate elicitor and purified proteinaceous elicitors from *Phytophthora* spp. *Plant Cell* 10:435-449.