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Integrated Signaling Network Involving Calcium, Nitric Oxide, and Active Oxygen Species but Not Mitogen-Activated Protein Kinases in BcPG1-Elicited Grapevine Defenses

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We have already reported the identification of the endopolygalacturonase 1 (BcPG1) from *Botrytis cinerea* as a potent elicitor of defense responses in grapevine, independently of its enzymatic activity. The aim of the present study is the analysis of the signaling pathways triggered by BcPG1 in grapevine cells. Our data indicate that BcPG1 induces a Ca^{2+} entry from the apoplast, which triggers a phosphorylation-dependent nitric oxide (NO) production via an enzyme probably related to a NO synthase. Then NO is involved in i) cytosolic calcium homeostasis, by activating Ca^{2+} release from internal stores and regulating Ca^{2+} fluxes across the plasma membrane, ii) plasma membrane potential variation, iii) the activation of active oxygen species (AOS) production, and iv) defense gene expression, including phenylalanine ammonia lyase and stilbene synthase, which encode enzymes responsible for phytoalexin biosynthesis. Interestingly enough, mitogen-activated protein kinase (MAPK) activation is independent of this regulation pathway that closely connects Ca^{2+} , NO, and AOS.

Additional keywords: depolarization.

In the course of their development, plants are frequently challenged by potential pathogens. However, disease development is more an exception than a rule, for plants possess preformed structures, constitutively produced chemicals, or both, which protect them from infections. Furthermore, when a microbe gets over these constitutive barriers, plants are able to activate inducible defense responses, including reinforcement of the cell wall, production of active oxygen species (AOS), and expression of defense-related genes that encode enzymes involved in the biosynthesis of phytoalexins and pathogenesis-related proteins such as chitinases or glucanases (Yang et al. 1997). These defense reactions are first expressed in the cells located at the very site of infection, and then, they extend to noninfected cells. This process, known as systemic acquired resistance, confers a protection to the whole plant against a broad spectrum of pathogens for several weeks (Durrant and

Dong 2004). Sometimes, defense reactions are associated with necrotic lesions at the pathogen penetration site. This hypersensitive response confines the pathogen at its infection site and prevents it from spreading to the whole host plant (Lam et al. 2001).

The activation of plant defense responses is initiated by the molecular recognition of the invading pathogen. Microbe perception is mediated thanks to receptors that detect some molecules synthesized by the pathogens or released from the microbe or plant cell walls after enzymatic hydrolysis. These molecules, known as elicitors, bind to plant plasma membrane or cytosolic proteins (Nimchuk et al. 2003). Protein phosphorylation and dephosphorylation through the activation of protein kinases and phosphatases is thought to transduce the defense signals to different downstream effectors, thus leading to defense responses (Felix et al. 1993; Lecourieux-Ouaked et al. 2000; Peck et al. 2001). Protein kinases are believed to regulate the transduction of the elicitor signal positively, whereas type 1 or 2A protein phosphatases (PP1 and PP2A), or both, are supposed to regulate it negatively to prevent a constitutive activation under normal conditions (Lecourieux-Ouaked et al. 2000). Among the different protein kinases activated during plant defense signaling, mitogen-activated protein kinases (MAPK) have been extensively studied (Romeis 2001), e.g., SIPK (salicylate-induced protein kinase) and WIPK (wound-induced protein kinase) in tobacco after infection or elicitor treatment (Lebrun-Garcia et al. 1998; Zhang et al. 1998). A modification of the plasma membrane permeability is one of the earliest responses detected. In particular, Ca^{2+} , H^+ , K^+ , and anion fluxes have often been reported (Nürnberg and Scheel 2001). Among these ions, Ca^{2+} plays a central role in the signal transduction leading to plant defense activation in response to microbial invasion (Yang et al. 1997). Elicitor-responsive Ca^{2+} -permeable ion channels located in the plasma membrane of plant cells may mediate elicitor-induced Ca^{2+} influx from the extracellular medium (Zimmermann et al. 1997). Moreover, the influx of extracellular Ca^{2+} into elicitor-treated cells has been shown to cause specific variations of free cytosolic calcium concentrations that are essential for the activation of defense responses (Knight et al. 1991; Lecourieux et al. 2002).

Besides Ca^{2+} influx, the production of superoxide anions (O_2^-) rapidly dismutated into H_2O_2 by a superoxide dismutase is another feature of plant defense. The major enzymatic source of AOS is an NADPH-oxidase, localized in the plasma membrane, which catalyzes the reduction of O_2 to O_2^- (Torres and Dangl 2005). The functions of AOS in plant defense are far

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from being understood. The release of AOS may have direct toxic effects on the pathogen. H₂O₂ also leads to the cross-linking of cell-wall structural components, rendering the cell wall more resistant to digestion by microbial enzymes (Bradley et al. 1992). Moreover, AOS play a role in elicitor signal transduction and in plant defense mechanisms. For instance, AOS could participate in phytoalexin production and could be involved in the hypersensitive response, depending on plant-pathogen systems (Apel and Hirt 2004). The seeming discrepancy between these results could be explained by the synergistic roles of H₂O₂ and nitric oxide (NO) (Delledonne et al. 2001).

Besides AOS, NO has been identified as an important messenger in plant defense signaling pathways (Lamotte et al. 2005). A rapid accumulation of NO has been observed in soybean cells inoculated with avirulent *Pseudomonas syringae* pv. *glycinea* (Delledonne et al. 1998), in *Arabidopsis* cells challenged with avirulent *Pseudomonas syringae* pv. *maculicola* race m6 (Clarke et al. 2000), and in tobacco leaves or cells treated with the fungal elicitor cryptogein (Foissner et al. 2000; Lamotte et al. 2004). Two structurally unrelated enzymes catalyzing the synthesis of NO in plants have been identified; one is a nitrate reductase (Desikan et al. 2002), the other is abscisic acid-activated AtNOS1 (Guo et al. 2003). Although AtNOS1 shares very little sequence identity with mammalian NO synthase (NOS), it oxidizes L-arginine to L-citrulline and NO through a Ca²⁺/calmodulin (CaM)- and NADPH-dependent reaction, like animal NOS. Using NO donors, NO has been shown to induce the expression of various genes related to defense responses (Delledonne et al. 1998; Durner et al. 1998; Polverari et al. 2003). In animals, the NO signal is partly mediated by two second messengers, cyclic GMP (cGMP) and cyclic ADP ribose (cADPR). cADPR is a Ca²⁺ mobilizing second messenger that binds to the ryanodin receptor (RyR)-type Ca²⁺-permeable channels and therefore activates Ca²⁺ release from intracellular stores into the cytosol. A similar transduction system appears to function in plants (Durner et al. 1998; Garcia-Mata et al. 2003; Lamotte et al. 2004).

In 2003, we reported that endopolygalacturonase 1 (BcPG1) from *Botrytis cinerea*, which is necessary for full virulence of *B. cinerea* (Ten Have et al. 1998), was an elicitor of defense responses in grapevine (Poinssot et al. 2003). BcPG1 triggers Ca²⁺ influx, H₂O₂ production, MAPK activation, defense related gene expression, and phytoalexin biosynthesis in grapevine cells. However, although the elicitor activity of BcPG1 can originate from the oligogalacturonides (OGA) that it released, we demonstrated that grapevine defense response activation was due to the recognition of specific motifs of the protein. Indeed, OGA (degree of polymerization of 9 to 20) are able to induce defense responses in grapevine cells, but the intensity and kinetics of the events triggered by OGA are very different from the effects of BcPG1. Moreover, chemical treatments of BcPG1 and desensitization assays have allowed us to discriminate between enzymatic and elicitor activities. The production of phytoalexins such as resveratrol is one of the major defense pathways in grapevine (Langcake 1981). Stilbene synthase is a key enzyme of phytoalexin biosynthesis; overexpressing the corresponding grapevine gene in tobacco plants leads to a better resistance of the transgenic plants to infection by *B. cinerea* (Hain et al. 1993). In addition, the genetic transformation of grapevine with the coding sequence of stilbene synthase under the control of a pathogen-inducible promoter increases resveratrol production during fungal infection, making plants more resistant to *B. cinerea* (Coutos-Thévenot et al. 2001). Furthermore, the treatment of grapevine leaves with NADPH-oxidase or Ser/Thr protein kinase inhibitors increases susceptibility to *Botrytis cinerea*, suggesting that the oxidative

burst and protein phosphorylation might play a major role in this resistance (Aziz et al. 2004). In this context, knowing more about BcPG1-induced signaling pathways will allow us to better understand the mechanisms of grapevine defenses.

In the present work, our data describe the origin and the involvement of free cytosolic calcium variations and NO production in BcPG1-elicited grapevine cells, with close links between calcium fluxes, NO synthesis, and AOS production. In our model, NO is shown to activate AOS production and defense gene expression, to regulate calcium fluxes through endomembranes but also the plasma membrane, whereas MAPK activation is independent of all monitored events.

RESULTS

Elevations of free cytosolic calcium concentration in response to BcPG1 are due to calcium influx from the extracellular medium and to calcium release from internal stores.

Calcium influx. The ability of BcPG1 to induce a Ca²⁺ influx in grapevine cells has been assessed using ⁴⁵Ca²⁺ as a tracer. BcPG1 triggers a Ca²⁺ influx reaching 0.12 ± 0.02 μmol per gram of fresh weight cell (FWC) after a 40-min treatment (Fig. 1A). The Ca²⁺ influx is suppressed by the addition of the Ca²⁺ chelators EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) (Fig. 1A) and BAPTA (1,2-bis(aminophenoxy)ethane-N,N,N',N'-tetra acetate) or the Ca²⁺ surrogate Gd³⁺ (data not shown) in the extracellular medium.

Free cytosolic calcium concentration variations. Given the role of free Ca²⁺ as a second messenger, we have investigated variations of free cytosolic Ca²⁺ concentration ([Ca²⁺]_{cyt}), using grapevine cells transformed with the gene encoding apoaequorin addressed to the cytosol. The luminescence intensity of this protein depends on free Ca²⁺ concentration in the targeted compartment. In control cells, the resting [Ca²⁺]_{cyt} is 150 ± 45 nM during the assay period. BcPG1 induces a biphasic elevation of [Ca²⁺]_{cyt} (Fig. 1B). A first elevation starts within 1 min, peaks at 1.3 ± 0.15 μM after 5 min, and then, decreases to 0.92 ± 0.03 μM after 15 min. This first peak is immediately followed by a second [Ca²⁺]_{cyt} increase, which reaches about 1 ± 0.1 μM 30 min after the beginning of the treatment and then decreases slowly but does not return to the background level before 2 h (data not shown).

Origin of the mobilized calcium. We next investigated the origin of the Ca²⁺ responsible for [Ca²⁺]_{cyt} elevations. To check the role of extracellular Ca²⁺ uptake on the Ca²⁺ signature, aequorin-transformed cells have been pretreated with Ca²⁺ chelators or a Ca²⁺ surrogate. The addition of Ca²⁺ chelators EGTA (Fig. 1C) or BAPTA or the Ca²⁺ surrogate Gd³⁺ (data not shown) suppresses the [Ca²⁺]_{cyt} increases induced by BcPG1, indicating that [Ca²⁺]_{cyt} elevations depend first on the Ca²⁺ influx from the extracellular medium. Moreover, the BcPG1-induced Ca²⁺ influx not only initiates [Ca²⁺]_{cyt} variations but is also involved in keeping [Ca²⁺]_{cyt} high during elicitation. Indeed, the addition of EGTA 15 min after BcPG1 treatment immediately abolishes the second rise (Fig. 1C). The involvement of Ca²⁺ from intracellular stores in BcPG1-induced [Ca²⁺]_{cyt} elevations has been checked using neomycin (25 μM), a phospholipase C antagonist, and U73122 (2 μM), a specific phospholipase C inhibitor, both inhibitors preventing IP₃-mediated effects on IP₃-gated Ca²⁺ channels in animal and plant cells (Berridge 1993). We have also tested the effect of ruthenium red (RR; 5 μM), which inhibits Ca²⁺ release via RyR-like channels when used at low concentration (Sanders et al. 1995). In BcPG1-treated cells, neomycin, U73122, and RR have the same effect on the Ca²⁺ signal (Fig. 1D); the first rise is limited to a sharp peak at 1 μM after 3 min, whereas the sec-

ond sustained peak is not affected. This result suggests that, in BcPG1-treated cells, the first $[Ca^{2+}]_{cyt}$ increase (0 to 15 min) may correspond to overlapping peaks of Ca^{2+} from different origins; the first one peaks after 3 min and corresponds to about 25% of the entire peak, probably corresponding to extracellular Ca^{2+} because of its insensitivity to neomycin, U73122, and RR, and the second one, inhibited by neomycin, U73122, and RR, occurring later (between 3 and 6 min) and corresponding to Ca^{2+} efflux from organelles. So, according to results obtained adding EGTA 15 min after BcPG1-treatment (Fig. 1C), the long-lasting sustained Ca^{2+} elevation that is insensitive to neomycin, U73122, and RR might be due to Ca^{2+} influx from the extracellular medium.

Calcium influx and subsequent $[Ca^{2+}]_{cyt}$ variations are regulated by one or more phosphorylation/dephosphorylation events.

Many reports indicate that the Ca^{2+} influx triggered by elicitors is regulated by upstream phosphorylation events (Conrath et al. 1991; Kohler and Blatt 2002). Surprisingly, in our model, staurosporine (Fig. 2A) and K252-a (data not shown), general Ser-Thr protein kinase inhibitors, increase by 50% the Ca^{2+} influx triggered by BcPG1.

Moreover, staurosporine (Fig. 2B) and K252-a (data not shown) modify the calcium signal induced by BcPG1 in

aequorin-transformed cells. The first $[Ca^{2+}]_{cyt}$ increase is reduced (from 1.3 to 1.0 μM) whereas the intensity of the second peak is increased (from 1 to 1.4 μM), in agreement with increased Ca^{2+} influx measured in presence of staurosporine or K252-a. Taken together, these results suggest that a protein kinase could negatively regulate Ca^{2+} influx and highlight a role for phosphorylation/dephosphorylation events in the control of Ca^{2+} fluxes and homeostasis in plant defense responses.

BcPG1-induced NO production is involved in plasma membrane depolarization, calcium homeostasis, AOS production, and gene expression.

NO production. NO production in grapevine cells has been detected using the specific probe 4,5-diaminofluorescein diacetate (DAF-2DA), a membrane-permeable derivative of the NO-sensitive fluorophore DAF-2. NO and its oxidized form, N_2O_3 , convert DAF-2 to DAF-2T (Kojima et al. 1998), whose fluorescence allows NO measurement both in plant tissues and cell suspensions (Foissner et al. 2000; Lamotte et al. 2004). Fluorescence increases within 10 min and then remains stable after about 30 min of BcPG1-treatment (Fig. 3A). Given the short half-life time of NO (3 s), values do not represent the actual amount of NO present in cells at any given moment but the accumulation of DAF-2T during the treatment. Therefore, our results indicate that elicitor-induced NO production is tran-

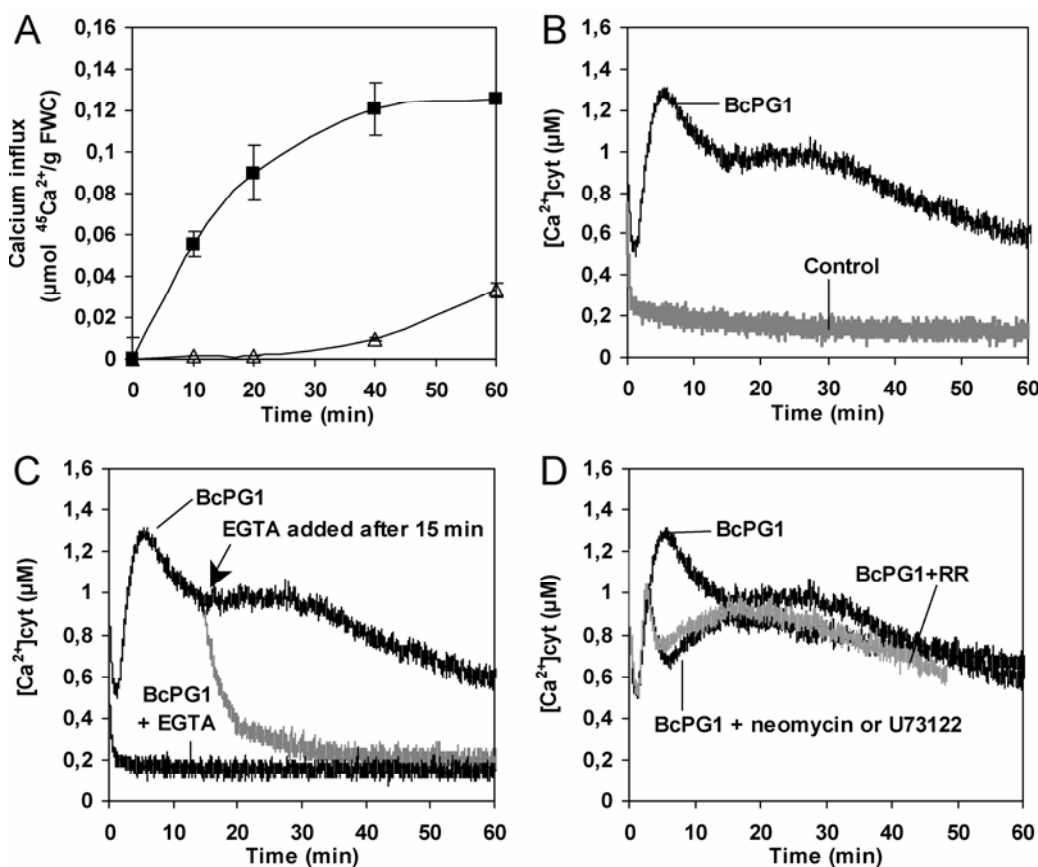


Fig. 1. BcPG1 induces calcium influx and variations of free cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) in grapevine cells depending on Ca^{2+} from extracellular medium and internal stores. **A**, Time course of Ca^{2+} influx induced by BcPG1 (5 μg per gram of fresh weight cell (FWC) and effect of a Ca^{2+} chelator in the extracellular medium. $^{45}Ca^{2+}$ (0.033 MBq per gram of FWC) used as a tracer was added in cell suspensions 7 min before BcPG1. Cells were treated with BcPG1 (■) or BcPG1 and 2 mM EGTA (Δ). The inhibitor was added to the cell suspensions 10 min before elicitation. Aliquots were withdrawn at the indicated times and influx was determined. Values shown are means \pm standard error of duplicate assays of three independent experiments. Values in control cells were subtracted. **B**, Variations of $[Ca^{2+}]_{cyt}$ in aequorin-transformed grapevine cells during a treatment with BcPG1 (5 μg per gram of FWC). **C**, Effect of a Ca^{2+} chelator on changes in $[Ca^{2+}]_{cyt}$ in aequorin-transformed grapevine cells treated with BcPG1 (5 μg per gram of FWC). EGTA (2mM) was added to the extracellular medium 10 min before BcPG1 or 15 min after BcPG1 (as indicated by arrow). **D**, Effects of neomycin, U73122, and ruthenium red (RR) on $[Ca^{2+}]_{cyt}$ changes in BcPG1-treated grapevine cells. Neomycin (25 μM), U73122 (2 μM), or RR (5 μM) were added 10 min before BcPG1. The curve obtained with U73122 was omitted because it was superimposed on that of neomycin. Data correspond to the mean of 15 independent experiments.

sient during the first half hour of treatment and the rate of production drops to zero 30 min after BcPG1 exposure. The addition of the specific NO scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) totally suppresses the fluorescence triggered by the elicitor, confirming that the increase of fluorescence is specifically due to NO (Fig. 3B).

To test whether a NOS-like enzyme is involved in BcPG1-induced NO production, we have assessed the effect of the NOS inhibitors L-NAME, a methyl ester derivative of the NOS substrate L-Arg (Delledonne et al. 1998; Durner et al. 1998) and pBITU, another potentially highly competitive inhibitor of NOS. These inhibitors reduce NO production by about 55 and 80%, respectively (Fig. 3B), suggesting that a NOS-like enzyme may be responsible for NO synthesis in grapevine. Both EGTA and staurosporine strongly reduce NO production in BcPG1-treated cells (Fig. 3B), indicating that NO synthesis in response to BcPG1 is strictly dependent on Ca^{2+} influx and one or more staurosporine-sensitive phosphorylation events.

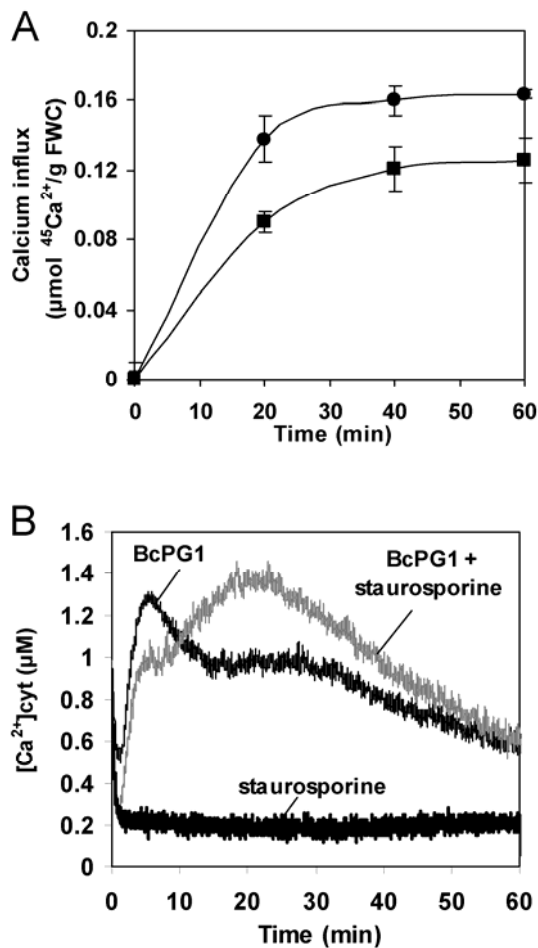


Fig. 2. Effect of a Ser-Thr protein kinase inhibitor in BcPG1-induced Ca^{2+} influx and free cytosolic calcium concentration ($[Ca^{2+}]_{\text{cyt}}$) changes. **A**, Effect of a general inhibitor of Ser-Thr protein kinase on the time course of Ca^{2+} influx induced by BcPG1 (5 μg per gram of fresh weight cell [FWC]). $^{45}\text{Ca}^{2+}$ (0.033 MBq per gram of FWC) used as a tracer was added in cell suspensions 7 min before BcPG1. Cells were treated with BcPG1 (■) or BcPG1 and 2 μM staurosporine (●). The inhibitor was added to the cell suspensions 10 min before BcPG1. Aliquots were withdrawn at the indicated times and influx was determined. Values shown are means \pm standard error of duplicate assays of three independent experiments. Values in control cells were subtracted. **B**, Effect of staurosporine on $[Ca^{2+}]_{\text{cyt}}$ changes in BcPG1-treated grapevine cells. Staurosporine (2 μM) was added 10 min before BcPG1. Data correspond to the mean of 15 independent experiments.

Because cPTIO is the most efficient compound in suppressing BcPG1-induced NO accumulation, we have used it to study the involvement of NO in downstream signaling events triggered by BcPG1.

Effect of NO on Ca^{2+} fluxes across endomembranes and the plasma membrane. We have analyzed the contribution of NO to BcPG1-induced Ca^{2+} influx and $[Ca^{2+}]_{\text{cyt}}$ variations by scavenging NO with cPTIO in elicitor-treated grapevine cells. In such conditions, the extracellular Ca^{2+} entry is increased 1.5-fold (Fig. 4A). In addition, the intensity and the duration of the first $[Ca^{2+}]_{\text{cyt}}$ rise are reduced (Fig. 4B) to the same levels as with neomycin or RR (Fig. 1D) and the duration of the second $[Ca^{2+}]_{\text{cyt}}$ peak is increased (Fig. 4B). Assuming that this second rise, which is insensitive to neomycin and RR, corresponds to

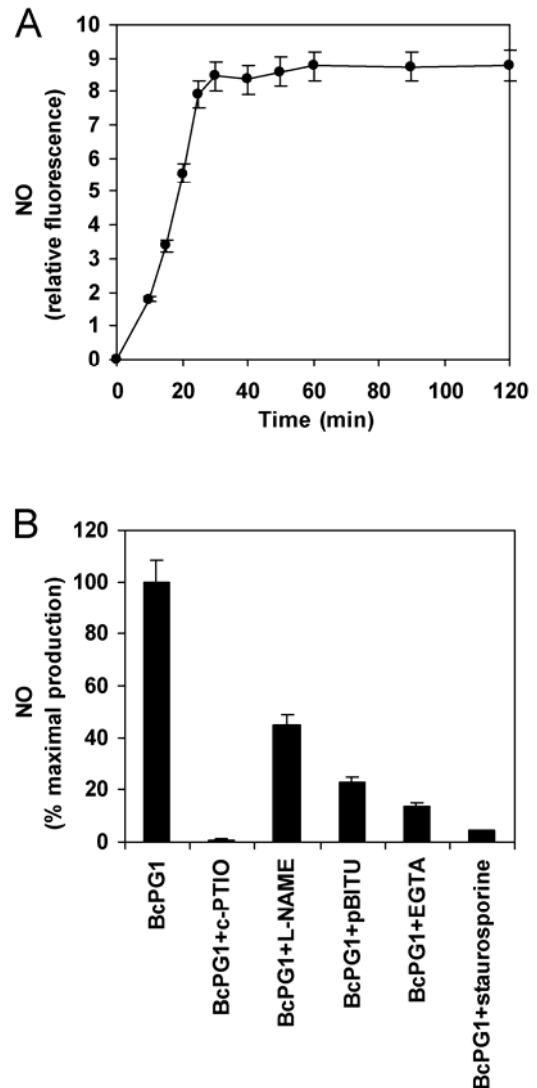


Fig. 3. Origin and dependence of nitric oxide (NO)-cumulated production in BcPG1-treated grapevine cells. **A**, Time course of NO-cumulated production induced by BcPG1. Cells were treated with BcPG1 (5 μg per gram of fresh weight cell [FWC]) (●). NO-cumulated production was monitored using 4,5-diaminofluorescein diacetate as a fluorescent probe. Values of control cells (H_2O) were subtracted. Values shown are means \pm standard error (SE) of duplicate assays of three independent experiments. **B**, Effect of different inhibitors and of a NO scavenger on BcPG1-induced NO-cumulated production. Cells were treated with BcPG1 (5 μg per gram of FWC) and 500 μM cPTIO or 10 mM L-NAME or 2 mM pBITU or 2 mM EGTA or 2 μM staurosporine. Inhibitors and the NO scavenger were added 10 min before BcPG1 treatment. NO-cumulated production was measured 30 min after BcPG1-treatment. Values shown are means \pm SE of duplicate assays of three independent experiments.

Ca²⁺ from the extracellular medium, this effect may originate from the increase in Ca²⁺ influx observed in the presence of cPTIO in elicitor-treated cells (Fig. 4A). Taken together, these results suggest that NO negatively regulates the elicitor-triggered Ca²⁺ influx or activates Ca²⁺ efflux while promoting Ca²⁺ release from internal stores through the activation of intracellular Ca²⁺-permeable channels sensitive to neomycin and RR.

Involvement of NO in membrane depolarization. Some Ca²⁺-permeable channels located on the plasma membrane are sensitive to membrane potential (Kurusu et al. 2004), and NO is known to modulate plasma membrane potential by acting on ion channels (Fischmeister et al. in press). So, we have hypothesized that NO could control Ca²⁺ fluxes across the plasma membrane (discussed above) via plasma membrane potential regulation. To test this hypothesis, we have used the voltage-sensitive dye DiBac₄ (Willmott et al. 2000). The efficiency of this probe to monitor plasma membrane depolarization in grapevine cells has been previously confirmed by adding increasing concentrations of extracellular K⁺ (data not shown). When applied on grapevine cells, BcPG1 induces a transient

membrane depolarization that occurs within a few seconds and peaks after 20 min (Fig. 4C). The cells then repolarize back to their initial basal level within 35 min. When the cells are co-treated with BcPG1 and NO scavenger cPTIO, depolarization is not significantly modified during the first 10 min (Fig. 4C). However, after about 15 min, fluorescence strongly decreases for at least 1 h, reflecting a hyperpolarization of the plasma membrane. Indeed, scavenging NO leads to a much more negative plasma membrane potential difference as compared with the control. Hence, this result suggests that NO could delay plasma membrane re-polarization and suppress hyperpolarization after BcPG1-induced plasma membrane depolarization.

Regulation of AOS production. We have tested the involvement of extracellular Ca²⁺, protein kinase activity, and NO on AOS production, measured using chemiluminescence of luminol or fluorescence of pyranine. EGTA, staurosporine, or cPTIO abolishes the H₂O₂ production triggered by BcPG1 (Fig. 5A). By contrast, no significant effect is observed with neomycin or RR. Taken together, these results suggest that AOS production depends on Ca²⁺ influx, protein kinase activ-

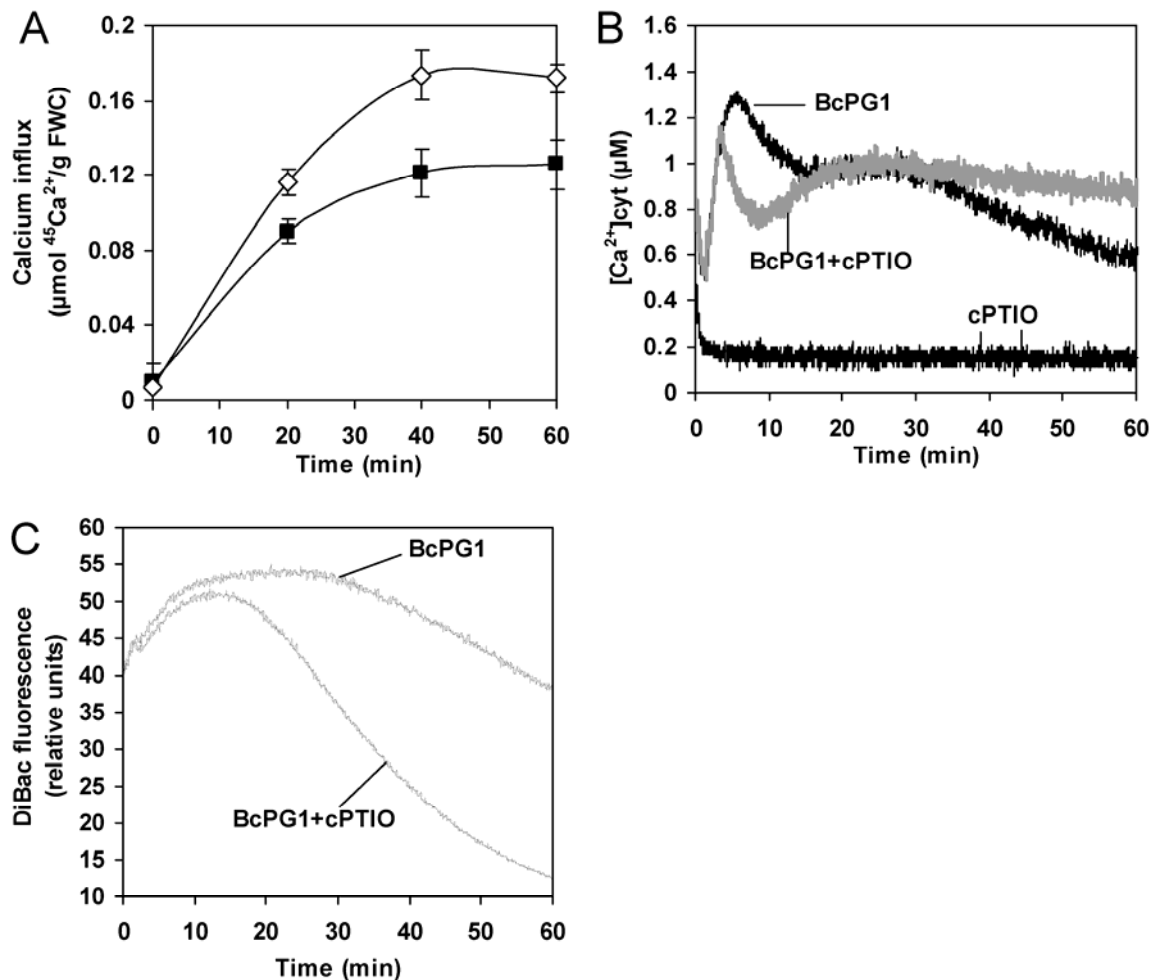


Fig. 4. Involvement of nitric oxide (NO) in Ca²⁺ fluxes and plasma membrane potential variations in BcPG1-treated grapevine cells. **A**, Ca²⁺ influx in BcPG1 grapevine cells in the presence of the NO scavenger cPTIO. Cells were treated with BcPG1 (5 μg per gram of fresh weight cell [FWC]) (■) or cotreated with BcPG1 and 500 μM cPTIO (◇). cPTIO was added 10 min prior to BcPG1. Values shown are means ± standard error of duplicate assays of three independent experiments. Values of control cells (H₂O or cPTIO) were subtracted. In control cells, cPTIO had no effect on Ca²⁺ influx. **B**, BcPG1-induced variations of free cytosolic calcium concentration in the presence of the NO scavenger cPTIO. Experiments were carried out using aequorin-transformed grapevine cell suspensions. cPTIO (500 μM) was added 10 min before treatment with BcPG1. Values shown are means of 15 independent experiments. **C**, Plasma membrane potential variations in the presence of the NO scavenger cPTIO in BcPG1-treated grapevine cells. Cells were treated with BcPG1 (5 μg per gram of FWC) or cotreated with BcPG1 and 500 μM cPTIO. cPTIO was added 10 min prior to BcPG1. cPTIO alone, omitted for clarity, did not induce any changes in fluorescence during the time of the experiment. Plasma membrane potential changes were monitored using the probe DiBac₄ (Bis(1,3-dibarbituric acid)-trimethine), whose fluorescence increased when accumulated in depolarized cells (λ_{ex} = 412nm; λ_{em} = 540nm). Values shown are means of three independent experiments.

ity, and NO but is independent of neomycin- and RR-sensitive Ca^{2+} release from internal stores.

The involvement of NO in AOS production has been verified using another fluorescent probe to monitor H_2O_2 production. Peroxynitrites are reported to increase the luminescence of luminol (Radi et al. 1993), and the NO donor DEA-NONOate interferes with the luminol assay (our data). Using the fluorescent probe pyranine, whose fluorescence decreases proportionally to the H_2O_2 amount (Lecourieux-Ouaked et al. 2000), we observed that the NO scavenger cPTIO suppresses the decrease in fluorescence monitored in BcPG1-treated cells (Fig. 5B). The capacity for NO to trigger H_2O_2 production has been confirmed by adding the NO donor DEA-NONOate to cell suspensions and measuring pyranine fluorescence changes. The NO donor induces a decrease in fluorescence, which is suppressed in the presence of cPTIO (Fig. 5B), indicating that NO could activate H_2O_2 production. Thus, NO, whether produced in response to BcPG1 or released by the NO donor, leads to AOS production in grapevine cells. As explained below, our

data support a NO-dependent activation of AOS production rather than the inhibition of AOS-degrading enzymes by NO.

Effect of NO on defense gene expression. Phenylalanine ammonia lyase (PAL-1) and stilbene synthase (VST-1) are two key enzymes of the biosynthesis pathway of phytoalexins, such as trans-resveratrol and its dimer ϵ -viniferin, produced by grapevine cells in response to BcPG1 (Poinssot et al. 2003). We studied the effect of NO on *PAL-1* and *VST-1* transcript accumulation during BcPG1 treatment by scavenging NO with cPTIO. Figure 5C shows that, in the presence of cPTIO, *PAL-1* transcript accumulation is deeply reduced and *VST-1* transcript accumulation is abolished after a 3-h treatment (when the accumulation of both transcripts is expected to peak; Poinssot et al. 2003). This result suggests that NO is involved in the activation of both genes and may participate in the regulation of phytoalexin biosynthesis. However, the NO donor DEA-NONOate does not induce *PAL-1* and *VST-1* transcript accumulation in grapevine cells (Fig. 5D). Taken together, these results suggest that, on the one hand, NO production in response to BcPG1 is involved in

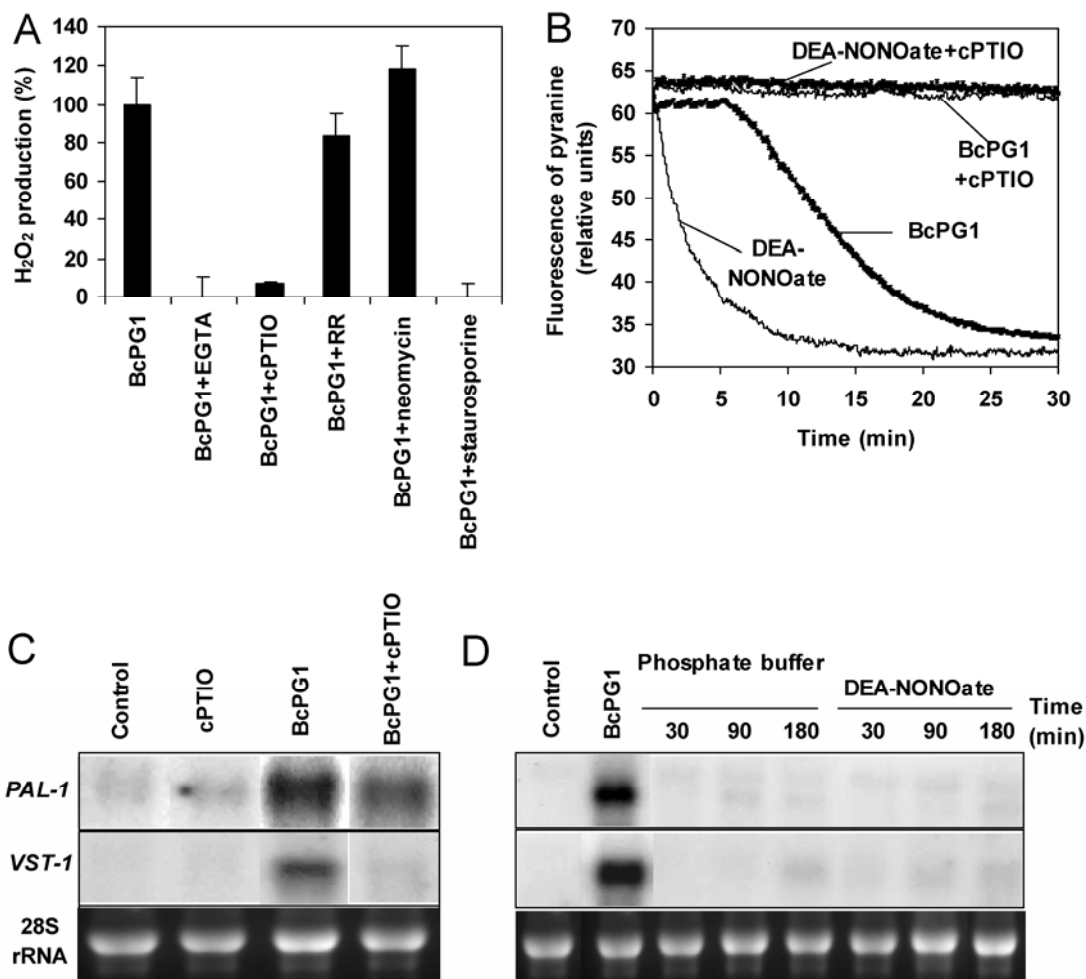


Fig. 5. Involvement of nitric oxide (NO) in H_2O_2 production and defense-gene expression in BcPG1-treated grapevine cells. **A**, Effects of different inhibitors on BcPG1-induced H_2O_2 production. Cells were treated with BcPG1 ($5 \mu\text{g}$ per gram of fresh weight cell [FWC]; control) in the presence of 2 mM EGTA or 500 μM cPTIO or 5 μM ruthenium red (RR) or 25 μM neomycin or 2 μM staurosporine. Inhibitors were added 10 min prior to BcPG1. H_2O_2 production was measured using chemiluminescence of luminol, 20 min after elicitation (750 ± 60 nmoles per gram of FWC in control cells). Values are means \pm standard error of three independent experiments. **B**, H_2O_2 production induced by the NO donor diethylamine-NONOate (DEA-NONOate) (5 mM) in grapevine cells. H_2O_2 production was measured monitoring the fluorescence decrease of pyranine. cPTIO (500 μM) was added 10 min prior to BcPG1. **C**, Effect of NO in BcPG1-mediated mRNA accumulation. The NO scavenger cPTIO (500 μM) was added 10 min before BcPG1 ($5 \mu\text{g}$ per gram of FWC). mRNA accumulation for *PAL-1* and *VST-1* was monitored by Northern blotting after a 3-h BcPG1 treatment. Total RNA was extracted from cells, and 10 μg was subjected to Northern blot analysis using *PAL-1* and *VST-1* cDNA clones as probes. Ethidium bromide-stained 28S rRNA is shown as a gel loading control. These results are representative of three independent experiments. **D**, Effect of the NO donor DEA-NONOate (5 mM) on *PAL-1* and *VST-1* transcript accumulation in grapevine cells. mRNA accumulation was monitored as described above. Cells were taken after a 3-h BcPG1 treatment or 30, 90, and 180 min after DEA-NONOate treatment. Phosphate buffer, used for NO-donor solubilization, is shown as a control for DEA-NONOate treatment.

the expression of both genes but, on the other hand, other events or second messengers are required and act synergistically with NO to trigger *PAL-1* and *VST-1* expression.

MAPK activation belongs to an independent pathway.

When applied on grapevine cells, BcPG1 induces the activation of two MAPK with apparent molecular masses of 45 and 49 kDa (Poinssot et al. 2003). Because of their major role in a number of signaling pathways, including elicitor-induced plant defense responses (Romeis 2001), we have analyzed their links with other events in BcPG1 signaling pathways. Extracellular Ca^{2+} chelation (EGTA), inhibition of cADPR- and IP_3 -dependent Ca^{2+} release from internal stores (RR and neomycin, respectively), and NO scavenging (cPTIO) do not modify MAPK activation (Fig. 6). But BcPG1-induced MAPK activation is inhibited by staurosporine (Fig. 6) as expected, since MAPK are activated by a phosphorylation cascade including Ser-Thr protein kinases.

DISCUSSION

We have already reported that BcPG1, the endopolygalacturonase 1 produced by the fungus *Botrytis cinerea*, triggered defense reactions in grapevine, including Ca^{2+} influx, AOS production, MAP kinase activation, defense gene transcript accumulation and phytoalexin production (Poinssot et al. 2003). In this study, we investigate the changes and the origin of $[\text{Ca}^{2+}]_{\text{cyt}}$, NO production, plasma membrane potential, and the links between Ca^{2+} influx, free cytosolic Ca^{2+} concentration elevations, AOS and NO production, variations of plasma membrane potential, MAPK activation, and defense gene expression.

In many physiological processes, changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ mediate cell stimulation and participate in signal transduction. The Ca^{2+} signal triggered by a specific stimulus is characterized by a lag time, magnitude, frequency, and duration (Trewavas 1999). Elicitors have been reported to induce $[\text{Ca}^{2+}]_{\text{cyt}}$ variations resulting from both the extracellular medium and intracellular Ca^{2+} pools (Lecourieux et al. 2002; Zimmermann et al. 1997). In order to analyze $[\text{Ca}^{2+}]_{\text{cyt}}$ variations, we have stably transformed *Vitis vinifera* cv. Gamay cells with the apoaequorin gene, the corresponding protein being addressed to the cytosol (Knight et al. 1991). This elicitor triggers a transient biphasic $[\text{Ca}^{2+}]_{\text{cyt}}$ rise, occurring within one minute after elicitation (Fig. 1B). These $[\text{Ca}^{2+}]_{\text{cyt}}$ peaks initially require a Ca^{2+} influx from the extracellular medium (Fig. 1A), and continuous Ca^{2+} influx is necessary to maintain the $[\text{Ca}^{2+}]_{\text{cyt}}$ rises (Fig. 1C). However, Ca^{2+} from the external medium is not solely responsible for $[\text{Ca}^{2+}]_{\text{cyt}}$ elevations. Indeed, neomycin,

which suppresses IP_3 production, and RR, which blocks the intracellular cADPR-dependent Ca^{2+} permeable channels (RZR-like), diminish the first $[\text{Ca}^{2+}]_{\text{cyt}}$ increase (Fig. 1D). Hence, the first BcPG1-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ corresponds to an influx of Ca^{2+} from the extracellular medium, which in turn mobilizes Ca^{2+} via the activation of IP_3 -dependent, RZR-type Ca^{2+} permeable channels, or both located in the membrane of internal stores, including endoplasmic reticulum and vacuoles (Allen et al. 1995). Because the second $[\text{Ca}^{2+}]_{\text{cyt}}$ is abolished by the addition of EGTA 15 min after BcPG1-treatment (Fig. 1C) and is not affected by neomycin and RR (Fig. 1D), we assume that the second phase of rise might be entirely due to the long-lasting Ca^{2+} influx. Nevertheless, we do not exclude that this second elevation can also involve endomembrane Ca^{2+} -permeable channels different from the IP_3 - and cADPR-dependent Ca^{2+} -permeable channels thought to be activated by BcPG1 or calcium transporters (i.e., vacuolar Ca^{2+} -ATPase, H^+ , and Ca^{2+} antiporters) inhibited by BcPG1 treatment. Our data are consistent with other recent studies indicating that elicitor-triggered $[\text{Ca}^{2+}]_{\text{cyt}}$ changes are mediated by Ca^{2+} -permeable channels located in the plasma membrane and endomembranes of plant cells (Lecourieux et al. 2002).

Strong evidence indicates that NO plays important roles in diverse physiological processes in plants. In particular, NO has been shown to be a crucial player in plant defense responses (Delledonne et al. 1998; Durner et al. 1998). In the present study, we show that BcPG1 induces a fast and transient NO production, probably due to a NOS-like enzyme since L-NAME and pBITU, two inhibitors of animal NOS, reduce by 55 and 80%, respectively, the BcPG1-induced NO production. The incomplete inhibition by the L-arginine competitor could be explained by the low accumulation of the competitive inhibitor in the cells or in a putative compartment, in which the NOS-like enzyme might be located. However, we do not exclude that a nitrate reductase could also participate in BcPG1-induced NO production (Desikan et al. 2002). The involvement of a nitrate reductase as a source of NO in response to pathogens has recently been reported by Modolo and associates (2005). Therefore, further investigations are required to identify one or more NO sources in BcPG1-treated cells.

Using a pharmacological approach, we have investigated the signaling events acting upstream of NO production. We have shown that both BcPG1-mediated Ca^{2+} influx and phosphorylation events are required for NO synthesis (Fig. 3B). These data suggest that, in elicitor-treated grapevine cells, the enzyme responsible for NO production is activated by free Ca^{2+} together with one or more protein kinases, whose activity could be Ca^{2+} -dependent or not.

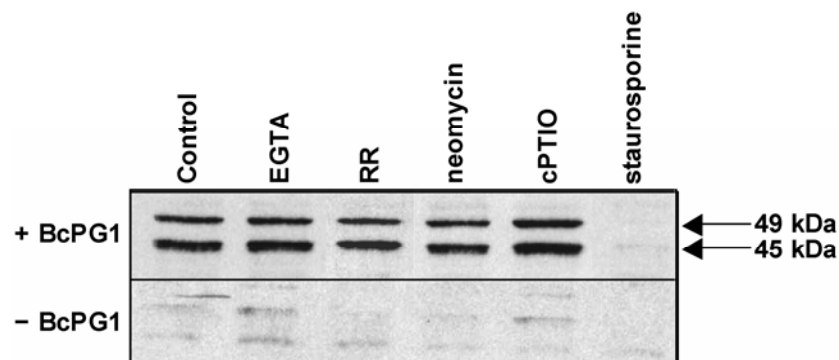


Fig. 6. Regulation of mitogen-activated protein kinase (MAPK) activation in BcPG1-treated grapevine cells. Cells were treated with BcPG1 (5 μg per gram of fresh weight cell; control) in the presence of 2 mM EGTA or 500 μM cPTIO or 5 μM ruthenium red (RR) or 25 μM neomycin or 2 μM staurosporine. Inhibitors were added 10 min prior to BcPG1. Proteins were extracted from cells 10 min after BcPG1 treatment and MAPK activation was analyzed by Western blot. Results are representative of three independent experiments.

In mammals, NO has been shown to regulate plasma membrane or intracellular Ca^{2+} -permeable channels either directly by *S*-nitrosylation of critical cysteines or indirectly via the second messengers cGMP, IP_3 , and cADPR (Clementi 1998). Several arguments suggest that NO is also a key Ca^{2+} -mobilizing compound in plants (Wendehenne et al. 2004). It promotes $[\text{Ca}^{2+}]_{\text{cyt}}$ rise during stomatal closure (Garcia-Mata et al. 2003) and in response to the elicitor cryptogein (Lamotte et al. 2004). It has been postulated that NO may mobilize Ca^{2+} from internal stores through the activation of RYR-like channels (Garcia-Mata et al. 2003; Lamotte et al. 2004) and plasma membrane Ca^{2+} -permeable channels (Lamotte et al. 2004). The process by which NO regulates Ca^{2+} release in plants is still unclear, but the possibility for NO to operate via cGMP, cADPR, or

both has been discussed (Lamotte et al. 2005). In our model, scavenging NO by using cPTIO results in a significant reduction of the first BcPG1-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ peak (Fig. 4B) and in a $[\text{Ca}^{2+}]_{\text{cyt}}$ signal similar to those obtained with neomycin and RR, which lead, respectively, to the inhibition of IP_3 - and cADPR-mediated Ca^{2+} release (Fig. 1D). These data suggest that NO might contribute to BcPG1-triggered $[\text{Ca}^{2+}]_{\text{cyt}}$ increases by promoting Ca^{2+} release from internal stores through the activation of IP_3 -dependent Ca^{2+} channels or RYR-like channels, as previously reported in cryptogein-treated tobacco cells (Lamotte et al. 2004). Furthermore, in our model, NO negatively regulates the elicitor-mediated Ca^{2+} entry in BcPG1-treated grapevine cells (Fig. 4A), since scavenging NO increases $^{45}\text{Ca}^{2+}$ influx in response to BcPG1. Increase in the Ca^{2+} influx

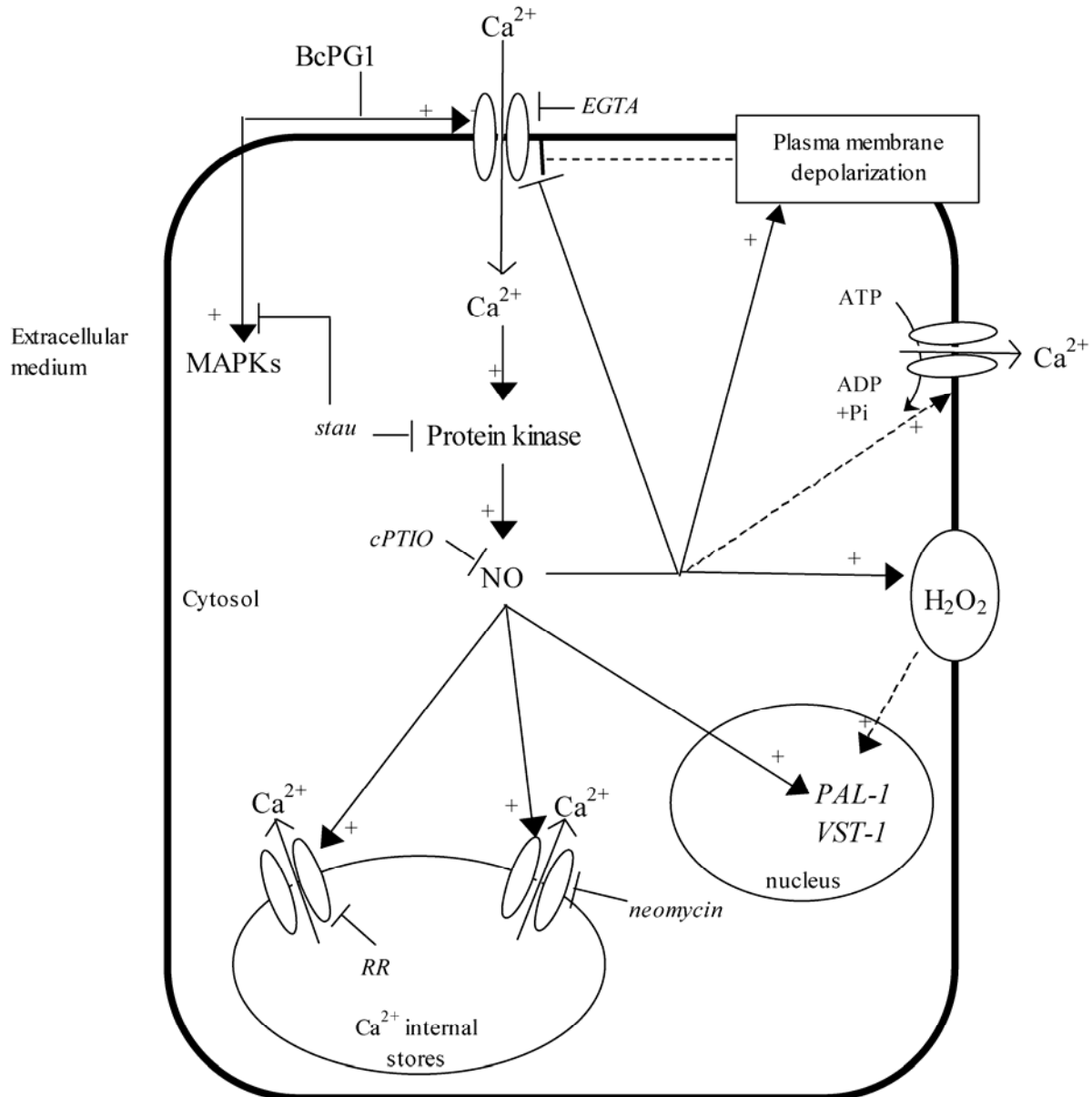


Fig. 7. Schematic model of signaling pathways activated in BcPG1-treated grapevine cells. BcPG1 activates Ca^{2+} influx from the external medium leading to a typical free cytosolic calcium concentration increase. Ca^{2+} rises are responsible for protein kinase activation and nitric oxide (NO) production. NO activates Ca^{2+} efflux from internal stores through IP_3 - and ryanodin receptor-like Ca^{2+} -permeable channels. NO also reduced Ca^{2+} rises either by inhibiting Ca^{2+} influx, by activating a plasma membrane Ca^{2+} -ATPase pump, or both. NO maintains elicitor-induced depolarization, which could explain the inhibitory effect of NO on Ca^{2+} influx through putative hyperpolarization-dependent Ca^{2+} -permeable channels. NO triggers H_2O_2 production and the expression of both defense genes *PAL-1* and *VST-1*. The NO-dependent activation of both defense genes could be mediated by H_2O_2 . BcPG1 induces the activation of two mitogen-activated protein kinases independently of the other indicated events. Lines with arrowheads indicate activation; lines with a perpendicular bar at the end indicate inhibition; and the dashed lines indicate alternative hypotheses. Inhibitors are indicated in italic, stau = staurosporine, and RR = ruthenium red.

and decrease in the $[Ca^{2+}]_{\text{cyt}}$ in the presence of cPTIO are not contradictory. Indeed, the Ca^{2+} influx reflects the activation of plasma membrane Ca^{2+} channels, whereas variations of the $[Ca^{2+}]_{\text{cyt}}$ result from the activity of many Ca^{2+} exchanger proteins and of Ca^{2+} binding by Ca^{2+} affine proteins. Moreover, previous data indicate that, during the Ca^{2+} influx, free $[Ca^{2+}]_{\text{cyt}}$ increases represent less than 1% of the total amount of Ca^{2+} that enters the cells (Lecourieux et al. 2002). The negative regulation of the Ca^{2+} influx by NO could result from an inhibition of plasma membrane Ca^{2+} -permeable channels or from an increase in the Ca^{2+} efflux via plasma membrane Ca^{2+} -ATPases. Indeed, in animal cells, it has been reported that NO could inhibit the *N*-methyl-D-aspartate receptor directly by *S*-nitrosylation (Stamler et al. 2001) and some L-type Ca^{2+} -permeable channels directly by *S*-nitrosylation or indirectly via a dependent cGMP pathway (Campbell et al. 1996; Poteser et al. 2001). Moreover, we demonstrate that NO delays plasma membrane repolarization and suppresses hyperpolarization after a transient plasma membrane depolarization (Fig. 4C). This effect could explain why the Ca^{2+} influx is increased when NO is scavenged by cPTIO in BcPG1-treated cells (Fig. 4A). Indeed, by inhibiting hyperpolarization, NO should reduce the driving force for Ca^{2+} entry and should suppress the activation of plasma membrane hyperpolarization-dependent Ca^{2+} channels (Gelli and Blumwald 1997). How NO controls plasma membrane potential in BcPG1-elicited grapevine cells is not yet elucidated, but recently, NO has been shown to inactivate outward-rectifying K^+ channels by *S*-nitrosylation in *Vicia* stomatal guard cells (Sokolovski and Blatt 2004), and it has also been demonstrated that the H^+ -ATPase of clathrin-coated vesicles is reversibly inhibited by *S*-nitrosoglutathione (Forgac 1999). Finally, taking into account that both the NO scavenger cPTIO and the protein kinase inhibitor staurosporine, which inhibits NO production (Fig. 3B), lead to a similar increase of the Ca^{2+} influx into BcPG1-treated cells (Fig. 1A and 4A), our data suggest that the effect of staurosporine on the Ca^{2+} influx should result from a collapse in NO synthesis.

Thus, in the BcPG1 transduction pathway, a close link exists between Ca^{2+} and NO. A free cytosolic Ca^{2+} concentration rise triggers NO production, which amplifies the Ca^{2+} signaling by activating Ca^{2+} -permeable channels of internal stores, thus mobilizing other actors in another location while, at the same time, NO suppresses the initial Ca^{2+} signal (Fig. 7).

To further investigate the close connection between NO and Ca^{2+} , we have analyzed the involvement of the two molecules in AOS production in BcPG1-treated cells. BcPG1-induced AOS production is dependent on NO, as demonstrated using different methods with the NO scavenger cPTIO, which suppresses H_2O_2 production (Fig. 5A and B). Our data also show that NO released by the NO donor DEA-NONOate activates H_2O_2 production (Fig. 5B). How NO controls AOS production or AOS levels is still unexplained in our model. NO could inhibit catalases as previously reported in plants (Clarke et al. 2000), explaining the high level of H_2O_2 in the absence of the NO scavenger cPTIO. Nevertheless, no difference is observed in catalase activity either in vitro or in vivo scavenging NO nor in BcPG1-treated cells (data not shown). In the same way, peroxidases, which possess a prosthetic heme group to which NO can bind, are not affected by NO in our assays (data not shown). Thus, taking into account that NO does not inhibit AOS-degrading enzymes and that NO released by DEA-NONOate triggers H_2O_2 production, we can assume that AOS production is activated by NO in BcPG1-treated grapevine cells. NO could activate i) directly the enzyme responsible for AOS production by posttranslational modification, or ii) indirectly through one or more protein kinases, which would be responsible for enzyme activation by phosphorylation. NO could

also activate AOS production via plasma membrane depolarization maintenance. Indeed, the plasma membrane depolarization triggered by anion efflux in cryptogin-elicited tobacco cells has been suggested to explain NADPH-oxidase activation, leading to AOS production (Wendehenne et al. 2002). Moreover, BcPG1-induced AOS production is dependent on Ca^{2+} influx but not on Ca^{2+} release from internal stores, as observed with EGTA and neomycin or RR, respectively (Fig. 5A). Thus, the activation of a NOS-like enzyme by Ca^{2+} on the one hand and the Ca^{2+} - or NO-dependent production, or both, of H_2O_2 on the other hand suggest that the Ca^{2+} effect on AOS production is mediated by NO. Alternatively, the enzyme responsible for AOS production could be a checkpoint for cross-talks involving both Ca^{2+} and NO.

To further understand the role of NO in BcPG1-induced defense responses, we have investigated the involvement of NO in defense gene expression. A role for NO in gene expression has been reported, in which the administration of NO donors induces the expression of genes related to defense, photosynthesis, secondary metabolism, and iron metabolism (Durner et al. 1998; Polverari et al. 2003). In comparison, only few genes have been shown to be up-regulated through a NO-dependent pathway in response to avirulent pathogens or elicitors (Delledonne et al. 1998; Lamotte et al. 2004). Here, we demonstrate that endogenous NO produced in response to BcPG1 regulates the expression of genes encoding PAL-1 and VST-1, two enzymes involved in phytoalexin biosynthesis; the NO scavenger cPTIO partly inhibits BcPG1-induced accumulation of *PAL-1* transcript, whereas it suppresses almost entirely the elicitor-mediated accumulation of *VST-1* mRNA (Fig. 5C). Therefore, NO might be involved in phytoalexin synthesis induced by BcPG1. Similarly, NO has been implicated in phytoalexin accumulation in soybean cotyledons challenged by an elicitor from *Diaporthe phaseolorum* f. sp. *meridionalis* (Modolo et al. 2002). The signaling pathway mediating the effect of NO on defense gene expression could involve the same second messengers responsible for intracellular Ca^{2+} mobilization. In tobacco, *PAL* expression is induced by a membrane-permeable analog of cGMP (Durner et al. 1998) and is suppressed by specific guanylate cyclase inhibitors. Moreover, it has been found that cADPR induces *PAL* expression and that this induction is blocked by RR (Durner et al. 1998). Hence, according to these studies, which demonstrate a link between Ca^{2+} release via RR-sensitive Ca^{2+} -permeable channels and phytoalexin accumulation, we could postulate that, in our model, NO regulates defense-gene expression by increasing Ca^{2+} release from internal stores via cGMP, cADPR, or both. However, we cannot dismiss that NO-dependent defense-gene expression could be mediated by H_2O_2 , as previously described (Jabs et al. 1997), since scavenging NO leads to the suppression of H_2O_2 accumulation. Moreover, since the NO released by a donor does not induce any *PAL-1* and *VST-1* expression, other second messengers present in elicited cells might act synergistically with NO or the spatial and temporal production of NO is a determining factor in the specificity of the response or both. Thus, we can assume that NO is necessary but not sufficient to activate *PAL-1* and *VST-1* expression in BcPG1-treated grapevine cells, supporting the idea of a complex signaling network that does not follow a simple linear pathway, in which each branch is controlled by a combination of several second messengers.

Finally, our study shows that MAPK activation does not depend on BcPG1-related NO production (Fig. 6), as shown by Lamotte and associates (2004) in cryptogin-treated tobacco cells. Moreover, like observations on parsley cells and pep13, tobacco cells and cryptogin, or *Cf9* transgenic tobacco cells and Avr9 systems (Ligterink et al. 1997; Lebrun-Garcia et al.

1998; Romeis et al. 1999), MAPK activation is not controlled by H₂O₂, since the NO scavenger that entirely inhibits H₂O₂ production does not modify MAPK activation in response to BcPG1. Finally, BcPG1-induced MAPK activation does not act downstream of [Ca²⁺]_{cyt} variations, as concluded by pretreatments with EGTA, RR, or neomycin of BcPG1-elicited grapevine cells (Fig. 6). This result differs from those previously reported in other plant-elicitor models (Lebrun-Garcia et al. 1998; Ligterink et al. 1997; Romeis et al. 1999) but is consistent with Ca²⁺-independent activation of SIPK by harpin_{PspH} in tobacco (Lee et al. 2001). MAPK are part of a module, in which a MAPK kinase kinase activates by phosphorylation a MAPK kinase, which finally phosphorylates MAPK, leading to their activation. In some models, complete MAPK modules have been elucidated, such as in the flagellin-treated *Arabidopsis* cells in which the elicitor-receptor interaction induces the successive activation of MEKK1, MKK4/MKK5, and two MAPK, i.e., MPK3 and MPK6 (Asai et al. 2002). The knowledge of such MAPK modules and their specific activation processes allow a better understanding of the MAPK role in stimulus-challenged cells. Indeed, studies carried out with loss- or gain-of-function mutants at different levels of the MAPK module highlight its important role in the transcriptional regulation of defense genes and disease resistance in tobacco and *Arabidopsis* (Asai et al. 2002; Kim and Zhang 2004). So, protein kinases acting upstream of MAPK and MAPK targets, such as transcription factors able to control defense-gene expression, have to be identified in this independent pathway induced by BcPG1 in grapevine cells.

Taken together, the results presented here enable us to describe a part of the signaling network induced by the proteinaceous elicitor BcPG1 in grapevine cells (Fig. 7). Although BcPG1 mobilizes the same molecules in grapevine as those mobilized by other elicitors in other plants, the signaling pathways are different. Hence, the knowledge of data coming from different plant-elicitor models will enable us, thanks to computer communication systems and models (Trevino Santa Cruz et al. 2005), to better understand the signal transduction networks involved in plant defense activation, and more precisely, as far as we are concerned, the way NO activates AOS production in BcPG1-induced signaling pathway.

MATERIALS AND METHODS

Grapevine cell culture conditions.

Grapevine (*Vitis vinifera* cv. Gamay) cell suspensions were maintained as described by Poinssot and associates (2003).

Transformed *Vitis vinifera* cv. Gamay cells expressing apoaquorin (Knight et al. 1991) were used to generate cell suspensions. Transformed cell suspensions were subcultured every 8 days by transferring 30 ml of aequorin-transformed cells to 70 ml of fresh liquid Nitsch-Nitsch medium (Nitsch and Nitsch 1969) and were maintained in suspension by continuous shaking (150 rpm at 24°C). Transgenic grapevine cell suspensions behaved similarly to the untransformed *Vitis vinifera* cv. Gamay cultures with respect to phenotype and growth kinetics.

Aequorin-transformed grapevine cells.

Transformation of *Vitis vinifera* cv. Gamay was carried out using the pBIN19 35S-apoaquorin vector integrated in the *Agrobacterium tumefaciens* EHA 105 strain. Briefly, 4 ml of an 8-day-old exponentially growing grapevine cell suspension were incubated at 25°C in the dark with 100 µl of the transformed *Agrobacterium tumefaciens* preculture. After 48 h of cocultivation, grapevine cells were washed and plated on Nitsch-Nitsch agar medium containing 300 µg of cefotaxim per milliliter and 10 µg of paromomycin per milliliter. After three weeks, trans-

formed calli were collected and subcultured on the same medium containing 50 µg of paromomycin per milliliter for 1 month at 25°C under constant light before transferring them into a liquid Nitsch-Nitsch medium containing 100 µg of paromomycin per milliliter at 25°C under constant light.

Cell culture treatments.

BcPG1 was purified from the culture filtrate of *Botrytis cinerea* T4, as described previously by Poinssot and associates (2003). Briefly, after filtration and dialysis, two steps of high pressure liquid chromatography were necessary to purify BcPG1 from *Botrytis cinerea* liquid culture.

The pharmacological compounds were purchased from Sigma-Aldrich (St. Quentin, Falavier, France), with the exception of carboxy-PTIO, coelenterazine (Calbiochem (Schwalbach, Germany), and DEA-NONOate (Cayman Chemical, Ann Arbor, MI, U.S.A.). All of the chemicals were dissolved in water, except staurosporine, K252-a, and L-NAME, which were prepared in dimethylsulfoxide (DMSO). DEA-NONOate was dissolved in phosphate buffer (50 mM, pH 7.2) and was freshly prepared before experiments. Equivalent volumes of DMSO or solubilization buffer were added to control cells to ensure they did not interfere with the experiments.

Grapevine cells were collected during the exponential growth phase, were washed with the M10 suspension buffer (175 mM mannitol, 0.5 mM K₂SO₄, 0.5 mM CaCl₂, and 10 mM morpholineethanesulfonic acid (MES), pH 5.3), and then was resuspended at 0.1 g of FWC per milliliter. After 1 h of equilibration (150 rpm, 24°C), grapevine cells were treated with elicitor at the rate of 5 µg per gram of FWC and measurements of NO and AOS production, calcium influx, or MAPK analysis were performed. Defense-gene transcript accumulation was determined using cells maintained under sterile conditions in their culture medium. For free cytosolic calcium concentration variations analysis, 8-day-old transgenic grapevine cell suspensions were collected and washed by filtration with M10 buffer before functional aequorin reconstitution and elicitor treatments. Cells were resuspended in the M10 suspension buffer to give a final concentration of 0.1 g of FWC per milliliter. In vivo reconstitution of aequorin was performed by the addition of 6 µl of coelenterazine (5 mM stock solution in DMSO) to 10 ml of cell suspension for at least 3 h in the dark (150 rpm at 24°C).

All chemicals used were tested for their nontoxicity on grapevine cells 4 h after treatment. They were added 10 min before elicitor treatment, except for experiments with apoaquorin-transformed grapevine cells, in which pretreatments with chemicals lasted until the return to the basal level, to avoid nonspecific effect of the compounds on calcium variations. Equivalent volumes of DMSO were added to control cells to ensure they did not interfere with the experiments. When used, final DMSO concentration did not exceed 0.25% (vol/vol).

Ca²⁺ influx measurement.

Seven minutes before treatment, cell suspensions were incubated with ⁴⁵Ca²⁺ (0.033 MBq per gram of FWC; Pharmacia Biosciences, Piscataway, NJ, U.S.A.). After different periods of treatment, duplicate 1.5-ml aliquots were filtered under vacuum on GF/A glass-microfiber filters (Whatman, Clifton, NJ, U.S.A.) and were washed twice with a total volume of 20 ml of buffer (175 mM mannitol, 0.5 mM K₂SO₄, 5 mM LaCl₃, and 10 mM MES, pH 5.3) before the cells were transferred to scintillation vials. After a 1-h period at 65°C, dry weight was determined and 10 ml of Ready Safe cocktail (Beckman Instruments, Fullerton, CA, USA) was added to the vials before counting in a scintillation counter (TRI-CARB 2100 TR, Packard, Rungis, France).

Free cytosolic calcium variation analysis.

Measurements of aequorin luminescence were carried out as described by Lecourieux and associates (2002), with minor modifications. The bioluminescence of 250- μ l aliquots of cells, transferred carefully to a luminometer glass, was recorded continuously at 1-s intervals using a digital luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany). The luminescence counts were recorded as relative light units per second and were exported simultaneously (using Win Term software; Berthold) into an Excel spreadsheet (Microsoft, Redmond, WA, U.S.A.) on a computer. At the end of the experiments, residual functional aequorin was quantified by adding 300 μ l of lysis buffer (10 mM CaCl₂; 2% Nonidet P40, vol/vol; 10% ethanol, vol/vol) and monitoring the resulting increase in luminescence. The luminescence data were transformed into Ca²⁺ concentrations as described by Lecourieux and associates (2002).

Nitric oxide measurement.

NO accumulation was determined as previously described (Lamotte et al. 2004) by using the specific fluorophore DAF-2DA (Sigma-Aldrich). After filtration, cell suspensions were incubated with 20 μ M DAF-2DA for 1 h in the dark at 24°C on a rotary shaker (150 rpm) and were then rinsed twice with fresh M10 suspension buffer to wash off excessive fluorophore. Cells were then transferred into 24-well plates (Costar, Corning Inc., Corning, NY, U.S.A.) containing 1 ml of cells per well and were treated with either BcPGI (5 μ g per gram of FWC), chemicals, or both. NO production was measured using a 24-well reader fluorimeter (Fluoroskan Ascent Fluorometer; Lab-systems, Helsinki, Finland) with λ_{ex} = 485 nm (excitation) and λ_{em} = 510 nm (emission) filters. Fluorescence was expressed as relative fluorescence units.

Plasma membrane depolarization measurement.

Cells were equilibrated in the dark for 2 h in suspension buffer supplemented with 1 μ M DiBac₄ (Bis(1,3-dibarbitoric acid)-trimethine) (Sigma-Aldrich). Then they were transferred into a quartz tank and DiBac₄ fluorescence was measured every 4 s for 1 h, using a fluorimeter (SAFAS flx-Xenius, Monte Carlo, Monaco) with λ_{ex} = 490 nm and λ_{em} = 510 nm. Fluorescence was expressed as relative fluorescence units.

H₂O₂ production measurement.

Luminol assay. Aliquots (250 μ l) of cell samples were analyzed for H₂O₂ production, using the chemiluminescence from luminol with a luminometer (Lumat LB 9507; EG&G Berthold, Evry, France), as previously described (Poinsot et al. 2003).

Pyranine assay. H₂O₂ production was measured using the fluorescence from pyranine (λ_{ex} = 402 nm, λ_{em} = 512 nm). Aliquots (1.5 ml) of cell samples, maintained under soft agitation, were mixed with an aqueous solution of pyranine (1.75 μ M). The fluorescence from pyranine was measured every 4 s for 30 min, using a spectrofluorimeter (SAFAS flx-Xenius, Monaco). Fluorescence was expressed as relative fluorescence units.

Protein analysis.

Protein concentration was determined according to Bradford (1976) with bovine serum albumin as the standard. Aliquots containing 15 μ g of protein were made soluble in Laemmli buffer (Laemmli 1970) and then submitted to Western blot analysis.

Western and Northern blot analyses.

MAPK analysis by Western blot was carried out as previously described by Poinsot and associates (2003).

Defense gene-encoded transcript accumulation was analyzed by Northern blot as previously described by Poinsot and associates (2003).

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