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Protein phosphorylation in *Nicotiana tabacum* cells in response to perception of lipopolysaccharides from *Burkholderia cepacia*

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Abstract

Bacterial LPS have the ability to act as modulators of the innate immune response in plants. Complex and largely unresolved perception systems exist for LPS on the plant cell surfaces that lead to the activation of multiple intracellular defense signaling pathways. The aim of the present study was to investigate the perception mechanism of cultured *Nicotiana tabacum* cells towards LPS from *Burkholderia cepacia* ($LPS_{B.cep.}$), with regard to the role of protein phosphorylation during signal perception-related responses to gain a better understanding of the chemosensory perception of LPS elicitor signals in plant cells. In vivo labeling of protein phosphorylation events during signal transduction indicated the rapid phosphorylation of several proteins with the hyperphosphorylation of two proteins of 28 and 2 kDa, respectively. Significant differences and de novo LPS-induced phosphorylation were also observed with two-dimensional analysis. The protein kinase inhibitor, staurosporine, totally inhibited the extracellular alkalinization response induced by $LPS_{B.cep.}$, while the oxidative burst was only partially inhibited by staurosporine. Inhibition of protein phosphatase activity by calyculin A intensified the $LPS_{B.cep.}$ responses. The results indicate that perception- and signal transduction responses during $LPS_{B.cep.}$ elicitation of tobacco cells require a balance between the actions of certain protein kinases and protein phosphatases.

Keywords: 2′,7′-Dihydrodichlorofluorescin-diacetate; Elicitor; Extracellular alkalinization; Lipopolysaccharides; Oxidative burst reaction; Protein phosphorylation

1. Introduction

Protein phosphorylation is an effective method of post-translational protein modification, which plays a fundamental role in the regulation of many cellular processes, particularly signal transduction during plant–pathogen interactions (Trewavas, 2000). Biochemically, phosphorylation can alter a protein's inherent biological activity, subcellular location and half-life and determines the interaction of the phosphoprotein with other proteins or cellular components which can be decisive in signal transduction to determine the extent, duration and ultimately the final effectiveness of defense mechanisms (Xing et al., 2002). Cellular responses that are reversibly controlled by protein phosphorylation requires a fine balance between the actions of protein kinases which phosphorylate target proteins at specific sites, and the actions of protein phosphatases, which remove the specific phosphates from the phosphorylated target proteins.

An ever-growing body of evidence implicates changes in protein phosphorylation as mediating a plethora of elicitor responses in plant cells. Elicitor treatment causes rapid changes in protein phosphorylation profiles. Protein phosphorylation and dephosphorylation reactions have been identified by Grab et al. (1989) in soybean cultures during the elicitation of phytoalexins by partially

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purified fungal β-glucan elicitor. Dietrich et al. (1990) reported that an elicitor from *P. megasperma* triggered rapid, transient and specific protein phosphorylation in parsley cells, and Farmer et al. (1990) found that oligogalacturonide treatment of tomato and potato leaves resulted in enhanced phosphorylation of plasma membrane proteins. Microbial elicitors have further been found to activate and phosphorylate the *Arabidopsis thaliana* MAPK6 (Nühse et al., 2000) as well as MAPKs in tobacco cells (Droillard et al., 2000).

Lipopolysaccharides (LPS) occur in the outermost layer in the capsule of gram-negative bacteria and may provide the key to recognition of potential pathogens by host plants. Through cell–cell interactions, plants can come into contact with LPS originating from root-associated rhizobacteria, bacterial endophytes as well as bacterial pathogens. LPS is composed of the hydrophobic lipid A, the covalently linked non-repetitive core oligosaccharide, divided into inner and outer core, and the O-antigen of oligosaccharide-repeating units. LPS is recognized as a cell surface derived microbe/pathogen-associated molecular pattern by mammalian cells, and has the ability to activate the innate host defense system by stimulating the synthesis of immunoregulatory molecules which act as mediators for microbial death (Nürnberger et al., 2004). The conserved lipid A region, in particular, has been implicated in the activation of defense-related responses in both vertebrates and invertebrates (Newman et al., 2000). Biologically active LPS molecules have been shown to act as determinants of induced systemic resistance and activators of the phenotypically related systemic acquired resistance, characterized by accelerated and enhanced defense responses (Dow et al., 2000).

Several reports have described defense-related responses in plants induced by LPS. Pre-treatment of pepper leaves with LPS altered gene expression patterns induced by subsequent challenge with bacterial pathogens. After sensitization of pepper leaves with LPS, marked changes in the kinetics and degree of gene expression following challenge of the leaves with *Xanthomonas campestris* pv. *campestris* and *X.c. vesicatoria* were obtained (Newman et al., 2000). Other changes induced by LPS include induction of antimicrobial activity, phytoalexin synthesis and changes in plant cell wall ultrastructure (Newman et al., 2001).

Burkholderia species are common in the rhizosphere and can control plant diseases (Cao et al., 2001). LPS extracted from an endophytic strain of *Burkholderia cepacia* have been shown to have a protective effect on the *Nicotiana tabacum–Phytophthora nicotianae* interaction when the plants were pre-treated with LPS and subsequently inoculated with zoospores of the pathogen. In addition, the LPS were found to induce PR-proteins and indicated an enhanced defensive capacity due to LPS-preconditioning of the plants (Coventry and Dubery, 2001). LPS pre-treatment also potentiated the expression of genes encoding PR proteins upon subsequent bacterial inoculation (Dow et al., 2000). Taken together, these results indicate that LPS induce quite specific alterations in plant gene expression and defense- and resistance-related responses, and suggest the existence of an LPS signal perception and response system in plant–pathogen interactions that could be part of a broad-spectrum defense mechanism against pathogens.

Previously, we have provided evidence that $LPS_{B.cep.}$ has specific effects on the biochemical perception systems involved in the interaction with cultured tobacco cells. The biochemical basis of the mechanism of action of $LPS_{B.cep.}$ as an elicitor involved in the triggering of plant defense responses was investigated and the results indicated that $LPS_{B.cep.}$ is perceived by mechanisms similar to that operating in the perception of yeast-derived elicitor (YE) (Gerber et al., 2004). Further research needs to be conducted to unravel the complex interactions of LPS with plants and to lay the foundations of exploitation of LPS as a mode of crop protection. In this communication, we report on the changes in protein phosphorylation following perception of the $LPS_{B.cep.}$ signal.

2. Results

2.1. $LPS_{B.cep.}$-induced changes in protein phosphorylation

[^{32}P]Orthophosphate uptake by *N. tabacum* cell cultures indicated a labeling time of 5 min for phosphorylation studies, as this was the shortest labeling time resulting in high enough phosphate uptake to easily visualize the phosphoprotein bands with autoradiography. Radiolabeling for times longer than 5 min resulted in high basal phosphorylation levels, making it difficult to distinguish between the phosphorylation levels of different protein bands induced by the elicitors and the bands phosphorylated in the control cells (data not shown).

Treatment of suspension cultured tobacco cells with LPS triggered phosphorylation of a number of proteins not phosphorylated in the control cells, as shown by electrophoretic analysis on one-dimensional (1D) SDS–PAGE gels and autoradiography. Time studies of the effect of LPS and YE elicitation on the protein phosphorylation pattern revealed that several proteins become hyperphosphorylated upon elicitation. Control experiments (receiving no LPS or YE, but only pre-incubation medium and [^{32}P]) were performed at time intervals of 5 and 60 min treatment with pre-incubation medium after 5 min of labeling the cells with [^{32}P]. This was done in order to assess the basal levels of protein phosphorylation of the cells over the entire time span of the experiment. Elicitation of the cells by 100 $\mu g ml^{-1}$

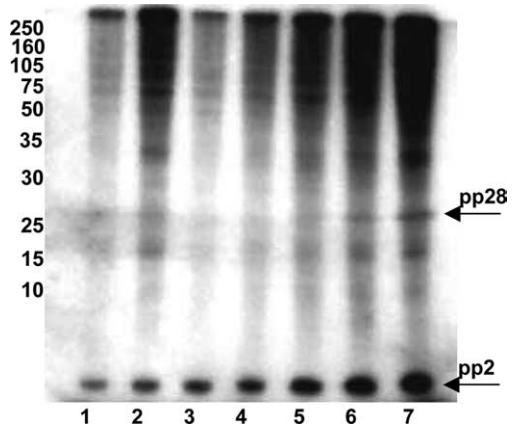

Fig. 1. Time-course of LPS-induced protein phosphorylation in tobacco cell cultures induced with LPS (100 $\mu g ml^{-1}$). Autoradiogram of total protein extracts from cells harvested at various times after elicitation. Lanes are as follows: (1) control at 5 min, (2) control at 60 min, (3) 5 min LPS, (4) 10 min LPS, (5) 20 min LPS, (6) 30 min LPS, and (7) 60 min LPS. The arrows indicate the positions and M_r of the induced phosphoproteins.

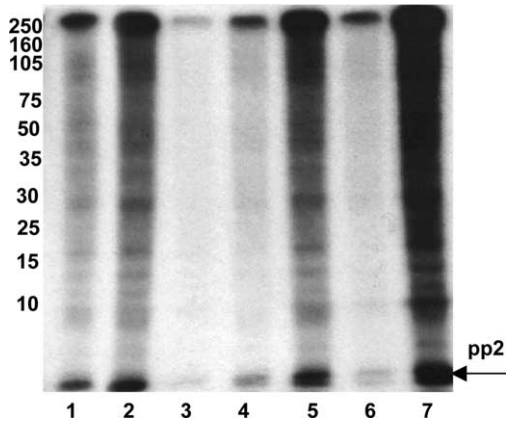

Fig. 2. Autoradiogram of total protein extracts of tobacco cell cultures treated with staurosporine (10 μM) or calyculin A (200 nM) and induced with LPS (100 $\mu g ml^{-1}$) and YE as a positive control. Cells were harvested 60 min after elicitation. Lanes are as follows: (1) control at 60 min, (2) YE, (3) staurosporine, (4) YE + staurosporine, (5) LPS, (6) LPS + staurosporine, and (7) calyculin A. The arrow indicates the position of the induced phosphoprotein, pp2.

LPS (Fig. 1) resulted in the phosphorylation of two proteins at higher levels than that of the control cells. The first of these is pp28, a 28 kDa protein, which appeared at a time interval of 30 min (lane 6) after LPS elicitation and increasing in intensity from 30 to 60 min (lanes 6 and 7). The second, most apparent phosphoprotein pp2, a small 2 kDa protein. The phosphorylation intensity of the pp2 protein increased extensively over the time course of the experiment. Although this protein also appeared in the control experiments (lanes 1 and 2), its intensity at 60 min is lower than that of even the 5 min LPS-elicitation time point (lane 3). This protein exhibited the highest amount of phosphorylation at 60 min after elicitation (lane 7).

Treatment of the cells with YE resulted in a phosphoprotein induction pattern similar to that induced by LPS, however, pp28 appeared hyperphosphorylated from 20 to 60 min after YE elicitation and the phosphorylation levels of YE-induced pp2 were less intense than the LPS-induced pp2 levels (data not shown).

2.2. The effect of protein kinase and protein phosphatase inhibitors on $LPS_{B.cep.}$-induced protein phosphorylation

The protein kinase inhibitor, staurosporine (10 μM) and the protein phosphatase inhibitor calyculin A (200 nM) were added to the pre-incubated *N. tabacum* cell cultures. The protein phosphorylation pattern is indicated in Fig. 2. The control, lane 1, represents the background levels of protein phosphorylation of the cells. The addition of YE and LPS (100 $\mu g\ ml^{-1}$ each) to the cells (lanes 2 and 5, respectively) increased the levels of protein phosphorylation of certain proteins. Treatment of the cells with staurosporine substantially reduced the levels of protein phosphorylation of the control cells as well as cells treated with YE and LPS. Lanes 3, 4 and 6 indicate the protein phosphorylation patterns of the control cells, YE-treated cells and LPS-treated cells in the presence of 10 μM staurosporine, respectively. The phosphorylation intensity of pp2, induced by YE and LPS (lanes 2 and 5) decreased considerably in the presence of staurosporine, indicating that the phosphorylation of this protein is dependent on staurosporine-sensitive protein kinases. The addition of the protein phosphatase inhibitor, calyculin A (200 nM), substantially increased the overall protein phosphorylation levels, as well as that of pp2, even in the absence of elicitor treatment or any other stimulus.

2.3. Two-dimensional PAGE analysis of the phosphoproteome

Separation of ^{32}P-labeled proteins by two-dimensional (2D) gel electrophoresis allowed visualization of the phosphoproteome, i.e. the total complement of rapidly phosphorylated proteins in the cells. The proteins were separated on the basis of their isoelectric points (p*I*) between pH 3 and pH 10 on a non-linear gradient. Several proteins showed highly reproducible increased or decreased incorporation of radioactive phosphate over the time course of the experiment, as indicated by triangles or circled groups of proteins (Fig. 3). Constitutively phosphorylated proteins (Fig. 3, arrows) served as internal controls for protein loading and phosphate incorporation.

2.4. $LPS_{B.cep}$-induced extracellular alkalinization

Extracellular alkalinization, due to a K^+ efflux and H^+ influx across the cellular membrane, is regarded as

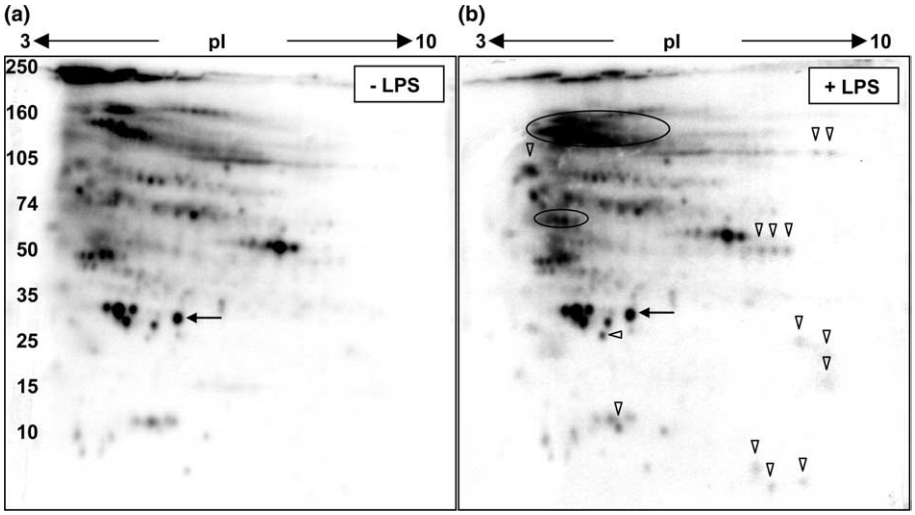

Fig. 3. 2D autoradiography image of the phosphoproteome of *N. tabacum* cells treated for 60 min with pre-incubation medium (a), indicating the basal phosphoprotein expression pattern and (b), the LPS-induced changes in the phosphoprotein expression profile after 60 min. Differentially expressed and de novo phosphoproteins are indicated by triangles or circulation of groups of proteins. Arrows indicate examples of constitutively expressed phosphoproteins.

one of the earliest cellular responses involved in signal perception and signal transduction leading to, or associated with the oxidative burst (Otte et al., 2001). Treatment of the tobacco cell cultures with 100 μg ml^{-1} $LPS_{B.cep.}$ resulted in an LPS-induced pH burst that started at a pH of 5.7 and reached a maximum pH of 6.0 after 10 min (Gerber et al., 2004). This pH was maintained over the next 10 min before declining by only 0.1 pH units to a pH value of 5.9, which was maintained for the duration of the experiment with very slight fluctuations. The constitutive pH level of the culture medium always averaged at 5.7 ± 0.1. Control cells were treated with pre-incubation medium alone and showed only very slight variation in pH during the entire experiment.

The addition of staurosporine (10 μM) to the LPS-treated cells resulted in a complete inhibition of the elicitor-induced extracellular alkalinization response. In contrast, the addition of calyculin A (200 nM) to the cells in the absence of LPS, resulted in a major increase in extracellular pH to a magnitude greater than that observed for the LPS reaction (Fig. 4). The calyculin A-induced extracellular pH burst reached a stable maximum pH of 6.5 over 35 min and remained at this pH for the entire time course of the experiment.

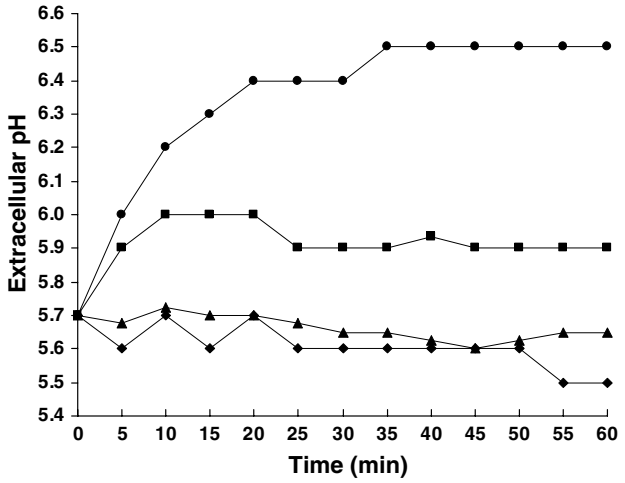

Fig. 4. The effect of protein kinase and protein phosphatase inhibitors on the extracellular pH levels of tobacco cell cultures in the absence of, or in combination with LPS (100 μg ml^{-1}). The extracellular pH increase of the LPS-treated cells (■) (Gerber et al., 2004) was completely suppressed by the addition of 10 μM staurosporine (◆) to the reaction. Addition of 200 nM calyculin A (●) caused a considerable increase in extracellular pH in the absence of LPS or any other stimulus. Control cells (▲) were treated with pre-incubation medium alone. Results represent the average of at least three independent experiments.

2.5. Protein kinase and protein phosphatase inhibitors affect the $LPS_{B.cep.}$-induced oxidative burst

The oxidative burst of tobacco cells in response to $LPS_{B.cep.}$ was previously described (Gerber and Dubery, 2002), using luminol-dependent chemiluminescence detection of peroxide. Staurosporine (10 μM) and calyculin A (200 nM) were added in combination with LPS to the tobacco cell cultures and the effect of these two inhibitors on the elicitor-induced oxidative bursts was assessed by the 2′,7′-dihydrodichlorofluorescin-diacetate (H_2DCF-DA) assay over time.

The addition of staurosporine to the LPS-induced tobacco cells resulted in only a partial suppression of the levels of ROS in comparison to the complete suppression observed with extracellular alkalinization, the suppression being 55% in comparison to the ROS levels

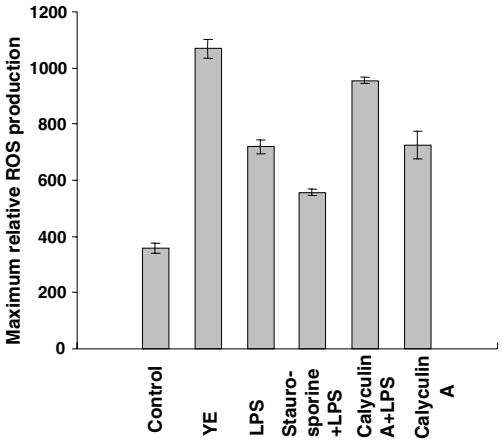

Fig. 5. The effect of protein kinase and protein phosphatase inhibition on the LPS-induced ROS production by tobacco cells. Staurosporine (10 μM) inhibited ROS production when added to the cells in combination with LPS (100 $\mu g ml^{-1}$), while addition of calyculin A (200 nM) alone, and in combination with LPS (100 $\mu g ml^{-1}$), resulted in increased ROS production to levels higher than the LPS control. The YE-induced ROS production is included as a positive control. The maximum relative ROS production at time period 60 min is indicated. Error bars represent the standard error of the mean, ($n = 9$).

induced by LPS treatment of the cells without staurosporine. The partial suppression can be ascribed to the fact that not all protein kinases, e.g. certain MAP kinases, are inhibited by staurosporine (Romeis et al., 1999). The addition of calyculin A to the cells resulted in an increase in the levels of ROS production to levels considerably higher (164%) than that of the LPS-induced ROS levels (Fig. 5).

3. Discussion

We have previously shown that LPS and YE induce considerable changes in signal perception and transduction and defense-associated responses in suspension cultured tobacco (*N. tabacum*) cells. These responses included a rapid influx of Ca^{2+} into the cytoplasm of aequorin-transformed tobacco cells, an oxidative burst, concomitant with the production of reactive oxygen and nitrogen species and alkalinization of the extracellular culture medium (Gerber et al., 2004). YE was used in this study for comparative purposes in the analysis of LPS elicitation on protein phosphorylation and the role of protein kinases and phosphatases.

3.1. Protein phosphorylation accompanies $LPS_{B.cep.}$ elicitation

Protein phosphorylation plays a fundamental role in the regulation of many cellular processes, particularly signal transduction during plant–pathogen interactions (Xing et al., 2002). Changes in protein phosphorylation mediate elicitor responses in plant cells and elicitor treatment causes rapid, transient and specific protein phosphorylation changes in protein phosphorylation profiles (Grab et al., 1989; Dietrich et al., 1990; Farmer et al., 1990; Felix et al., 1991).

In the present study, the effect of LPS elicitation on the protein phosphorylation pattern of *N. tabacum* cell suspension cultures was examined by in vivo labeling of the cells with [^{32}P]orthophosphate, extraction of the radiolabeled phosphoproteins and analysis on 1D and 2D SDS–PAGE and autoradiography. Because it is known that elicitor treatment can affect the phosphate uptake of the cells (Strasser et al., 1983), a [^{32}P]orthophosphate uptake study was performed to determine the time-dependent uptake of the radiolabeled phosphate by the cells. With reference to the data obtained, a phosphate labeling time of 5 min before LPS addition was chosen, since radiolabeling of the cells for longer times resulted in excessively high background levels of protein phosphorylation (data not shown). Control experiments were performed in parallel with elicitor treatment for the shortest (5 min) as well as for the longest time point (60 min) during the experiments. This eliminated any discrepancies and uncertainties as to whether the observed hyperphosphorylated protein bands were due to elicitor treatment or increasing [^{32}P] uptake by the cells.

LPS elicitation of *N. tabacum* cells revealed the prominent hyperphosphorylation of two proteins on 1D gels, one of ~28 kDa (labeled pp28), and another protein(s) of ~2 kDa (pp2) not hyperphosphorylated in the control experiments (Fig. 1). pp2 migrated well above trace levels of the unincorporated radiolabeled phosphate and sometimes appeared as a doublet. The pp2 doublet (Fig. 2) could be due to differential phosphorylation status of a protein fragment upon proteolysis of a larger protein.

The effect of the protein kinase inhibitor, staurosporine and the protein phosphatase inhibitor calyculin A, on the LPS- and YE-induced protein phosphorylation pattern in *N. tabacum* cells was also assessed. Addition of staurosporine, 30 min prior to LPS or YE elicitation completely prevented the elicitor-induced changes in protein phosphorylation observed in Fig. 2, as well as the background levels of protein phosphorylation by the control cells. In contrast, treatment of the cells with calyculin A resulted in hyperphosphorylation of several proteins to such a degree that individual bands are mostly indistinguishable from each other on the autoradiogram. Hyperphosphorylation of a protein in the region of pp2, was also observed in the calyculin A-treated protein extract (Fig. 2). Although not clearly visible, pp28 is in all likelihood also hyperphosphorylated in the calyculin A-treated protein extract. Taken together, these data indicate that phosphorylation of certain proteins by staurosporine-sensitive protein kinases

is essential for the continuous phosphorylation of the proteins induced by LPS elicitation. In addition, the hyperphosphorylation of specific proteins by calyculin A, including those phosphorylated by LPS and YE induction, indicates that LPS action could be based on inhibition of a protein phosphatase(s) as well as activation of a protein kinase(s) (Felix et al., 1991, 1994).

The superior resolution capability of 2D PAGE allowed the detection of several phosphoproteins that could not be sufficiently separated on conventional 1D SDS–PAGE. The 2D autoradiogram revealed the abundance of radiolabeled phosphoproteins, including several proteins hyperphosphorylated in response to $LPS_{B.cep.}$ elicitation as well as a number of de novo phosphorylated proteins not seen in the control experiment. The representation of the $LPS_{B.cep.}$ induced differentially expressed phosphoproteins over an extensive range of molecular weights and p*I*s (Fig. 3) demonstrates the intricate involvement of protein phosphorylation in $LPS_{B.cep}$. signal perception and transduction responses in plant cells.

3.2. Protein phosphorylation supports $LPS_{B.cep.}$-induced extracellular alkalinization

Several reports have been published regarding elicitor-induced alkalinization of the extracellular culture medium of plant cells (Felix et al., 1991, 1994; Pachten and Barz, 1999; Albus et al., 2001; Otte et al., 2001). In the present study, clear differences in the magnitude and duration of the respective LPS- and YE-induced extracellular pH bursts was observed. Although YE induced an extracellular pH burst of greater magnitude than LPS, the YE-induced burst terminated and declined back to almost basal pH levels of the control experiments, while the LPS-induced burst was a more sustained and long-duration burst that did not decline back to control pH levels for the entire time course of the experiment (Fig. 4). During the measurement of cytoplasmic Ca^{2+} influxes after YE and LPS elicitation of *N. tabacum* cell suspension cultures, it was also observed that the YE-induced influx of Ca^{2+} was of a greater magnitude than that of LPS, but the LPS-induced Ca^{2+} influx started later than that of YE. In addition, although the LPS-induced Ca^{2+} influx was lower than that induced by YE, it was a more sustained, long-duration signal maintaining low levels of Ca^{2+} for the duration of the experiment (Gerber et al., 2004).

Bollwell (1999) used buffering conditions and ionophores to demonstrate that the oxidative burst was absolutely dependent upon extracellular alkalinization, due to the pH dependence of H_2O_2 generation by a cell wall peroxidase in bean cells. These data are in contrast to results obtained by Pachten and Barz (1999), who reported that elicitor-induced signal transduction of the oxidative burst and the extracellular alkalinization in cultured chickpea protoplasts seem to be independent of the cell wall. Otte et al. (2001), reported that an 80% inhibition of the extracellular alkalinization after a complete inhibition of the oxidative burst by diphenylene iodonium indicates that the elicitor induced increase of extracellular pH is mainly based on proton consumption for superoxide anion radical ($O_2^{-\cdot}$) dismutation and further suggested a simultaneous deactivation of the plasma-membrane H^+-ATPase during the oxidative burst and extracellular alkalinization. Although there are conflicting reports on the dependence of the oxidative burst and the extracellular alkalinization on each other, both processes seem to be regulated by protein phosphorylation.

When the protein kinase inhibitor, staurosporine, was added to tobacco cells before elicitation by $LPS_{B.cep.}$ it resulted in inhibition of the pH increase of the extracellular medium and at times, the pH levels for the reaction with LPS and staurosporine were lower than the pH levels of the control experiments. This total suppression of the LPS-induced extracellular pH burst by staurosporine indicates that certain protein kinases are involved in initiating and/or maintaining of the extracellular alkalinization response. Addition of the protein phosphatase inhibitor, calyculin A, to the cells resulted in a considerable increase in extracellular pH even in the absence of LPS or any other stimulus (Fig. 4), which correlates with the ROS results (Fig. 5). Staurosporine also suppressed the YE-induced extracellular alkalinization response to pH levels of the non-induced control experiments (data not shown).

Otte et al. (2001) reported that elicitation of cultured chickpea cells stimulates a signal transduction pathway leading to several rapid responses like the transient K^+ efflux and H^+ influx; extracellular alkalinization followed by extracellular acidification, the oxidative burst, and the activation of defense-related genes. Previous research by different groups has proved that pharmacological inhibitors of protein kinases block a broad spectrum of early defense responses, while protein phosphatase inhibitors induce many of the same responses induced by elicitors (Boller, 1995; McDowell and Dangl, 2000). On the whole, these data, in accordance with the results from the present study regarding the oxidative burst and extracellular alkalinization in tobacco cells induced by $LPS_{B.cep.}$, confirm that dynamic changes in protein phosphorylation are involved in early plant defense responses.

3.3. Protein kinase and protein phosphatase inhibitors modulate the $LPS_{B.cep.}$-induced oxidative burst

LPS was previously thought not to trigger all plant defense responses including the induction of an oxidative burst and associated ROS production linked to the HR (Dow et al., 2000). However, recent reports indi-

cate that LPS from various sources (Meyer et al., 2001; Albus et al., 2001), including *B. cepacia* (Gerber et al., 2004), have the capability to induce an oxidative burst in tobacco cells.

In an effort to better understand the mechanisms involved in the production of the oxidative burst reaction, the role of protein kinases and phosphatases during the oxidative burst was examined, using the H_2DCF-DA assay in tobacco cells. Protein kinase inhibitors (K-252a and staurosporine) blocked the oxidative burst in cultured soybean cells in a concentration-dependent manner, and protein phosphatase inhibitors (calyculin A and okadaic acid), induced the oxidative burst in the absence of any additional stimulus (Chandra and Low, 1995). Mathieu et al. (1996) also found that calyculin A induced extracellular alkalinization and an oxidative burst reaction in tobacco cells, while staurosporine strongly reduced extracellular alkalinization and suppressed the oxidative burst.

Similar results were obtained in this study regarding the suppression of the oxidative burst by staurosporine, and an induction of the oxidative burst reaction even in the absence of elicitor treatment by calyculin A (Fig. 5). These data, in accordance to findings by Felix et al. (1994), suggests that certain kinases are constitutively active and indispensable for production of ROS during the elicitor-induced oxidative burst in plant cells. The findings that calyculin A notably increased LPS-induced ROS production indicates that phosphorylated proteins which are dephosphorylated by calyculin A-sensitive protein phosphatases are actively involved in the production and/or maintenance of the LPS-induced oxidative burst. The lower levels of ROS obtained by LPS elicitation alone in comparison to the ROS levels of LPS and calyculin A combined, suggests that a certain degree of dephosphorylation of proteins involved in the LPS-induced oxidative burst takes place by the action of calyculin A-sensitive protein phosphatases. Treatment of the cells with LPS directs the equilibrium of protein phosphorylation/dephosphorylation more in favour of the phosphorylation of certain proteins required for the onset or maintenance of the LPS-induced oxidative burst. Altogether, these results suggests that the on/off switch for the oxidative burst is likely to be controlled by a delicate balance between constitutive protein kinases and phosphatases, and that this balance can be upset or manipulated by the interaction of various elicitors with their hosts.

These documented early responses to $LPS_{B.cep.}$ make a new contribution to understand the biochemical basis of the mechanism of action of LPS as an elicitor involved in the triggering and modulation of plant defense responses. The results obtained in this study provide evidence that *B. cepacia* LPS has specific effects on reversible protein phosphorylation events underlying the perception systems involved in the interaction of plant cells with $LPS_{B.cep}$. A more comprehensive proteomic study of the $LPS_{B.cep.}$-induced phosphoproteome including mass spectrometry-acquired phosphoprotein sequence data and phosphorylation site mapping will be performed in order to contribute to the understanding of the biochemical action mechanism of $LPS_{B.cep.}$ as a resistance elicitor or possible triggering agent of innate immunity.

4. Experimental

4.1. Plant cell cultures and culture conditions

N. tabacum cv. Samsun cell suspension cultures were established from seeds obtained from the ARC Institute for Industrial Crops, South Africa, and were grown at 25 °C in the dark in Murashige and Skoog (1962) medium containing 0.25 $mg\,l^{-1}$ kinetin (Sigma) and 0.50 $mg\,l^{-1}$ 2,4-dichlorophenoxy acetic acid (Sigma) on a shaker at 120 rpm. All experiments were performed using cells in the logarithmic growth phase, 3–5 d after sub-cultivation.

4.2. Extraction and purification of *B. cepacia* lipopolysaccharides

An endophytic strain of *B. cepacia*, (ASP B 2D), (Gerber et al., 2004) was cultured for 10–14 d at 25 °C in sterile liquid nutrient broth (BioLab) at 16 $g\,l^{-1}$on an orbital shaker at 110 rpm. LPS were extracted from *B. cepacia* cultures as previously described (Gerber et al., 2004). This procedure included an RNA degradation step and a 12 kDa cut-off dialysis step. The average yield of LPS was 10% of the total starting weight of lyophilized *B. cepacia*. No contaminating protein could be detected in the pure $LPS_{B.cep.}$ fractions.

4.3. Extraction and purification of the yeast elicitor

Yeast elicitor (YE) was prepared from baker's yeast (*Saccharomyces cerevisiae*) as described (Gerber et al., 2004).

4.4. In vivo protein labeling with [^{32}P]orthophosphate

Labeling of tobacco cell aliquots (0.2 $g\,ml^{-1}$) with 100 $\mu Ci\,ml^{-1}$ carrier free [^{32}P]orthophosphate (370 $Mbq\,ml^{-1}$; Amersham Biosciences) was carried out prior to any other treatment to avoid interference with phosphate transport or metabolism. The cell suspensions were labeled with [^{32}P]orthophosphate for 5 min, before the addition of $LPS_{B.cep.}$ or YE (100 $\mu g\,ml^{-1}$ each) for time intervals of 5, 10, 20, 30 and 60 min. Control experiments, that received no $LPS_{B.cep.}$ or YE but pre-incubation medium alone, were performed for time intervals of

5 and 60 min in order to assess the basal levels of phosphorylation of the proteins in the cells over the entire time study. At the desired times, the [^{32}P]orthophosphate labeling and elicitor-induced phosphate incorporation was terminated by rapidly freezing the cells in liquid nitrogen. For 2D gel electrophoresis analyses, the cells (0.2 g ml^{-1}) were treated with 100 μg ml^{-1} $LPS_{B.cep.}$ for 60 min, followed by in vivo labeling with 100 μCi ml^{-1} carrier free [^{32}P]orthophosphate (370 Mbq ml^{-1}, Amersham) for 5 min. The [^{32}P]orthophosphate labeling was terminated by rapidly freezing the cells in liquid nitrogen. Control experiments, that received no $LPS_{B.cep.}$ treatment but pre-incubation medium alone, were similarly performed for 60 min.

Proteins from the frozen cell aliquots were extracted by adding 500 μl of 0.1 M Tris-buffered phenol (pH 8.0) and sonifying the cells at intervals of 3×10 s at 65% power (Bandelin Sonopuls). The cell aliquots were kept on ice in between sonifying intervals. After sonification, the extracts were centrifuged at 13,000*g* for 5 min at 4 °C. The bottom phenol phase was removed and kept aside while the remaining two phases were again mixed with an equal volume of phenol, vortexed for 20 s and centrifuged at 13,000*g* for 5 min at 4 °C. The two phenol phases were combined and the proteins were precipitated by the addition of an equal volume of methanol (−20 °C), followed by centrifugation at 13,000*g* for 5 min at 4 °C. The resulting protein pellet was washed twice with methanol (−20 °C), dissolved in 1× SDS-sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol) and boiled in a water bath for 90 s. The protein extracts to be used in 2D gel electrophoresis were further purified and desalted using the ReadyPrep™ 2D Cleanup Kit (BioRad) as per manufacturer's instructions. The final protein pellets were resuspended in 50 μl 2D rehydration/sample buffer [8 M urea; 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); 0.5% IPG buffer pH 3–10 NL containing carrier ampholyte (Amersham Biosciences), trace amounts of bromophenol blue and dithiotreitol (DTT, 7 mg/2.5 ml buffer)]. The samples were left in the buffer for 1 h at room temperature to ensure that all proteins are fully denatured and solubilized prior to centrifugation and sample application. Following resuspension, the amount of protein in each sample was quantified using the Amido Black protein dye assay described by Sheffield et al. (1987).

4.5. One-dimensional electrophoretic analysis

SDS–PAGE gel electrophoresis of the [^{32}P]-labeled protein extracts was performed on 15% polyacrylamide gels (Laemmli, 1970). Tricine–SDS–PAGE analysis was also used for the separation of low molecular weight peptides (Schägger and von Jagow, 1987). Following electrophoresis, the protein bands on the gel were visualized by silver staining, adapted and modified from Bassam et al. (1991). The proteins were fixed in the gels by addition of a solution of 40% ethanol and 10% acetic acid for 30 min on a shaker. Following washing with distilled water for 15 min (three changes of water), the gels were subsequently sensitized with a solution of 1.5 mM sodium dithionite ($Na_2S_2O_4$) and rinsed with 3× 20 s changes of distilled water. The gels were exposed to silver nitrate solution (0.2% $AgNO_3$, 1 mM formaldehyde) for 30 min and rinsed in distilled water before the developing solution [0.48 M $Na_2CO_3 \cdot 10H_2O$, 20 mM sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$), 6 mM formaldehyde] was added until the protein bands were sufficiently stained. The reaction was terminated by placing the gels in 5% acetic acid for 15 min.

4.6. First-dimension isoelectric focusing for 2D electrophoresis

Two immobilized pH-gradient strips (IPG strips, 13 cm × 0.3 cm × 0.05 cm, Amersham Biosciences) were rehydrated with 250 μl 2D rehydration/sample buffer containing equal amounts of protein form the control and the LPS-induced extracts, respectively. Rehydration loading of the strips was allowed for at least 10–12 h at room temperature. The isoelectric focussing (IEF) was performed on an Ettan IPGphor IEF apparatus (Amersham Biosciences). Proteins were focused according to their isoelectric points in the gel by applying a potential of 500 V for 1 h, followed by 1000 V for 1 h, and finally 8000 V for 2 h, as recommended by the manufacturers.

4.7. Second-dimension SDS–PAGE

After IEF separation, the second-dimension SDS–PAGE was performed in order to separate the proteins according to their molecular weights. The IPG strips were equilibrated in two separate 15 min steps in equilibration solution. The first equilibration solution contained: 50 mM Tris–HCl, pH 8.8; 6 M urea; 30% glycerol; 2% SDS; DTT, 100 mg per 10 ml equilibration solution. The second equilibration solution contained all of the above components, except that DTT is substituted for by 250 mg per 10 ml equilibration solution of iodoacetamide. The strips were subsequently transferred to a vertical Hoefer SE 600 gel apparatus containing a 10% SDS–polyacrylamide gel (13 cm × 18 cm × 0.15 cm) employing the Tris–glycine system (Laemmli, 1970). Electrophoresis was performed at 18 °C at 10 mA per gel for the first 45 min, followed by 25 mA per gel until the bromophenol blue tracking dye had migrated to within 1 mm from the bottom edge of the gel. The gels were subsequently silver stained as described above and subjected to autoradiography on Bio-Rad MS film at −80 °C.

4.8. Extracellular alkalinization measurements

N. tabacum cell suspension cultures were incubated in 4 ml aliquots at 0.2 $g ml^{-1}$ pre-incubation medium in open vials on an orbital shaker at 120 rpm for 3 h (Gerber et al., 2004). The pH before elicitation was constantly monitored to ensure that the observed elicitor-induced alkalinization response was in fact due to elicitor treatment and not other stress factors or external components. $LPS_{B.cep.}$ or YE was then added to the cells at a final concentrations of 100 $\mu g ml^{-1}$ and the extracellular pH of the culture medium was measured with a pH electrode over 60 min. The pH electrode was attached to a 'PC Turtle' (Hanna Instruments), which was used as a data collector to register pH readings once a minute for the duration of the experiment. Control cells were treated with 1 ml pre-incubation medium alone.

The effect of protein kinase and protein phosphatase inhibitors on the extracellular alkalinization was investigated by pre-incubation of cell cultures as described above. The protein kinase inhibitor, staurosporine (10 μM; Sigma), and the protein phosphatase inhibitor, calyculin A (200 nM; Sigma), were added to the cell cultures either alone, or in combination with LPS (100 $\mu g ml^{-1}$). Control cells were treated with 1 ml pre-incubation medium alone.

4.9. Detection of the oxidative burst reaction using an H_2DCF-DA fluorescence microplate assay

The procedure of using 2′,7′-dichlorofluorescein fluorescence to measure the oxidative burst reaction of elicitor-challenged *N. tabacum* cell suspension cultures in multiwell microplate assays was previously described (Gerber and Dubery, 2004). Aliquots (1 ml) of diluted cell suspensions (0.2 $g ml^{-1}$) were treated with YE or $LPS_{B.cep.}$ at final concentrations of 100 $\mu g ml^{-1}$. The induced cell suspensions (200 μl) were transferred to the wells of a 96-well black fluorescence plate (Labsystems) and 20 μl of a 1 mM H_2DCF-DA stock in DMSO were added to obtain a final concentration of 100 μM. Control cells were mock-treated with 100 μl medium only. The fluorescence resulting from the oxidative burst reaction produced by the cells was measured continuously for 1 h on a fluorometer (Fluoroskan Ascent, Labsystems) at an excitation wavelength of 485 nm and emission wavelength of 538 nm. The resulting fluorescence was expressed as relative ROS production.

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