Available online at www.sciencedirect.com
ScienceDirect
Plant Physiology and Biochemistry 44 (2006) 369–379
Plant Physiology and Biochemistry
www.elsevier.com/locate/plaphy

Lipopolysaccharide-responsive phosphoproteins in *Nicotiana tabacum* cells

I.B. Gerber[a], K. Laukens[b,c], E. Witters[b,c], I.A. Dubery[a,*]

[a] *Department of Biochemistry, University of Johannesburg, Kingsway Campus, P.O. Box 524, Auckland Park, 2006 Johannesburg, South Africa*
[b] *Laboratory of Plant Biochemistry and Plant Physiology, Department of Biology, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerpen, Belgium*
[c] *Center for Proteome Analysis and Mass Spectrometry, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerpen, Belgium*

Received 22 December 2005
Available online 05 July 2006

Abstract

Mounting evidence is merging to affirm the effectiveness of bacterial lipopolysaccharides (LPS) as biological control agents, inducers of innate immunity, and to stimulate/potentiate the development of defense responses in plants through protein phosphorylation-mediated signal perception/transduction responses. In vivo labeling of protein phosphorylation events during signal transduction indicated the rapid phosphorylation of several proteins. Substantial differences and de novo LPS-induced phosphorylation were also observed with two-dimensional analysis. In this study, qualitative and quantitative changes in phosphoproteins of *Nicotiana tabacum* suspension cells during elicitation by LPS from the Gram-negative bacteria, *Burkholderia cepacia*, were analyzed using two-dimensional electrophoresis in combination with a phosphoprotein-specific gel stain. Trypsin digested phosphoproteins were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) and nano-electrospray-ionization liquid chromatography tandem mass spectrometry (nano-ESI-LC/MS/MS). A total of 27 phosphoproteins were identified from 23 excised gel spots. The identified phosphoproteins indicate that $LPS_{B.cep}$-induced signal perception/transduction involves G-protein coupled receptor signaling, Ca^{2+}/calmodulin-dependent signaling pathways, H^{+}-ATPase regulation of intracellular pH, thioredoxin-mediated signaling and phosphorylation of 14-3-3 regulatory proteins. Other targets of $LPS_{B.cep}$-responsive phosphorylation included NTP pool maintenance, heat shock proteins, protein biosynthesis and chaperones as well as cytoskeletal tubulin. The results add novel insights into the biochemical process of LPS perception and resulting signal transduction.

Keywords: Innate immunity; Lipopolysaccharides; Phosphoproteins; Plant defense; Proteomics; Signal transduction

1. Introduction

Plant disease resistance to infection often depends on whether the plant is able to recognize the pathogen early and trigger inducible defense responses. Defense responses in higher plants involve protective mechanisms against a wide variety of stresses such as viral, bacterial and fungal pathogen attack; wounding, chemicals in water, soil and air and ultraviolet radiation. One key feature of all living organisms is the ability to discriminate between many different potential pathogens (non-self) that they may be faced with at any given time. This forms the basis for the activation of innate defense mechanisms [1]. Pathogen recognition and the concomitant activation of disease resistance responses in plants occur either at the non-cultivar-specific level (i.e. non-host or species resistance, non-cultivar-specific host resistance) or at the cultivar level (i.e. cultivar-specific host resistance). In the gene-for-gene hypothesis, complementary pairs of dominant pathogen-encoded avirulence (*Avr*) genes and dominant plant resistance (*R*) genes play the major role in cultivar-specific resistance, which is only expressed by particular plant cultivars against certain races of pathogen species. *Avr* gene products/proteins are considered to be virulence factors during the colo-

Abbreviations: 2D, two dimensional; ESI, electrospray ionization; Flg22, flagellin-22 peptide; IEF, isoelectric focusing; IPG, immobilized pH gradient; LC, liquid chromatography; LPS, lipopolysaccharides; MALDI, matrix assisted laser desorption ionization time of flight; MS, mass spectrometry; NO, nitric oxide; PAMP, pathogen-associated molecular pattern; p*I*, isoelectric point; PMF, peptide mass fingerprinting; RuBP, ruthenium II tris (bathophenanthroline disulfonate); TOF, time of flight.

* Corresponding author. Tel./fax: +27 11 489 2401.
E-mail address: iad@na.rau.ac.za (I.A. Dubery).

0981-9428/$ - see front matter © 2006 Elsevier SAS. All rights reserved.
doi:10.1016/j.plaphy.2006.06.015

nization of susceptible host plants by a pathogen, but in resistant host cultivars, these proteins act as specific elicitors of plant defense responses to betray the presence of the pathogen to the plants surveillance system. In contrast, the absence of either gene (R or Avr) or the failure thereof to produce a functional product, results in disease [2].

In response to pathogen attack, plants have met this challenge by the evolution of a family of receptors that recognize conserved surface components of microbial pathogens, called pathogen-associated molecular patterns (PAMPs). In the mammalian innate immune system PAMPs bind to pattern recognition receptors (that distinguish self from conserved microbial structures that are shared by different pathogens) to trigger the expression of antimicrobial compounds [3–5]. Lipopolysaccharides (LPS) from Gram-negative bacteria, peptidoglycans from Gram-positive bacteria, eubacterial flagellin, unmethylated bacterial DNA fragments, as well as glucans, chitins, mannans, and proteins derived from the fungal cell wall; are PAMPs that are able to trigger innate immune responses in various vertebrate and invertebrate organisms [4,5]. Moreover, many of these molecules have been shown to act as general elicitors of defense responses in various plant species [6,7]. LPS from Gram-negative bacteria have been shown to be inducers of plant defense reactions [8–15]. In addition flg22, a highly conserved amino-terminal fragment of flagellin, the main building block of eubacterial flagellae, triggers defense reactions in diverse plant species. Previously, a proteomics approach utilizing ^{32}P orthophosphate protein labeling to identify phosphorylated plant proteins during PAMP responses elicited by flg22 has been performed [16]. Together with evidence for LPS as an inducer of plant defense responses, this data indicates that plants have acquired and maintained the ability to recognize microbe-associated patterns [17].

Plant protein phosphorylation/dephosphorylation in response to pathogen attack is an important, intricate process interweaved with a multitude of other regulatory processes involved in defense- and resistance-related responses. There is no doubt that protein phosphorylation plays a pivotal role as one of the most dynamic and effective post-translational protein modifications. Phosphorylation serves to activate or deactivate critical enzymes and other proteins, alter subcellular protein location, half-life, interaction in protein complexes and the cross-talk and integration of distinct signals within the cell.

We have previously reported on the role of $LPS_{B.cep}$ in the molecular mechanisms and components involved in signal perception and signal transduction during defense-associated responses in *N. tabacum* cell suspension cultures. $LPS_{B.cep}$ induced a rapid influx of Ca^{2+} into the cytoplasm of aequorin-transformed tobacco cells, as well as the production of reactive oxygen and nitrogen species during an oxidative burst, and K^+/H^+ exchange during alkalinization of the extracellular culture medium [14]. In addition, we have found that $LPS_{B.cep}$ elicitation of tobacco cells has specific effects on the reversible phosphorylation events underlying the perception systems involved in the interaction of these plant cells with $LPS_{B.cep}$ [13]. These results indicated that the perception- and signal transduction responses during $LPS_{B.cep}$ elicitation of tobacco cells require a fine balance between the actions of certain protein kinases and protein phosphatases.

In this study, we utilized a proteomic approach to analyze changes in $LPS_{B.cep}$-induced phosphoprotein profiles of tobacco. The main goal was to identify proteins whose phosphorylation status is responsive to $LPS_{B.cep}$ treatment. The advantages of such an approach are that differentially phosphorylated proteins that are actually present at a desired time interval can be distinguished and different forms of the same protein can be resolved. The results reveal aspects of the dynamic nature of protein phosphorylation during $LPS_{B.cep}$ elicitation of *N. tabacum* cells and improves our understanding of the biological processes involved in the triggered responses.

2. Materials and methods

2.1. Extraction and purification of Burkholderia cepacia lipopolysaccharides ($LPS_{B.cep}$)

An endophytic strain of *Burkholderia cepacia*, (ASP B 2D), 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 *Burkholderia cepacia* cell walls using the hot-phenol procedure as previously described [14]. This procedure included an RNA degradation step, protein denaturation and removal, 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.

2.2. Plant cell cultures and LPS treatment

Nicotiana tabacum cv. Samsun cell suspension cultures were established and grown as previously described [10]. All experiments were performed using cells in the logarithmic growth phase, 3–5 d after sub-cultivation. Cell cultures (0.3 g ml^{-1}) were treated with 100 μg ml^{-1} $LPS_{B.cep}$ dissolved in 1 mM $CaCl_2$/2.5 mM $MgCl_2$ for 2 h at room temperature with gentle agitation on an orbital shaker. Control experiments received no $LPS_{B.cep}$, but an equivalent volume of 1 mM $CaCl_2$/2.5 mM $MgCl_2$ solution for 2 h.

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

For radiolabeled 2D phosphoproteome analyses, *N. tabacum* cells (0.3 g ml^{-1}) were treated with 100 μg ml^{-1} $LPS_{B.cep}$. for 2 h, followed by in vivo labeling with 3.7 MBq 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 an equivalent volume of 1 mM $CaCl_2$/2.5 mM $MgCl_2$ solution, were similarly performed. Two-dimensional gel electrophoresis, gel staining and autora-