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Differential display profiling of the *Nicotiana* response to LPS reveals elements of plant basal resistance

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Abstract

The identification of cDNAs, representing up-regulated genes induced by lipopolysaccharides from *Burkholderia cepacia*, was achieved by differential display of mRNAs isolated from tobacco cells. In addition to up-regulation of superoxide dismutase, involved in the production of the signalling and defense molecule, hydrogen peroxide; differentially expressed cDNAs, indicative of the operation of an innate immune recognition system and expression of basal resistance, were identified. These include homologs to a receptor-like protein kinase; a binding protein for the type III secreted effector protein, harpin; a virus resistance N gene; an endogenous pararetrovirus and the *Pto* kinase. The altered gene expression may be responsible for activation of surveillance mechanisms and enhancement of the non-self recognition capacity. The putative roles of these transcripts in LPS-induced responses are discussed in relation to emerging concepts of innate immunity.

Keywords: Basal resistance; Defense response; Innate immunity; Lipopolysaccharides (LPS); Pathogen-associated molecular pattern molecules (PAMPs)

Plants have a broad, basal mechanism of non-self recognition and utilize an active immune system to combat attack from pathogenic microbes [1]. Penetration by a potential pathogen rapidly activates a system of 'non-self' recognition based on specifically adapted plant receptors. These receptors recognize characteristic and invariant pathogen-borne surface molecules or pathogen-associated molecular pattern (PAMP) molecules. The resulting intracellular signals are transduced to the nucleus through a network of signalling cascades that orchestrate defense-oriented transcriptional reprogramming of cellular metabolism and dynamic, structural rearrangements associated with an activated defense response. Similarities exist between the molecular organization of animal and plant systems for non-self recognition and anti-microbial defense [1–4]. Innate immunity is a phylogenetically ancient form of immunity common to all Metazoa and Viridiplantae, where plants have acquired and maintained the ability to recognize PAMPs. Current models on innate immunity and non-host resistance postulate that PAMPs are recognized by Toll-like receptors in animals and by receptor-like protein kinases in plants. In both cases, signalling pathways are activated that ultimately lead to a state of induced resistance [1,5].

LPS is an indispensable component of the outer cell surface of Gram-negative bacteria and plays a crucial role in bacterial interactions with eukaryotic hosts where it acts as stimulant of innate immunity in diverse eukaryotic species [6]. LPS has no structural homolog among multicellular organisms, making it a target for innate immune recognition. This tripartite molecule consists of a poly- or oligosaccharide region that is anchored in the outer bacterial membrane by lipid A, a specific carbohydrate lipid moiety. In mammals, lipid A acts as a potent stimulator of the innate immune system via the induction of inflammatory mediators released by host cells. According to current models, the specific cellular recognition of LPS leads to the rapid activation of an intracellular signalling

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network that is homologous to the interleukin signalling systems [6,7]. Previously we reported that LPS can be recognized by plants to directly trigger or potentiate basal plant defense responses [8,9] and recent studies provided insights into the mechanism of plant defense activation by LPS [10,11]. Although much remains to be established, it is suggested to be analogous to the innate immunity system of mammals, based on the recognition of PAMPs [1].

In an extension to biochemical studies [10,11] we have set up a model system for LPS from *Burkholderia cepacia* ($LPS_{B.cep.}$) perception to screen for induced genes associated with recognition. mRNA differential display has been used successfully to identify and isolate genes involved in plant-pathogen interactions [12]. Here we report on the identification of genes that are induced in tobacco cells following the perception of $LPS_{B.cep.}$.

Methods

LPS preparation and cell elicitation. $LPS_{B.cep.}$ was isolated from the endophytic strain of *Burkholderia cepacia* (ASP B 2D) [8]. *Nicotiana tabacum* cv. Samsun cell cultures were grown at 25 °C, in the dark, in Murashige and Skoog (MS) cell suspension medium containing 0.25 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 0.25 mg l^{-1} kinetin (pH 5.8) [8], whilst continuously shaking at 120 rpm. LPS, dissolved in 1 mM $CaCl_2$ and 2.5 mM $MgCl_2$, was added to a final concentration of 100 μg ml^{-1}. After 6 h induction time, the cells were filtered under sterile conditions to remove the suspending medium.

Differential display of RNA. Total RNA was isolated 6 h after elicitation from the $LPS_{B.cep.}$-treated cells and control cells, using the Fast-Green RNA extraction kit (BIO101/Q-Biogene). DDRT-PCR was performed on Dnase I-treated total RNA using the RNAimage kit (GenHunter Corp., Nashville, TN, USA). The HAP1–8 primers used were in combination with the anchor primers $HT_{11}M$ (where H = AAGC and M = A/G/C), as described [13]. cDNA fragments amplified were separated on a 6% denaturing polyacrylamide gel in TBE buffer as used for DNA sequencing. The gel was fixed, dried, and exposed to BioMax MR film (Kodak). cDNA of interest was excised from the gel and re-amplified [14]. The products were analyzed on 1.5% agarose gels stained with ethidium bromide. DNA was recovered, using GenElute™ Minus EtBr Spin columns (Sigma), and quantified using Hoechst Dye#33258 (Polysciences Inc, Warrington, PA) on a Fluoroskan II (Biosystems).

Reverse Northern analysis. One microgram cDNA of the differentially displayed PCR amplicons was dot-blotted onto positively charged nylon membranes (Roche). cDNAs from transcripts that did not exhibit differential expression were included as controls. ^{32}P-Labelled probes were transcribed from mRNA (5 μg total RNA) obtained from the control and $LPS_{B.cep.}$-treated samples in parallel reactions. Hybridization was performed using Rapid-Hyb buffer (Amersham Pharmacia Biotech). The sealed membrane was exposed to MS film (Kodak) at −80 °C overnight with an intensifying screen.

Cloning, colony PCR, sequencing, and database analysis. The PCR fragments were ligated and transformed into JM109 cells using the pGEM-T Easy vector kit (Promega, Madison USA). Colony PCR was performed to determine insert size and correct colony selection before sequencing. Subsequently, plasmid DNA was sequenced using a DYEnamic ET Dye terminator cycle sequencing kit for MegaBACE (Amersham) in a MegaBACE 200 automated capillary DNA sequencing system. Resulting nucleotide sequences were analyzed for homology using the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/), with the BLAST N search algorithms (National Centre for Biotechnology Information). Alignments were further compiled using DNAssist software (www.dnassist.org/patterton/software.htm).

Results and discussion

Cultured *N. tabacum* cv. Samsun suspension cells were treated with 100 μg ml^{-1} $LPS_{B.cep.}$ for 6 h. Subsequent to LPS treatment, total RNA was isolated from the cells, as well as media-treated control cells. Differential display was performed using random primer combinations as described. An overview of typical differential display gel autoradiographs is shown in Fig. 1. The RNA was effectively reverse transcribed and resulted in PCRs with identical patterns in duplicate samples. The results indicate significant changes in the pattern of gene expression, both quantitatively and qualitatively, indicative of the broad transcriptional re-programming associated with a plant defense response [1]. Selected bands were cut from the gel and used for re-amplification. These PCR products were subsequently probed via reverse Northern analysis to identify true positives (Figs. 2A′ and B′). The differentially expressed transcripts (DETs) were cloned, their sizes confirmed by colony PCR and sequenced. More than 250 DETs were identified using the primer combinations as stated and those related to innate immunity selected. Using this procedure, five cDNAs that may be indicative of the operation of an innate recognition system were identified as representing genes whose expression was responsive to $LPS_{B.cep.}$ treatment (Table 1). The sequences were analyzed using the BLAST homology search facility at NCBI. The sequence alignments are shown in Figs. 3A–E. The DETs were found to have homology to superoxide dismutase, an *Arabidopsis* gene encoding a receptor-like protein kinase, a binding protein for harpin, a type III secreted effector protein from Gram-negative bacteria, the *Nicotiana glutanosa* virus resistance (N) gene, the flanking region of a tobacco endogenous pararetrovirus (TEPRV), and the tomato *Pto* locus.

In mammals, the innate immune response to LPS is triggered through Toll-like receptor (TLR) 4, which consists of an extracellular leucine-rich repeat domain and a cytoplasmic Toll interleukin 1 receptor domain. During the recognition process, LPS binds to soluble LPS-binding protein and then forms a complex with GPI-anchored protein CD14, MD-2 and the transmembrane TLR4 [3]. Mammals possess only approximately 10 TLRs to recognize PAMPs. There is increasing evidence that the activation of plant innate immune responses upon recognition of PAMPs partially resembles the mechanism of activation of the innate responses in mammals and insects systems. In plants, which do not have an adaptive immune system, more than 600 members of the family of transmembrane receptor-like kinases have been identified in *Arabidopsis thaliana* [15].

Perception of $LPS_{B.cep.}$ by tobacco cells was previously shown to result in an increase in intracellular Ca^{2+} [10]. Modest, but prolonged, increases of cytosolic Ca^{2+} levels appear to be essential for elicitation of innate defense responses in plants [1]. $LPS_{B.cep.}$ was also previously shown to elicit an oxidative burst in cultured tobacco and Arabidopsis cells with an accompanying production of both