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Molecular characterisation and regulation of a *Nicotiana tabacum* S-domain receptor-like kinase gene induced during an early rapid response to lipopolysaccharides ☆

Natasha M. Sanabria, Henriëtte van Heerden [1], Ian A. Dubery *

Department of Biochemistry, University of Johannesburg, Auckland Park, South Africa

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ABSTRACT

The isolation, characterization and regulation of the first lipopolysaccharide (LPS)-responsive S-domain receptor-like kinase (RLK) in *Nicotiana tabacum* are reported. The gene, corresponding to a differentially expressed LPS-responsive EST, was fully characterised to investigate its involvement in LPS-induced responses. The full genomic sequence, designated *Nt-Sd-RLK*, encodes for a S-domain RLK protein containing conserved modules (B-lectin-, S- and PAN-domains) reported to function in mediating protein–protein and protein–carbohydrate interactions in its extracellular domain, as well as the molecular architecture to transduce signals intracellularly through a Ser/Thr kinase domain. Phylogenetic analysis clustered *Nt-Sd-RLK* with S-domain RLKs induced by bacteria, wounding and salicylic acid. Perception of LPS induced a rapid, bi-phasic response in *Nt-Sd-RLK* expression with a 17-fold up-regulation at 3 and 9 h. A defence-related W-box *cis* element was found in the promoter region of *Nt-Sd-RLK* and the transient induction of *Nt-Sd-RLK* in cultured cells by LPS exhibited a pattern typical of early response defence genes. *Nt-Sd-RLK* was also responsive to salicylic acid induction and was expressed in differentiated leaf tissue, where LPS elicited local as well as systemic up-regulation. The results contribute new knowledge about the potential role that S-domain RLKs may play within interactive signal transduction pathways associated with immunity and defence.

1. Introduction

Lipopolysaccharides (LPS) contribute to the cocktail of microbe-derived molecules, which plant cells are exposed to upon bacterial infection (Boller and Felix, 2009; Segonzac and Zipfel, 2011; Zhang and Zhou, 2010). LPS are major constituents of the Gram-negative outer membrane and are released from the bacterial cell wall into the plant apoplast through shedding by living cells or dissolution of dying or dead cells. In addition, LPS are important components of outer membrane vesicles, released from bacterial membranes (Sidhu et al., 2008). When plant cells perceive LPS, it can lead to the triggering of defence responses (Gerber et al., 2004; Zeidler et al., 2004), or to the priming of the plant (Madala et al., 2012; Newman et al., 2007).

Species of the *Burkholderia* genus occupy a wide range of ecological habitats in the environment. Some species are found in association with plants and are effective in stimulating plant growth or act as biocontrol agents (Vandamme et al., 2007). Our ongoing research has shown that the LPS from *Burkholderia cepacia*, strain ASP B 2D, exhibit activity as an inducer of defence-related responses (Coventry and Dubery, 2001; Gerber et al., 2004; Zeidler et al., 2004). This LPS was found to trigger a rapid influx of Ca^{2+} into the cytoplasm of tobacco cells, as well as the production of reactive oxygen and nitrogen species (ROS and NO) during an oxidative burst reaction and K^+/H^+ exchange and alkalization of the extracellular culture medium (Gerber et al., 2004). LPS also has specific effects on reversible protein phosphorylation events underlying the perception systems involved in its interaction of plant cells (Gerber and Dubery, 2004; Gerber et al., 2006, 2008), e.g. the phosphorylation and activation of an extracellular signal-related MAP kinase (Piater et al., 2004).

Positive feedback regulation operates in plant innate immunity with transcriptional activation of the components involved in perception and signalling (Navarro et al., 2004). The up-regulated expression of

Abbreviations: At, *Arabidopsis thaliana*; DDRT-PCR, differential display reverse transcription polymerase chain reaction; DUF, domain of unknown function; EST, expressed sequenced tag; LPS, lipopolysaccharide(s); LRR, leucine-rich repeat; MAMP, microbe-associated molecular pattern; Nb, *Nicotiana benthamiana*; Ng, *Nicotiana glutinosa*; Nt, *Nicotiana tabacum*; *Nt-Sd-RLK*, *Nicotiana tabacum* S-domain receptor-like kinase; OPS, O-polysaccharide; PAMP, pathogen-associated molecular pattern; PAN, plasminogen/apple/nematode domain; PRR, pattern recognition receptor; R, resistance; RACE, rapid amplification of cDNA ends; RLK, receptor-like kinase; RLP, receptor-like protein; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance; SI, self incompatibility; SD, S-domain; SLG, S-locus glycoprotein; SGLP, S-glycoprotein like; SRK, S-receptor kinase; TSS, transcription start site.

☆ Note: Nucleotide sequence data is available in the GenBank database under the accession number GU196248.

* Corresponding author. Tel.: +27 11 5592401; fax: +27 11 559 2605.
E-mail address: idubery@uj.ac.za (I.A. Dubery).
[1] Present address: Department of Veterinary Tropical Diseases, University of Pretoria, South Africa.

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receptor-like kinase (RLK) and resistance (*R*) genes presumably leads to an enhanced sensitivity of the plant to further stimuli, sensing the presence of invading micro-organisms, i.e. a primed or sensitised state (Newman et al., 2007; Sanabria et al., 2010). This enables the plant to respond more rapidly, as well as to a greater degree, to subsequent pathogen challenge by eliminating or containing the potential pathogens (Erbs and Newman, 2003).

Elicitation of *Nicotiana tabacum* cell suspensions with LPS from *B. cepacia* resulted in the differential expression of a putative S-domain *RLK* (Sanabria and Dubery, 2006). In addition, three S-domain *RLK* genes (At5g60900, At5g18470 and At1g70530) were found to be up-regulated in a transcriptional microarray analysis of *Arabidopsis thaliana* in response to elicitation by LPS (TAIR, expression set 100808727), suggesting a function in LPS-induced responses (Sanabria et al., 2008).

S-domain RLKs form a distinct class within the large plant RLK gene family (Shiu and Bleecker, 2001). The proteins are single-pass transmembrane Ser/Thr kinases displayed on the plasma membrane. The extracellular domains of S-domain RLKs include a B-lectin domain, a cysteine-rich EGF-like S-domain and a PAN (plasminogen/apple/nematode) domain. The PAN domain refers to a conserved module that functions by mediating protein–protein interactions, similar to leucine-rich repeat (LRR) domains, but also protein–carbohydrate (e.g. mannose binding) interactions (Shiu and Bleecker, 2001; Tordai et al., 1999).

The role of S-domain RLKs and S-domain receptor-like proteins (RLPs) during defence responses has not been thoroughly explored. The S-receptor kinases (SRKs) were the first of the Ser/Thr receptor protein kinase (RPK) groups to be associated with a specific trait, i.e. SI in *Brassica* reproduction (Kachroo et al., 2002; Takayama and Isogai, 2003). However, a number of S-domain *RLK* genes have been found to show up-regulated expression in response to pathogen infection and wounding, or, treatment with salicylic acid (SA), a metabolite that plays an important role in potentiating local and systemic acquired resistance (SAR) (Ohtake et al., 2000; Pastuglia et al., 1997, 2002). More recent reports link genes encoding S-domains to *R* gene function in *Oryza sativa* (Chen et al., 2006), disease resistance signalling in *A. thaliana* (Kim et al., 2009), pathogen perception in *Nicotiana glutinosa* (Kim et al., 2010) and induction of basal resistance in *N. tabacum* (Maimbo et al., 2010). It is thus plausible that S-domain RLKs could be utilised to function as sentinel pattern recognition receptors (PRRs) during self/non-self perception of MAMPs (Sanabria et al., 2008, 2010).

Further research is warranted in order to broaden our understanding of the involvement of S-domain RLKs in surveillance, perception and signal transduction events during plant-microbe interactions. In this report we describe the identification and characterization of an inducible S-domain RLK gene in *N. tabacum* that exhibits responsiveness towards elicitation by LPS, a lipoglycan MAMP molecule.

2. Materials and methods

2.1. Plant material

N. tabacum cv Samsun cell suspensions were grown under controlled conditions as previously described Sanabria and Dubery, 2006). All experiments were performed on cells in the logarithmic growth phase, 3–5 days after sub-cultivation. Plants were grown in a green house at 20–25 °C with a 16 h day/8 h night cycle.

2.2. MAMP preparation and elicitation

LPS was purified from *B. cepacia* (ASP B 2D) as previously described (Coventry and Dubery, 2001; Leone et al., 2006). A LPS stock solution (20 mg ml^{-1} dissolved in 1 mM $CaCl_2$, 2.5 mM $MgCl_2$), was freshly prepared, diluted and added to the cell suspensions to a final concentration of 100 μg ml^{-1}. Control cells in MS medium were treated with the diluted $CaCl_2/MgCl_2$ solution (final concentration of 5 μM/12.5 μM respectively).

Cells were similarly treated with 0.2 μM flg22 (synthesised by GL Biochem Laboratories, Shanghai, China, according to Navarro et al., 2004) and 100 μg ml^{-1} chitosan (purified from chitin as previously described, Louw and Dubery, 2000).

2.3. Gene-walking

Primers were designed for the *HAP3–15* transcript using Primer3 software (http://frodo.wi.mit.edu/). Table S1 lists all primers and adapters/oligonucleotides used. Genomic DNA was isolated using the DNeasy® Plant Mini kit (QIAgen, Germany). The genomic DNA samples were constructed from 2.5 μg DNA digested with *Dra*I, *Eco*RV, *Pvu*II, *Stu*I, *Eco*47III, *Nae*I, *Sna*BI and *Ssp*I restriction enzymes (Fermentas Life Sciences, USA). Adapters 1 and 2 were ligated to the digested ends using T4 DNA Ligase (Roche, Germany). Primary PCR was performed using Expand Long Template PCR System and Buffer Set 1 (1.75 mM $MgCl_2$ final concentration) (Roche, Germany), with 0.2 μM 1F25(1) or RevK1, 2R25(1) or RevS1, Adapter primers 1 and 2, 0.05 U enzyme, 0.2 mM dNTPs and 1 μl DNA digest product. The cycling parameters were as follows; 30 s at 94 °C, 35 cycles of 94 °C for 10 s and 68 °C for 6 min and finally 15 min at 70 °C, using an Eppendorf Gradient Master Cycler. Nested PCR was performed using a 50× dilution of the primary PCR product as template with 2F25(1) or RevK2, and 1R25(5), 1R25(9), 1R25(12) or RevS2 primers, as described above. A final concentration of 5% (v/v) DMSO was used to disrupt the secondary structure of the gene. All DNA samples and PCR products were separated on 1% (w/v) agarose gels immersed in 1× TBE buffer containing 0.36 μg ml^{-1} ethidium bromide. PCR fragments of interest were purified using the MinElute Gel Extraction kit (Qiagen, Germany), followed by ligation and transformation into *Escherichia coli* JM109 high efficiency competent cells using the pGEM®-T Easy vector kit (Promega, USA). Plasmid DNA was isolated using the QIAprep Spin Miniprep kit (QIAgen, Germany) and sequencing was performed in both directions using a DYEnamic ET Dye terminator cycle sequencing kit for MegaBACE (Amersham), in a MegaBACE 200 automated capillary DNA sequencing system.

2.4. RT-PCR and 3′/5′-RACE

Primers were designed (as described above) for the gene based on the experimentally obtained genomic sequence. Total RNA was isolated at various time intervals from cell suspensions treated with LPS (100 μg ml^{-1}) using QIAzol (QIAgen, Germany). Total RNA (2 μg) was treated with RQ1 DNase (Promega, USA). First strand synthesis was performed using an oligo-dT anchor primer and Transcriptor Reverse Transcriptase (Roche, Germany). All of the resulting cDNA was amplified using Expand Long Template PCR System (Roche, Germany), 0.625 μM of a 5′-specific primer, the PCR anchor primer, 0.05 U enzyme and 0.2 mM dNTPs. The cycling parameters were as follows: 2 min at 94 °C, 10 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 40 s), followed by 25 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 40 s, with a 20 s addition per extension cycle and finally 7 min at 72 °C. Nested PCR was performed using a 5′-specific primer and the PCR anchor primer, and visualised on a 1% (w/v) agarose gel. PCR fragments of interest were purified, followed by transformation into *E. coli* JM109 cells as described above.

RACE was performed using the 5′/3′-RACE kit (Roche, Germany). The 5′-RACE was performed with a specific primer, 1R25(1), to synthesise the first stand cDNA, according to manufacturer's instructions. A poly(A)-tail was added and the PCR amplification was performed using an oligo dT primer and a second specific primer, 2R25(1) or 143RHS. A nested PCR reaction was performed with the anchor primer and a third specific primer, 1R25(5) or 85RHS. The 3′-RACE was