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Short communication

An antagonist of lipid A action in mammals has complex effects on lipid A induction of defence responses in the model plant *Arabidopsis thaliana*

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Abstract

Lipopolysaccharides, the ubiquitous part of the outer membrane of Gram-negative bacteria, and their derivatives are recognised by plants to trigger or potentiate particular defence responses such as induction of genes encoding pathogenesis-related proteins. The molecular mechanisms of LPS perception that underpin these effects in plants are, however, unknown. Here, lipid A from *Halomonas magadiensis*, which is an antagonist of lipid A action in human cells, was used to investigate lipid A action in plants. Our findings offer an insight into the different structural requirements for direct induction and potentiation of plant defences by lipid A.
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1. Introduction

Lipopolysaccharides (LPS), a major component of the outer membrane of Gram-negative bacteria, have diverse roles in microbial interactions with eukaryotic hosts. LPS are one of a group of bacterial components known as Pathogen Associated Molecular Patterns (PAMPs) that trigger innate immunity in mammals [1]. Conversely, LPS as endotoxins are the main initiators of sepsis and septic shock in mammals. LPS comprise an O-antigen polysaccharide, a core oligosaccharide and a lipid A moiety, whose structure is generally conserved in different bacteria. The endotoxic activity of LPS has been shown to reside in the lipid A moiety. In mammals, lipid A is recognised by the Toll-like receptor *TLR4* [1]. Interference with lipid A action through the use of structurally-related antagonists is one potential approach for the treatment of LPS-induced sepsis [2]. The agonistic or antagonistic activity of lipid A in humans has been related to its conical or cylindrical conformation [3]. The mode of perception of LPS in plants is far less understood than in mammals and insects. It is now established that LPS can act as a PAMP in plants, triggering a wide range of basal defence responses such as the oxidative burst, nitric oxide generation, cell wall alterations and pathogenesis-related (PR) protein induction, reviewed in Ref. [4]. In addition, LPS can act to ''prime'' plants to respond by more rapid or greater level of expression of certain defences on subsequent pathogen challenge [5], whilst in parallel suppressing the hypersensitive response (HR), a defence

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response associated with programmed cell death [4]. LPS from a range of bacteria can trigger these responses. Although involvement of the lipid A structure in eliciting plant responses has been demonstrated in several cases [6,7], other structures within LPS also have activity [8,9]. Plant receptors recognising other bacterial PAMPs such as flagellin and Ef-Tu elongation factor have recently been described, while those involved in perception of LPS or LPS derivatives remain elusive [7,9]. Consequently, it is not clear to what extent LPS perception by plants mechanistically resembles that in animals.

Here we have compared LPS perception mechanisms in plants and animals by an examination of the effects of an established antagonist of LPS action in human cells on lipid A induction of a defence response in plants. *Halomonas magadiensis* is a Gram-negative extremophilic and alkaliphilic bacteria isolated from a soda lake in an East African Rift Valley. Determination of the structure of lipid A from *H. magadiensis* (Fig. 1a) led to the suggestion that the molecule may act as an LPS antagonist in human cells [10]. *Escherichia coli* lipid A, composed of a bis-phosphorylated hexa-acylated disaccharide backbone with an asymmetric distribution of the acyl residues (Fig. 1b), represents a very effective agonistic structure of immune responses in mammalian cells, while lipid A from *H. magadiensis*, characterized by a peculiar and very low degree of acylation, has been verified to inhibit this enteric LPS-induced human monocyte activation [11]. Structural differences on the lipid A skeleton, for example, acylation can strongly affect its agonist/antagonist activity. In fact, besides the effects of the most variable LPS portions in triggering the responses of the mammalian acquired immune system, the biological activity depends on the acylation pattern of lipid A. The activation of the mammalian innate immune system is performed by the natural blend of variously acylated lipid A species produced by a bacterium; indeed pathogenic strains usually have a degree of highly acylated lipid A [12].

We have examined the effects of *H. magadiensis* lipid A on induction of the *PR1* gene in *Arabidopsis thaliana* by lipid A from *E. coli*.

2. Materials and methods

2.1. Lipid A preparations from H. magadiensis and E. coli

The LPS of *E. coli* was purchased from Sigma Aldrich. Cultivation of bacteria and isolation of *H. magadiensis* LPS were performed as previously described [10]. Purification of the free Lipid A from LPS of *H. magadiensis* and *E. coli* was prepared as described [11].

2.2. Plant tests

Lipid A from both *H. magadiensis* and *E. coli* were tested for their ability to induce *PR1* gene expression in *A. thaliana* accession Columbia (Col-0). Lipid A, 50 μg/ml, from *H. magadiensis* and *E. coli*, respectively, were dissolved in water. Six-week-old *Arabidopsis* leaves were treated with either *H. magadiensis* lipid A or *E. coli* lipid A alone or

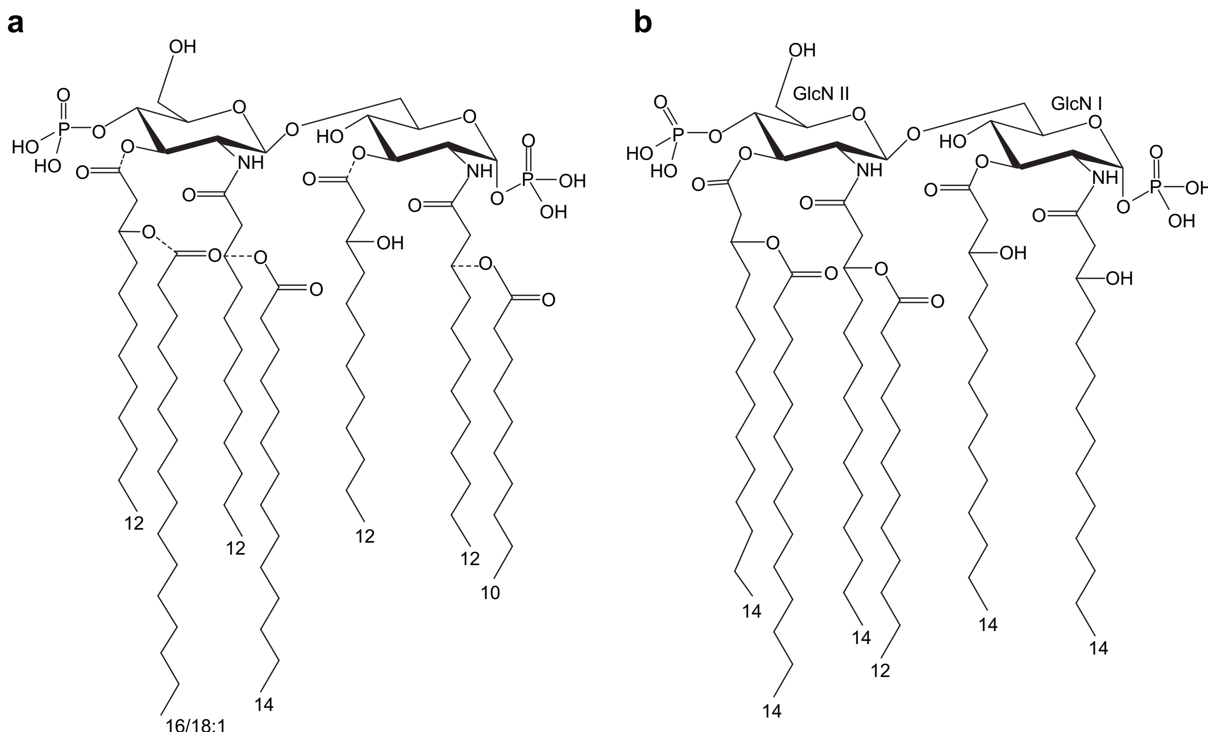

Fig. 1. Lipid A from (a) *Halomonas magadiensis* and (b) *Escherichia coli*. The dotted lines refer to non-stoichiometric substitutions and clearly show the low degree of acylation of lipid A from *H. magadiensis*.

infiltrated with *H. magadiensis* lipid A followed by *E. coli* lipid A infiltration at 0, 30, 60 and 180 min. Control leaves were infiltrated with water. Plants were placed at 25 °C with 8 h of light, and leaves were harvested 4, 12, 20 and 24 h after the last inoculation. All experiments were repeated three times.

2.3. Total RNA extraction, cDNA synthesis, quantitative real-time RT-PCR and statistical evaluations

The induction of *PR1* gene expression was analysed by quantitative real-time RT-PCR.

2.3.1. Total RNA

It was extracted using RNAwiz (Ambion, UK) following the manufacturer's instructions. Contaminating genomic DNA was removed by treatment with DNase using DNA-free (Ambion, UK) according to the manufacturer's recommendations. *cDNA* synthesis was performed using iscript (BioRad, USA) as described by the manufacturer. The final concentration of reversely transcribed total RNA was 35 ng/μl. For each sample, a negative control was made without adding reverse transcriptase (no-RT control).

2.3.2. Primers

The 18S rRNA gene was used as reference gene; the primers for this gene are described in Ref. [8]. The *PR1* primer pair (*PR1* Forward: gtgggttagcgagaaggcta; *PR1* Reverse: actttggcacatccgagtct) was designed using the gene sequence for *PR1* assigned AT2g14610 in the Arabidopsis Genome Initiative3 and the primer3 program (http://www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi). The primers were in general designed to flank an intron in order to be able to discriminate between cDNA or gDNA amplification products, respectively. Gene specificity was tested by blasting analyses of the amplicon. The amplicon was also tested for secondary structure on the Web site: http://www.bioinfo.rpi.edu/applications/mfold/. Both primer pairs were initially tested in the Mx3000P (Stratagene, USA) real-time PCR machine by making standard and dissociation curves on a gDNA dilution series ranging from 35 ng to 35 pg (see below for real-time RT-PCR conditions). Only primers yielding PCR efficiencies between 90 and 110% and with high linearity ($r > 0.98$) were accepted. The amplicons were furthermore run in a 2% agarose gel and stained with EtBr in order to verify the expected size of the PCR product.

2.3.3. Quantification by real-time RT-PCR

Polymerase chain reaction was performed in the Mx3000P (Stratagene, USA) with 35 ng cDNA, 10 pmol of each primer, 12.5 μl 2× SYBR Green master mix (Stratagene, USA) and 0.4 μl of a 500× diluted reference dye (Stratagene, USA) in a final volume of 25 μl. Prior to amplification, an initial denaturation step was performed (95 °C for 10 min) ensuring complete denaturation of cDNA and activation of the Taq polymerase (included in the SYBR Green master mix). This was followed by 40 cycles for 15 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C. Fluorescence was detected after each cycle. After the last amplification cycle, PCR was specified in a melting curve analysis by increasing the temperature from 58 to 95 °C while measuring the fluorescence for every 0.5 °C increase. For every sample, two parallel cDNA syntheses were made, each of which was run in duplicates in the real-time PCR experiments, thus yielding four replicates of each sample. The no-RT controls were run with one primer pair, to confirm that there was no contaminating chromosomal DNA in the original sample. Furthermore, a no-template control was run with every primer pair.

2.3.4. Statistical evaluations

The relative expression level of the *PR1* gene was evaluated in lipid A treated material compared to water treated material and normalized to the 18S rRNA expression level. The analyses were performed using the relative expression software tool (REST©) [13].

3. Results

Leaves of *A. thaliana* were infiltrated with either *H. magadiensis* lipid A or *E. coli* lipid A or infiltrated with *H. magadiensis* lipid A followed by *E. coli* lipid A after an interval of 0, 30, 60 and 180 min. Induction of *PR1* gene expression in the treated leaves was analysed by real-time RT-PCR on extracted RNA. An early accumulation of *PR1* transcript (after 4 h) was observed in response to treatment with *E. coli* lipid A. The level of transcript decreased over time with no accumulation evident at 24 h. In contrast, no *PR1* transcript accumulation was detected in response to *H. magadiensis* lipid A alone (Table 1).

Table 1
Induction of *PR1* gene expression in *Arabidopsis thaliana* as a result of 50 μg/ml *Halomonas magadiensis* lipid A or 50 μg/ml *Escherichia coli* lipid A treatment and pre-infiltration with 50 μg/ml *H. magadiensis* lipid A followed by 50 μg/ml *E. coli* lipid A

Time after treatment (h)	*E. coli* lipid A	*p*-Values	*H. magadiensis* lipid A	*p*-Values	Time after *E. coli* lipid A treatment (h)	0 Time between treatments	*p*-Values	30 min between treatments	*p*-Values	60 min between treatments	*p*-Values	180 min between treatments	*p*-Values
4	202[a]	0.001	0		4	0		0		5	0.001	2202	0.001
12	12	0.001	1	0.673[ns]	12	0		40	0.001	101	0.001	24	0.001
20	10	0.001	1	0.669[ns]	20	2	0.161[ns]	26	0.001	3	0.001	0	
24	1	0.662[ns]	3	0.579[ns]	24	0		11	0.001	11	0.001	15	0.171[ns]

ns: not significant.
Three independent repetitions have been done with similar results.
[a] Fold increase compared to water treated tissue, after normalization to 18S rRNA.

When *A. thaliana* leaves were co-infiltrated with *H. magadiensis* lipid A and *E. coli* lipid A, no accumulation of the *PR1* transcript was observed over a 24-h time course (Table 1). In plant tissue pre-treated with *H. magadiensis* lipid A for 30 min before treatment with *E. coli* lipid A, a transient accumulation of *PR1* transcript was observed at 12 h; this accumulation decreased over time. If the time between the *H. magadiensis* lipid A and *E. coli* lipid A treatments was increased to 60 min, a transient accumulation of *PR1* transcript was observed at 4 h after the *E. coli* lipid A treatment. A substantial level of transcript was observed at 12 h, then the level decreased considerably at 20 h followed by a slight increase at 24 h (Table 1). Remarkably, in plants pre-treated for 180 min with *H. magadiensis* lipid A before treatment with *E. coli* lipid A, levels of *PR1* transcript at 4 h after *E. coli* lipid A treatment were over 10-fold higher than those in plants without *H. magadiensis* lipid A treatment. Thereafter, the level of *PR1* transcript decreased dramatically, with no transcript at 20 h followed by a slight increase in transcript accumulation at 24 h.

4. Discussion

H. magadiensis lipid A alone did not induce *PR1* gene expression in *A. thaliana* but inhibited *PR1* gene expression induced by *E. coli* lipid A when the two preparations were applied simultaneously. However, when *H. magadiensis* lipid A was applied several hours prior to *E. coli* lipid A, *PR1* expression was strongly potentiated. Thus *H. magadiensis* lipid A demonstrates divergent effects in plants, being partially antagonistic to and synergistic with the action of *E. coli* lipid A.

The ability of *H. magadiensis* lipid A to antagonise the action of *E. coli* lipid A in inducing *PR1* gene expression in *A. thaliana* is consonant with its activity in blocking enteric LPS-induced human monocyte activation [11]. These observations suggest that *A. thaliana* is sensitive to the same structures of lipid A that determine biological activity in humans. An added complexity of lipid A action in plants appears to be that although *H. magadiensis* lipid A is unable to directly trigger *PR1* induction, it can potentiate the *PR1* induction caused by *E. coli* lipid A. This activity of *H. magadiensis* lipid A suggests that it may be a useful tool to dissect the molecular events underpinning potentiation from those associated with direct induction of plant defences. Furthermore, it will be of interest to generate derivatives of *H. magadiensis* lipid A to establish whether the structural requirements for the potentiation and antagonistic actions of lipid A derivatives are identical.

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