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Induction of Callose Deposition in Tobacco (*Nicotiana tabacum*) by Bacterial Lipopolysaccharide *Pseudomonas syringae* pv. *tabaci* and *Pseudomonas syringae* pv. *glycinea*

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Abstract

Lipopolysaccharide (LPS) is a major component of outer-membrane gram-negative bacteria, and it can act as a Pathogen-Associated Molecular Pattern (PAMP) for perception of pathogens by plants. LPS can be recognized by plants, triggering certain plant defense-related responses, including callose deposition. This study investigated induction of callose deposition by bacterial LPS in tobacco. Tobacco leaves were infiltrated with 400 µg/mL and 800 µg/mL LPS extracted from *Pseudomonas syringae* pv. *tabaci* (Pta) and *Pseudomonas syringae* pv. *glycinea* (Pgl) and incubated for 24 h or 48 h. To detect callose deposition, tobacco leaves were cleared in lactophenol solution, stained with aniline blue, and visualized by fluorescence microscopy. Results showed that LPS from Pgl induced more callose deposition in tobacco leaves than did that from Pta. In addition, a Pearson correlation test revealed that incubation period was the most significant factor in callose deposition, followed by the type of LPS bacteria. However, LPS concentration was not significantly corelated to callose deposition in tobacco leaves.

Abstrak

Induksi Deposisi *Callose* pada Tanaman Tembakau (*Nicotiana tabacum*) oleh Lipopolisakrida Bakteri *Pseudomonas syringae* pv. *tabaci* dan *Pseudomonas syringae* pv. *glycinea.* Lipopolisakarida (LPS) adalah komponen utama permukaan sel bakteri gram negatif. LPS dapat berperan sebagai Pathogen-Associated Molecular Pattern (PAMP), yaitu molekul yang menjadi target pengenalan patogen oleh tanaman. Pengenalan LPS oleh tanaman dapat menginduksi respon pertahanan tanaman, termasuk deposisi *callose*. Penelitian bertujuan untuk mengetahui induksi deposisi *callose* pada tanaman tembakau oleh LPS bakteri yang diekstraksi dari bakteri *Pseudomonas syringae* pv. *tabaci* (Pta) dan *P. syringae* pv. *glycinea* (Pgl). Untuk pengamatan deposisi *callose*, daun tembakau diinfiltrasi LPS Pta dan Pgl, dengan konsentrasi 400 µg/ml dan 800 µg/ml, diinkubasi selama 24 dan 48 jam. Selanjutnya, klorofil daun diluruhkan menggunakan larutan laktofenol dan diwarnai dengan *aniline blue*. Deposisi *callose* diamati menggunakan mikroskop fluoresen. Hasil pengamatan menunjukkan LPS bakteri Pgl menginduksi deposisi *callose* lebih banyak dibandingkan LPS bakteri Pta. Lebih lanjut, berdasarkan uji korelasi *pearson* diketahui bahwa waktu inkubasi adalah faktor yang berkorelasi paling signifikan terhadap deposisi *callose*, diikuti oleh jenis bakteri LPS. Namun, konsentrasi LPS tidak berkorelasi signifikan dengan deposisi *callose* pada daun tembakau.

Keywords: callose deposition, lipopolysaccharide, Pseudomonas syringae pv. tabaci, Pseudomonas syringae pv. glycinea, tobacco

1. Introduction

Plants, like other living organisms, are surrounded in their environments by potential pathogens of various species, including bacteria [1-2]. *Pseudomonas syringae* are gram-negative bacteria that cause disease in many plant species, with the symptoms of these diseases ranging from leaf spots to stem cankers. *P. syringae* has a very narrow host range [3-4] and *Pseudomonas syringae* pv. *tabaci* (Pta) and *P. syringae* pv. *glycinea* (Pgl) are two examples of phytopathogens that cause problems in crop plants. Pta produces Tabtoxin, which causes wildfire

disease in tobacco, causing characteristic chlorotic halos on tobacco leaves [5]. In addition, Pgl causes bacterial blight on soybean plants, with symptoms including water-soaked lesions that develop into necrotic leaf spots surrounded by chlorotic halos [6].

Plants protect themselves against pathogens by using a variety of passive and active defense mechanisme. Active defense mechanisms include inducing Reactive Oxygen Intermediate (ROI) or oxidative burst, synthesizing Nitric Oxide (NO), Hypersensitive Response (HR), producing phytoalexins, activating pathogenesis-related protein (PR), inducing defense-related genes, and synthesizing and depositing callose [1,7,8]. Callose is a polysaccharide in the form of β-1,3-glucan with some β-1,6-branches, and it exists in the cell walls of a wide variety of higher plants. Callose plays an important role in the growth and differentiation processes of plants and in response to biotic and abiotic stressors. Deposition of callose is an effective barrier induced at the site of attack during relatively early stages of pathogen invasion, inhibiting penetration of pathogens into plant cells. In addition, callose deposition serves as a matrix in which anti-microbial compounds can be deposited, thereby providing focused delivery of chemical defenses at the cellular sites of attack [9-11].

Active defenses, including callose deposition, are triggered or induced when a plant recognizes pathogens. Plant recognition of pathogens, a process known as Pathogen/Microbe-Associated Molecular Patterns (PAMP/ MAMP), is accomplished by Pattern-Recognition Receptors (PRRs) on plant cell surfaces [12-13]. Types of PAMP or MAMP include flagellins, Elongation factor-Tu (EF-Tu), harpin, cold-shock protein, peptidoglycan, and lipopolysaccharides (LPS) [14-15]. Flagellin is one PAMP/MAMP molecule that has been reported to induce callose deposition on the plants *Arabidopsis thaliana* [11,16] and *Nicotiana benthamiana* [17]. In addition, on *Arabidopsis thaliana*, callose deposition can be induced by chitin, peptidoglycan [16], and chitosan [11]. However, data are rare about induction of callose deposition by LPS in tobacco plants (*Nicotiana tabacum*). *Nicotiana tabacum* and *Arabidopsis thaliana* [18] are widely used as models to study plant-pathogen interactions.

LPS are significant MAMP molecules. LPS are ubiquitous components of the outer membrane in gram-negative bacteria [19]. LPS may play a number of important roles in the interactions of bacterial pathogens with eukariotic hosts. The general structure of LPS consists of three parts, lipid A, core oligosaccharide, and a chain of oligosaccharide repeating units called O-chain, or O-antigen [19-22]. It has been reported that O-antigen oligosaccharides, core oligosaccharide, and lipid A moieties can induce plant defense responses [24,32,33]. However, the mechanisms of LPS perception by plants still are obscure. Therefore, this study analyzed LPS-induced defense responses in tobacco to identify mechanisms of bacterial LPS perception. Tobacco is known to be used to study disease resistance induced by LPS bakteri [25].

2. Methods

The plant sample is tobacco (*Nicotiana tabacum* L. cv. Xanthi). Tobacco plants were grown from seed at 26 ºC in a growth chamber for about six weeks. LPS was extracted from the tobacco pathogen *Pseudomonas syringae* pv. *tabaci* (Pta) (host interaction) and the soybean pathogen *P. syringae* pv. *glycinea* (Pgl) (non host interaction) using an LPS extraction kit (INTRON).

Tobacco leaves were infiltrated with 400 µg/mL and 800 µg/mL Pta or Pgl and incubated for 24 h or 48 h. As a control treatment, leaves were infiltrated with H_2O. Leaf chlorophyll was cleared in lactophenol solution (phenol: glycerol: lactid acid: dH_2O = 1:1:1:1) diluted to two times volume with 50% alcohol. Then, leaves were incubated for 15 min at room temperature, followed by incubation at 65 ºC for 30 min. These steps were repeated with new lactophenol solution until all chlorophyll was destroyed decayed. Then, leaves were soaked in 50% alcohol for 15 min and in sterile dH_2O for 15 min. Next, leaves were soaked in a solution of 0.01% aniline blue dissolved in 150 mM K2HPO4 (pH 9.5) for 30 min. Finally, leaves were placed on glass, a few drops of 50% glycerol solution were added, and the leaves were covered with glass. Callose deposition was observed by fluorescence microscopy [23].

Data were analized qualitatively and quantitatvely. Quantification of callose deposition was analyzed using Photoshop CS 5 software [11]. Then, data were analyzed qualitatively by statistical tests (ANOVA, Duncan). Statistical tests were conducted using SPSS 16 software.

3. Results and Discussion

The study used young tobacco leaves, about six weeks old, because a preliminary study showed that chlorophyll was easier to kill in younger leaves. Qualitatively, callose deposition in tobacco leaves stained with aniline blue appeared green when observed under fluorescent microscopy. Observation of photo samples showed that both bacterial LPS and H_2O treatment induced callose deposition in tobacco leaves. However, callose deposition induced by bacterial LPS was much more extensive than that induced by H_2O. This indicated that Pta and Pgl LPS both induced callose deposition, Pgl LPS moreso than Pta (Figure 1).

Quantitative data on callose deposition was obtained by measuring the total area of callose deposition in every leaf. The results showed that Pgl LPS produced a

greater total area of callose deposition than did Pta LPS (Table 1). Statistical analysis was conducted using a Duncan test. As Figure 2 shows, in general, callose deposition in leaves infiltrated by Pta or Pgl LPS was greater than in leaves infiltrated with H_2O. These results indicate that tobacco plant cells can recognize bacterial Pta and Pgl LPS molecules and take defensive actions.

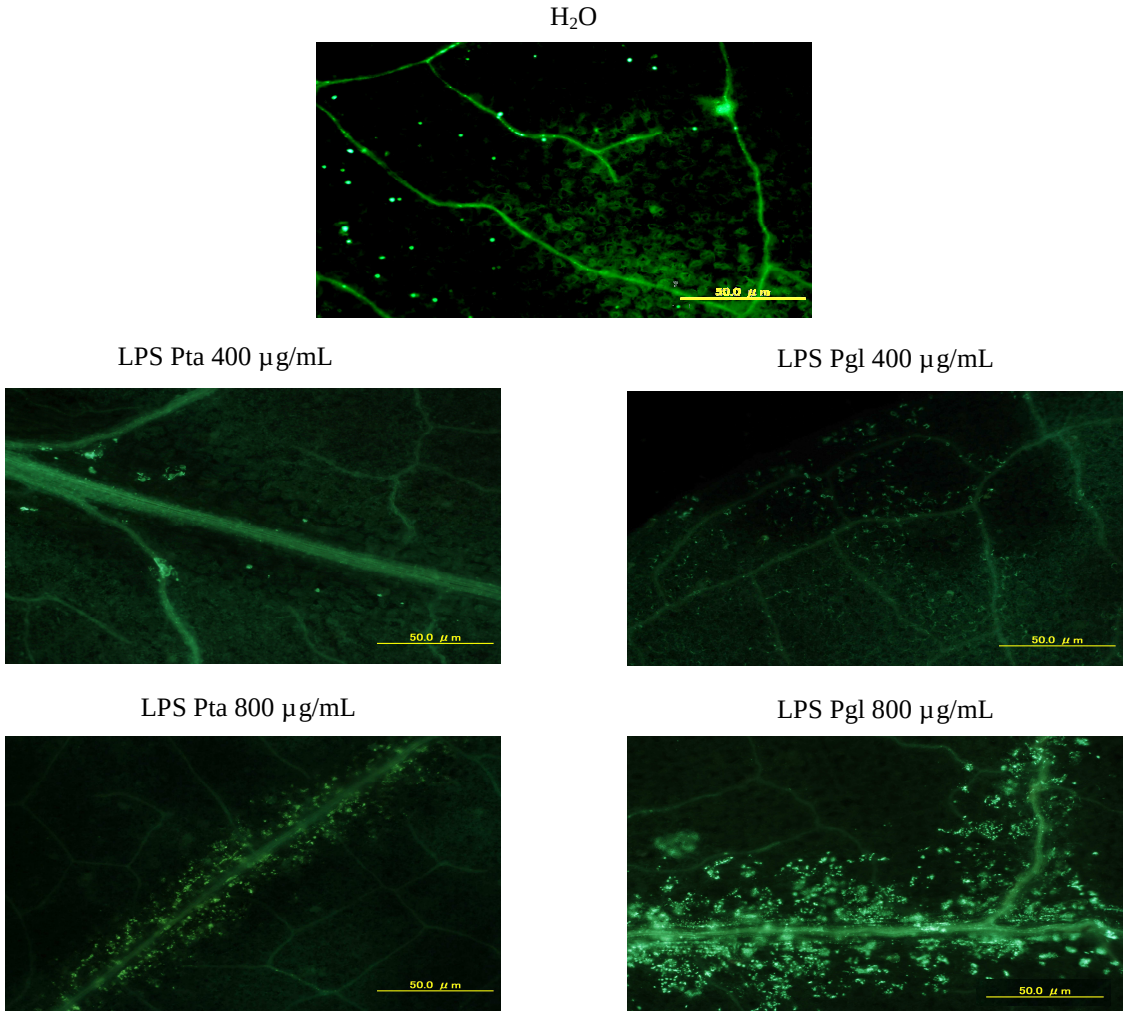

Figure 1. Microscopic Observation of Typical Callose Deposition in Tobacco Leaves Treated with LPS Bacteria *Pseudomonas syringae* pv. *tabaci* (Pta) and *Pseudomonas syringae* pv. *glycinea* (Pgl) after 48 h Incubation

Table 1. Total Area of Callose Deposition (μm^2) in Tobacco Leaf

Treatment	24 h Incubation					48 h Incubation				
	H_2O	400 µg/mL LPS		800 µg/mL LPS		H_2O	400 µg/mL LPS		800 µg/mL LPS	
		Pta	Pgl	Pta	Pgl		Pta	Pgl	Pta	Pgl
1	11	120	108	109	237	61	99	337	693	987
2	37	231	282	184	290	162	119	426	1194	3001
3	69	244	294	418	771	583	184	2589	1260	3105
4	413	311	313	571	1344	1325	187	3024	1328	3511
5		482	400	682	1826		251	3439	14093	9194
6		753	3700	1132	1887		1409	3842		13501

Notes: * Data on total area of callose deposition per leaf in each treatment, sorted from lowest to highest.
(Pta = *Pseudomonas syringae* pv. *tabaci; Pgl = Pseudomonas syringae* pv. *glycinea).*

Recognition of MAMP molecules by PRRs is an important factor in inducing defense responses [13,24]. Plant cell recognition of LPS induces certain signal transductions and effects changes in cellular activity associated with the defense response [25]. LPS of *Pseudomonas solanacearum* infiltrated to tobacco leaves binds to mesophyll cell walls, inducing ultrastructural changes like vesiculation [25]. Vesicles form in cells associated with protein secretion or material transport [26]. In addition, vesicle formation is one of the processes involved in inhibiting or stopping growth of pathogens [15]. The purified LPS *Burkholderia cepacia* has been found to trigger a rapid influx of Ca^{2+} into the cytoplasm of tobacco cells [27]. Ca^{2+} is one of the most important second messengers in eukaryotes and is involved in signal transduction processes that induce plant defense responses [15].

As shown in Figure 2, Pgl LPS was better at inducing callose deposition than Pta LPS, which could indicate that Pgl LPS induces stronger tobacco defense responses than does Pta LPS. Differences in response induction between the two may be related to differences in recognition by of each by tobacco cells. LPS has no structural homolog among multicellular organisms, making it a target for defense response recognition [28]. LPS structures consist of three main parts: lipid A, core oligosaccharide, and a polysaccharide chain with a repeating unit called the O-antigen [1]. It has been reported that parts of the LPS, (lipid A, core oligosaccharide and O-antigen) are involved in inducing plant defense responses [24,32,33].

Recently, the structure of O-antigen repeating units has been identified [Unpublish data]. The O-antigen repeating units of Pta LPS are [→3)-α-L-Rha*p*-(1→4)-β-L-Rha*p*-(1→3)-α-D-Rha*p*-(1→]$_n$, whereas those of Pgl LPS comprise only D-rhamnan and are [→3)-α-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→]$_n$. The fact that LPS Pgl produces a greater defense response than does Pta indicates that O-antigen repeating units composed of D-rhamnose alone are more recognizable to tobacco plants than are those compposed of L-rhamnose (L-6-deoxy-mannose). L-rhamnose is a deoxy sugar that is enriched in some parts of plant biomass, such as hemicellulose and pectin. The L-form of rhamnose is more common in nature than the D-form [29]. Many micro-organisms living on decaying plant material are able to use L-rhamnose as a carbon source. These micro-organisms can cause the structure of D-rhamnose to be more recognizable as a foreign molecule than L-rhamnose. Therefore, tobacco plant cells more readily recognize Pgl LPS as foreign molecules compared to Pta LPS, so Pgl LPS induces a stronger defense response than Pta LPS.

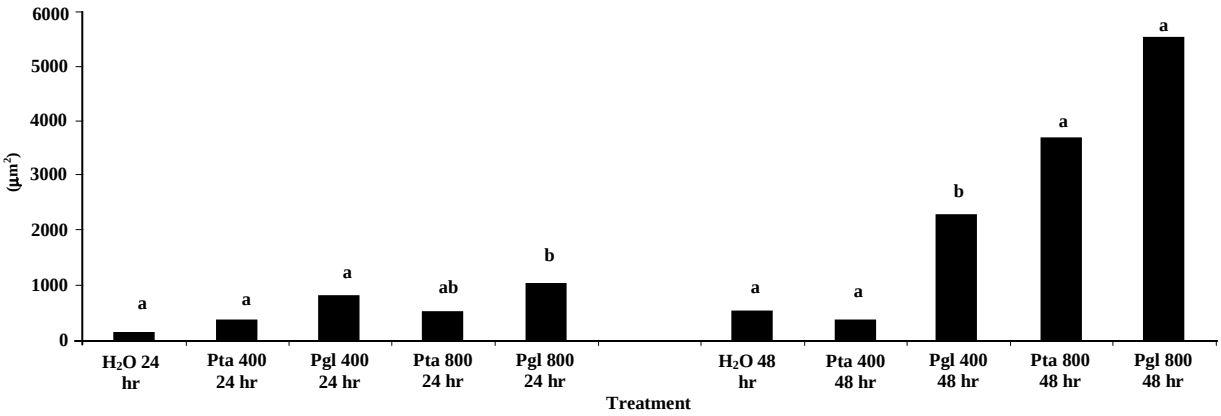

Figure 2. Graph of Duncan Test on the Effect of Two Types of Bacterial LPS (*Pseudomonas syringae* pv. *tabaci* (Pta) and *Pseudomonas syringae* pv. *glycinea* (Pgl)) on Callose Deposition in Tobacco Leaves. Value with the Same Letter are not Significantly Different at P=0.05

Table 2. Pearson Correlation Test between Callose Area and Incubation Time, LPS Concentration and Type of LPS Bacteria

		Callose area	Incubation time	LPS concentration	LPS bacteria
Callose area	Pearson correlation	1	0.349**	0.044	0.278*
	Sig. (2-tailed)		0.009	0.748	0.040
	N	55	55	55	55

**Correlation is significant at the 0.01 level (2-tailed).
*Correlation is significant at the 0.05 level (2-tailed).

The important role of the O-antigen LPS structure in inducing plant defense response is illustrated by the fact that mutant *Pseudomonas fluorescens* bacteria that have lost their O-antigen structure produce a different effect in Induced Resistance Systemics (ISRs) than do wild type *P. fluorescens* [30] Other studies have shown that the LPS structures lipid A and core oligosaccharide also play a role in recognition by the receptor plant [30,32,33].

A Pearson correlation test was done to correlate callose area with types of treatment (LPS bacteria type, LPS concentration, and incubation time). Table 2 shows: 1) correlation between callose area and incubation time was highly significant (Pearson correlation of 0.349); 2) correlation between callose area and type of LPS bacteria was significant (Pearson correlation of 0.278); and 3) correlation between callose area and concentration of LPS was not significant (Pearson correlation of 0.044). These results suggest that incubation period has the most influence on inducing callose deposition in tobacco leaves, followed by the type of LPS bacteria (Pgl or Pta). Concentration of LPS appears to have no significance.

The influence of incubation time on callose deposition can be understood when one recognizes that callose deposition is one of the basal defense responses in plants [19] and provides a physical barrier to prevent and inhibit pathogen penetration [9,31]. This means that the longer pathogens infect the plant, the deeper and more broadly they will penetrate. In response, more callose will be synthesized and deposited.

4. Conclusions

Many studies have been done on induction of plant defense responses by PAMPs. Here, we described induction of callose deposition as a defense response by tobacco leaves against bacterial lipopolysaccharide extracted from *Pseudomonas syringae* pv. *tabaci* (Pta) and *Pseudomonas syringae* pv. *glycinea* (Pgl). Results showed that tobacco plant cells can recognize bacterial LPS Pta and Pgl molecules and can induce defense responses. In addition, results indicated that LPS from Pgl induced more callose deposition in tobacco leaves than did LPS from Pta. Statistical tests showed that incubation period has the highest significance level in correlation with callose deposition area, followed by the type of LPS bacteria. LPS concentration is not significantly correlated to callose deposition area in tobacco leaves. Further work is needed to understand the role of LPS in inducing defense response in tobacco plants.

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