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Protective effect of bacterial lipopolysaccharides in the grapevine-*Agrobacterium vitis* interaction

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Summary

Cell-associated lipopolysaccharides (LPS) were extracted by the phenol-chloroform-petroleum ether extraction method (PCP) from a nopaline strain of *Agrobacterium vitis*, purified by treatment with DNase/RNase, proteinase K and dialysis, characterized by polyacrylamide gel electrophoresis (SDS-PAGE), and bioassayed on grapevine shoot nodal segments. LPS preparation used for the experiments *in planta* was a mixture of rough-type LPS, obtained from precipitation with water after PCP-extraction, and some smooth- and rough-type LPS from the remaining phenol phase. Infiltration of an aqueous dispersion of the mixture in concentrations of 25-1000 g·ml $^{-1}$ did not cause grapevine tissue necrosis, and callus formed within one month. When the LPS dispersions were infiltrated in the grapevine nodal segments, 24 h before challenge inoculation with *A. vitis* (5x10^2 cells per 5 l droplet), they prevented tumorgenesis and tissue necrosis; but, when the pathogen was inoculated at a higher concentration (5x10^5 cells per 5 l droplet), these LPS applications were active in protecting plant tissue from necrosis and did not prevent tumor induction. The potential role of LPS as candidate molecules in the protection of grapevine from *A. vitis* infection is discussed.

K e y w o r d s : grapevine, crown gall, *Agrobacterium vitis*, lipopolysaccharides, protection.

Introduction

Bacterial lipopolysaccharides (LPS, endotoxins) (BRADE *et al.* 1999) are amphiphilic essential outer membrane constituents occurring in almost all Gram-negative bacteria. Chemically, they comprise three regions, *i.e.* the O-specific polysaccharide, the core region and the lipid A, all of which form a smooth(S)-form LPS. Rough(R)-form LPS occurs in wild-type and laboratory strains that possess mutations in the genes encoding the O-specific polysaccharide biosynthesis. LPS play an important protective role for the bacteria and are essential for their viability. On the other hand, they represent the O-antigens of Gram-negative bacteria and are responsible for various pathophysiological effects (*e.g.*, fever, hypotension, shock) in mammals including humans. These effects are associated with the lipid A, which represents the toxic moiety of LPS. The structures of lipid A from various LPS have been investigated in detail (ZÄHRINGER *et al.* 1994, 1999), and it was shown that biological activity of lipid A is sensitive to even slight modifications of its chemical structure.

In interactions between plants and Gram-negative bacteria, LPS are thought to be involved in various processes. The unique structure of lipid A in *Rhizobium leguminosarum* may reflect functional roles in symbiosis (RAETZ 1993). Protein-lipopolysaccharide complexes (pr-LPS), extracted from *Pseudomonas syringae* pathovars and purified, acted as signal molecules by modifying *in planta* the heterologous tobacco leaf tissue-*P. syringae* pv. *aptata* interaction and the homologous one with *P. syringae* pv. *tabaci* (MAZZUCCHI *et al.* 1979, 1982). Moreover, the induced resistance to *P. syringae* pv. *tabaci* in the tobacco leaf mesophyll could be transmitted to plants directly regenerated from tissue where primary induction took place (BAZZI *et al.* 1994; BIZARRI *et. al.* 1996).

Localized LPS pretreatments may interfere during the plant-*Agrobacterium* interaction by inhibiting the pathogen's cell attachment to competent sites, which is crucial for tumorgenesis (MATTHYSSE *et al.* 1978). In particular, a LPS fraction prepared from virulent strains prevented tumor formation if applied to the wound prior to the application of the pathogenic bacterium (WHATLEY *et al.* 1976), and only the polysaccharide portion of LPS (not the lipid A component) was responsible for interference with tumorgenesis (LIPPINCOTT and LIPPINCOTT 1976).

The use of "information-rich molecules", acting as chemical messengers or signals, specifically recognized by several procariotic and eucariotic proteins, might have a potential in inhibiting tumour formation in grapevine. This paper deals with the purification and characterization of LPS, extracted from a virulent *A. vitis* strain, by polyacrylamide gel electrophoresis (SDS-PAGE), and their effect on grapevine tissue. The activity of these purified LPS in preventing tissue necrosis (BURR *et al.* 1987; BAZZI *et al.* 1999) and/or tumor formation was tested after challenge inoculation with the pathogen.

Material and Methods

A g r o b a c t e r i u m v i t i s a n d g r o w t h c o n d i t i o n s: The tumorgenic strain CG 49 (having a nopaline

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type Ti plasmid) was provided by T. J. BURR of the Department of Plant Pathology, New York State Agricultural Experiment Station, Geneva, N.Y. The bacterium was grown at 28 °C to the early stationary phase in an aerated liquid YM medium (for mass cultivation) or on YM agar plates containing mannitol, yeast extract and salts, at pH 7.2 (MILLER *et al.* 1990).

LPS extraction: After harvesting by centrifugation (16,000 x *g*, 20 min, 4 °C) the whole bacterial cells were washed with cold distilled water. The cell pellet (100 g) was lyophilized until ready to use. LPS were extracted by the phenol-chloroform-light petroleum (PCP) extraction method (GALANOS *et al.* 1969), specific for R-form lipopolysaccharides. Part of the lyophilized bacterial pellet (25 g) was used to extract LPS avoiding the ethanol, acetone, and diethyl ether washing step. The isolated R-and S-type LPSs were purified by treatment with DNase/RNase (6-8 h, 37 °C in a buffer solution containing 10 mM $MgCl_2$ and 20 mM phosphate buffer, pH 8.0) and proteinase K (overnight, 37 °C in distilled water) and dialysis into regenerated cellulose tubes (Cellu Sep™, MWCO 12,000-14,000) against deionized water (48 h, 4 °C). The dialyzed products were concentrated in rotavapor and lyophilized. Glucosamine (GlcN), 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and fatty acids were determined as described (RUND *et al.* 1999).

Polyacrylamide Gel Electrophoresis: LPS preparations were analysed by discontinuous slab gel electrophoresis using sodium dodecyl sulphate (SDS-PAGE) and detected in the gels by silver staining according to the protocols of HITCHCOCK and BROWN (1983).

Grapevine material: Canes of the crown gall susceptible *Vitis vinifera* cv. Cabernet-Sauvignon were collected from asymptomatic dormant mother vines in the Cooperative Rauscedo Nurseries (Pordenone, Italy) and stored at 4 °C in moist dark conditions. Whenever vegetative material was required, two-bud cuttings were forced in sterile perlite in the greenhouse. For *in vitro* experiments, young actively growing shoots were cut into one-node segments and surface sterilized with 70 % ethanol for 2 min, followed by 1.3 % sodium hypochloride (containing one drop of Tween 20/100 ml) for 20 min and rinsed with sterile distilled water until all visible traces of detergent had disappeared. The explants were trimmed aseptically at both ends and placed apex down in Phytacon jars (Sigma) containing 70 ml growth regulator-free NITSCH & NITSCH medium (1969) with 1.5 % sucrose. Cultures were maintained in growth chambers illuminated by cool-white fluorescent lamps with a photoperiod of 16 h light at a mean temperature of 26-28 °C.

Inoculum preparation: The tumorgenic *A. vitis* strain CG 49 was grown on YM agar plates for 48 h at 28 °C, suspended in sterile distilled water (SDW) at an absorbance of A_{660} = 0.1, containing about 10^8 CFU ml^{-1}. Tenfold dilutions of the mother suspension were prepared to inoculate grapevine nodal segments.

Grapevine bioassays: Groups of 10 nodal segments were vacuum-infiltrated (100 kPa, 20 min) with a mixture of LPS, obtained from precipitation with water after PCP-extraction, and from the remaining phenol phase. LPS dispersions in SDW were infiltrated at different concentrations (1000, 100, 50, 25 g·ml^{-1}); for each LPS concentration, 20 segments were infiltrated and transferred into new Phytacon jars. After 24 h, the basal ends of nodal segments were pierced through a depth of 1.0-1.5 mm by a no. 20 sterile needle and droplets (5 l) of bacterial inoculum were placed on wound sites (ca. $5x10^5$ and $5x10^2$ cells per segment). Nodal segments treated with LPS, *A. vitis* CG 49 and SDW were used as controls.

Detection of opines: Hyperplasias at the inoculation sites were assayed one month after LPS application for the presence of the tumour marker nopaline, according to the high voltage paper electrophoresis (HVPE) procedure of OTTEN and SHILPEROORT (1978).

Results

The PCP-extraction method, combined with specific purification steps, was suitable to obtain LPS preparations free of nucleic acids, proteins, phospholipids and other bacterial cell components. Compositional analyses revealed the presence of GlcN, and 3-hydroxytetradecanoic acid as characteristic LPS constituents. The Figure shows the SDS-PAGE patterns of LPS from nopaline strain CG 49 of *A. vitis*, the rough-type LPS (Re-chemotype) from *Escherichia coli* F 515 and the smooth-type (S-LPS) from *Salmonella enterica* sv. Abortus-equi A 11-78 included for comparison purposes. In the tumorgenic strain CG 49, R-LPS prevailed (yield 0.5 % of bacterial dry mass), even if a small amount of S-LPS was found in the phenol phase.

The infiltration in grapevine nodal segments of aqueous dispersions of the mixture of LPS in different concentrations did not cause by itself tissue necrosis, and only callus formation was observed as in negative controls treated with SDW. The localized inoculation of *A. vitis*

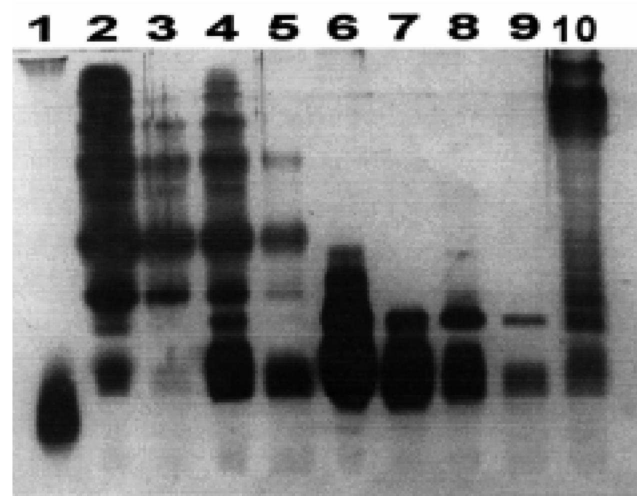

Figure: Gel electrophoresis of *A. vitis* LPS (nopaline strain CG 49). The gel (15 % acrylamide) was run and stained as described by HITCHOCK and BROWN (1983). The well samples were as follows: **1**: 5 g of *E. coli* F 515 R-LPS (control); **2**: 20 g S-LPS; **3**: 5 g S-LPS; **4**: 20 g S-LPS; **5**: 5 g S-LPS; **6**: 20 g R-LPS; **7**: 5 g R-LPS; **8**: 20 g R-LPS; **9**: 5 g R-LPS; **10**: 5 g S-LPS of *S. enterica* sv. Abortus-equi A 11-78 (control). Wells 2 through 5 contain LPS extracted from the phenol phase; wells 6 through 9 contain LPS extracted according to the standard procedure of GALANOS *et al.* (1969). LPS, S-LPS, R-LPS: see Introduction.

CG 49 suspension at low concentration (5x10^2 bacteria per 5 l droplet) to nodal segments 24 h after LPS application (concentrations ranging from 25 to 1000 g·ml $^{-1}$) gave rise to callus formation. In all cases, nopaline was not detected in the extracts from these hyperplasias by HVPE analysis. LPS application in different concentrations did not prevent tumor formation at the basal ends of grapevine nodal segments inoculated 24 h later with the challenge bacterium at high concentration (5x10^5 bacteria per 5 l droplet), and nopaline was found in all the analysed extract samples (Table).

Table

Effect of bacterial lipopolysaccharides (LPS) in preventing tumor formation and tissue necrosis on grapevine segments

Treatments	Number of nodal segments	Number of bacteria per basal end (strain CG 49)	Tumor formation (HVPE)	Tissue necrosis
LPS (g·ml $^{-1}$)				
25	10	5 x 10^2	-	-
25	10	5 x 10^5	+	-
50	10	5 x 10^2	-	-
50	10	5 x 10^5	+	-
100	10	5 x 10^2	-	-
100	10	5 x 10^5	+	-
1000	10	5 x 10^2	-	-
1000	10	5 x 10^5	+	-
25-1000	20		-	-
CG 49 (contr. +)	10	5 x 10^2	+	-
”	10	5 x 10^5	-	+
SDW (contr. -)	10		-	-

(-) absence and (+) presence of nopaline in the extracts from hyperplasias. SDW: Sterile distilled water; HVPE: High voltage paper electrophoresis.

After about 10 d, the application of CG 49 suspensions at low and high concentration (positive controls) caused visible tumors and tissue necrosis, respectively; after one month, necrotic tracts reached a mean length of about 0.5 cm. Very small galls were sporadically observed in the apparently healthy tissue adjacent to the necrotic streaks.

Discussion

The protocols used to extract and purify LPS were suitable to obtain protein and nucleic acid-free preparations, ready to disperse in water. Applications of aqueous dispersions of R- and S-form LPS from the nopaline *A. vitis* strain CG 49 were not phytotoxic, protected grapevine tissue from necrosis and, to a certain extent, prevented tumor induction. In the pretreated nodal segments (controls), these complex polymers did not cause *per se* visible effects up to a concentration of 1000 g·ml $^{-1}$. A ”differential” protective effect was observed when the challenge pathogen was inoculated 24 h after their infiltration down to a concentration of 25 g ml $^{-1}$: necrosis and tumor formation did not occur when a low number of bacterial cells (5x10^2) was inoculated at the wound sites; at a high inoculum concentration (5x10^5) tissue necrosis was prevented, but not tumorgenesis.

In this experimental approach it is difficult to refer the observed phenomena to induced resistance or interference. The pretreatment might have evoked physico-chemical alterations of plant cell walls around competent sites, in the absence of an active host response. As shown in other systems (Medeghini Bonatti *et al.* 1985; Stefani and Rudolph 1989), macromolecular complexes such as pr-LPS and EPS infiltrated in plant tissues accumulated in intercellular spaces and formed cell wall appositions. Alternatively, a role of LPS fractions acting as primary signals for plant cell receptors and defense responses could be hypothesized. Evidence that the O-specific chain of *P. fluorescens* LPS was an important determinant of induced systemic resistance (ISR) against carnation and radish *Fusarium* wilt was given by Van Peer and Shippers (1992) and Leeman *et al.* (1995); nevertheless, a mutant of the same strain lacking of such a region was active in inducing systemic resistance in the systems *Arabidopsis thaliana*-*P. syringae* pv. *tomato* and *F. oxysporum* f. sp. *raphani* as a proof of the existence of other ISR determinants (Van Wees *et al.* 1997). The strong biological activity of *Ralstonia solanacearum* LPS was highlighted in inducing synthesis of some antitumoral cytokinins (*e.g.*, tumor necrosis factor, TNF, interleukin-1, IL-1 and ϒ-interferon, IN) in animals (Varbanets *et al.* 1997). This was primarily attributed to O-specific polysaccharides and lipid A. As in *Rhizobium* K-antigens involved in nodulation, could have a role in the *Agrobacterium*-host interaction (Denny 1995).

Speculative hypotheses were made (Bazzi *et al.* 1999) on the factors controlling necrogenesis in grapevine: the lethal effect of excessive levels of accumulated auxin on plant cells and the pectolytic activity of strain CG 49 which has the chromosomal gene *pehA*, coding for a single polygalacturonase (PG) that degrades plant cell walls. Could the prevention of tissue necrosis by means of LPS application be attributed, at least in part, to inactivation of PG or by eukaryotic metabolic inhibitors triggered by signal molecules? Among monosaccharides originated from plant cell wall degradation, D-galacturonic acid has the strongest effect on *vir* genes induction (Ankenbauer and Nester 1990): a lack of PG or its inactivation could lead to a drastic decrease of ”saturable” receptor sites, negatively affecting bacterial attachment, multiplication and tumorgenesis. However, recent data indicated that *A. vitis* PG is insufficient by itself to cause grapevine-specific necrosis, and the pathogen’s ability to elicit an HR response in tobacco may be related to the mechanism of necrosis (Herlache and Burr, pers. comm.). In the treatments (positive controls) with low concentrations of *A. vitis*, tumor induction took place in the absence of tissue necrosis; on the other hand, tumors developed on nodal segments where necrosis was prevented after LPS application and inoculation of the challenge bacterium at high concentration.

Therefore, the necrosis factor does not seem to play a key role in tumorgenesis: this and the protective effect shown by LPS were differentially modulated by the level of challenge inoculum. *A. vitis* and *A. rubi* strains, unlike *A. tumefaciens*, produce primarily R-LPS and this would indicate a non-essential pathogenetic role (WEIBGEN *et al.* 1993). In the LPS preparation from CG 49 strain a certain amount of S-form was found, consistent with the expression of *rfb* genes (WHITFIELD and VALVANO 1993). Our results partially agree with those obtained by HERLACHE *et al.* (unpubl.), that is, LPS *per se* did not elicit any visible plant reaction and tissue necrosis; on the contrary, these authors found that none of the R-LPS, obtained from CG 49 or non-tumorgenic biological control strains (CG 523, F2/5) of *A. vitis* according to the protocol of DARVEAU and HANCOCK (1983) and exogenously applied to grapevine tissues, inhibited tumorgenesis and prevented necrosis induction.

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