Exopolysaccharides of *Pantoea agglomerans* have different priming and eliciting activities in suspension-cultured cells of monocots and dicots

Imke Ortmann[a], Uwe Conrath[b,1], Bruno M. Moerschbacher[a,*]

[a] *Department of Plant Biochemistry and Biotechnology, Westfälische Wilhelms-Universität Münster, Hindenburgplatz 55, 48143 Münster, Germany*
[b] *Plant Biochemistry and Molecular Biology Unit, Department of Plant Physiology, RWTH Aachen University, 52056 Aachen, Germany*

Abstract Induced disease resistance of plants is often associated with an enhanced capacity to activate cellular defense responses to pathogen attack, named the "primed" state of the plant. Exopolysaccharides of *Pantoea agglomerans* have recently been reported as the first priming active component of bacterial origin in wheat cells. We now show that *Pantoea* exopolysaccharides also prime rice cells for better elicitation of a rapid oxidative burst. In contrast, in tobacco and parsley cell cultures *Pantoea* exopolysaccharides activate the oxidative burst response directly. Our results point to a different recognition and/or mode of action of *Pantoea* exopolysaccharides in monocot and dicot plants.
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1. Introduction

In plants, induced disease resistance (IR) provides long-lasting protection against a broad spectrum of pathogens [1] and, therefore, is of agricultural interest. Various types of IR have been described in plants and include systemic acquired resistance (SAR) [1], induced systemic resistance (ISR) [2,3], and β-aminobutyric acid (BABA)-IR [4]. Over the past years, progress has been made in elucidating molecular and physiological mechanisms that are associated with the various types of IR in dicotyledonous plants. One mechanism that is common to SAR, ISR, BABA-IR, and also to other types of IR in plants is "priming" [5]. In the primed state, plant cells have a higher capacity to mobilize their various defense responses, thus resulting in faster and/or stronger deployment of defenses upon biotic or abiotic stress.

Recently, we detected the primed state in suspension-cultured cells of wheat by demonstrating that an elicitor-induced oxidative burst and the activity of extracellular peroxidases were strongly enhanced when the cells had been pretreated with priming-inducing molecules [6]. Using this assay, we identified exopolysaccharides (EPS) isolated from the spent growth medium of the gram-negative bacterium *Pantoea agglomerans* (formerly known as *Erwinia herbicola* or *Enterobacter agglomerans*) as a novel bacterial inducer of the primed state in a monocot [6].

To investigate whether the EPS of *P. agglomerans* are active at priming also in species other than wheat, we compared the ability to EPS prime for augmented induction of the oxidative burst in suspension-cultured cells of wheat, rice, tobacco, and parsley. The oxidative burst was chosen as an assay, because the rapid release of H_2O_2 is generally assumed to be a key event in the orchestration of various cellular defense responses [7].

2. Material and methods

Chitin hexamer (Accurate Chemical & Scientific Corporation, New York, USA) was used as the elicitor of the oxidative burst in suspension-cultured cells of wheat [8] and rice [9]. EPS were precipitated from the spent growth medium of *P. agglomerans* by addition of 4 vol. of 95% (v/v) ethanol supplemented with 1% (w/v) potassium chloride overnight at 4 °C. Subsequently, EPS were dissolved in water and dialyzed (molecular weight cut-off 12–14 kDa) against distilled water, followed by lyophilisation and electrophoretic verification of the absence of LPS and proteins, as described previously [6].

Suspension-cultured cells of wheat (*Triticum aestivum* L. cv Prelude-Sr5), rice (*Oryza sativa* L.), tobacco (*Nicotiana tobaccum* L. cv PC-120), and parsley (*Petroselinum crispum* L.) were grown in 50 ml cell culture medium [wheat cells in MS [10] medium supplemented with 2,4 D (2 mg l^{-1}), casein hydrolysate (1 g l^{-1}), and sucrose (30 g l^{-1}); rice cells in MS [10] medium supplemented with 2,4 D (1 mg l^{-1}) and sucrose (30 g l^{-1}); tobacco cells in LS [11] medium; parsley cells in modified B5 [12] medium] at 25 °C on a rotary shaker in the dark (120 rpm). Cells were transferred to fresh growth medium every seven (rice, tobacco, parsley) or ten days (wheat).

Prior to experiments, cells were gently separated from the cell culture medium through a sintered glass filter at the third day after subculturing. Aliquots of cells (300 mg) were transferred to 5 ml of assay medium [5% (v/v) cell culture medium in 10 mM MES supplemented by 3% (w/v) sucrose] in a six well micro-titer plate and resuspended on a rotary shaker. When wheat or rice cells were used for the experiments, the wells of the microtiter plate already contained EPS at the indicated concentrations from a stock solution (4 mg ml^{-1} EPS in water), or water as a control, before cell transfer. After a six-hour adaptation period, elicitor or EPS were added to pretreated or non-pretreated cells.

The generation of H_2O_2 upon addition of either chitin hexamer or EPS was measured by luminol-dependent chemiluminescence as described previously [13]. A calibration of the assay was done after each measurement, using a stock solution of H_2O_2 (100 μM for wheat and parsley cells or 1 mM for rice and tobacco cells in the above mentioned potassium phosphate buffer), diluted to different concentrations (corresponding to 0–30 μM of H_2O_2 for wheat and parsley cells

*Corresponding author. Fax: +49 251 83 28371.
E-mail addresses: uwe.conrath@bio3.rwth-aachen.de (U. Conrath), moersch@uni-muenster.de (B.M. Moerschbacher).

[1] Fax: +49 241 80 22181.

or 0–300 μM of H_2O_2 for rice and tobacco cells in 200 μl of cell suspension) in potassium phosphate buffer to a final volume of 600 μl. For each calibration, a fresh stock solution of H_2O_2 was prepared immediately before the calibration-measurement from a 12 mM solution of H_2O_2 in water, the concentration of the stock solution of H_2O_2 was checked regularly at 240 nm.

3. Results

In case of wheat and rice, direct application of EPS of *P. agglomerans* to the cell cultures even at the highest concentration tested (80 μg ml^{-1}) did not induce an oxidative burst response (Figs. 1 and 2). Rather, the EPS treatment primed the cells for augmentation of the H_2O_2 accumulation that had subsequently been induced by the chitin hexamer elicitor. In fact, after preincubation for 6 h of wheat or rice cell cultures with EPS at different concentrations (2–80 μg ml^{-1}), the cells responded with enhancement of the oxidative burst induced by chitin hexamer (0.1 μg ml^{-1}) compared to the burst that was elicited in cells pretreated with water (Figs. 1 and 2). The potentiation of the oxidative burst in EPS-pretreated cells was dependent of the EPS concentration that was used for the pretreatment. Enhancement at the highest concentration of EPS tested (80 μg ml^{-1}) was 2.6-fold for wheat and 7.3-fold for rice cells. In contrast to wheat and rice, cells of the dicots tobacco and parsley directly responded to the exposure to EPS of *P. agglomerans* by induction of an oxidative burst (Figs. 3 and 4). Application of EPS alone (2–80 μg ml^{-1}) to tobacco or parsley cells caused an EPS concentration-dependent accumulation of H_2O_2 that was evident already at the lowest EPS concentration tested (2 μg ml^{-1}; Figs. 3 and 4). In case

Fig. 2. Augmentation of an elicitor-induced oxidative burst after preincubation of rice cells with EPS of *P. agglomerans*. Suspension-cultured cells of rice were preincubated for six hours with 2 (◁), 10 (◆), 40 (▽) or 80 (▲) μg ml^{-1} of EPS prior to addition of chitin hexamer (0.1 μg ml^{-1}). As a positive control, rice cells were pretreated with water for 6 h before chitin hexamer (●, 0.1 μg ml^{-1}) was added. As a further control, the direct elicitor activity of the highest concentration of EPS (80 μg ml^{-1}) was elucidated (■). The generation of H_2O_2 was quantified using a luminol-dependent chemiluminescence assay. Data shown are from one representative of three independent experiments.

of parsley cells, this direct defense response inducing activity was confirmed by investigating the production and secretion of furanocoumarins which represent the phytoalexins of parsley. Furanocoumarins were extracted with chloroform and subsequently determined spectrophotometrically. Even at low EPS dose (2 μg ml^{-1}) there was significant direct accumulation
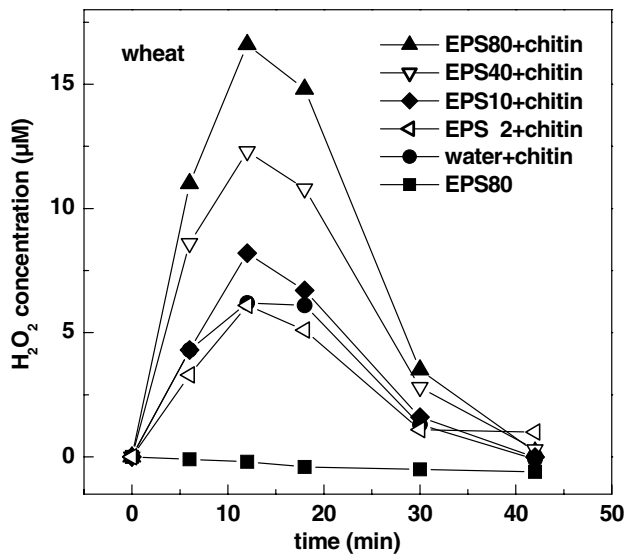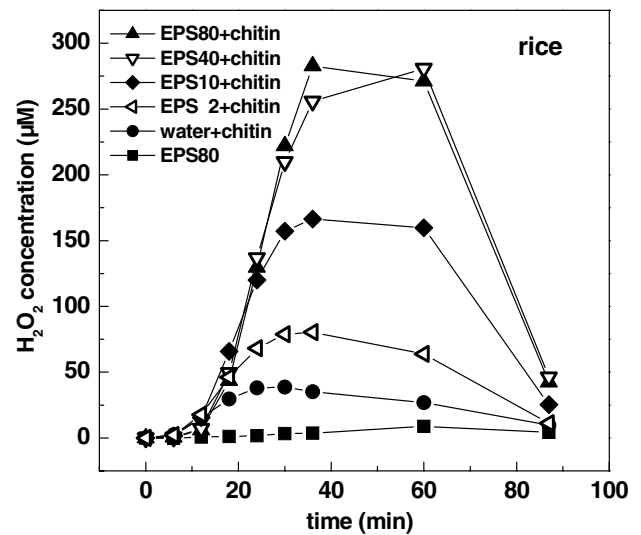
Fig. 1. Potentiation of an elicitor-induced oxidative burst after preincubation of wheat cells with EPS of *P. agglomerans*. Suspension-cultured wheat cells were preincubated for 6 h with 2 (◁), 10 (◆), 40 (▽), or 80 (▲) μg ml^{-1} of EPS prior to elicitation with chitin hexamer (0.1 μg ml^{-1}). As a control, wheat cells were pretreated with water for 6 h before chitin hexamer (●, 0.1 μg ml^{-1}) addition. As a further control, direct elicitor activity of the highest concentration of EPS (80 μg ml^{-1}) was determined (■). The generation of H_2O_2 was quantified using a luminol-dependent chemiluminescence assay. Data shown are from one of three independent experiments with similar results.
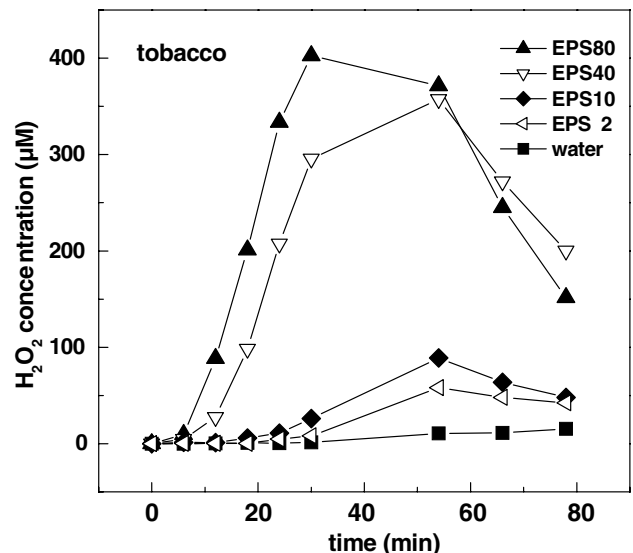
Fig. 3. Induction of an oxidative burst in tobacco cells by EPS of *P. agglomerans*. Suspension-cultured tobacco cells were treated with 2 (◁), 10 (◆), 40 (▽), or 80 (▲) μg ml^{-1} of EPS. As a control, water instead of EPS was added to the cells (■). The generation of H_2O_2 was quantified using a luminol-dependent chemiluminescence assay. Data shown are from one representative out of three independent experiments.
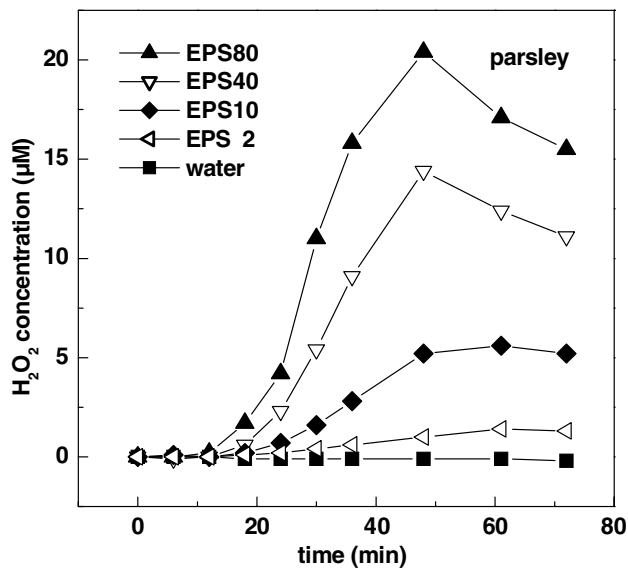
Fig. 4. Induction of an oxidative burst in parsley cells by EPS of *P. agglomerans*. Parsley cells in culture were treated with 2 (◁), 10 (◆), 40 (▽) or 80 (▲) μg ml^{-1} of EPS. As a negative control, water rather than EPS was added to the cells (■). Quantification of H_2O_2 was by luminol-dependent chemiluminescence. Data are from one representative of three independent experiments.

of furanocoumarins, as indicated by increased absorbance of the organic chloroform phase at 320 nm (data not shown).

4. Discussion

The above results revealed that EPS of *P. agglomerans* exhibit different bioactivities in wheat and rice vs. tobacco and parsley cells, suggesting different activities in monocotyledonous and dicotyledonous plant species. Obviously, they prime wheat and rice for potentiation of the subsequently elicited H_2O_2 defense response without acting as elicitors themselves, while they are active at directly eliciting this response in tobacco and parsley. Two hypotheses may be considered to explain these class-specific bioactivities. Firstly, the priming activity of EPS in wheat and rice may be a consequence of a specific co-evolutionary adaptation process in the non-pathogenic rhizosphere bacterium *P. agglomerans* and specific plants. Secondly, the class-specific bioactivities of EPS may be due to a general difference between cells from monocot and dicot species in their IR strategies.

Several examples for adaptation of bacteria to a specific plant species by adaptation of a signaling molecule have been described previously. Flagellin, a subunit of the bacterial flagellum, was reported to rapidly elicit defense responses in suspension-cultured tomato cells [14] and also to cause growth inhibition, callose deposition, and the activation of *PR* genes in *Arabidopsis* [15]. In contrast to the flagellins of other bacteria, flagellins of plant-associated *Agrobacterium tumefaciens* and *Rhizobium meliloti*, even at high concentrations, did not display direct elicitor activity in tomato and *Arabidopsis*, indicating that these bacteria avoid recognition by the plants probably by synthesizing flagellin variants [14,15]. Likewise, ergosterol, the typical sterol in membranes of most higher fungi, elicits rapid alkalinization in tomato cells, while plant-asso-

ciated fungi, such as biotrophic rust and mildew pathogens, contain modified ergosterols that do not act as elicitors of early defense responses [14,16]. Moreover, the bacterial elongation factor-Tu (EF-Tu), the most abundant bacterial protein, was shown to elicit early defense responses in both cell cultures and whole plants of *Arabidopsis* [17]. By contrast, EF-Tus of some plant pathogenic bacteria such as *Pseudomonas syringae* pv. *tomato* and *Xanthomonas fastidiosa* exhibit reduced elicitor activity.

A different response of monocots and dicots to bacterial components with a role in signaling has been proposed previously, when bacterial flagellin was shown to directly activate defense-related responses in a number of species of dicot plants, but not in rice [14]. Moreover, there is evidence that monocotyledonous plants recruit different mechanisms for IR responses than dicots [18]. In both monocots and dicots, IR can be induced by the same chemicals [19], such as salicylic acid (SA), 2,6-dichloroisonicotinic acid (INA), and a benzothiadiazole (BTH). But, few of the so-called "chemically induced genes" in the monocot species wheat [20] and barley [21] showed homology to typical *IR* genes known from dicotyledonous plants. Moreover, in contrast to most dicots, rice contains high levels of endogenous SA and these are not further elevated in response to pathogen attack [22]. Thus, SA is unlikely to be a key molecule in the IR response in rice, and possibly also in other monocotyledonous plants.

The 'non-expresser of *PR* genes-1' (NPR1) protein is known to be a key mediator of IR in dicotyledonous plants [23,24]. Overexpression of the *NPR1* gene in *Arabidopsis* led to disease resistance which has been associated with accelerated and/or enhanced defense gene expression [25]. In contrast, transgenic rice plants overexpressing the gene *OSNH1*, the closest homologue to *NPR1* in rice, displayed constitutive expression of *PR* genes in the absence of prior induction by chemicals or pathogens [26]. This finding indicates different regulatory activities of the *OSNH1* gene in rice and possibly other monocots compared to *NPR1* from *Arabidopsis* and possibly other dicots.

Further analysis of the activity of EPS from other bacterial species and/or of other bacterial components such as flagellin and EF-Tu for possible differences in their bioactivities in cells of monocots and dicots may shed light on the possibility of different IR strategies between the plant classes. This knowledge may eventually lead to the development of specific disease resistance strategies for cereal crop protection.

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