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BRIEF COMMUNICATION

Progesterone moderates damage in *Arabidopsis thaliana* caused by infection with *Pseudomonas syringae* or *P. fluorescens*

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Abstract

Brassinosteroids are known to protect plants against various abiotic and biotic stresses, however, very limited information is available about the role of progesterone. Therefore the effects of *Pseudomonas syringae* pv. *syringae* (*P.s.*) wild type strain 61, its *hrcC* mutant, and the saprophytic *P. fluorescens* (*P.f.*) strain 55 were investigated in wild type *Arabidopsis thaliana* cv. Columbia and its *rbohF* knock-out mutant with and without progesterone pre-treatment. The reactions of wild type and *rbohF* mutant *Arabidopsis* to bacterial inoculations were similar although 2 h after injection of *P.s.* a larger increase of electrolyte leakage was measured in wild type than in *rbohF* knockout mutant leaves. The *hrcC* mutant caused weak necrotic symptoms and increased leakage in both types of *Arabidopsis* although to a much lesser extent than *P.s.* The *P.f.* did not induce any visible symptom but slightly increased the electrolyte leakage in both types of *Arabidopsis.* Inoculation by all *Pseudomonas* bacteria led to significant alterations in photosystem II efficiency as compared to control plants. Pre-treatment of leaves with progesterone diminished the necrotic symptoms, the electrolyte leakage and improve the efficiency of photosystem II caused by *Pseudomonas* bacteria.

Additional key words: *Arabidopsis rbohF* knockout mutant, ion leakage, PS II efficiency, *Pseudomonas syringae pv. syringae hrcC* mutant.

Brassinosteroids (BRs) are involved in the regulation of cell elongation, fertility, flowering, senescence, and photomorphogenesis (Janeczko *et al*. 2003, Kesy *et al*. 2003). They stimulate plant growth and protect plants against various abiotic and biotic stressors (Krishna 2003, Nakashita *et al*. 2003, Bajguz and Hayat 2009, Skoczowski *et al*. 2011). Mutant plants deficient in BR biosynthesis or sensitivity showed marked developmental defects (Clouse and Sasse 1998, Yang *et al*. 2005). Pre-treatment of rape cotyledons with BR substantially decreased the damage caused by inoculation with *Pseudomonas* bacteria and by cold stress (Janeczko *et al*. 2007a,b, Skoczowski *et al*. 2011). The presence of progesterone, a C-21 steroid (pregn-4-ene-3β-ol-3,20-dione), a well-known mammalian gonadal hormone in plants was first reported by Gawienowski and Gibbs (1968). Later, the occurrence of progesterone and its related steroids was demonstrated in various higher plants (Iino *et al*. 2007, Simersky *et al*. 2009, Pauli *et al*. 2010, Janeczko 2012). In *Arabidopsis thaliana*, progesterone was detected in both shoot and inflorescence tissues in the amounts of 160 and 400 ng kg^{-1}(f.m.), respectively (Iino *et al*. 2007). The growth of *Arabidopsis* seedlings was promoted by progesterone at low concentrations but suppressed at

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Abbreviations: BRs - brassinosteroids; F_v/F_m - variable to maximum fluorescence ratio; hpi - hours post inoculation; *P.f.* - *Pseudomonas fluorescens*; *P.s.* - *Pseudomonas syringae* pv. *syringae*; PS - photosystem.
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higher concentrations under both light and dark growth conditions. Progesterone, depending on its concentration, promoted or inhibited growth of shoots and roots of sunflower (Bhattacharya and Gupta 1981) and it stimulated tube growth of *in vitro* matured tobacco pollens (Ylstra *et al*. 1995). Progesterone induced flowering in both winter wheat and *Arabidopsis* (Janeczko and Filek 2002, Janeczko *et al*. 2003).

Recently, molecular studies revealed the presence of progesterone receptors both in plant cell membranes and in the cytosolic fraction (Yang *et al*. 2005, Janeczko *et al*. 2008). An apoplastic oxidative burst, the accumulation of reactive oxygen species (ROS) in the extracellular space of plant tissues is an early response of plants to abiotic stresses (Joo *et al*. 2005) and to pathogens (Doke 1983, Wojtaszek 1997). Plasma membrane bound NADPH oxidases and cell wall peroxidases are considered as the main sources of ROS in the apoplast. In recent years, knock-out mutants or sense- and anti-sense transgenic lines provided evidence for the significance of these enzymes in the response of plants to pathogens. In accordance with these results, we observed an increased spread of cell death at the macroscopic level on leaves of *Arabidopsis rbohD* (for respiratory burst NADPH oxidase homologue D) knock-out mutant upon inoculation with the fungus *Alternaria brassicicola* (Pogány *et al*. 2009). In addition, Torres *et al*. (2002) found reduced ion leakage and increased cell death especially in *rbohF*, but also in *rbohD* mutants of *Arabidopsis* after infection with incompatible bacteria. The question arises whether progesterone has any protective effect in plants against biotic stress. Therefore the aim of our investigations was to compare the effects of inoculations with three bacteria, an incompatible wild type *Pseudomonas syringae* pv. *syringae* (*P.s.*), a *P.s. hrcC* mutant, and the saprophytic *P. fluorescens* (*P.f.*), between wild type *Arabidopsis* and the *rbohF Arabidopsis* knock-out mutant, with and without progesterone pre-treatment. In order to characterize the stress responses of plants, changes in membrane permeability and in the efficiency of photosystem (PS) II were measured.

Wild type (WT) *Arabidopsis thaliana* L. (Columbia ecotype) and the *Arabidopsis* T-DNA insertion line (for respiratory burst NADPH oxidase homologue F mutant having third intron insertion; *AtrbohF*) were ordered from the *Salk* (La Jolla, CA, USA) collection. Seeds were sown into pots and kept 4 d in a growth chamber at temperature of 5 °C, 8-h photoperiod, and irradiance of 190 μmol m^{-2} s^{-1}. Then the temperature was increased to 20 ^{o}C and the plants were grown for the next 36 d. A 4.1 mM stock solution of progesterone (*Sigma-Aldrich*, Poznań, Poland) was prepared in 50 % ethanol and diluted with distilled water to obtain the final concentration of 1 μM used in the experiments. Leaves of 40-d-old plants were brushed with 1 μM progesterone solution by using a paintbrush. The leaves of the control plants were brushed with distilled water. Two hours after pre-treatment, suspensions of *Pseudomonas* bacteria (10^8 cfu cm^{-3}) were injected into the leaves with to use a plastic syringe without a needle (Szatmári *et al*. 2006). Three *Pseudomonas* strains were used: *P.s.* strain 61, *P.s. hrcC* mutant (61-143 1530B, *Alan Collmer*, Cornell Univer-sity, Ithaca, USA), and *P.f.* strain 55. These *Pseudomonas* strains encompass plant pathogens with differing host specificities and corresponding pathovar designations. Mutant analysis showed that *P.s.* requires the Hrp (type III protein secretion) system encoded by a 25-kb cluster of *hrp* and *hrc* genes in order to elicit the hypersensitive response (HR) in nonhosts or to be pathogenic in hosts (Deng *et al*. 1998). In contrast to wild type, mutation in a *hrc* gene causes the lack of HR in WT *Arabidopsis* plants. *P.f.* is a common saprophytic bacterium without pathogenic or HR inducing activity in plants.

Visual evaluation of necrotic symptoms on infected and progesterone-treated leaves were made 72 hours post inoculation (hpi). After pre-treatment/inoculation (when the water evaporated from the infiltrated leaves during about 1 h), leaves were periodically cut and placed on the surface of 10 cm^3 of distilled water in Petri dishes. Membrane permeability was determined by measuring ion leakage from the cotyledons with an *OK-102/10* conductivity meter (*Radelkis*, Budapest, Hungary) according to the method described by Barna *et al*. (1993). Measurements were made 2, 26, 40, 46, 66, and 72 hpi in 6 repetitions (1 repetition = 3 leaves). PS II efficiency was characterized by measuring the parameters of chlorophyll *a* fast fluorescence using a *Plant Efficiency Analyzer* (*PEA*; *Hansatech*, King's Lynn, UK) according to a method described earlier (Janeczko *et al*. 2005, Skoczowski *et al*. 2011). Measurements were done with the excitation irradiance of 3 mmol m^{-2} s^{-1}. The leaves were measured after 30 min of adaptation to darkness at a temperature of 20 °C. Fluorescence was measured with a *PIN*-photodiode and changes in fast fluorescence were registered from 10 μs to 1 s. Based on the fluorescence curves obtained, the following fluorescence parameters were calculated according to equations of Strasser *et al*. (2000) using the *Handy PEA v. 1.30* software (*Hansatech*): F_v/F_m - variable to maximum fluorescence ratio, which characterizes the photochemical efficiency of PS II, ABS/CSm - energy absorption by antennas; TRo/CSm - energy trapped in reaction centres; ETo/CSm - energy flux for electron transport; DIo/CSm - energy dissipated as heat; where CSm is a cross section of the sample. These measurements were made 24 h after inoculation in 15 repetitions (1 repetition = 1 leaf). At least three independent experiments were conducted in each case. Statistical analysis was performed using Duncan's *t*-test. Differences were considered to be significant at $P < 0.05$.

Table 1. Ion leakage [μS g^{-1}(f.m.)] from leaves of WT and *rbohF* mutant *Arabidopsis* 2, 26, 40, 46, 66, and 90 h after injection with water, *P.s., P.s.* hrcC mutant, and *P.f.* of leaves with or without progesterone pre-treatment. Means from 6 repetitions ± SD; cont - control (water-brushed leaves); prog - leaves brushed with a 1 μM progesterone solution; Wi - leaves injected with water.

A. thaliana	Treatments	2 h	26 h	40 h	46 h	66 h	90 h
WT	cont	37±5	139±41	176±36	190±17	229±6	260±19
	prog	45±8	181±32	212±22	232±12	290±20	348±8
	Wi	129±25	237±35	238±36	276±29	336±31	341±37
	prog/Wi	137±15	259±11	286±25	354±37	427±29	440±31
	P.f.	72±8	290±33	344±40	363±42	473±79	545±88
	mutant	84±17	372±104	416±108	428±94	553±69	711±5
	P.s.	679±156	2787±374	2915±380	2973±374	3188±200	3976±443
	prog/*P.s.*	553±46	2270±111	2571±113	2589±88	2819±91	3227±123
rbohF mutant	cont	32±9	123±9	162±9	174±11	240±9	299±18
	prog	58±20	189±19	247±39	273±44	362±62	439±76
	Wi	174±27	222±28	253±7	254±8	293±14	317±20
	prog/Wi	186±10	332±6	345±24	386±1	442±11	452±13
	P.f.	79±21	409±71	441±63	453±64	547±86	653±145
	mutant	72±6	399±61	436±35	468±4	571±6	681±25
	P.s.	243±21	3301±452	3519±387	3590±364	3795±332	4381±489
	Prog/*P.s.*	199±12	2702±248	2926±175	2972±210	3120±186	3387±73

Table 2. Parameters of chlorophyll fluorescence in WT and mutant *Arabidopis* leaves 24 h after pretreatment with progesterone and infection with *P.s., P.f.*, and *P.s. hrcC* mutant. Values marked with the same letters are not significantly different (within columns - for WT and mutant separately) according to Duncan test ($P \leq 0.05$). Abbreviations as in Table 1.

A. thaliana	Treatments	F_v/F_m	ABS/CSm	TR_o/CSm	ET_o/CSm	DI_o/CSm
WT	cont	0.838 a	685 a	534 a	282 a	151 a
	prog	0.842 a	626 ab	492 a	261 ab	134 ab
	P.s.	0.764 b	276 d	202 c	105 d	73 d
	prog/*P.s.*	0.814 a	495 c	379 b	198 c	116 bc
	mutant	0.837 a	486 c	381 b	199 c	105 c
	P.f.	0.835 a	510 bc	396 b	209 bc	114 bc
rbohF mutant	cont	0.842 a	614 a	482 a	256 a	132 ab
	prog.	0.844 a	667 a	527 a	282 a	140 a
	P.s.	0.765 c	249 c	181 c	90 c	68 d
	prog/*P.s.*	0.797 bc	433 b	334 b	174 b	100 c
	mutant	0.835 a	488 b	381 b	197 b	108 bc
	P.f.	0.830 ab	475 b	369 b	193 b	105 c

P.s. caused strong, visible symptoms (HR) within 24 h in leaves of both types of *Arabidopsis*. The *hrcC* mutant bacteria caused slight necrosis development only on leaves of both *Arabidopsis* types after 48 h. *P.f.* did not induce any visible symptom on any *Arabidopsis* leaves. The pre-treatment with progesterone significantly attenuated the symptoms of HR caused by *P.s.* Leakage of electrolytes from leaves of WT and mutant corresponded to symptoms and these data characterized more exactly the reaction of plants to the inoculations. Two hours after injection of *P.s.* into *Arabidopsis* leaves, a significant increase of electrolyte leakage was measured in WT and somewhat later in *rbohF* mutant in accordance with the results of Torres *et al*. (2002) with *Pseudomonas syringae* pv. *tomato*. This increase continued rapidly until 26 hpi and than reached a plateau (Table 1) indicating the end of the hypersensitive response. Not only the *P.s.* but also the *hrcC* mutant and even *P.f.* increased the leakage of electrolytes significantly in both types of *Arabidopsis* although to much lesser extent than *P.s.* (Table 1). Progesterone treatment alone slightly elevated the membrane permeability in both WT and mutant leaves and increased electrolyte leakage. On the other hand, in accordance with the symptoms, progesterone pre-treatment decreased the leakage of electrolytes induced by *P.s.* in leaves of both types (Table 1).

Changes in the parameters of PS II efficiency were in accordance with data on leakage of electrolytes. Alterations of fluorescence parameters after inoculation with bacteria were similar in WT and *rbohF* mutant with or without progesterone pre-treatment. The most substantial changes 24 h after infiltration of bacterial suspensions were detected in ABS/CSm and TRo/CSm but all the other parameters (F_v/F_m, ETo/CSm, DIo/CSm) changed as well. Again, the *P.s.* caused the largest changes. Inoculations with the *hrcC* mutant and with *P.f.* also led to significant alterations in the measured fluorescence parameters (except F_v/F_m) as compared to control plants (Table 2). Progesterone pre-treatment alone did not cause significant changes in PS II efficiency, however, decreased the harmful effect of *P.s.* (Table 2).

It is noteworthy that progesterone treatment alone slightly, but significantly, increased the leakage of ions as compared to control leaves brushed only with water, similarly to oilseed rape leaves treated with brassinosteroids (Janeczko *et al*. 2007a,b, Skoczowski *et al*. 2011). We suggest that the increased membrane permeability in progesterone treated plant cells is probably due to effect on cell elongation. In accordance, Zhang *et al*. (2005) reported that BR-initiated cell expansion was correlated with plasma membrane hyperpolarisation and regulation of anion channels and proton pump. However, again similarly to BRs, progesterone treatment attenuated the increase of ion leakage induced by *P.s.* (Table 1). In accordance with our earlier results (Skoczowski *et al.* 2011), not only *P.s.* but also the *hrcC* mutant and even *P.f.* significantly increased the leakage of electrolytes in both types of *Arabidopsis*. It is noteworthy that *P.f.* can induce systemic resistance in plants as well (Vanitha and Umesha 2011). Although infiltration of leaves with water itself resulted in slightly elevated membrane permeability, it cannot be responsible for the mutant and *P.f.* induced ion leakage (Table 1). Progesterone pre-treatment decreased the elevated leakage caused by mutant and saprophytic bacteria (data not shown) but not in the case of water infiltration. These results demonstrate that the stress reducing effect of progesterone is stronger than its capacity to increase membrane permeability.

Several studies reported a decrease in photosynthesis after pathogen infection (Berger *et al*. 2004, Bonfig *et al*. 2006, Freeman and Beattie 2009). In addition, the maximum PS II quantum yield, effective PS II quantum yield and nonphotochemical quenching were decreased in *Arabidopsis* leaves infected with compatible and incompatible *P. syringae* strains (Bonfig *et al*. 2006). Our results are in accordance with these findings since not only *P.s*. but also mutant and *P.f.* caused a decrease in PS II efficiency in WT and mutant *Arabidopsis* (Table 2). Progesterone treatment alone had no effect on PS II efficiency, but it effectively diminished the destructive effect of incompatible bacteria. Our earlier experiments showed that BRs can ameliorate the negative impact of biotic and abiotic stresses on PS II efficiency but they do not influence it in non-stressed plants (Janeczko *et al*. 2005, Skoczowski *et al.* 2011). Our present results revealed the similarity between the impacts of BRs and progesterone on PS II functioning.

In general, progesterone treatment had protective effect on *Arabidopsis* plants against stress induced by incompatible bacteria similar as BRs had on oilseed rape. As regards the mechanism of the stress protective effects of progesterone and BRs, one can only propose a hypothesis. It has been found very recently that progesterone in plants can stimulate the activity of antioxidative enzymes (Erdal and Dumlupinar 2011) which may play an important role in the protective effect of this steroid. The protective action of BRs on photosynthetic parameters may be connected with the regulation of biosynthesis of heat shock proteins as well (Dhaubhadel *et al*. 2002). In case of progesterone, however, this problem would require further studies.

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