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BRIEF COMMUNICATION

Influence of brassinosteroids on initiation of the root gravitropic response in *Pisum sativum* seedlings

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Abstract

In roots of *Pisum sativum* seedlings, the average lag-time required for initiation of the gravitropic response was reduced proportionally to the concentration of 24-epibrassinolide (EBL) added to the root solution (range of 10^{-13} to 10^{-8} M concentrations). A treatment with clotrimazole, a compound inhibiting steroid synthesis, prevents initiation of the gravitropic response. This effect was partly reverted by addition of EBL. From analysis of variability in the populations, it is suggested that BR conditions the root curvature through a gravitropic-induced change in sensitivity to the PGRs regulating cell elongation.

Additional key words: clotrimazole, 24-epibrassinolide, pea.

Brassinosteroids (BRs) are informative compounds influencing a large range of developmental and metabolic processes (for review, see, Khripach *et al.* 1999, Bishop and Koncz 2002, Krishna 2003, Sasse 2003). However, the precise mode of action of BRs remains quite obscure, especially because they interfere with almost all the plant growth regulators (Kauschmann *et al.* 1996, Chory and Li 1997, Ephritikhine *et al.* 1999, Suzuki *et al.* 2001) and on highly integrated physiological processes, such as phytochrome/hormonal signalling networks (Halliday and Fankhauser 2003) and expression of the reproductive development (Kęsy *et al.* 2003/4).

An effect of BR on gravitropism has been observed for a long time. In seedlings positioned horizontally, BR accelerates the upward curvature of hypocotyls in bean (Meudt 1987) and tomato (Park 1998). Kim *et al.* (2000) described a modification of the gravitropic response in maize roots treated in which BR induced a change in velocity of the root curvature. The anisotropy generating the root curvature has been classically interpreted as a difference in PGRs production between the two sides of the root cap. However, recent finding have revealed a more complex situation. In soybean hypocotyl, gravitropism appears to be modulated by an anisotropic change in tissue sensitivity rather than an anisotropy in auxin concentration (Rorabaugh and Salisbury 1989). It is, therefore, possible that the gravitropic response involves a change in sensitivity to PGRs. These considerations stimulated us to test whether the effect of BR on gravitropism may be related to a change in sensitivity to PGRs in the root elongation zone.

Video-imaging of the root response revealed that gravitropism results from a succession of processes. It starts with a combination of enhancing growth along the upper and inhibiting growth along the lower surface of the root, followed by a transient backward curvature (Ishikawa *et al.* 1991). Moreover, even within the first stage, the rate of curvature is determined by the difference between emergence of the anisotropy and its dissipation (Zieschang and Sievers 1991, Evans and Ishikawa 1997). From the diversity of responses involved in gravitropism and their interrelation, it looks difficult to analyse the effect of brassinosteroids on the whole process. This is why we decided to focus our attention on the effect of BR on initiation of the process, the response directly resulting from anisotropy in growth between the upper and lower part of the elongation zone.

Seeds of pea (*Pisum sativum* L. cv. Alaska) were purchased from Ferry Morse Inc., Mountain Views, CA, USA. They were germinated in vermiculite moistened

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Abbreviations: BR - brassinosteroid; CLZ - clotrimazole; EBL - 24-epibrassinolide; GCI - gravitropic curvature initiation; PIGR - percentage of initiation of gravitropic response per time interval.

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with tap water, in a growth chamber (temperature of 20 °C, 13-h photoperiod with irradiance of 400 µmol m^{-2} s^{-1}. The seedlings were used for bioassay 6 d following imbibition, when the seminal root had a length of 7 to 9 cm.

At the beginning of the test (at 08:00), seminal roots were transferred in a dark room maintained at the same temperature. Seedlings were placed horizontally on plastic supports floating on the root solution, so that roots but not shoots were immersed in the solution because a contact of the shoot with the root solution modifies the root gravitropic response (unpublished observations). Populations of 20 seedlings were exposed together to the same treatment. Each root was examined every 30 min from beginning of the experiment. The gravitropic response was considered to start when the angle of curvature from horizontality (hand-measured) was about 30°. This response was defined as a gravitropic curvature initiation (abbreviated as GCI). The GCI_{50} was defined as the time (min) required for curvature initiation in 50 % of the population. It was here determined graphically from the kinetics of response of the population.

24-epibrassinolide (EBL, purchased from *Sigma*, St. Louis, USA) was dissolved in pure ethanol and aliquots of these stock solutions were dissolved in tap water to prepare final solutions. Clotrimazole (purchased from *Sigma*) was firstly dissolved in an aliquot of DMSO before adding water. The 10 h pre-treatment with clotrimazole started at 22:00 by flooding for 1 h the pots with seedlings with a solution of clotrimazole. Then, the solution in excess was drained and the plants were used, 9 h later, for gravitation experiments. A 1-h flooding with water (plus aliquot of DMSO) at the same time had no incidence on GCI (not shown).

Sets of 20 seedlings of *Pisum* were exposed to various concentrations of 24-epibrassinolide (EBL) from beginning of the experiment. This experiment was reproduced four times and the four GCI_{50} calculated for each treatment were averaged. This reveals that the GCI_{50} is reduced proportionally to the concentration of EBL added (Table 1). GCI_{50} is affected by concentrations as low as 10^{-13} M EBL, but a statistically significant difference with GCI_{50} of control plants (two-tailed *t*-test, $P < 0.05$) is observed only for seedlings exposed to an EBL concentration of at least 10^{-9} M (Table 1).

Minor differences in GCI_{50} are observed between the four populations of control plants (not shown). For this reason, the effect of treatments has been compared to that of control for each one of the four sets of experiments, and a relative GCI_{50} has been calculated as the ratio between GCI_{50} of EBL-treated population and GCI_{50} of the corresponding population of control plants. For each treatment, an average and CV value of relative GCI_{50} has been calculated. The decrease in relative GCI_{50} clearly appears to be proportional to the EBL concentration applied (Table 1). However, as shown by fluctuations of the CV values, reliability of the effect of EBL on populations also depends on the concentration applied. A maximum CV value is observed for exposure to 10^{-10} M EBL (Table 1).

Table 1. Influence of EBL concentration on GCI_{50} in roots of *Pisum sativum*. The GCI_{50} has been calculated on basis of populations of 20 individuals as described in Methods. Average values are calculated from GCI_{50} calculated on four distinct experiments. For each EBL concentration, the relative GCI_{50} has been calculated as the percentage of the GCI_{50} of the corresponding population of control plants. The four relative values were averaged, and the relative variation was calculated as the CV of relative GCI_{50}. ns - non significant difference with control (two-tailed *t*-test, $P > 0.05$); * - $P < 0.01$; ** - $P < 0.001$.

EBL [M]	GCI_{50} [min]	Relative GCI_{50}	Relative variation
0	142.7 ± 27.9		
10^{-13}	128.5 ± 9.7ns	91.5	10.1
10^{-12}	121.0 ± 12.6ns	85.7	6.9
10^{-11}	110.6 ± 10.3ns	78.9	12.4
10^{-10}	103.9 ± 22.2ns	75.0	24.6
10^{-9}	95.3 ± 15.8*	68.8	21.5
10^{-8}	86.6 ± 10.4**	62.4	18.5
10^{-7}	84.0 ± 10.1**	60.1	15.3

In order to analyse this phenomenon, the structure of the population has been investigated through calculation of the percentage of plants initiating the gravitropic response (abbreviated as PIGR) at each time-interval of 60 min. A bimodal distribution frequency is observed for

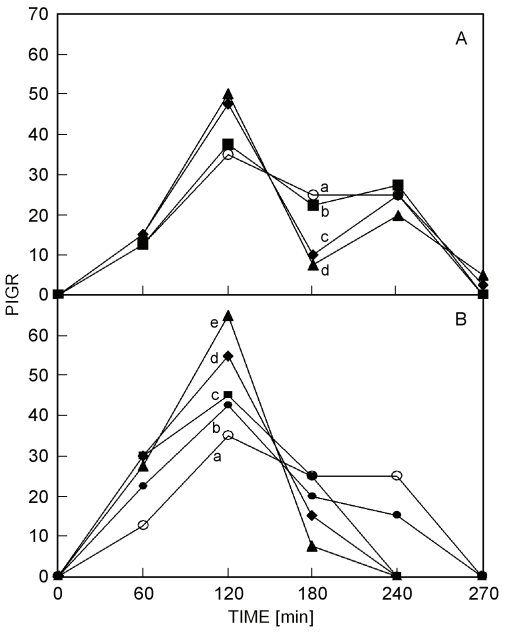

Fig. 1. Effect of EBL on kinetics of gravistimulation (PIGR) in *Pisum sativum.* PIGR are calculated on time intervals of 60 min for populations of 40 individuals tested simultaneously. *A*: *curves a*: control, *b*: 10^{-13} M EBL, *c*: 10^{-12} M EBL, *d*: 10^{-11} EBL. *B*: *curves a*: control, *b*: 10^{-10} M EBL, *c*: 10^{-9} M EBL, *d*: 10^{-8} M EBL, *e*: 10^{-7} M EBL. Two sets of 20 plants exposed simultaneously to identical conditions were pooled, so that distribution frequencies were calculated on basis of 40 individuals.

control and low EBL concentrations (Fig. 1*A*). It confirms a previous observation about the high variability inherent to gravitropic response in pea seedlings (Konings 1964).

The late-responding subpopulation is considerably reduced following treatment with 10^{-10} M EBL, and it completely disappears towards exposure to 10^{-7} M EBL (Fig. 1*B*). However, the time required for GCI response in the early-responding subpopulation was unchanged by the EBL treatment. This effect suggests that the main difference between these two subpopulations concerns their level of sensitivity to EBL: a high-sensitivity for the early-responding subpopulation and a low sensitivity for the late-responding one (threshold of about 10^{-10}M). A comparative analysis of root response under conditions of constant gravistimulation revealed the existence of two different areas of root curvature apparently not synchronized nor regulated by the same hormonal processes (Wolverton *et al.* 2002). It is, therefore, possible that the variability in response observed here corresponds to individuality in the BR-dependent threshold of induction of these coexisting responses to gravity.

Clotrimazole is known to inhibit steroid synthesis in animals (Ayub and Levell 1990, Nakajin *et al.* 1991, Gemzik and Parkinson 1992). It also prevents accumulation of steroids in plant tissues when added to the root medium at a concentration of 29 µM (Bach 1985). Clotrimazole probably affects the BR metabolism, since it target, the cytochrome P_{450}, is involved in BR biosynthesis (Choe *et al.* 1998).

Clotrimazole had no influence when added at the beginning of the experiment at a concentration of 1.45 µM. However, from 3.62 µM to 29 µM, clotrimazole reduced the GCI_{50} proportionally to the concentration added (not shown). In populations treated with 29 µM clotrimazole, about 75 % of the individuals did not react to gravitropism during the 5 h of the experiment. This inhibited pool of the population was reduced to 40 and 30 % when EBL was also added at a concentration of 10^{-9} and 10^{-7} M, respectively (not shown). This reveals a counteracting effect of clotrimazole and EBL on gravitropism.

Addition of clotrimazole may perturb many metabolic processes indirectly involved in expression of the gravitropic response. In order to test this point, plants were treated and/or pre-treated for 10 h with clotrimazole (29 µM) before starting the gravistimulation. If clotrimazole induces metabolic perturbations, a cumulative effect was expected for plants both treated and pre-treated. However, no significant difference in GCI_{50} was observed from comparison between populations pre-treated or not pre-treated by clotrimazole (Table 2). An effect of clotrimazole pre-treatment may be hidden by the inhibition induced by clotrimazole treatment. However, no pre-treatment effect is observed on GCI_{50} for plants untreated or exposed to EBL during the experiment (Table 2). In parallel, structure of the population was not affected by the pre-treatment (not shown). The counteractive effect of EBL and clotrimazole confirms the direct involvement of brassinosteroids in initiation of the gravitropic response.

Table 2. Influence of EBL treatment, clotrimazole treatment and pretreatment on GCI_{50} of roots in *Pisum sativum*. Seedlings were exposed to 29 µM clotrimazole (CLZ) at the beginning of the gravitropic experiment (treatment) or 10 h before (pre-treatment) as described in Methods. EBL was added at the beginning of the treatment. GCI_{50} are means of three independent experiments. A significant effect ($P < 0.05$, two-tailed *t*-test) of clotrimazole pretreatment is not observed in any case.

Treatment		GCI_{50} [min]	
CLZ [µM]	EBL [M]	no pre-treatment	CLZ pre-treatment
0	0	162.8 ± 13.2	175.0 ± 41.0
	10^{-9}	104.6 ± 3.6	121.0 ± 21.7
	10^{-7}	78.4 ± 6.0	92.3 ± 15.3
29	0	> 300	> 300
	10^{-9}	232.0 ± 22.7	255.0 ± 39.3
	10^{-7}	190.5 ± 22.8	216.3 ± 52.1

Still *et al.* (1997) emphasized that "biochemical variation of this magnitude among individual seeds has significant implications for design and interpretation of experiments in seed biology." The presented study is an example of misleading interpretations emerging from dismissing the importance of variability (Amzallag 2001). An extensive variability has been reported concerning the time required for initiation of the gravitropic response (Konings 1964, Zieschang and Sievers 1991, Ishikawa *et al.* 1991). It has been generally related to the phenomenon of circumnutation. However, even though EBL may influence the rhythm of circumnutation, it has to reduce the time lag of the late-responding subpopulation, but not to eliminate it, as it is currently observed (Fig. 1). A calculation of GCI_{50} raises to the conclusion that BR accelerates initiation of the gravitropic response. But an analysis of structure of the population (Fig. 1) reveals that two modes of response spontaneously coexist. The BR treatment induces preferentially one of these modes, without accelerating the rate of initiation of the gravitropic response. This mechanism is not yet identified, but a similar influence of steroids on phenotypic variability has been already reported concerning germination of barley seeds (Gregory 1981). As previously suggested (Amzallag 2002), changes in tissue sensitivity to PGRs may be a main effect of BR at physiological concentrations. Initiation of gravitropic response represents a simple experimental system for testing this point.

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