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Endogenous brassinosteroids in wheat treated with 24-epibrassinolide

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Abstract

The aim of the study was to examine the effect of exogenous 24-epibrassinolide on its uptake and content of endogenous brassinosteroids in wheat seedlings. 24-Epibrassinolide was applied at two concentrations (0.1 and 2.0 μM) and in three different methods: by soaking seeds, by drenching and by spraying plants. Brassinosteroids were determined by high-performance liquid chromatography combined with electrospray mass spectrometry. Three important brassinosteroids, 24-epibrassinolide, brassinolide and castasterone, were detected in the wheat leaves, but their contents varied with leaf insertion and plant age. Increased 24-epibrassinolide content in the leaf tissue was found when this hormone was applied by soaking or drenching. Additionally the seed treatment influenced brassinosteroid balance in seedlings. The growth response of wheat seedlings treated with 24-epibrassinolide has been also investigated.

Additional key words: brassinolide, castasterone, growth response, homobrassinolide, HPLC/MS.

Introduction

Brassinosteroids (BRs) were first discovered in oilseed rape pollen in 1979 (Grove *et al*. 1979) and nowadays they are considered to function as a separate class of steroid phytohormones (Bishop and Yokota 2001, Zullo and Adam 2002). BRs receptors are located on a plant cell surface (Bishop and Yokota 2001, Kinoshita *et al.* 2005). BRs regulate the expression of numerous genes, influence the activity of different metabolic pathways and contribute to the regulation of cell division and differentiation. In *in vitro* cultures, BRs stimulated callus proliferation in *Arabidopsis thaliana*, regeneration in cauliflower and *Spartina patens*, and embryogenesis in conifers, rice and coconuts (Hu *et al.* 2000, Sasaki 2002, Azpeitia *et al*. 2003, Lu *et al.* 2003, Pullman *et al*. 2003). The application of BRs increased the yield of wheat, potatoes, rice and mustard (Ramraj *et al.* 1997, Zullo and Adam 2002) and improved quality of ground nut (Vardhini and Rao 1998). BRs interacted synergistically with auxins, stimulated ethylene production, were involved in the root gravitropic response, and increased resistance to temperature, water or salinity stresses and biotic stresses (Mazorra *et al*. 2002, Krishna 2003, Núñez *et al*. 2003, Upreti and Murti 2004, Janeczko *et al*. 2005, 2007, Amzallag and Vaisman 2006). BRs can be applied to plants in many ways. Seeds may be soaked in a solution containing brassinosteroids, growing plants may be drenched or sprayed, plants growing *in vitro* may be exposed to brassinosteroids contained in the culture medium. However, little is known about BRs uptake and content in plants exogenously treated with these regulators. Moreover, in spite of extensive studies of BR occurrence in the plant kingdom, there are very few data concerning the natural content of brassinosteroids in wheat (Bajguz and Tretyn 2003). Therefore, the aim of this study was to identify and quantify endogenous brassinosteroids in wheat seedlings using high-performance liquid chromatography-electrospray mass spectrometry [HPLC-(+)ESI-MS] technology, and to study the effect of three different ways of 24-epi-brassinolide application (soaking seeds, drenching and spraying of seedlings) on the BR content in plant tissues of wheat.

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Abbreviations: BR - brassinosteroid, 24-epiBL - 24-epibrassinolide.
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Materials and methods

24-Epibrassinolide (24-epiBL) was purchased from *Sigma*, Poznań, Poland. The stock solution contained 4.1 mM 24-epiBL in 50 % ethanol while working solutions were diluted with distilled water. In all 24-epiBL solutions, as well as in the control (water-ethanol solution), the ethanol concentration was adjusted to 0.1 % (v/v). Other unlabelled brassinosteroids were obtained from *Olchemim* (Olomouc, Czech Republic) or CIDtech Research (Waterloo, Canada). [$^2H_6$]-brassinolide and [$^2H_6$]-castasterone were gifts from Dr. B. Schneider (Jena, Germany). Bovine serum albumin, dimethyl-formamide, dimethylsulfoxide, and *DEAE-Sephadex* were from *Sigma* (St. Louis, USA). C-18 reversed phase columns and cartridges were from *Waters* (Prague, Czech Republic), *Strata X* reversed phase columns (33 μm, surface modified styrene divinylbenzene) were from *Phenomenex* (Torrance, USA). All other chemicals were obtained from *Lachner* (Neratovice, Czech Republic).

The experiment was conducted on spring wheat (*Triticum aestivum* L. cv. Cytra). Three types of experiments were carried out. In the first experiment, 24-epiBL was applied by soaking seeds. Seeds were sown on Petri dishes (9 cm in diameter) with filter paper (25 seeds per dish). The seeds were drenched with 10 cm^3 0.1 or 2 μM 24-epibrassinolide solution. Control seeds were watered with dist. water containing 0.1 % ethanol. The seeds were kept 24 h in darkness (25 °C). Subsequently, the seeds were transferred to new Petri dishes and watered firstly with 10 cm^3 H_2O, and then with 5 cm^3 H_2O daily (to keep the filter paper wet). The plants were grown in a growth chamber for 9 d under a 16-h photoperiod with irradiance of 150 μmol m^{-2} s^{-1} (PAR), day/night temperature of 20/17 °C and relative humidity 70 %.

In the second experiment, 24-epiBL was applied by drenching the root system of the seedlings. Seeds were sown on Petri dishes on a moist filter paper and kept in darkness at 25 °C. After 24 h the seeds were moved into growth chamber and after 48 h the seedlings (with coleoptiles and a few roots) were moved to new Petri dishes and drenched once with 0.1 or 2 μM 24-epiBL solution. Control plants were watered with dist. water containing 0.1 % ethanol. Then the filter paper was kept moist by daily application of 5 cm^3 dist. H_2O to each Petri dish. The plant growth response was evaluated in forty 10-d-old seedlings. The two upper leaves were cut off and immediately prepared for BR analysis (fresh mass 3 × 1.2 g; 15 plants per sample).

In the third experiment, the germinated seeds were transferred into pots with soil (16 plants per pot) and grown in growth chamber. 10-d-old seedlings at the two-leaf stage were sprayed with a solution of 24-epiBL (0.1 or 2 μM) containing *Tween 20* (0.0013 % v/v). Control plants were sprayed with a solution of distilled water (0.1 % ethanol) and *Tween 20*. The growth response was measured in 21-d-old seedlings (n = 25). In this case BR content was analysed in the third developed (unsprayed) leaf (fresh mass 3 × 0.5 g; 15 leaves per sample). In all experiments, the exogenous contamination of analyzed leaves with 24-epiBL was carefully prevented.

Endogenous brassinosteroids were extracted and quantified as described previously (Swaczynová *et al*. 2007). The extracts were purified by passing them through a C-18 column (0.5 g) and a *DEAE Sephadex A-25* column (1 cm^3 g^{-1}) coupled with a *Strata-X* reversed phase column (33 μm). The purified samples were evaporated, dissolved in 100 % methanol, vortexed and sonicated in an ultrasonic bath. The samples were then analysed by high performance liquid chromatography using an *Alliance 2695* separation module (*Waters*, Milford, MA, USA) linked to a diode-array detector (*Waters PDA 996*) and a *ZMD 2000* single quadrupole mass spectrometer equipped with electrospray interface (*Micromass*, Manchester, UK). The samples were dissolved in 100 % methanol, filtered through a micro-filter (*PTFE*, 4 mm, 0.45 μm, *Waters*) and 0.005 cm^3 of each sample (25 % of total sample) was injected onto a 150 mm × 2.1 mm, 5 μm *Symmetry C-18* reversed phase column (*Waters*). The column thermostat was set at 30 °C and the mobile phase had the following binary gradient of solvent A (methanol) and solvent B (5 mM formic acid) at a flow rate of 0.25 cm^3 min^{-1}: 0 min, 70 % A; 0 - 12 min 75 % A; 12-16 min 100 % A. The column was equilibrated at initial conditions for 10 min. Using post-column splitting (1:1), effluent was simultaneously introduced into the diode-array detector (scanning range 210 - 400 nm; with 1.2 nm resolution) and electrospray source (source temperature 100 °C, capillary voltage +3.2 kV, cone voltage +20 kV, desolvation temperature 250 °C). Nitrogen was used as both the desolvation gas (550 dm^3 h^{-1}) and the cone gas (50 dm^3 h^{-1}). The detector parameters were set to a span size 0.5 m/z and an interchannel delay of 0.02 s. Quantification was performed by SIM of molecular $[M + Na]^+$. The dwell time of each SIM channel was calculated to obtain 16 scan points per peak and individual mass.

Results and discussion

Three important brassinosteroids were detected in the leaves of the wheat plants: 24-epibrassinolide, brassinolide and castasterone (Tables 1, 2). The identity of all brassinosteroid metabolites was verified by comparison of the mass spectra and chromatographic retention times with those of authentic standards. Using a post column split of 1/3, the effluent was introduced from HPLC into the electrospray source and data were obtained from

Table 1. The content of brassinosteroids [ng g^{-1}(f.m.)] in the first and second leaf of 10-d-old wheat seedlings after application of 24-epibrassinolide by 24 h soaking of seeds and by drenching of 3-d-old plants. Means ± SE. Data in columns, marked with the same letter, are not significantly different according to the Duncan test at $P < 0.05$; TR - traces, ND - not detected. 24-Epicastasterone and 28-homocastasterone were never detected.

Application	24-epiBL [μM]	24-epiBL	Brassinolide	Castasterone	28-homobrassinolide
Seed soaking	0.0	0.287 ± 0.014 a	0.421 ± 0.035 a	0.289 ± 0.045 a	0.204 ± 0.011
	0.1	0.280 ± 0.027 a	0.497 ± 0.016 a	0.316 ± 0.032 a	ND
	2.0	0.924 ± 0.248 b	0.364 ± 0.083 a	0.324 ± 0.017 a	ND
Drenching	0.0	0.258 ± 0.060 a	0.303 ± 0.056 a	TR	ND
	0.1	0.696 ± 0.252 b	0.272 ± 0.112 a	ND	ND
	2.0	1.109 ± 0.265 b	TR	ND	ND

Table 2. The content of brassinosteroids [ng g^{-1}(f.m.)] in the third leaf of 21-d-old wheat seedlings and plant height [cm] after 11 d from spraying plants at the two-leaf stage with 24-epiBL. Means ± SE. Data in columns, marked with the same letter, are not significantly different according to the Duncan test at $P < 0.05$; TR - traces, ND - not detected. 24-Epicastasterone and 28-homocastasterone were never detected.

24-epiBL [μM]	24-epiBL	Brassinolide	Castasterone	28-homobrassinolide	Height
0.0	ND	0.885 ± 0.050 a	0.785 ± 0.079 a	ND	25.8 a
0.1	ND	1.099 ± 0.161 a	1.030 ± 0.048 b	ND	24.3 a
2.0	TR	0.875 ± 0.091 a	0.585 ± 0.162 a	TR	23.8 a

selective ion recording. (+)ESI-MS-chromatograms of real samples (*T. aestivum*) showed two peaks at a retention time of 8.40 min and 9.13 min in channel *m/z* 503, which belong to the quasi-molecular ions of 24-epiBL and brassinolide, respectively. One peak was also recorded in the channel expected for castasterone (*m/z* 487) at retention time 12.5 min. All brassinosteroids yielded quasi-molecular stable ions of $[M+Na]^+$ in electrospray-positive mode (ESI+ for brassinolide and 24-epiBL: 503.24) as the most abundant ion in the mass spectrum. This phenomenon is most likely due to the strong and stable attachment of sodium to the ketone and hydroxyl groups of steroids (Ma and Kim 1997, Swaczynová *et al*. 2007). In the positive ion mode, *m/z* 427.26, 445.29, 463.28, 481.28, 519.28 ions corresponding to $[M-3H_2O+H]^+$, $[M-2H_2O+H]^+$, $[M-H_2O+H]^+$, $[M+H]^+$ and $[M+K]^+$, respectively, were also detected. However, under the conditions tested, strong and specific $[M+Na]^+$ ions were mainly observed. Quantification was performed using the standard isotope dilution method. Final concentrations were calculated from the areas of the *m/z* $[M+Na]^+$ peak for labelled and authentic brassinosteroids in the SIM chromatograms. It seems that the occurrence of brassinolide and 24-epiBL proved in this work in wheat seedlings has not been reported previously (Bajguz and Tretyn 2003). BRs have been found in many monocotyledons and dicotyledons (Grove *et al*. 1979, Bajguz and Tretyn 2003). In the *Graminae*, brassinosteroids have been found in rice seeds and bran, roots of *Zea mays* L. and in rye seeds (Park *et al*. 1994, Abe *et al*. 1995, Schmidt *et al*. 1995, Kim *et al*. 2000). The BRs most frequently encountered are castasterone, teasterone, secasterone and typhasterol. In wheat grains, teasterone, typhasterol, castasterone, 6-deoxocastasterone and 3-dehydroteasterone have been detected (Yokota *et al*. 1994). Wheat seeds contain campestanol, which may be one of the early precursors in brassinosteroid biosynthesis (Takatsuto *et al*. 1999). The content of BRs detected in our experiments varied with leaf insertion, plant age and method of application of an exogenous hormone (Table 1, Table 2). 24-epiBL was found in the first and second leaves of 10-d-old control plants, but not in the third leaf of 21-d-old control plants. The content of brassinolide and castasterone in the third leaves of 21-d-old plants was about twice the content in the first and second leaves of 10-d-old seedlings. 28-Homobrassinolide was present in seedlings whose seeds had previously been soaked with control solution. According to the literature, in pea plants, the content of 6-deoxocastasterone, castasterone and typhasterol differed depending on the organ examined (Symons and Reid 2004). In another study on grapes, the content of 6-deoxocastasterone and castasterone also changed during berry development (Symons *et al*. 2006). Interestingly, castasterone in our work was detected in 10-d-old seedlings whose seeds had previously been soaked (in plants of control and 24-epiBL treated) (Table 1). This metabolite, however, did not occur in the same age seedlings after drenching with 24-epiBL and occurred in trace amounts in drenched control. Apparently, the method of seed treatment itself influences hormonal balance in seedlings. In literature there is reported beneficial effect of presowing seed treatment (seed priming) on plant germination and growth (Harris

et al. 2001, Rashid *et al*. 2006). Alternations in hormonal regulation (concerning probably not only BR) in the beginning of plant development may be significant for further growth. Generally, higher 24-epiBL content in the leaf tissue was found when 24-epiBL was applied by soaking seeds or drenching plants than by spraying (Tables 1, 2). When 24-epiBL (lower concentration,

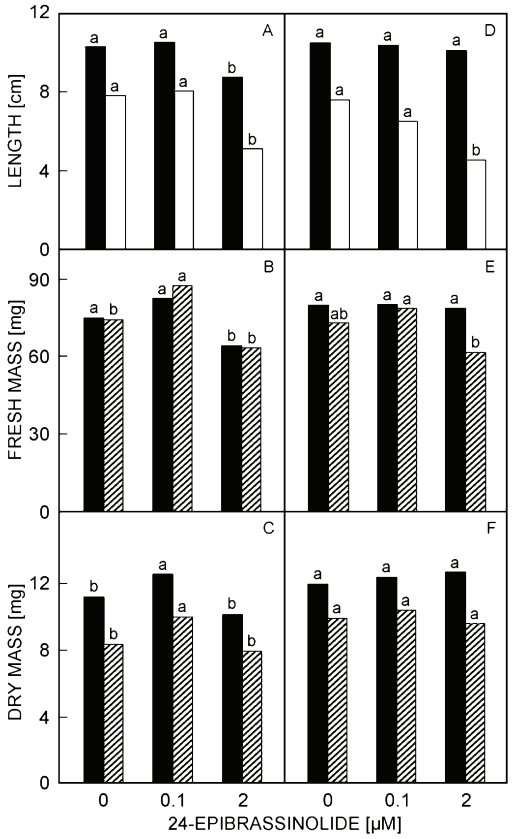

Fig. 1. Growth response of wheat after application of 24-epibrassinolide by 24 h soaking of seeds (*A*, *B*, *C*) and by drenching of 3-d-old plants (*D*, *E*, *F*). *Black columns* - shoots, *white columns* - the longest root and *stripped columns* - all roots. Means marked with the same letters (separately for each parameter; for shoots and for roots) are not significantly different according to the Duncan test at $P < 0.05$.

0.1 μM) was sprayed on the first and second leaves of 10-d-old seedlings, its content in the third leaves of 21-d-old plants was not affected (Table 2). On the other hand, when 24-epiBL was applied by drenching the root systems, its endogenous content in the leaf tissues was significantly increased. In fact, there was no significant difference whether 24-epiBL was applied at concentrations of 0.1 and 2.0 μM, which differ twenty times. The transport of 24-epiBL from the growth medium to the roots may have been limited by a certain mechanism which inhibits uptake when the concentration of 24-epiBL exceeds a certain physiological limit. The effect of the dosage at which 24-epiBL was applied was more evident in the experiment in which seeds were soaked with a BR containing solution. The content of 24-epibrassinolide was significantly affected only after the application of 2.0 μM 24-epiBL. When 24-epiBL was applied at a dose of 0.1 μM, there was a significant difference after drenching only. In other studies, the effectiveness with which brassinosteroids were transported was also dependent on the way in which they were applied. When [^{14}C]24-epiBL was applied to wheat leaves, it was transported only towards the apex of the leaves, and not from the leaves to other leaves or organs (Nishikawa *et al*. 1994, 1995). However, when [^{14}C]24-epiBL was applied to the roots of wheat seedlings that were only a few days old, it was efficiently taken up and distributed throughout the seedlings (Nishikawa *et al*. 1995) and it was probably transported through the xylem (Nishikawa *et al*. 1994). However, according to Symons and Reid (2004), BRs are not transported over long distances under natural conditions and they are probably synthesised close to the site of action. Exogenous BRs moved only slowly, if at all, after application to leaves (Symons *et al*. 2008). When ^{3}H-brassinolide and ^{3}H-castasterone were applied to the mature leaves or apical buds of pea plants, they were not transported far away from the application site (Symons and Reid 2004). Only at the cellular level, endogenous BRs appeared to be transported (Symons *et al*. 2008). The enzymes for BR biosynthesis are located within the cell but BR reception is thought to occur on the outer side of cell membrane. Therefore, BRs must move from the interior of the cell to the exterior, where they are perceived by the same cell or by neighbouring cells (Symons *et al*. 2008).

Many results have been published which describe BR dose-response in many plants including *Graminae* (Braun and Wild 1984, Fujii and Saka 2001, Chon *et al*. 2000). BR activity in growth response tests depends not only on dosage, but also on plant cultivar, the plant organ treated and the method of BR application. Our earlier experiments show that the same BR concentration may stimulate growth of the upper part of wheat seedlings if applied once to the leaves, but it may decrease the growth if applied *via* the roots and for a longer time of exposure, *i.e*. a few days continuously. This may be connected with overaccumulation of exogenously applied BR in the plant tissue. In this experiment we show that the application of higher concentration (2.0 μM) of 24-epibrassinolide *via* the seeds or roots significantly increased (3 - 4 times) the content of this hormone in plants and it resulted in the inhibition of plant growth (Fig. 1). In plants whose seeds were treated with 24-epiBL at this concentration, the growth of leaves and roots, as well as the fresh mass of the upper part of the plants, were negatively affected. Interestingly, BR applied by soaking at 0.1 μM concentration stimulated dry mass accumulation of leaves and roots of wheat, although 24-epibrassinolide content in the tissue was not changed in comparison with the control. The effect caused by BR (concentration 2.0 μM) but applied *via* drenching of the root system, was weaker (Fig. 1). In plants drenched with 24-epiBL (2.0 μM) the root length decreased but the length of the shoot was not

affected. There is no effect of 24-epiBL on plant growth if applied by drenching at 0.1 μM concentration. There is also no statistically important effect on the growth of 21-d-old seedlings after the spraying of 24-epiBL (Table 2).

To conclude, three important brassinosteroids: 24-epibrassinolide, brassinolide and castasterone were detected in the leaves of wheat plants, but their contents varied with leaf insertion and plant age. The effectiveness with which brassinosteroid was transported in the plant depended on the way in which it was applied. Higher 24-epibrassinolide content in the leaf tissue was found when the hormone was applied by soaking or drenching than by spraying. The method of seed and plant treatment itself may influence brassinosteroid content in seedlings. The content of 24-epibrassinolide, 3 - 4 times higher than in control tissue, resulted in a decrease of plant growth and biomass accumulation.

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