

Brassinosteroids Regulate Plasma Membrane Anion Channels in Addition to Proton Pumps During Expansion of *Arabidopsis thaliana* Cells

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Brassinosteroids (BRs) are involved in numerous physiological processes associated with plant development and especially with cell expansion. Here we report that two BRs, 28-homobrassinolide (HBL) and its direct precursor 28-homocastasterone (HCS), promote cell expansion of *Arabidopsis thaliana* suspension cells. We also show that cell expansions induced by HBL and HCS are correlated with the amplitude of the plasma membrane hyperpolarization they elicited. HBL, which promoted the larger cell expansion, also provoked the larger hyperpolarization. We observed that membrane hyperpolarization and cell expansion were partially inhibited by the proton pump inhibitor erythrosin B, suggesting that proton pumps were not the only ion transport system modulated by the two BRs. We used a voltage clamp approach in order to find the other ion transport systems involved in the PM hyperpolarization elicited by HBL and HCS. Interestingly, while anion currents were inhibited by both HBL and HCS, outward rectifying K⁺ currents were increased by HBL but inhibited by HCS. The different electrophysiological behavior shown by HBL and HCS indicates that small changes in the BR skeleton might be responsible for changes in bioactivity.

Keywords: *Arabidopsis thaliana* — Brassinosteroid — Ion currents — Plasma membrane — Proton pump — Suspension cells.

Abbreviations: ABP, auxin-binding protein; 9-AC, anthracene-9-carboxylic acid; BL, brassinolide; BR, brassinosteroid; DIDS, 4,4-diisothiocyano-2,2-stilbene disulfonate; EB, erythrosin B; FC, fusicoccin; HBL, 28-homobrassinolide; HCS, homoethylcastasterone; K⁺ ORC, potassium outward rectifying current; LRR, leucine-rich repeat; PM, plasma membrane; RLIT, rice lamina inclination test; SITS, 4-acetamido-4-isothiocyano-2,2-stilbene disulfonate; STG, stigmaterol; TEA, tetraethylammonium.

Introduction

Plant sterols are primarily components of cellular membranes. A minor proportion of them are precursors of steroid derivatives, which have the ability to elicit biological responses

(Clouse 2002a, Schaller 2003); brassinosteroids (BRs) come into this category. They were discovered in *Brassica napus* pollen (Mitchell et al. 1970) and have been found throughout the plant kingdom. Like others plant hormones, BRs were shown to participate at very low concentrations in the control of numerous processes associated with plant embryogenesis and development (Mandava 1988, Clouse and Sasse 1998, Friedrichsen and Chory 2001). Brassinolide (BL), the first brassinosteroid to be identified (Grove et al. 1979), causes cell elongation and cell division in stems, inhibits root growth, promotes xylem differentiation and delays abscission (Mandava 1988, Clouse 2002b, Nemhauser et al. 2004). BRs have been shown to promote microtubule reorganization in a transverse orientation, allowing longitudinal cell growth (Catterou et al. 2001). They have also been shown to control several processes working towards cell expansion (Thummel and Chory 2002). Plant cells are enclosed in a rigid pectocellulosic wall. Thus cell expansion is achieved only if the cell wall loses rigidity and becomes extensible. In *Arabidopsis thaliana* hypocotyls or soybean epicotyls, BR application increases cell wall extensibility by inducing expression of genes encoding xyloglucan endotransglycosylases (Zurek et al. 1994, Xu et al. 1995).

The mechanism by which BRs control plant cell expansion has not been completely elucidated so far. Interestingly, both BRs and auxin promote cell expansion (Nemhauser et al. 2004). BRs act more in concert with auxin than with other plant hormones (Mandava 1988). An interdependency of BRs and auxin signaling has been shown in *A. thaliana*, and many responses induced by BRs have been reported to be similar to those induced by auxin (Sasse 1990, Zurek et al. 1994, Nemhauser et al. 2004). In contrast to auxin, a BR receptor has been identified as well as a two leucine-rich repeat (LRR) receptor kinase involved in BR signal transduction in *A. thaliana* (Clouse 2002a, Thummel and Chory 2002, Kinoshita et al. 2005).

In tobacco mesophyll protoplasts and maize cells, it has been reported that plasma membrane (PM) perception of the auxin by auxin-binding proteins (ABPs) induced cell hyperpolarization (Barbier-Brygoo et al. 1991, Felle et al. 1991, David et al. 2001). Evidence has been found that the PM ion current activation contributes to the initial phase of the hyperpolarization. For example, Thomine et al. (1997) found that

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anion channel blockers were able to counteract the physiological functions caused by auxin, and suggested that an anion channel might be involved in the auxin signal transduction. Furthermore, it was shown that the activities of the PM H⁺-ATPase and anion channels were involved in the auxin-induced electrical responses (Lohse and Hedrich 1992, Zimmermann et al. 1994).

Osmotic and electrical relationships in plants are closely linked by the ion transporters in the plasma membrane. The proton pump generates an H⁺ electrochemical gradient, and provides a driving force for the rapid ion fluxes required for the uptake of various nutrients such as K⁺, Cl⁻, NO₃⁻, amino acids and sucrose across the PM (Serrano 1989, Sze et al. 1999). The regulation of H⁺-ATPase activity (Palmgren 2001, Kasamo 2003) not only allows nutrient uptake in plant cells but also controls water fluxes (Sondergaard et al. 2004). Water uptake is one of the motors required for cell expansion, presumably by controlling activities of PM and tonoplast aquaporins (Morillon et al. 2001, Ozga et al. 2002).

However, little is known about the roles of ion transport systems during BR-induced cell expansion. Previous studies reported a proton secretion induced by BL when applied to azuki bean epicotyls or apical root segments of maize. This proton secretion was accompanied by an early hyperpolarization of the PM, indicating that proton pumps could be targets of BRs (Cerana et al. 1983, Romani et al. 1983). Schumacher et al. (1999) have shown that tonoplast H⁺-ATPase (V-ATPase) played an important role in hypocotyl elongation promoted by BRs. V-ATPases are supposed to translocate osmolytes from the cytosol to the vacuole. Mutation in the *DET3* gene encoding a V-ATPase reduced the effects of BR (Schumacher et al. 1999) and it would be interesting to see the impact of BRs on the recently described tonoplast proteomic analysis of *A. thaliana* suspension cells (Shimaoka et al. 2004).

Ion transport systems are often associated with plant hormone signaling pathways as an important early component of plant cell responses to specific stimulation, including growth and development, but little is known about the action of BRs on ion fluxes through the PM.

In this study, we investigate the component necessary to explain BR-induced hyperpolarization in *A. thaliana* cell suspensions and its link with cell elongation. This material is very appropriate for electrophysiological studies because the measurements are made on cells whose physiological wall functions are retained.

We present experiments using combined voltage clamping and pharmacological approaches during BR signaling on cells where BRs induced cell enlargement. The effects of two BRs on the activities of the ion transport systems present in the PM of *A. thaliana* cells were studied. We used the 28-homobrassinolide (HBL) and its direct biosynthetic precursor 28-homoethylcastasterone (HCS). The only difference between these two BRs resides within the B ring of the BR skeleton.

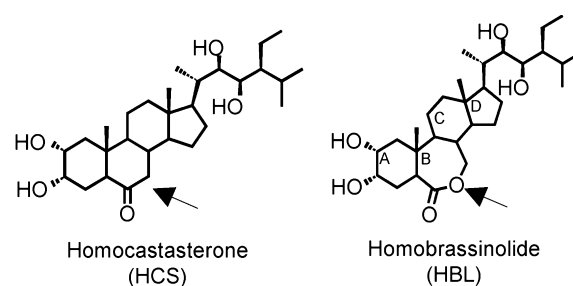


Fig. 1 Chemical structures of HBL and its precursor HCS. The black arrow points to the only difference existing between the two brassinosteroids.

The B ring of HBL bears a lactone function, whereas HCS contains a ketone group (Fig. 1).

Our results clearly show that both BRs modulate activities of proton pumps, anion and K⁺ channels and that these modulations were associated with cell expansion. We show, for the first time, that the typical early membrane hyperpolarization triggered by BRs might be mediated by the reduction of anion channels in addition to the activation of the H⁺-ATPase activity in *A. thaliana* cells.

Results

BRs promote plant growth and enlargement of A. thaliana suspension cells in a dose-dependent mode

The rice lamina inclination test (RLIT) is very sensitive to BRs. It has therefore been widely used to evaluate biological potency of natural and synthetic BRs. In the present study, the bioassay was performed first on whole seedlings. BRs were inoculated at the insertion of the second leaf, and the degree of the maximum leaf inclination angle caused by HBL or HCS was used to indicate the growth-promoting bioactivity of both BRs (Maeda 1965, Galagovsky et al. 2001). The results are shown in Table 1. It was found that both compounds caused inclination of rice laminae. HBL bioactivity was, however, slightly stronger than HCS bioactivity. Anatomic studies confirmed that lamina inclinations induced by both BRs were due to the increase in the size of the parenchymatic tissues, rather than to the induction of cell divisions (data not shown).

Table 1 Inclination angles induced by HBL and HCS in the rice lamina inclination test

Dose (ng/plant)	Inclination angles (°)	
	HBL	HCS
5	35 ± 3	30 ± 2
50	90 ± 4	84 ± 5
500	101 ± 4	97 ± 4

The indicated angles correspond to the average inclination measured for 23–25 replicates (mean ± SE). Controls containing the same amounts of ethanol as for HBL and HCS showed angles of 5 ± 3°.

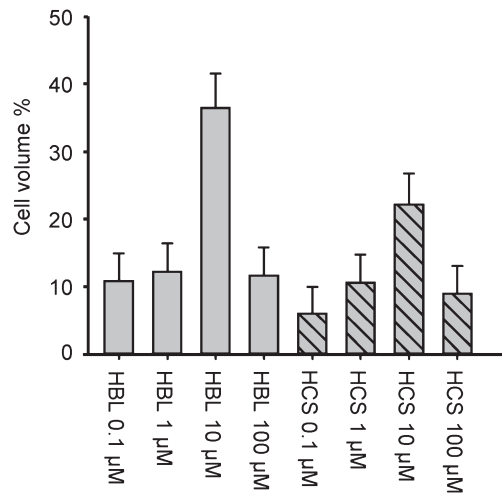


Fig. 2 Effects of HBL and HCS on *A. thaliana* suspension cell growth. Cell growth was estimated by measuring the increase in cell volume. Cell diameters were measured after 24 h treatment with various concentrations of HBL or HCS. Control cell diameters ranged from 40 to 55 μm . Volumes of cells treated either by HBL or HCS are expressed as a percentage of the control value. For each condition, 250 cell diameters were determined. Means \pm SE are represented.

Next, we assessed the effects of both BRs at a cellular level. *A. thaliana* suspension cells were used as a model. Effects of HBL and HCS on cell growth were estimated by comparing the volume of cells treated for 24 h by HBL or HCS (0.1 to 100 μM) with the volume of cells submitted to a control treatment. Whatever the concentrations used, HBL and HCS promoted cell enlargement by comparison with control treatment (Fig. 2). For both BRs, maximum effects were observed at 10 μM . Cells treated with HBL presented a volume increase by $28.9 \pm 4.6\%$ ($n = 233$). This increase was $16.6 \pm 3.8\%$ ($n = 276$) for HCS. The HCS-induced cell enlargement was statistically less effective (*t*-test, $P < 0.05$).

BRs provoked PM hyperpolarization and medium acidification in *A. thaliana* suspension cells

In order to determine if BRs modulate the activities of ion transport systems and to identify what systems they modulate, the impacts of HBL and HCS were first studied on the PM potential since this reflects the global activity of the whole ion transport system.

The distribution of the membrane potentials of cells conserved in resting conditions followed a Gaussian curve centered on the mean value -43.8 ± 11 mV ($n = 145$).

In the range of 0.1–100 μM BRs, PM hyperpolarizations were found to be dependent on the concentration used (Table 2). For both BRs, the largest effect was monitored with the 10 μM concentration. Since 10 μM was again the most effective concentration for both BRs, it was used for all subsequent experiments.

Treatment with HBL or HCS led to rapid PM hyperpolarization (ΔE_m about -12 and -8 mV; Fig. 3B) in about 80%

Table 2 Variations of the PM potential elicited by HBL and HCS

Concentration (M)	ΔE_m (mV)	
	HBL	HCS
0.1	-5.5 ± 1.0	-3.0 ± 0.6
1	-7.5 ± 0.8	-6.5 ± 0.7
10	-12.0 ± 1.3	-8.6 ± 1.1
100	-11.3 ± 1.1	-6.7 ± 0.8

Arabidopsis thaliana suspension cells were impaled with microelectrodes. Changes in the PM potential after addition of BRs were monitored (resting membrane potential -43.8 ± 11 mV). In the table, each value represents the average change \pm SE monitored for at least five independent experiments. The biologically inactive STG (10 μM) promoted a membrane potential variation of -0.5 ± 0.3 mV ($n = 4$).

of cells. An example of an electrical record is given for HBL (Fig. 3A).

To highlight the contribution of H^+ -ATPase pumps in the PM hyperpolarization due to HBL and HCS, we used erythrosin B (EB) (Wach and Gräber 1991). Application of 25 μM EB induced the PM depolarization in <1 min ($\Delta E_m = 6.4 \pm 1.1$ mV; $n = 7$; Fig. 3A, B). These data confirmed that PM proton pumps of *A. thaliana* suspension cells were active and had an electrogenic component. The PM hyperpolarizations elicited by BRs were reduced by about 40% (Fig. 3B) when EB was added after either HBL or HCS. This indicates that proton pumps might be involved in the BR-induced hyperpolarizations and that other EB-insensitive systems might contribute to these hyperpolarizations.

In addition to electrophysiological studies, the pH of the medium was continuously monitored. Before any treatment, it ranged from 5.4 to 5.6. HBL was shown to induce both medium acidification ($\Delta\text{pH} \sim 0.45$ units in <10 min) and PM hyperpolarization ($\Delta E_m = -12 \pm 1.3$ mV, $n = 4$) (Fig. 3B, C). These data revealed the HBL induced activation of the PM proton pumps of *A. thaliana* suspension cells.

BR-induced cell enlargement is mediated by proton pump activity

Osmotic and electrical relationships in plant cells are closely linked by the H^+ -ATPase and the ion channels in the PM. It was tempting to hypothesize that cell volume expansions induced by BRs were related to the modifications of ion fluxes. Thus we investigated whether ion transport systems were involved in the cell enlargement observed in response to BRs by pharmacological approaches. Therefore, we first analyzed whether proton pumps were involved by using EB. Cell volume expansion was strongly reduced after 24 h ($-28.1 \pm 2\%$; $n = 260$) in the presence of 25 μM EB alone (Fig. 4). In contrast, cell volume expansion induced by HBL or HCS in the presence of EB were less reduced: about -24% for HCS and only -7% for HBL, statistically the most active BR (*t*-test, $P = 0.05$). These results indicate that proton pumps are involved in cell volume increases elicited by both BRs.

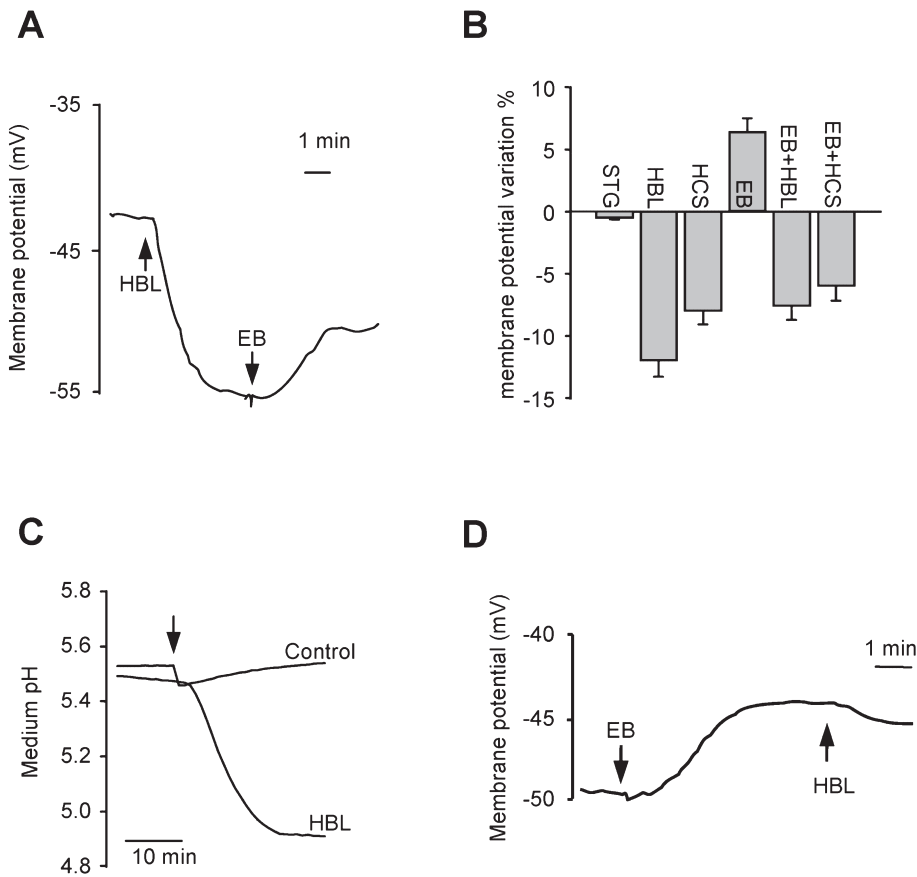


Fig. 3 BRs cause PM hyperpolarization and medium acidification of *A. thaliana* suspension cells even in the presence of the H^+ -ATPase inhibitor. (A) An example of an electrical record showing the hyperpolarization of the PM potential elicited by 10 μ M HBL and the depolarization by a subsequent addition of the proton pump inhibitor EB (25 μ M) (treatments are indicated by arrows). The resting potential was -42 mV. The diameter of the cell was 48 μ m. The experiment was repeated 10 times; similar results were obtained and a representative result is shown. (B) Variations of the PM potential elicited by 25 μ M EB, 10 μ M HBL, 10 μ M HCS or a combination of HBL or HCS with EB. The inactive BR stigmasterol (10 μ M) had no significant effect on the plasma membrane potential. Bars represent the average variation expressed as a percentage of the control value of at least four independent experiments. SD values are indicated. (C) The pH of the culture medium was monitored over time. Addition of HBL (10 μ M) or culture medium (Control) is indicated by the arrow. A representative result out of three independent experiments is shown. (D) An example of an electrical record showing the hyperpolarization of the PM potential elicited by HBL (10 μ M) even after addition of EB (25 μ M) (treatments are indicated by arrows). The resting potential was -49 mV. The diameter of the cell was 45 μ m.

Contribution of anion channels to the BR-induced cell enlargement and PM hyperpolarization

In cells pre-treated with 25 μ M EB for 5 min, 10 μ M HBL triggered a slight hyperpolarization of the PM: $\Delta E_m = -4.8 \pm 0.7$ ($n = 6$). Fig. 3D gives an example showing that this slight hyperpolarization induced by HBL occurred even in the presence of the H^+ -ATPase inhibitor. In fact, EB did not completely block the effects of HBL, even when proton pumps were inhibited, suggesting that other ion transport systems were involved. Anion channels might be some of these other candidates as activation of slow anion channels (S-type) favored the turgor loss responsible for stomatal closure (Schroeder et al. 2001).

Addition of 200 μ M 9-AC, a potent inhibitor of anion channels (Schwartz et al. 1995, Forestier et al. 1998), to the suspension mimicked BR effects since 9-AC increased the diameter of the cells (Table 3). We observed an additive effect of HBL and 9-AC when they were added simultaneously to the cell suspension, but no additive effect of HCS and 9-AC was observed, although 9-AC and HCS had similar effects (Table 3). BRs should therefore be able to modulate the activities of anion channels. These results show that anion channels and

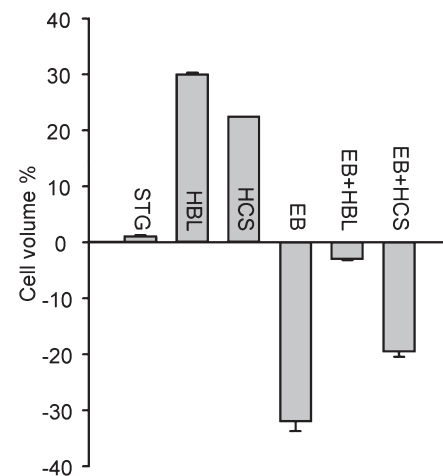


Fig. 4 Effect of 25 μ M EB, a proton pump inhibitor, on the cell expansion induced by HBL and HCS (both 10 μ M). *A. thaliana* suspension cells were incubated either in the presence of HBL or HCS alone or associated with EB. Cell expansion was estimated by measuring increases in cell volume. Diameters were measured after 24 h treatment. Volumes of cells treated were expressed as a percentage of the control value. STG (10 μ M) had no significant effect on the cell volume increase. Values are means \pm SE ($n =$ at least 180).

Table 3 Effects of 9-AC, an anion channel inhibitor, on the cell volume expansion induced by HBL and HCS

BRs ± 9-AC	Cell volume expansion (%)
STG	1.0 ± 0.1; <i>n</i> = 180
HBL	28.9 ± 4.6; <i>n</i> = 233
HCS	16.6 ± 3.8; <i>n</i> = 276
9-AC	33.8 ± 5.0; <i>n</i> = 166
HBL + 9-AC	40.5 ± 2.1; <i>n</i> = 180
HCS + 9-AC	19.1 ± 1.1; <i>n</i> = 180

Arabidopsis thaliana suspension cells were incubated in the presence of HBL or HCS alone or associated with 200 µM 9-AC. Cell volume expansion was estimated by measuring the cell diameter after 24 h treatment. Volumes of cells treated are expressed as a percentage of the control value ± SE (*n* from 180 to 276).

proton pumps may be at least two mechanisms involved in BR-promoted cell enlargement.

PM hyperpolarization induced by HBL and HCS involves activation of both H⁺ proton pumps and ion channels

PM hyperpolarizations may result from the modulations of multiple ion transport systems, such as the activation of PM K⁺ outward rectifying current (K⁺ ORC) or inhibition of anion currents. We investigated which ion channels functioned in the PM of *A. thaliana* suspension cells, and whether or not they were involved in the BR-induced hyperpolarizations. This was achieved by using a voltage-clamp technique to investigate the modulations of both outward and inward currents across the PM in intact cells, namely in cells where the cell wall functions are conserved (Jeannette et al. 1999, Kurkdjian et al. 2000, Hallouin et al. 2002).

After application of pulses from –200 to +80 mV, most of the cells (68%) exhibited both outward and inward currents (Fig. 5A). The outward current was activated by depolarizing pulses and displayed the time- and voltage-dependent rectifying activation characteristic for the K⁺ ORC. This K⁺ ORC could be fitted using a Hodgkin–Huxley type model (Roberts and Tester 1995) and was, moreover, sensitive to K⁺ channel blockers. Both Ba²⁺ (5 mM, BaCl₂) and tetraethylammonium (10 mM, TEA-Cl) (not shown) inhibited the time-dependent K⁺ ORC by approximately 84 and 95%, respectively, after a 4 min exposure at +80 mV clamping (Fig. 5B). The present results clearly show that a large part of the time-dependent K⁺ ORC observed was due to K⁺ efflux.

The remaining inward current confirmed the slow voltage-dependent deactivation (Fig. 5D) of S-type currents previously shown by Schroeder and Keller (1992). Moreover, this inward current is sensitive to 200 µM 9-AC (Fig. 5E). It corresponded to the S-type current we previously characterized in *A. thaliana* cells (Brault et al. 2004).

We next analyzed the effects of HBL and HCS on both currents. Treatments with HBL or HCS (10 µM) strongly

decreased anion currents in a similar manner to that which we observed after the addition of the anion channel inhibitor 9-AC (Fig. 6C, 5F). Inhibition of anion currents after a –200 mV pulse was 70 ± 14%; *n* = 7 (Fig. 6A) and 40 ± 9%; *n* = 10 (Fig. 6B) for HBL and HCS, respectively. As observed on RLIT, *A. thaliana* cell enlargement and membrane potential, HBL was found to be more efficient than HCS in inhibiting anion current.

Nevertheless, HBL and HCS had opposite effects on K⁺ ORC since HBL increased K⁺ ORC by 35 ± 14%; *n* = 7 (Fig. 7A, C) whereas HCS decreased it by 51 ± 12%; *n* = 10 (Fig. 6B, C). Taken together, these results show that HBL and HCS are active on PM ion transport systems other than proton pumps. HBL induced two hyperpolarizing processes, it reduced anion currents and activated K⁺ ORC; whereas HCS simultaneously induced a hyperpolarizing and a depolarizing process, a reduction of anion current and reduction of K⁺ ORC.

Discussion

BRs may serve as one of the critical signals controlling plant growth and development (Clouse 2002a, Thummel and Chory 2002), mainly driven by accumulation of ions in the cells. Then it is reasonable to think that cell expansion induced by BRs is related to the modifications of ion fluxes. Therefore, growth regulation by osmolytes such as ions and sugars promotes water uptake, cell volume modifications and variations of PM electrical polarization.

The aim of this work was to identify the nature of the PM hyperpolarization induced by BRs which precedes cell expansion of *A. thaliana* cells. The results presented herein are the first, to our knowledge, to detail the effects of two BRs (28-HBL and 28-HCS) on the electrophysiological characteristics of intact (i.e. with cell walls) *A. thaliana* cells in suspension (Fig. 1, 3, 5). *A. thaliana* cells are a very practical plant material for the dissection of the action of BRs on the PM mechanisms. First, these cells are as sensitive to BR as the other plant materials commonly used for studying BR responses. As we showed, optimal BR concentrations for RLIT and *A. thaliana* cell expansion are the same (10 µM; Table 1, Fig. 2). Secondly, they allow electrophysiological studies. Unlike most intact cells in higher plant tissues, with the exception of guard cells, suspension cells are not linked to others through plasmodesmata. Membrane potential recordings stayed stable for >20 min under our experimental conditions, and there was very little current leakage (Fig. 5). Most importantly, protoplast preparation is not required, so physiological experiments are performed on cells, without osmotic stress, where the physiological functions of the cell wall are maintained.

BRs cause PM hyperpolarization and cell expansion

Both BRs induced PM hyperpolarization (Fig 3B) and promoted cell volume increase, but the responses monitored for each of them differed slightly in their amplitudes. HBL caused a larger hyperpolarization (Fig. 3B) and a larger cell expansion

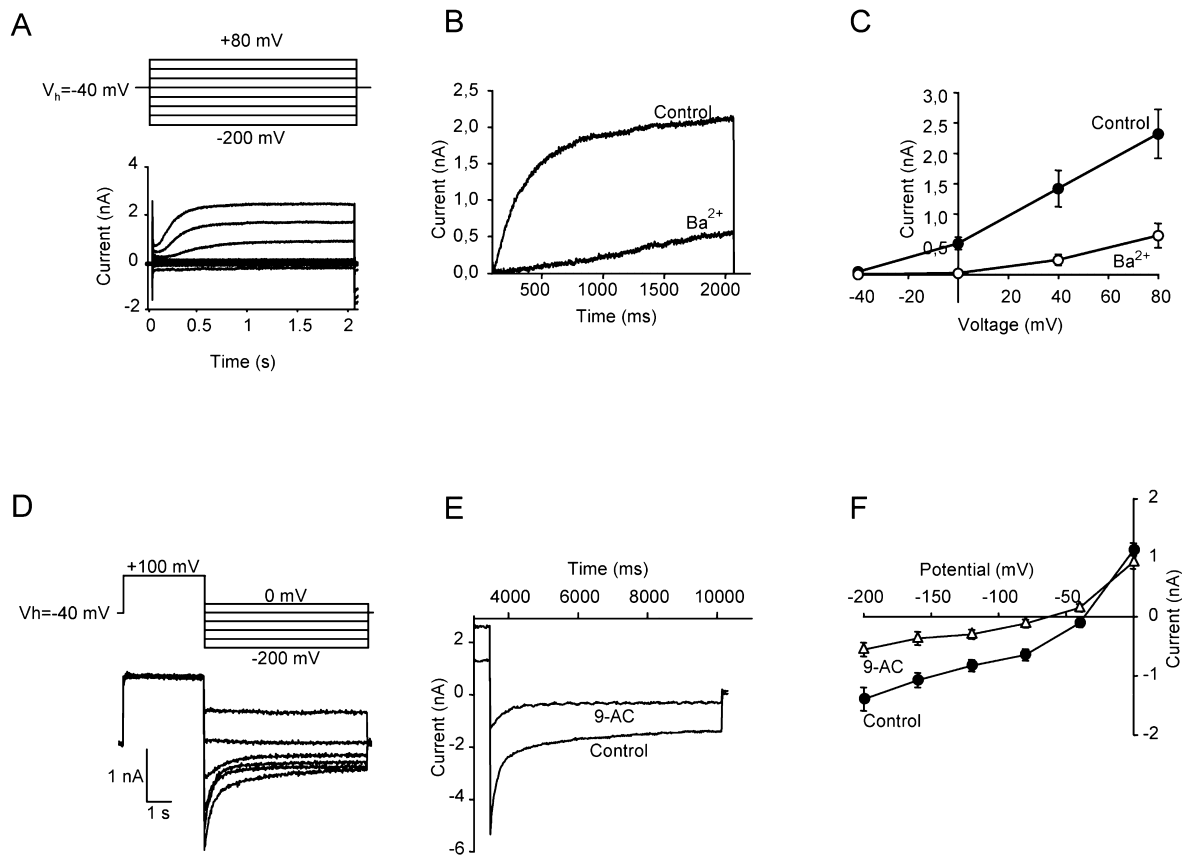


Fig. 5 Identification of the ion currents monitored through the PM of *A. thaliana* suspension cells. Cells were impaled by a single electrode and whole-cell currents monitored by a discontinuous voltage-clamp method described in Materials and Methods. (A) Typical whole-cell inward and outward currents recorded across the plasma membrane of intact cells polarized close to the average membrane potential. Plasma membranes were submitted to voltage pulses from -200 to $+80$ mV (in 40 mV steps for 2 s). Representative resulting currents are shown. The holding potential was -40 mV. (B) Effect of extracellular Ba^{2+} , an inhibitor of K^+ channels, on the whole-cell current measured across the PM of intact *A. thaliana* suspension cells. Currents were recorded under $+80$ mV pulse. The holding potential was -40 mV. The cell diameter was 40 μ m. A representative result out of three independent experiments is shown. (C) Corresponding current–voltage relationships are given at 2 s. In the presence of Ba^{2+} (circle), outward rectifying currents are reduced as compared with the control current (filled circle), showing that the outward current was a K^+ ORC. Mean variations of at least three independent experiments \pm SD are shown. Similar results were obtained with 10 mM TEA-Cl (not shown). (D) Inward currents were activated by a depolarizing pre-pulse ($+100$ mV for 4.5 s). Then, 9.5 s hyperpolarizing pulses ranging from -200 to 0 mV were applied (in 40 mV steps). The holding potential was -40 mV. The cell diameter was 38 μ m. (E) Effects of 9 -AC, an inhibitor of anion channels, on inward currents under the clamp protocol described in D. A -200 mV hyperpolarizing pulse was applied for 9.5 s before (filled circle) and after (triangle) addition of 100 μ M 9 -AC. The holding potential was -40 mV. Only the final 250 ms of the 3.5 s pre-pulse are shown. (F) Corresponding current–voltage relationships are given at steady state (10 s). Bars represent the mean variation of at least four independent experiments. SD values are indicated.

(Fig. 4) than HCS. Furthermore, cell cultures of *A. thaliana* responded to the addition of HBL with a rapid acidification of the medium (Δ pH ~ 0.45 units after 10 min, Fig. 3C) during the hyperpolarization (Jeannette et al. 1999). This HBL effect is similar to that of the proton pump activator fusicoocin (FC), although the medium acidification promoted by the proton pumps activator was about 95% higher in magnitude at 2 μ M (Brault et al. 2004). This suggests that the target of BRs which leads to the medium acidification could either be the proton pumps themselves or a signaling pathway controlling their activity. PM hyperpolarization appeared therefore to be of general importance in the responses to BRs as it was observed with

auxin (Barbier-Brygoo et al. 1991, Lohse and Hedrich 1995, Nemhauser et al. 2004) and also for the fungal toxin FC (Zingarelli et al. 1999, Tode and Lüthen 2001).

Contribution of proton pumps to the hyperpolarization

Activation of proton pumps leads to the generation of a pH gradient across the PM, and to its hyperpolarization. This pH gradient provides the driving force for the uptake by co-transporters of ions or organic compounds (sugars, amino acids) against their electrochemical gradients (Sze et al. 1999, Palmgren 2001, Kasamo 2003). In response to HBL, tomato pericarp cells exhibited high levels of total reductive sugars

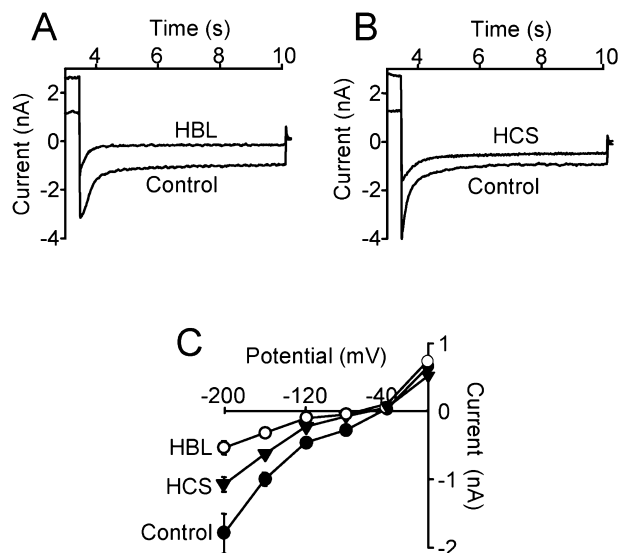


Fig. 6 Effects of HBL and HCS on the whole-cell inward anion current measured across the PM of intact *A. thaliana* suspension cells. For (A) and (B), currents were activated by a depolarizing pre-pulse (+100 mV for 3.5 s), then, a -200 mV hyperpolarizing pulse was applied for 9.5 s before and after addition of $10 \mu\text{M}$ HBL or HCS. The holding potential was -40 mV. Only the final 250 ms of the 3.5 s pre-pulse are shown. (A) Reduction by $70 \pm 14\%$ ($n = 7$) of anion current caused by $10 \mu\text{M}$ HBL at -200 mV as compared with the control current. (B) Reduction by $40 \pm 9\%$ ($n = 10$) of anion current caused by $10 \mu\text{M}$ HCS at -200 mV as compared with the control current. (C) Current–voltage relationships corresponding to the compilation of six, seven and 10 independent experiments for control (filled circle), HBL (circle) and HCS (filled triangle), respectively. Anion current inhibition exerted by the inactive STG was $7 \pm 1\%$ ($n = 6$).

(Vardhini and Rao 2002). In the BR biosynthesis mutant *det2* (Goda et al. 2002), it is interesting to note that expression of the *AAP3-4* gene, coding for an amino acid transporter, *KUP1* coding for a high-affinity potassium transporter, and *AKT2*, coding for a potassium channel, were down-regulated.

Our results clearly indicate that hyperpolarization and cell expansion are decreased in the presence of the proton pump inhibitor EB (Fig. 3, 4). These data show that proton pumps were required for both responses. This is consistent with previous reports where BRs were shown to favor electrogenic proton extrusion and growth in bean epicotyls or maize root segments (Cerana et al. 1983, Romani et al. 1983). The involvement of proton pumps during cell expansion is well known. It is interesting to note that the electrical response of the PM to auxin is characterized, as for BRs, by a sustained hyperpolarization linked to the proton pump activity (Bates and Goldsmith 1983, Barbier-Brygoo et al. 1991).

Inhibition of anion current may be a crucial step in BR-induced cell growth

Proton pumps contributed only to a part of the hyperpolarization elicited by BRs (Fig. 3A). We showed that BRs were

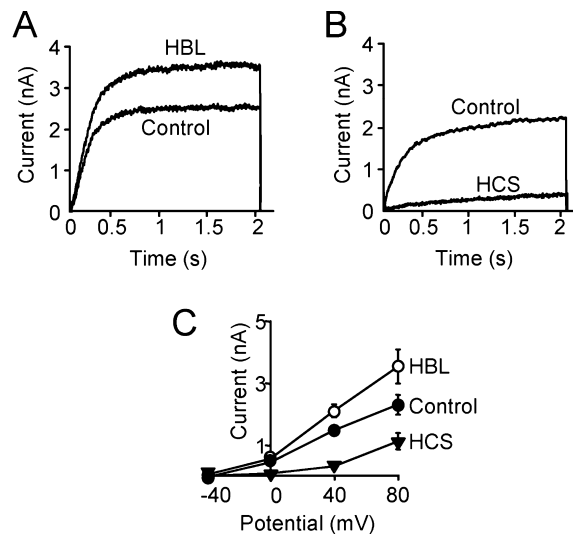


Fig. 7 Effects of HBL and HCS on the K^+ ORC measured across the PM of intact *A. thaliana* suspension cells. (A) Typical K^+ ORC traces at $+80$ mV recorded before and after addition of $10 \mu\text{M}$ HBL. HBL activated K^+ ORC by $35 \pm 11\%$ at $+80$ mV ($n = 7$). (B) Typical K^+ ORC traces at $+80$ mV recorded before and after addition of $10 \mu\text{M}$ HCS. HCS inhibited K^+ ORC by $51 \pm 12\%$ at $+80$ mV ($n = 10$) (C) Current–voltage relationships corresponding to the compilation of six, seven and 10 independent experiments for control (filled circle), HBL (circle) and HCS (filled triangle), respectively. The inactive STG elicited a small increase in K^+ ORC $5 \pm 1\%$, $n = 6$.

still able to hyperpolarize the PM even in the presence of EB (Fig. 3D), indicating that BRs recruited one or more other hyperpolarizing mechanism. Electrophysiological studies have demonstrated that besides an auxin-stimulated activity on the PM H^+ -ATPase (Lohse and Hedrich 1992), direct modulation of the guard cell anion channel could be observed (Marten et al. 1991, Lohse and Hedrich 1995). Thus, anion channels were also good candidates for hyperpolarization since a reduction of anion currents with HBL in the presence of EB (about -7% after 1 min at -200 mV, not shown) was observed.

Two highly distinct types of anion channels operate in plants, a rapid (R-type) and a slow (S-type) anion channel (Schroeder and Keller 1992). Based on their kinetic parameters, it can be assumed that the S-type anion channel could be involved in the reduction of anion effluxes induced by PM hyperpolarization. We clearly established that HBL and HCS reduced the anion currents monitored across the PM of *A. thaliana* cells (Fig. 6). Like 9-AC, an anion current inhibitor, they also increased their volume (Table 3). Anion channel inhibitors and BRs therefore had common effects: they reduced anion currents and increased cell expansion (Fig. 5, 6). This suggests that the inhibition of anion channels provoked by BRs could be involved, in addition to activation of proton pumps, in the BR-induced cell expansion. Since anion currents we measured are inward currents, the reduction of such currents corresponds to the reduction of anion effluxes. Furthermore, such a reduction is known to decrease cellular osmotic potential which

represents the driving force for cell growth (Cleland 1995). Hence, cell expansion is related to the inhibition of anion currents by BRs which should therefore be a crucial step in the control exerted by BRs on cell expansion. This conclusion is in agreement with previous reports which highlight the role of anion channels in the control of cell volume.

Blue light limits etiolation by restraining cell expansion. It activates anion channels favoring anion effluxes (Cho and Spalding 1996) and, in consequence, favors cell shrinking (Wang and Iino 1998). The effect of blue light on cell expansion was shown to be sensitive to anion channel inhibitors. When protoplasts of *A. thaliana* hypocotyls or corn coleoptiles are incubated in the presence of an anion channel inhibitor, the blue light-induced cell shrinking is abolished, indicating that anion channels are key elements in the control of cell volume (Wang and Iino 1997, Wang and Iino 1998). This was confirmed in intact *A. thaliana* seedlings. These results showed that activation of anion channels would impair cell expansion. In contrast, the reduction of anion channel activity would lead to cell expansion. Zonia et al. (2002) reported that anion channel blockers, when applied to pollen tubes, rapidly increased the volume of the apical region of tobacco pollen tubes.

The similarity between the S-type current described in guard cells and the current recorded in this study (Fig. 5C, 6) suggests that they may be involved in similar functions.

Furthermore, a small stimulating effect on cell elongation was observed for 9-AC, DIDS (4,4-diisothiocyano-2,2-stilbene disulfonate) and SITS (4-acetamido-4-isothiocyano-2,2-stilbene disulfonate) when applied alone (Thomine et al. 1997). Thus, the S-type current could be involved in long-term anion efflux which, when it is associated with K^+ efflux, can regulate the osmotic potential of cells.

In this study, both BRs were able to increase the cell volume within a period of 24 h. This indicates that both BR treatments could contribute to cell turgor, and suggests the involvement of anion channel current inhibition by BRs in the regulation of cell osmosis.

It is now well known that anion channels may be involved in signal transduction of biotic or abiotic factors (Schroeder 1995). For example, anion channels were shown to participate in the control of anthocyanin accumulation in response to blue light (Noh and Spalding 1998) and in the ABA-induced expression of the *RAB18* gene (Ghelis et al. 2000, Hallouin et al. 2002). Similarly, anion channels could also be implicated in some signaling pathways controlling cell expansion. The etiolation of *A. thaliana* seedlings was shown to be reversed by application of auxin, which reduced the length of hypocotyl cells (Thomine et al. 1997). If an anion channel inhibitor such as 9-AC is applied together with auxin, the reduction exerted by auxin on cell length is eliminated. One could assume that anion channel inhibitors hinder auxin effects by stimulating a general process leading to cell expansion. For example, they could limit anion effluxes and thus increase cell length. However, anion channel inhibitors did not block the inhibition of

hypocotyl elongation induced by other phytohormones such as ethylene or cytokinins. This suggested that anion channel inhibitors in fact would not interfere with the basic machinery of cell elongation but rather with an auxin signaling pathway (Thomine et al. 1997). Thus, in the case of BRs which block anion channels, the reduction of anion currents could be one step in the BR signaling pathway leading to cell expansion.

Differences in the molecular structure result in distinct bioactivities

The two BRs we used for this study displayed similar effects on cell expansion and anion currents, although the amplitude of the responses differed slightly. They have, however, opposite effects on K^+ ORC. Whereas HBL activated K^+ ORC (Fig. 7A), HCS reduced it (Fig. 7B). This difference could explain, at least in part, why the amplitudes of the PM hyperpolarizations elicited by HBL and HCS were different. Whereas K^+ ORC activation might contribute to the PM hyperpolarization elicited by HBL, K^+ ORC reduction elicited by HCS might oppose it.

It is interesting to note that opposite effects are observed in response to HBL and HCS on K^+ ORC. This shows that minor changes in BR structure not only changed the amplitude of responses, but also changed the K^+ ORC response. As HCS is the direct precursor of HBL, one could assume that the apparent biological activity of HCS could result from its conversion into HBL. However, this did not tally with our results since HBL and HCS induced opposite K^+ ORC responses. Thus both BRs should have the ability to promote specific biological responses by themselves, meaning that both BRs are recognized by a receptor. So far, a single BR receptor, BRI1, has been identified in *A. thaliana* (Li and Chory 1997). Interestingly, the tomato homolog of BRI1 was shown, probably in interaction with other proteins, to recognize not only BRs but also the peptide hormone systemin. Interactions of these two ligands with the receptor elicited responses specific to each of them (Wang and He 2004). One can therefore suppose that HBL and HCS could be perceived by BRI1 and, according to the structure of the perceived BR, BRI1 could activate or reduce K^+ ORC. The dissection of the signaling pathway triggered by HBL and HCS and leading to the modulation of K^+ ORC will lead to a better understanding of the molecular mechanisms of action of BRs.

In this study, we report different electrophysiological behaviors for HCS and HBL. This result establishes, for the first time, that small changes in the structural skeleton of BRs might be responsible for their distinct final bioactivities, by triggering alternative early origins of the signal transduction cascades. Recognition of different steroidal structures by putative membrane BR receptors may be one of the crucial starting points for those different signal transduction cascades (Clouse 2002b). The results reported in this work suggest that differential electrophysiological behavior of BR analogs could allow the study of structure–activity relationships in order to identify

the BR analogs that could act as better ligands for optimum BR receptor perception.

Materials and Methods

Chemicals

HBL and HCS were synthesized according to Teme Centurión and Galagovsky (1998). Other chemicals were purchased from Sigma-Aldrich Corporation (Saint-Quentin Fallavier, France).

Plant material

Arabidopsis thaliana L. (ecotype Columbia) suspension cells used for this study were derived from the suspension established by Axelos et al. (1992). Cell suspensions were maintained as previously described (Brault et al. 2004). Briefly, they were cultured at $22 \pm 2^\circ\text{C}$ under continuous white light and a 130 rpm agitation in a 1 liter round-bottom flask containing 300 ml of Gamborg medium (Gamborg et al. 1968). The cells were subcultured by a 10-fold dilution in fresh medium. Experiments were conducted on 4-day-old cell suspensions; the pH of the medium was pH 5.8.

Rice lamina inclination test

RLITs were conducted as previously described by Ramirez et al. (2000). Rice seeds (*Oryza sativa*, variety Chui) were washed with ethanol and water and then left to germinate in water at 30°C for 2 d under a 16 h photoperiod. Germinating seeds were then cultivated on agar for 4 d (16 h photoperiod). Intact seedlings (4–5 cm long) were inoculated with $0.5 \mu\text{l}$ of BR solution (1 mg ml^{-1} in ethanol) just under the second leaf joint. After inoculation, seedlings were kept at 30°C in the dark for 48 h. Inclinations induced by BRs were evaluated by measuring the angle formed by the second leaf and the sheath. At least 20 seedlings were used for each condition.

Measurement of cell diameter

The *A. thaliana* cell suspensions were treated for 24 h with BRs and/or inhibitors. During treatment, normal culture conditions were maintained. Aliquots of treated cells were mounted under a white light microscope and images were digitalized with a CCD-IRIS Sony camera driven by the control module of the Kappa ImageBase software version 2 (Kappa Optoelectronics GmbH, Germany). Images were then analyzed and the largest diameter of the cells was determined using the Metro module of the Kappa software. For each condition, diameters of 180–276 cells were determined.

Electrophysiology

A discontinuous single electrode voltage clamp method (Bouteau et al. 1999) was used because in small cells (Ghelis et al. 2000) this method causes less membrane injury as compared with the classical voltage-clamp method which uses two electrodes. The cells were equilibrated for 24 h in fresh Gamborg culture medium (24.73 mM KNO_3 , 1.02 mM CaCl_2 , 1.01 mM MgSO_4 , pH 5.8) before electrophysiological experiments. Voltage-clamp measurements of whole-cell currents from intact cells were carried out at room temperature ($22 \pm 2^\circ\text{C}$). K^+ ORC and anion currents were measured as previously described (Jeannette et al. 1999, Brault et al. 2004). Briefly, cells were immobilized by means of a microfunnel and were impaled with a borosilicate capillary glass microelectrode filled with 600 mM KCl (electrical resistance from 50 to $80 \text{ M}\Omega$). Membrane potentials were held at -40 mV . K^+ ORCs were activated by depolarizing pulses from -40 to $+80 \text{ mV}$, in 40 mV steps during 2 s, and anion currents were activated by a depolarizing pre-pulse ($+100 \text{ mV}$ for 4.5 s), then hyperpolarizing pulses from -200 to 0 mV were applied for 9.5 s. We sys-

tematically checked that cells were correctly clamped by comparing the protocol voltage values with those actually imposed.

pH response measurements

Continuous measurements of extracellular pH were performed in 5 ml of cultured cells. The experiments were run simultaneously in $2 \times 10 \text{ ml}$ flasks (control and test) each containing 1 g FW per 5 ml of suspension medium, at 24°C , with orbital shaking at 60 rpm. For each condition, the medium pH of the experiment was between 5.4 and 5.8 at the beginning. Simultaneous changes in pH were followed on both suspension media by using 2 Metrohm 632 pH meters with pH-sensitive combined electrodes working in parallel and values were monitored during 30 min. HBL was added when a stable pH was obtained. The buffer capacity of the culture medium was $4 \mu\text{eq OH}^- \text{ pH unit}^{-1}$.

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