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Effect of brassinolide, alone and in concert with abscisic acid, on control of stomatal aperture and potassium currents of *Vicia faba* guard cell protoplasts

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The essential role of brassinosteroids (BRs) in normal plant growth, development and physiology has been established by the analysis of biosynthesis and signal transduction mutants. Some of the BR-related mutants also display altered sensitivity to the phytohormone abscisic acid (ABA) suggesting that BRs normally counteract the effects of ABA on root growth, seed germination, and possibly stomatal movement. In this study, the effect of a specific BR, brassinolide (BL), on guard cell function of *Vicia faba* was examined alone and in conjunction with ABA. Unlike other described plant responses, BL did not oppose the effect of ABA in regulation of stomatal movement. On the contrary, BL modulated stomatal aperture by promoting stomatal closure and inhibiting stomatal opening, functions of this hormone that were previously undescribed. This study also demonstrated a role for plant steroidal hormones in ion channel regulation: BL inhibited inwardly rectifying K^+ currents of *V. faba* guard cell protoplasts in a manner similar to ABA. In both stomatal movement assays and whole-cell patch clamp experiments, the effects of BL and ABA applied together were not additive, suggesting that these two hormones may function in interacting pathways to regulate stomatal apertures and guard cell physiology.

Introduction

Brassinosteroids (BRs) are the only known class of plant steroid hormones, and they are essential for normal plant development and physiology. Mutant plants that are deficient in either the biosynthesis or the perception of BRs are typically dark green dwarfs with epinastic leaves, have reduced or no fertility, and exhibit delayed development (Bishop and Koncz 2002). For most BR-deficient mutants, a wild-type phenotype can be partially or fully restored with the addition of brassinolide (BL), one of the most potent BRs. Plants with defects in BR perception or signal transduction are not rescued by exogenous BL treatment.

Results of several types of bioassays suggest that BRs play a role in fine-tuning plant responses to other phytohormones. For example, exogenous BL is additive with gibberellin (GA) (Mayumi and Shiboaka 1995) and synergistic with auxin in promoting elongation of stem or hypocotyl segments in bean and cucumber (Yopp et al. 1981, Cohen and Meudt 1983, Katsumi 1985). It has been suggested that exogenous BL opposes abscisic acid (ABA) in promotion of bean stem

Abbreviations – ABA, abscisic acid; BL, brassinolide; BR, brassinosteroid; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis (aminoethylether)-tetraacetic acid; EtOH, ethanol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; K^+, potassium ions; MES, 2-(N-morpholino)-ethanesulphonic acid.

elongation, an effect briefly described in a review (Mandava 1988).

Detailed evidence for the interaction of BL and other hormones is found in the characterization of various BR-related mutants of *Arabidopsis*. A BR biosynthetic mutant, *sax1* (Ephritikhine et al. 1999a, 1999b), was originally identified based on its hypersensitivity to ABA and auxin in primary root elongation assays. When *sax1* was further characterized, it was found that only addition of 24-epibrassinolide, but not auxin, GA, or ABA, could restore normal root responses and seedling growth (Ephritikhine et al. 1999a). Thus while a defect in BR biosynthesis is the primary lesion associated with *sax1*, responses to multiple hormones are affected.

BR signal transduction mutants such as *bri1, bin2/dwf 12* (*brassinosteroid insensitive 2/dwarf 12*), and *bin5* also displayed hypersensitivity to ABA inhibition of primary root growth (Clouse et al. 1996, Li et al. 2001, Choe et al. 2002, Yin et al. 2002). On contrary, roots of plants that overexpressed the positive regulator *BEE1* (*Brassinosteroid Enhanced Expression*) were more responsive to exogenous BL and partially insensitive to ABA (Friedrichsen et al. 2002). This is consistent with the observation that expression of *BEE1*, *BEE2*, and *BEE3* transcripts in seedlings was enhanced by BL and decreased by ABA treatment (Friedrichsen et al. 2002). Based on the physiological characteristics of these various BR-related mutants, it is possible that BL has a role in downregulating some responses to ABA. When BL (or the ability to respond to it) is lacking, the ABA responses are amplified.

BL also interacts with other plant hormones in the processes of seed germination and breaking of seed dormancy, which are promoted by GA and antagonized by ABA. In *Arabidopsis*, the germination of severe GA biosynthetic mutants can be rescued by application of GA or 24-epibrassinolide. Further, seeds of the BR biosynthetic mutant *det2* (*de-etiolated 2*) and *bri1* are more sensitive to ABA as compared with wild-type seeds (Steber and McCourt 2001). This again suggests that BL normally has a role in counteracting ABA responses.

Guard cells and plant stomata provide a unique, isolatable system for studying the effect of phytohormones on plant function; the role of ABA in regulation of guard cell physiology has been especially studied. The coordinated passage of ions and solutes across the guard cell plasma membrane creates turgor changes that drive stomatal movement and control of the pore aperture. In the process of stomatal opening, activation of H^+-ATPases hyperpolarizes the guard cell plasma membrane, driving the influx of K^+ ions through inwardly rectifying K^+ channels (K^+_{in} channels) and osmotic swelling of the guard cell pair. Conversely, activation of anion channels that mediate anion efflux helps to depolarize the plasma membrane, driving K^+ efflux through outwardly rectifying (K^+_{out}) channels, leading to guard cell shrinkage and stomatal closure. An increase in cytosolic Ca^{2+} levels is also associated with stomatal closure. ABA can prevent stomatal opening by inhibiting the function of H^+-ATPases and K^+_{in} channels. Conversely, ABA can promote stomatal closure by activating anion and K^+_{out} channels, as well as Ca^{2+}-permeable channels (reviewed in Blatt 2000, Assmann and Wang 2001, Schroeder et al. 2001).

To date, there are no published studies that specifically address the effects of BL on stomatal aperture, or whether BL also modulates ABA responses of guard cells as it does for ABA responses of other plant tissues. One *Arabidopsis* mutant that exhibits defects in BR responses such as inhibition of root growth and seed germination may also show alterations in guard cell function. Ephritikhine et al. (1999a) reported that in the BR-deficient *sax1* mutant, promotion of stomatal closure by ABA was enhanced as compared with wild-type controls.

In this study, bioassays were performed with *Vicia faba* to address the question of whether BL affects stomatal movement. *V. faba* is a model plant for studying stomatal responses and guard cell electrophysiology, with well-defined experimental protocols. Dose–response curves were generated for *V. faba* stomatal responses to ABA and BL. Further stomatal movement assays combining ABA and BL were then performed to test the hypothesis that BL counteracts the effect of ABA in promoting stomatal closure and inhibiting stomatal opening. Finally, whole-cell K^+ currents of *V. faba* guard cell protoplasts were measured in response to treatment with ABA alone or in combination with BL.

Materials and methods

Plant material and chemicals

V. faba (cv. Longpod) seeds were sown in a soil mixture of three parts Metro-Mix 350 to one part Perlite (Scotts, Marysville, OH) and watered to saturation with 1/4 strength Hoaglands solution three times a week. Plants were grown in a growth chamber with a photoperiod of 10 h light and 14 h dark with temperatures of 22°C and 18°C, respectively. The light quality consisted of both fluorescent and incandescent bulbs, with a total fluence rate of 200 μmol m^{-2} s^{-1}. For all experiments, leaves were harvested from 3- to 4-week-old plants. Unless otherwise noted, all chemicals were from Sigma (St. Louis, MO).

Assays of stomatal movement

To assess promotion of stomatal closure, whole *V. faba* leaves were incubated in the light (four fluorescent tubes, GE, 180 μmol m^{-2} s^{-1}) for 1 h to initially open the stomata. Abaxial epidermal fragments were peeled with forceps, floated on incubation solution [30 mM KCl, 10 mM 2-(N-morpholino)-ethanesulphonic acid (MES), pH 6.15] with or without hormone treatment, and returned to the light for 1 h. To assess inhibition of stomatal opening, abaxial epidermal peels from *V. faba* were first floated on incubation solution (30 mM KCl, 10 mM MES, 1 mM Ca^{2+}, pH 6.15) in the dark to close stomata. The peels were rinsed with distilled water and transferred to Ca^{2+}-free incubation solution with or without hormone treatment, and then exposed to light. After 1 h, stomatal apertures were measured using a compound microscope and an ocular micrometer (Leica brand, Fisher Scientific, Pittsburgh, PA). The innermost walls of the guard cells were chosen as the focal plane.

ABA was made as a 50 mM stock in 95% ethanol (EtOH), and BL (CIDTech, ON, Canada) was prepared as a 10 mM stock in dimethyl sulfoxide (DMSO). Control treatments equivalent to the highest amount of solvent vehicle (v/v) were included in each assay (0.004% EtOH and 0.01% DMSO). Experiments were carried out as blind trials with the treatments coded and revealed after measurements were taken. Within a single experiment, 20 or 30 apertures were measured per treatment; each experiment (with multiple treatments) was performed three times. MS Excel was used to calculate average stomatal apertures and standard errors, perform 2-tailed *t*-tests, and create graphs. Error bars represent standard errors.

Electrophysiology

V. faba guard cell protoplasts were isolated as previously described (Miedema and Assmann 1996). After preparation, protoplasts were resuspended in bath solution and incubated on ice in the dark for at least 2 h before use in patch-clamp experiments. The pipette solution contained 98 mM K-glutamate, 2 mM KCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 2 mM ethylene glycol-bis (aminoethyl-ether)-tetraacetic acid. The pH was adjusted to 7.2 with 1 M Tris, and the final osmolality was adjusted with sorbitol to 500 mmol kg^{-1}. A stock of 0.5 M Mg-ATP was prepared daily in 1 M Tris and added to the pipette solution at a final concentration of 2 mM. The bath solution contained 10 mM K-glutamate, 1 mM $CaCl_2$, 4 mM $MgCl_2$, and 10 mM HEPES. The pH was adjusted to 6.0 with 1 M Tris, base and the final osmolality was adjusted with sorbitol to 450 mmol kg^{-1}. After achieving a whole-cell configuration and measuring stable currents, hormone was added to the bath solution surrounding the guard cell protoplast. The time-activated currents elicited by a set of voltage pulses were measured repeatedly at 5-min intervals, up to 20 min after treatment. Hormone and solvent concentrations paralleled those used in the stomatal movement assays. No treatment controls were also carried out to detect any change in current over the time period of this protocol (i.e. current decrease or 'rundown' independent of any treatment).

Patch pipettes were pulled from Kimax 51 capillary tubes (VWR Scientific, Boston, MA). Using the above solutions, pipette resistance was typically approximately 20 MΩ. Data were acquired using an Axopatch 1B amplifier and analyzed with pCLAMP software (Axon Instruments, Foster City, CA). Protoplasts were held at a resting potential of −50 mV, and whole-cell data were sampled at 1 kHz. For each family of currents, the voltage protocol (corrected for liquid junction potential) started at a potential of −147 mV and was stepped to a final voltage of 67 mV in either 20 mV or 30 mV increments. Each voltage pulse lasted 1.8 s followed by a return to resting potential for 2 s between runs. Whole-cell currents were normalized for protoplast surface area (pA/pF) with whole-cell capacitance as measured by the amplifier. The time-activated current was calculated as the magnitude of the average steady-state current (between 1500 and 1700 ms) at the end of a voltage pulse minus the instantaneous current at 8 ms.

Results

Promotion of stomatal closure by BL and ABA

The range of BL concentrations tested was based on other studies where the hormone was used (e.g. Li and Chory 1997, Hu et al. 2000), and higher levels of BL were not more effective (data not shown). In contrast to what we predicted based on BL antagonism of ABA responses in other tissues, BL promoted stomatal closure. As shown in Fig. 1A, all concentrations of BL induced stomatal closure in the light ($P \leq 0.001$), whereas the no treatment and solvent controls did not ($P \geq 0.10$). The greatest change in aperture, approximately 3 μm, resulted from the 5 μM BL treatment. As expected, ABA also induced stomatal closure. Maximum response occurred in peels exposed to 20 μM or 50 μM ABA, where stomata closed approximately 5 μm (Fig. 1B).

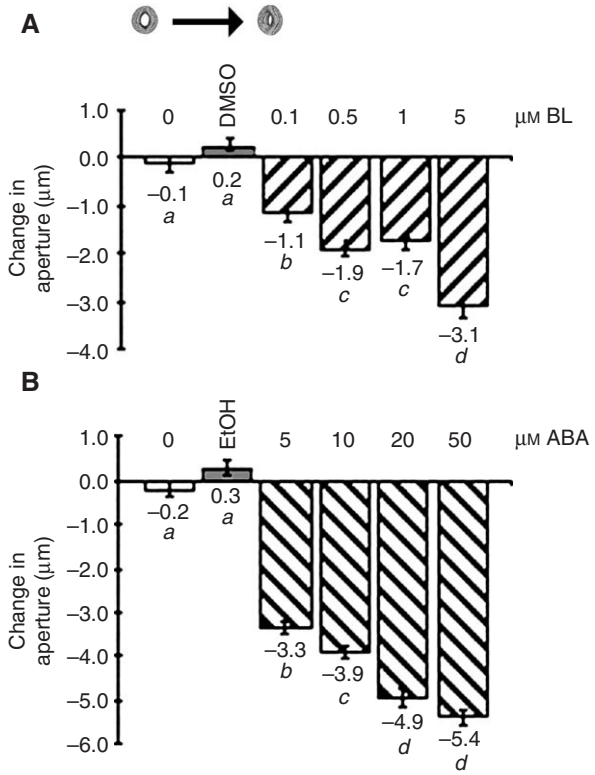

Fig. 1. Promotion of stomatal closure in *Vicia faba* by brassinolide (BL) and abscisic acid (ABA). Stomatal apertures were measured before and 1 h after hormone treatment in the light. Each bar is the mean of three trials, with 20 stomata measured per trial. For both (A) and (B), the solvent and no treatment controls were not significantly different ($P > 0.10$). All hormone treatments (*b, c, d*) were significantly different from the controls ($P < 0.001$). DMSO, dimethyl sulfoxide; EtOH, ethanol.

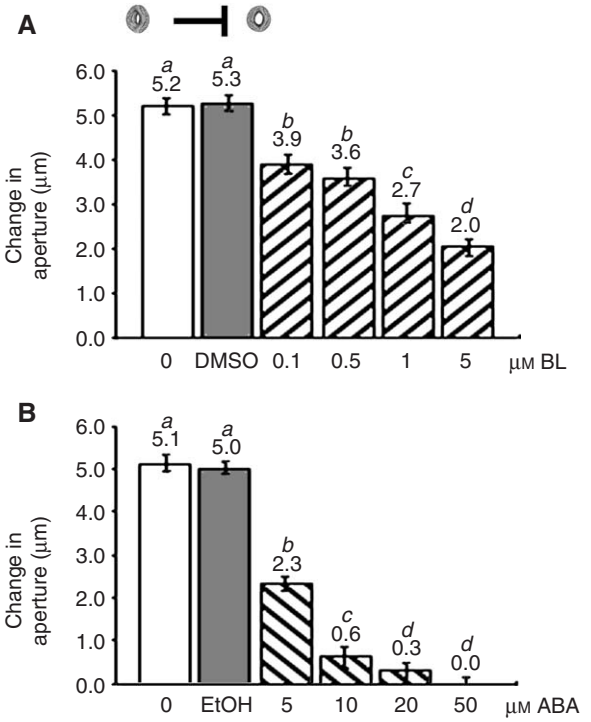

Fig. 2. Inhibition of stomatal opening by brassinolide (BL) and abscisic acid (ABA). Stomatal apertures were measured before and 1 h after hormone treatment in the light. Each bar is the mean of three trials, with 20 stomata measured per trial. For both (A) and (B), the solvent and no treatment controls were not significantly different from each other ($P = 0.10$, *a*). All hormone treatments were significantly different from the controls ($P = 0.001$, *b, c, d*). DMSO, dimethyl sulfoxide; EtOH, ethanol.

Inhibition of stomatal opening by BL and ABA

A corresponding set of experiments focused on the separate process of stomatal opening. As shown in Fig. 2A, all concentrations of BL tested significantly inhibited stomatal opening ($P \leq 0.001$, *b, c, d*) as compared with the solvent and no treatment controls, which were not different from each other ($P > 0.10$, *a*). At the highest BL concentration, stomatal opening was only 2 μm, as compared with approximately 5 μm of opening for both controls. All concentrations of ABA also inhibited stomatal opening (Fig. 2B, $P \leq 0.001$ *b, c, d*).

The data from each type of stomatal assay (promotion of closure and inhibition of opening) were fitted to logarithmic curves ($R^2 \geq 0.92$ for all), and the concentrations that yielded a half-maximal response were calculated using the equation of the curve (data not shown). For both promotion of closure and inhibition of opening, the concentrations for half-maximal response were approximately 2 μM ABA and 0.25 μM BL. These values were used as the basis for the next set of experiments combining both hormones.

Effect of BL combined with ABA on stomatal movement

In both closure and opening experiments, when BL was combined with ABA, there was no opposition of the stomatal response to ABA (Figs 3 and 4). In Fig. 3A, stomatal closure in response to 2 μM ABA, alone and with varying concentrations of BL (0, 0.1, 0.5, and 1 μM), is shown. Stomata from the no treatment and solvent controls remained open in the light and were not different from each other ($P > 0.5$, *a*). Stomatal aperture decreased approximately 3.5 μm with all types of hormone treatment, which were statistically equal ($P \geq 0.2$, *b*). These results are consistent with those from experiments utilizing higher levels of hormones (Fig. 3B). The average change in aperture promoted by 20 μM ABA alone (4.3 μm closure) was not different from that induced by 20 μM ABA with 5 μM BL (4.4 μm closure) ($P > 0.1$, *c*).

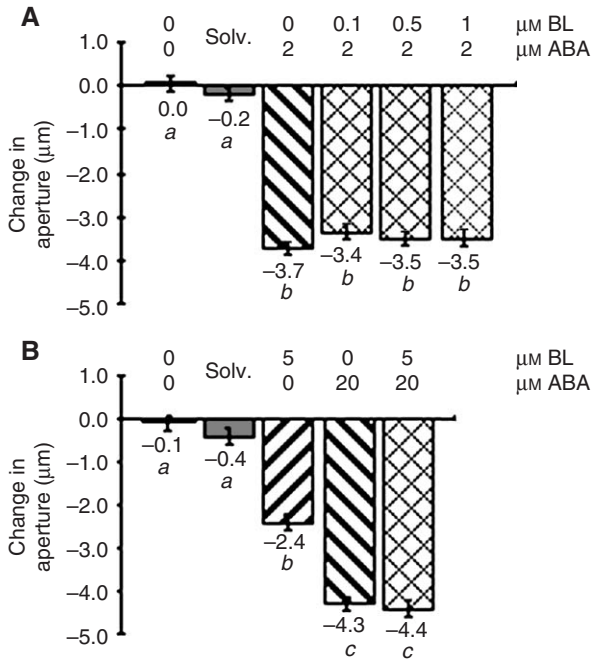

Fig. 3. Promotion of stomatal closure by brassinolide (BL) in conjunction with abscisic acid (ABA). Each bar is the mean of three trials, with either 20 stomata (A) or 30 stomata (B) measured per trial. The solvent and no treatment controls were not significantly different ($P = 0.10$, *a*). All hormone treatments were significantly different from the controls ($P = 0.001$ *b, c*). Solv., solvent.

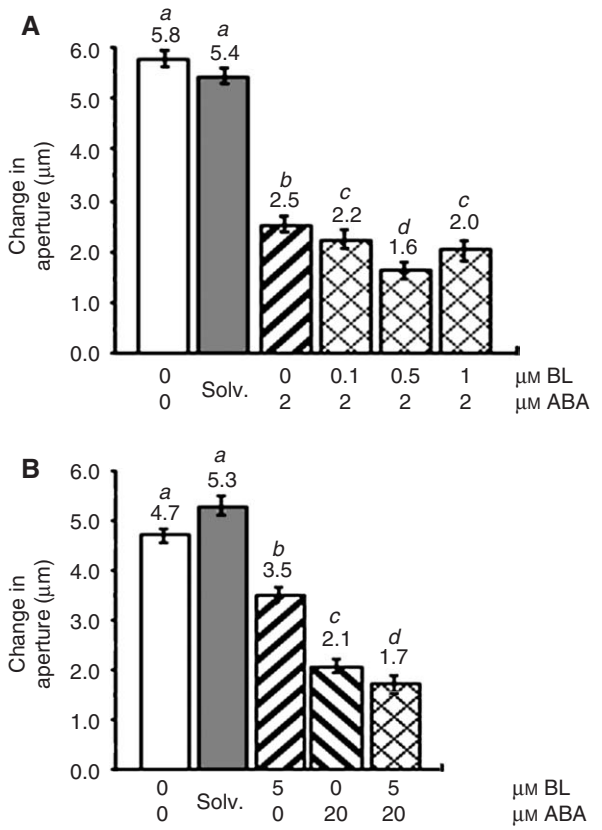

Fig. 4. Inhibition of stomatal opening by brassinolide (BL) in conjunction with abscisic acid (ABA). Each bar is the mean of three trials, with either 20 (A) or 30 (B) stomata measured per trial. The solvent and no treatment controls were not significantly different ($P = 0.10$, *a*). All hormone treatments were significantly different from the controls ($P = 0.001$, *b, c, d*). For inhibition of stomatal opening, there was a significant difference between 20 µM ABA alone and 20 µM ABA with 5 µM BL ($P = 0.015$, *d*). Solv., solvent.

When varying concentrations of BL were combined with 2 µM ABA in assays for inhibition of stomatal opening, there was also no consistent trend in stomatal response as a function of BL concentration. Stomata from all sets of hormone-treated epidermal peels showed significant inhibition of opening as compared with the controls ($P \leq 0.001$, Fig. 4A). There was slightly more inhibition of stomatal opening when 5 µM BL was added in conjunction with 20 µM ABA (2.1 µm, *d*), as compared with 20 µM ABA alone (1.7 µm, *c*) ($P = 0.015$, Fig. 4B).

Effect of BL and ABA on whole-cell K^+ currents of guard cell protoplasts

Because BL promoted stomatal closure and inhibited opening, whole-cell patch clamp experiments were carried out with guard cell protoplasts of *V. faba* to see if BL also affected K^+ currents in a manner similar to ABA. Representative current families are shown in Fig. 5. By convention, the downward-deflecting current traces represent K^+ entry into the cell (K^+_{in}), and the upward-deflecting current traces represent K^+ exit from the cell (K^+_{out}).

Even when normalized for cell size, initial K^+ currents were variable across all treatment sets, ranging from −21 pA/pF to −65 pA/pF for K^+_{in} at −147 mV (data not shown). Even larger variability has been reported before for *V. faba* guard cell protoplasts, for example −22 pA/pF to −266 pA/pF at −167 mV (Romano et al. 2000). Not all cells displayed K^+_{out} currents, and the presence or absence of K^+_{out} currents could not be correlated with any treatment. Because K^+_{out} current was not consistently present, it was not further analyzed.

K^+_{in} currents from the no treatment (rundown control) and solvent controls were similar, showing slight decrease over a time course of 15 min. Two µM ABA, 0.25 µM BL, or the combination of both hormones all decreased K^+_{in} current over the same time period, to a significantly greater extent than was observed for the controls. No shift in voltage activation of currents was

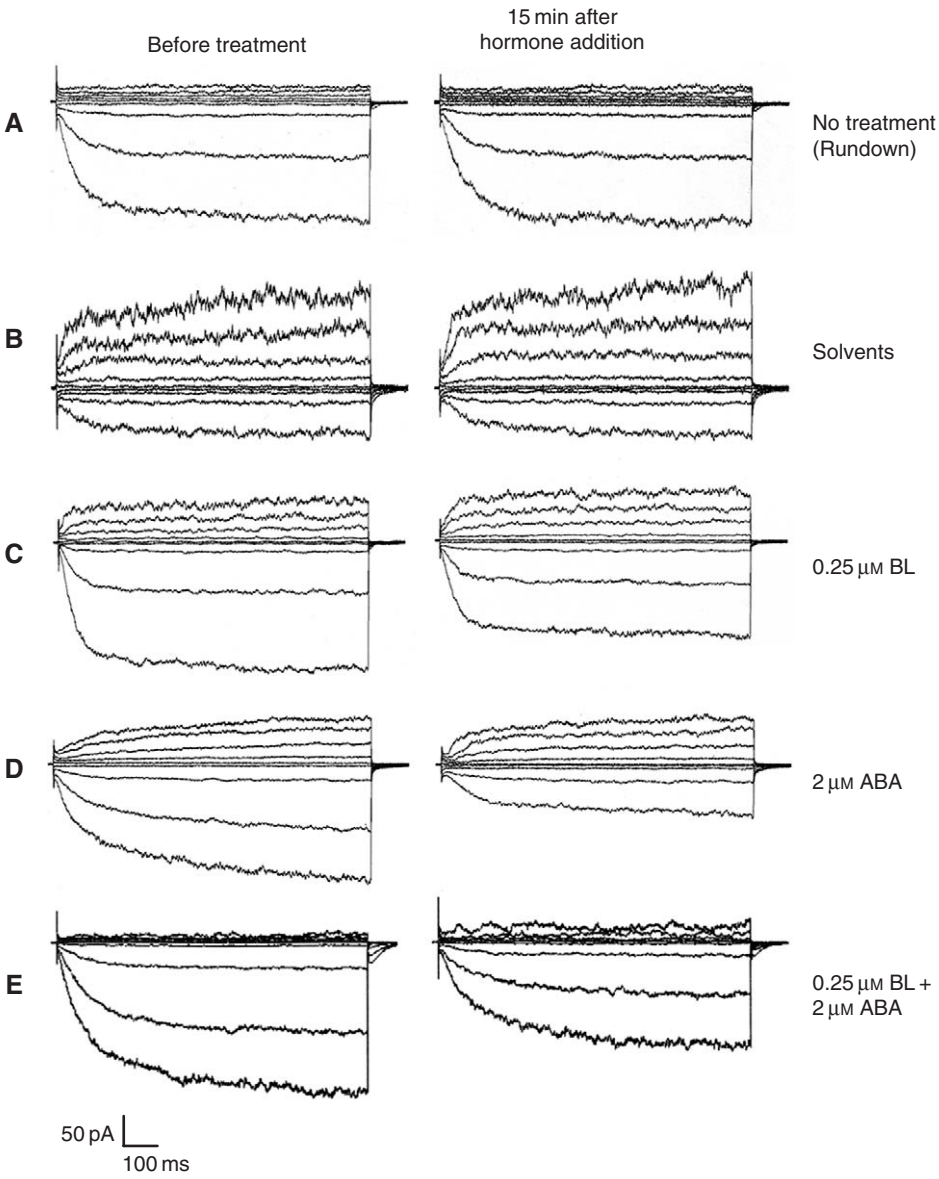

Fig. 5. K^+ currents from *Vicia faba* guard cell protoplasts. Representative current families are shown. Treatment is indicated to the right of each pair of traces. By convention, upward-deflecting traces indicate ion movement out of the cell (K^+_{out}). Downward-deflecting traces indicate ion movement into the cell (K^+_{in}). At 15 min, K^+_{in} currents were stable for solvent and no treatment (rundown) controls. All hormone treatments resulted in a decrease in K^+_{in} currents. ABA, abscisic acid; BL, brassinolide.

detected after the different hormone treatments (2 μM ABA, 0.25 μM BL, or 2 μM ABA + 0.25 μM BL, data not shown). Tail current analysis confirmed that the reversal potential (E_{rev}) was appropriate for potassium (E_K) (data not shown).

The time-activated K^+_{in} current resulting from the −147 mV pulse was quantified for each cell, before and 15 min after treatment. The average percent change in K^+_{in} current was then calculated for each population. As shown in Fig. 6, the decrease of K^+_{in} current in solvent-treated cells was comparable to the no treatment (rundown) controls ($P > 0.5$, *a*). For each set of hormone-treated guard cell protoplasts (0.25 μM BL, 2 μM ABA, and 2 μM ABA combined with 0.25 μM BL), the average decrease in K^+_{in} current was significant ($P < 0.05$). There was no significant difference between the different hormone treatments ($P > 0.5$, *b*). All hormone treatments showed significantly greater decrease in K^+_{in} currents than in the rundown or solvent controls (Fig. 6, *b* vs. *a*).

Discussion

Overall, the results of our experiments indicate that the plant steroid BL can modulate stomatal aperture by promoting

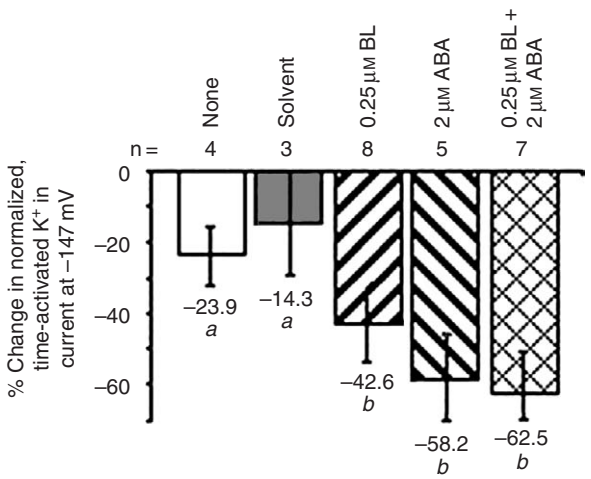

Fig. 6. Change in normalized, time-activated K^+_{in} currents of *Vicia faba* guard cell protoplasts 15 min after hormone treatment. To account for variability in protoplast size, the current values were divided by the whole-cell capacitance. The time-activated current resulting from a voltage step to −147 mV was quantified for each cell, before and 15 min after hormone treatment. The percent change in current was averaged for each treatment set. The change in no treatment cells was not significantly different from solvent-treated cells ($P > 0.50$, *a*, and data not shown). All cells treated with hormone showed a significant percent decrease in K^+_{in} current as compared with control cells ($P < 0.05$, *b*). BL, brassinolide; ABA, abscisic acid.

stomatal closure and inhibiting stomatal opening, functions of this hormone that were previously undescribed. These results also represent the first report that shows an effect of a plant steroid on ion channel activity.

BL promoted stomatal closure in the light in a dose-dependent manner, similar to ABA (Figs 1 and 3). Additional assays with higher concentrations of BL indicated that saturation of the closing response occurred at 5 μM BL (data not shown). The amount of stomatal closure elicited by 20 μM ABA, approximately 5 μm, corresponded to that observed in previous work using *V. faba* (Jacob et al. 1999).

In the separate process of stomatal opening, BL was inhibitory, again similar to ABA. At saturating hormone concentrations, ABA completely inhibited stomatal opening as compared with the controls, while BL inhibited opening by approximately 60% (Fig. 2). Overall, the absolute amount of stomatal aperture change induced by BL was less than that of ABA. The concentrations needed to generate a half-maximal stomatal response, 0.25 μM for BL and 2 μM for ABA are both within physiological ranges (Zhang and Outlaw 2001, Bajguz and Tretyn 2003).

If BL opposed the action of ABA in guard cell function (Ephritikhine et al. 1999a), then the combination of BL with ABA should have resulted in a reduced amount of stomatal closure as compared with ABA treatment alone. This was not observed. BL itself promoted stomatal closure, and there was no subtractive, additive, or synergistic effect found in conjunction with ABA (Fig. 3). BL also inhibited stomatal opening by itself, and it did not oppose or enhance the ABA response in this process (Fig. 4). There was slightly more inhibition of opening observed in epidermal peels treated with 2 μM ABA and 0.5 μM BL, but this effect is not consistent with the overall trend of the response (Fig. 4). Therefore, while BL may inhibit or oppose the effects of ABA in root elongation and seed germination, it apparently does not necessarily counteract all plant responses to ABA.

It is known that ABA can inhibit the K^+_{in} currents involved in stomatal opening (see *Introduction*). Because BL inhibited stomatal opening, it was hypothesized that BL could also inhibit K^+_{in} currents. The response of K^+ currents of isolated *V. faba* guard cell protoplasts was measured in patch-clamp experiments. Hormone-treated guard cell protoplasts (0.25 μM BL, 2 μM ABA, or 2 μM ABA combined with 0.25 μM BL) displayed a decrease in K^+_{in} currents (Fig. 5C, D, E). When the decrease in time-activated current K^+_{in} at −147 mV was quantified for each set of hormone-treated guard cell protoplasts, there was no significant difference ($P > 0.5$) between the different hormone treatments (Fig. 6). Experiments using higher concentrations of hormone were also carried out. Normalized K^+_{in} current of *V. faba* guard cell protoplasts decreased approximately 40% on average after treatment with 15 μM ABA, a level consistent with previous reports (Jacob et al. 1999) and significantly different from the solvent and rundown controls (data not shown). Given the inherent cell to cell variation, there was no statistically significant difference between the average decrease observed with 15 μM ABA alone (38.8 ± 6.0%, n = 9) and 15 μM ABA + 5 μM BL (24.0 ± 7.2%, n = 8) ($P = 0.14$, data not shown). Overall, it appears that BL can decrease K^+_{in} currents, similar to the effect of ABA. This is consistent with the results of stomatal movement assays where BL inhibited stomatal opening.

Addition of BL to ABA-treated epidermal peels or isolated guard cell protoplasts did not alter the ABA responses of this cell type under most conditions suggesting that the two hormones may function in the same or in interacting pathways. A possible target of both hormones could be cytosolic Ca^{2+} oscillations, which can be inhibited by ABA and may also be regulated by BL. The partially BL-insensitive Arabidopsis mutant, *det3*, shows altered Ca^{2+} responses under some conditions, thus affecting stomatal closure (Allen et al. 2000). Aquaporins might also be involved; one study has suggested that BL affects water permeability of cell membranes (Morillon et al. 2001).

Our results contrast with those obtained with the *Arabidopsis* BR biosynthetic mutant, *sax1*. In *sax1*, an enhancement of ABA-induced stomatal closure was reported (Ephritikhine et al. 1999a) suggesting that BR and ABA have opposing effects on guard cell function. However, our results are consistent with observations in sorghum, in which coapplication of epibrassinolide enhanced the drought-protective effects of ABA, in part by reducing stomatal transpiration (Xu et al. 1994a, 1994b). Such observations suggest that guard cells of different species may respond differently to BL application. The idea of species or family specificity in BL response is supported by the observation that BR biosynthesis mutants of the leguminous species *V. faba* and pea (Symons et al. 2002, Fukuta et al. 2004) do not exhibit the de-etiolation phenotype observed in similar mutants of Arabidopsis, tomato, and rice (Chory et al. 1991, Szekeres et al. 1996, Koka et al. 2000, Mori et al. 2002). The idea of family-specific hormonal responses is also supported by the discovery that the BR receptor, BRI1, is also the receptor for systemin, a peptide hormone involved in wound signaling that has only been found in Solanaceaeous species (Szekeres 2003, Wang and He 2004). Alternatively, the developmental status of the plant may also affect hormonal responsiveness (Bradford and Trewavas 1994, Amzallag 2002). As the *sax1* mutant is a dwarf, the functional developmental stage of the *sax1* leaves may differ from that of the fully expanded wild-type *V. faba* leaves used in the present study.

BL affected stomatal apertures relatively quickly, within 1 h (Figs 1–4), and a decrease in K^+_{in} currents of BL-treated guard cell protoplasts occurred within 15 min (Figs 5 and 6) or less (data not shown). The rapid timeframe of these effects may imply a change in signal transduction as opposed to changes in gene expression. There are 'non-genomic' mechanisms of steroid action proposed for animal cells, whereby perception of the steroid at the plasma membrane initiates changes in signal transduction that can yield a response within minutes (Lösel and Wehling 2003, Hewitt et al. 2005). However, at present, we also cannot exclude the possibility that BL induced rapid changes in gene expression in guard cells, as has been observed, for example, for ABA-induction of dehydrin gene expression (Li et al. 2002).

In this study, BL affected the physiology of guard cells; it may also play a role in their development. Some BR-related *Arabidopsis* mutants with defects in light or hormone signaling exhibit alterations in stomatal patterning and development. Clustering of stomata has been noted for *ucu2* mutants (similar to *ultracurvata 1*, a BR-signaling mutant). UCU2 encodes an FK506-binding protein, and the *ucu2* mutation results in decreased BL sensitivity in inhibition of primary root growth, leaf epinasty, and clustered stomata on abaxial leaf surfaces (Pérez-Pérez et al. 2004). The BR-biosynthetic mutant *bul1/dwf 7 has* five to six times more leaf stomata as compared with wild-type. Although there is an increased density of stomata, they are also smaller on average (Catterou et al. 2001). The mechanisms underlying these phenotypes remain unknown. Future work should address the role of BL in stomatal development as well as guard cell physiology.

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