Abaxial and adaxial stomatal density, stomatal conductances and water status of bean primary leaves as affected by paclobutrazol

I. TARI

Department of Plant Physiology, University of Szeged, POBox 654, H-6701 Szeged, Hungary

Abstract

The plant growth retardant, paclobutrazol at 8.5 or 17.0 μM concentrations effectively inhibited the stem elongation and primary leaf expansion of bean seedlings. Although the retardant reduced the relative water content in well-watered plants, the water and pressure potentials remained high in the primary leaves. K⁺, Na⁺, Mg²⁺ and Ca²⁺ contents in the primary leaves of the paclobutrazol-treated plants were not significantly different from those in the control. The stomatal density increased on both surfaces but the length of guard cells was not reduced significantly on the adaxial epidermes of the paclobutrazol-treated primary leaves. The inhibitory effect of paclobutrazol on the abaxial stomatal conductances became more pronounced with time during the light period but the adaxial surfaces displayed similar or slightly higher conductances than those of the control. The transpiration rate on a unit area basis did not change significantly or increased in the treated leaves thus the reduced water loss of paclobutrazol-treated plants was due to the reduced leaf area. Stomatal conductances of the adaxial surfaces responded more intensively to exogenous abscisic acid and the total leaf conductance decreased faster with increasing ABA concentration in the control than in the paclobutrazol-treated leaves. Paclobutrazol, an effective inhibitor of phytosterol biosynthesis, not only amplified the stomatal differentiation but increased the differences between the adaxial and abaxial stomatal conductances of the primary leaves.

Additional key words: abscisic acid, abaxial/adaxial surface, Phaseolus vulgaris L., primary leaf, stomatal index, stomatal size, water relations.

Introduction

The fitness of plants under water-limited conditions can be improved by the application of triazole growth retardants which are effective inhibitors of gibberellic acid and sterol biosynthesis (Grossmann 1990).

Paclobutrazol (PB) [(2RS, 3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1, 2, 4-triazol-1-yl)pentan-3-ol] decreased the water loss of apple seedlings under water-stressed (Asamoah and Atkinson 1985) or non-stressed conditions (Steffens et al. 1983) and induced drought resistance in jack pine (Marschall et al. 1991). White spruce seedlings maintained relatively high pressure potential in the tissues of needles and withstood prolonged drought after PB treatment by changing the elastic properties of the cell walls (Marschall and Dumbroff 1999).

The reduction of the leaf area by growth retardants often results in an increased stomatal density (Yim et al. 1997) and the size or density of the stomata may vary over the surface of the leaf. Stomatal heterogeneity is manifested in different average conductances in different regions of the leaf (Terashima et al. 1988, Pospíšilová and Šantrůček 1994, Lawson et al. 1998a). In combination with hydraulic mechanisms, spatial changes in the concentrations of different diffusible signals such as IAA, ABA, potassium ion or internal CO₂ concentration (c_i), determine the steady-state stomatal aperture (Lawson et al. 1998b, Mott and Buckley 1998). Although it is commonly accepted, that stomatal conductance (g_s) is largely linearly related to stomatal aperture, it has recently

Received 12 August 2002, accepted 7 January 2003.

Abbreviations: ABA - abscisic acid; E - transpiration rate; g_s - stomatal conductance; IAA - indole-3-acetic acid; PB - paclobutrazol; ψ_s - pressure potential; ψ_o - osmotic potential; ψ_w - water potential.

Acknowledgements: The author would like to thank Anna Novák for excellent technical assistance. This work was supported partially by a grant from the Hungarian National Scientific Foundation and by Ministry of Education (OTKA T/038392; FKFP 1P057).

Fax: (+36) 62 544307, e-mail: tari@bio.u-szeged.hu

215
been demonstrated, that the steepest increase of gs occurred at small degrees of stomatal opening in *Sambucus* leaves (Kaiser and Kappen 2001). Moreover, the stomatal conductances may be different for the two surfaces (Mott et al. 1993).

External factors and plant hormones generally affect not only the function, but also the differentiation of the stomata. The theory of Bünning (1956) concerning the stomatal differentiation of dicotyledonous plants postulates that the existing stomata inhibit the differentiation of new ones, but stomatal differentiation may also be determined by long-distance signals originating from the mature leaves (Brownlee 2001). There are stomatal density mutants in *Arabidopsis* which display a highly increased stomatal density and have a mutation in a gene of a putative subtilisin-like protease converting an inactive peptide signals into an active form (Berger and Altmann 2000). The differentiation of stomata depends on the external CO2 concentration, the stomatal density being lower in plants that grow in high concentrations of CO2 (Woodward and Kelly 1995). Exposure of *Vigna sinensis* L. plants from shade to bright light for 1 d also increased the stomatal indices on the upper epidermis of the third trifoliate leaves (Schoch et al. 1980).

In contrast, water stress and abscisic acid (ABA) reduced the stomatal index, increased the stomatal density and decreased the stomatal conductance on the adaxial surface of spring wheat leaves (Quarrie and Jones 1977). Similar results have been found for stomatal conductances after short-term ABA treatment in tobacco plantlets acclimatizing to ex vivo conditions (Pospíšilová 1996, Pospíšilová et al. 1998). Cytokinins have an opposite effect: natural and synthetic cytokinins have been shown to promote stomatal opening (Jewer and Incoll 1980). Indoleacetic acid (IAA) and synthetic auxins increased the stomatal apertures and acted antagonistically to ABA (Dunleavy and Ladley 1995). IAA was remarkably effective in producing wide adaxial apertures and eliminated the normal disparity in the stomatal opening between abaxial and adaxial stomata (Pemadasa 1982a), and the responses of adaxial and abaxial stomata to light, particularly in the blue region, also differed in the epidermal peels of *Commelina* (Pemadasa 1982b).

Plant growth retardants modify the concentrations of different hormones, including those which are effective in the regulation of stomatal movement. In short-term experiments, the ABA content exhibited a transient increase followed by a decline in oilseed rape after triazole treatment (Häuser et al. 1990). According to Asare-Boamah et al. (1986), triadimefon protected bean plants from water stress by increasing the concentration of ABA. In contrast, reduced ABA contents have been found after PB treatment in non-stressed or water stressed apple (Wang et al. 1987) and wheat seedlings (Buta and Spaulding 1991).

The concentration of IAA in the primary leaves of bean was elevated by PB (Nagy et al. 1991) and the cytokinin contents in different parts of soybean seedlings were also increased by triazole (LAB 150987) treatment (Grossmann et al. 1987). Thus, the changes in the endogenous hormone concentrations favour the stomatal opening in PB-treated plants.

The present paper describes the effects of PB on the stomatal development of bean primary leaves with respect to the differences between the abaxial and adaxial surfaces, and examines the effects of changes in the stomatal frequency and water status on the stomatal conductance and transpiration rate of bean primary leaves. We were also interested in the sensitivity of the stomatal conductance to exogenous abscisic acid in PB-treated plants.

### Material and methods

*Seeds of Phaseolus vulgaris* L. cv. Juliska were immersed in 8.5 or 17.0 μM PB solution for 2 h and then germinated in a thermostat at 25 °C. After 48 h the seeds were sown in garden mould and the plants were grown in a chamber (*Controlled Environments*, Winnipeg, Canada) under a 14-h photoperiod, 25/20 °C day/night temperatures, and 65 % relative humidity. The plants were watered daily by sub-irrigation. Measurements were made on the fully expanded primary leaves of 14-d-old seedlings. The experiments were repeated twice. The stomatal index was defined as the number of stomatal complexes per 100 epidermal cells.

The epidermal morphology of the leaves of 15-d-old plants was examined by using polystyrene replicas of both leaf sides (Quarrie and Jones 1977). The leaf surfaces were divided into three regions at equal distances on the main vein: the apical, middle and basal regions. The leaf imprints were examined under a light microscope, and the cell dimensions and stomatal indices were recorded for 9 different primary leaves. The peristomal groove distance was defined and measured after Lawson et al. (1998b). One experiment was conducted with epidermal peels, which did not reveal significant differences from the data for the imprints.

Stomatal conductance was determined in the middle part of the abaxial and adaxial sides of fully expanded primary leaves at an irradiance of 180 μmol m⁻² s⁻¹ with a diffusion porometer (*Model VP-2000*, *Cayuga Development Research Instruments*, New York, USA). The water potential of primary leaves (∂ψw) was measured.
in a pressure chamber (PMS Instrument Co., Corvallis, USA). The osmotic potential ($\psi_o$) of the expressed sap was determined with an osmometer via determination of the freezing point depression. The pressure potential ($\psi_p$) was calculated from the difference between $\psi_o$ and $\psi_t$. The appropriate concentrations of (±)-ABA were dissolved in 0.05 % Tween 20 solution and sprayed onto both surfaces of the primary leaves. The control leaves were treated with 0.05 % Tween 20. Stomatal conductances were measured 24 h after ABA treatment.

Whole leaf transpiration was determined gravimetrically (Schubert et al. 1991). The detached leaves were allowed to dry adaxial side up in the growth chamber in which the plants were growing and their mass was determined for 10 min at 1-min and for the next 3 h at 30-min intervals. The transpiration rate ($E$) was calculated from the initial slope of the water loss curves recorded immediately after leaf excision. Leaf areas were also measured at the harvest with a digital planimeter (Tamaya Technics Inc., Tokyo, Japan).

Concentrations of K\(^+\), Na\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\) were determined after wet digestion of 100 mg dried plant material in a mixture of nitric acid and 30 % H\(_2\)O\(_2\) (5:4; v/v) at 200 °C for at least 3 h. The metal contents of the primary leaves were measured with a Hitachi Z-8200 Zeeman polarized atomic absorption spectrophotometer (Hitachi Ltd., Tokyo, Japan).

### Results and discussion

PB has proved to be very effective in the reduction of leaf expansion in a number of species, including bean (Tari and Nagy 1994). A significant reduction in the growth rate occurred in the linear phase of the expansion of primary leaves, the leaf area was 35 and 55 % lower in plants treated with 8.5 and 17.0 μM PB, respectively. Differentiation of the stomata took place mainly during the exponential phase of primary leaf growth and further

<table>
<thead>
<tr>
<th>PB [μM]</th>
<th>Stomatal density [mm(^{-2})]</th>
<th>Stomatal index [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>adaxial</td>
<td>abaxial</td>
</tr>
<tr>
<td>0</td>
<td>47.4 ± 6.6</td>
<td>207.1 ± 19.0</td>
</tr>
<tr>
<td></td>
<td>middle</td>
<td>40.4 ± 8.8</td>
</tr>
<tr>
<td></td>
<td>base</td>
<td>39.5 ± 6.6</td>
</tr>
<tr>
<td>8.5</td>
<td>apex</td>
<td>166.7 ± 36.6</td>
</tr>
<tr>
<td></td>
<td>middle</td>
<td>83.7 ± 9.1</td>
</tr>
<tr>
<td></td>
<td>base</td>
<td>101.8 ± 9.1</td>
</tr>
<tr>
<td>17.0</td>
<td>apex</td>
<td>165.8 ± 9.2</td>
</tr>
<tr>
<td></td>
<td>middle</td>
<td>88.6 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>base</td>
<td>98.3 ± 14.1</td>
</tr>
</tbody>
</table>

Table 1. Stomatal densities, abaxial/adaxial ratios and stomatal indices in different zones of the two surfaces of control and PB-treated primary leaves in 15-d-old bean plants. Means ± SE (3 x 3 leaves from 3 pots, 10 microscopic areas per leaf).

<table>
<thead>
<tr>
<th>PB [μM]</th>
<th>Guard cell length [μm]</th>
<th>Peristomatal groove distance [μm]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>adaxial</td>
<td>abaxial</td>
</tr>
<tr>
<td>0</td>
<td>26.0 ± 1.2</td>
<td>24.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>middle</td>
<td>28.4 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>base</td>
<td>29.5 ± 0.3</td>
</tr>
<tr>
<td>8.5</td>
<td>apex</td>
<td>26.7 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>middle</td>
<td>29.5 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>base</td>
<td>29.0 ± 0.8</td>
</tr>
<tr>
<td>17.0</td>
<td>apex</td>
<td>26.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>middle</td>
<td>28.1 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>base</td>
<td>29.0 ± 0.6</td>
</tr>
</tbody>
</table>

Table 2. Effect of PB on the length of guard cells and on the peristomatal groove distance in different zones of the adaxial and abaxial epidermes of primary leaves in 15-d-old bean plants. Stomatal characteristics were determined on the imprints prepared 2 h after the beginning of the light period. Means ± SE (3 x 3 leaves from 3 pots, 10 microscopic areas per leaf).
expansion decreased the stomatal density (unpublished results).

In control plants stomatal density of primary leaves decreased from the apical to the basal region of both the adaxial and the abaxial surfaces (Table 1). The stomatal frequency was highly increased by PB and the differences between the leaf regions were more significant than in the control. The differentiation of the stomatal complexes was also modified. PB increased the stomatal indices, the percentage of guard cell pairs among the epidermal cells on the apical and middle parts of both surfaces, which suggested, that in accordance with Bünning's theory, the size of the inhibition field around the existing stomatal complex might have been reduced. The length of the stomata on the adaxial surface did not change significantly after PB treatment (Table 2), although other stress factors, such as water stress or ABA (Quarrie and Jones 1977), which reduced the expansion of the leaves, were also known to reduce the stomatal size. The decrease in the peristomal groove distance, which had been introduced as a surrogate measure for stomatal aperture in bean primary leaves (Lawson et al. 1998b), was obvious only on the abaxial surface at 17.0 μM PB treatment. This suggests that the control of gs by pore area reduction is substantially higher in abaxial than in adaxial stomata in retardant-treated leaves.

High concentrations of triazole retardants such as PB inhibit not only the biosynthesis of gibberellins, but also that of plant sterols, e.g. campesterol (Grossmann 1990). Theoretically, these inhibitors should reduce the concentrations of brassinosteroids but the decrease in the accumulation of common phytosterols also plays an important role in the regulation of membrane fluidity, permeability and other membrane-coupled processes. It was found, that the cpd (constitutive photomorphogenesis and dwarfism) mutant of Arabidopsis deficient in the biosynthesis of brassinosteroids, exhibited an enhanced stomatal differentiation on the hypocotyl, cotyledon and leaf epidermis (Molnár 2001). PB induced similar changes in stomatal differentiation to those of brassinosteroid-deficient mutant plants in the bean primary leaves. Moreover, PB induced similar changes in the distribution of IAA, ethylene production, ethylene precursor 1-aminoacyclopropane-1-carboxylic acid between apical and basal parts of bean hypocotyls, as light and increased the IAA content in the leaf blades in our system (Nagy et al. 1991, Tari and Mihalik 1998). These hormonal changes contribute to the control of cell cycle and differentiation of epidermal cells complementing the primary effects of the retardant.

Table 3. The effects of PB on the relative water content (RWC), water potential (ψw), osmotic potential (ψs), calculated leaf pressure potential (ψp) and transpiration rate (E) in primary leaves of bean. The measurements were made 2 h after the beginning of the light period. Values are means ± SE. n = 18. Means denoted by * or *** are significantly different from the control at P ≤ 0.05 or 0.001 levels, respectively, as determined by Student’s t-test.

<table>
<thead>
<tr>
<th>PB [μM]</th>
<th>RWC [%]</th>
<th>ψw [MPa]</th>
<th>ψs [MPa]</th>
<th>ψp [MPa]</th>
<th>E [mmol leaf⁻¹ min⁻¹]</th>
<th>E [mmol m⁻² s⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90.0 ± 1.1</td>
<td>-0.55 ± 0.10</td>
<td>-0.67 ± 0.04</td>
<td>0.122</td>
<td>0.24 ± 0.01</td>
<td>0.68 ± 0.05</td>
</tr>
<tr>
<td>8.5</td>
<td>87.9 ± 1.5</td>
<td>-0.40 ± 0.06</td>
<td>-0.56 ± 0.03*</td>
<td>0.156</td>
<td>0.11 ± 0.02***</td>
<td>0.85 ± 0.30</td>
</tr>
<tr>
<td>17.0</td>
<td>84.1 ± 0.6***</td>
<td>-0.36 ± 0.05</td>
<td>-0.58 ± 0.05</td>
<td>0.224</td>
<td>0.14 ± 0.02***</td>
<td>1.05 ± 0.05***</td>
</tr>
</tbody>
</table>

Table 4. Ion contents [μmol g⁻¹ (d.m.)] in primary leaves of 15-d-old bean plants after PB treatment. Means ± SE. n = 3. Means were not significantly different from the control as determined by Student’s t-test.

<table>
<thead>
<tr>
<th>PB [μM]</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>Na⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>593.0 ± 38.7</td>
<td>607.7 ± 16.2</td>
<td>312.3 ± 62.0</td>
<td>36.1 ± 2.0</td>
</tr>
<tr>
<td>8.5</td>
<td>689.7 ± 41.5</td>
<td>611.0 ± 20.0</td>
<td>275.7 ± 96.7</td>
<td>31.2 ± 0.4</td>
</tr>
<tr>
<td>17.0</td>
<td>651.7 ± 19.5</td>
<td>588.0 ± 50.6</td>
<td>184.7 ± 7.2</td>
<td>30.9 ± 1.0</td>
</tr>
</tbody>
</table>

The water status of tissues may determine the stomatal conductance and transpiration rate of the leaf. In well-watered 15-d-old plants, PB treatment resulted in constant but not necessarily significant increases in water, osmotic and pressure potential (Table 3). The tissue concentrations of those macroelements which play a role in some way or another in the regulation of stomatal movement (K⁺, Ca²⁺, Mg²⁺ and Na⁺) did not change significantly in the treated leaves (Table 4), although the Mg²⁺ content was lower in the treated tissues.

The stomatal conductance of bean primary leaves exhibits a daily pattern (Davies and Van Volkenburg 1983): the stomata open at the beginning and close gradually during the last third of the light period. PB treatments affected gs on adaxial and abaxial surfaces differently. Depending on the phase of the light period, the retardant reduced gs on the abaxial surface, but on the adaxial surface gs remained similar to that of the control or increased slightly as a function of time (Fig. 1). The observed changes in gs were not directly related with the changes in stomatal frequency. The two surfaces responded also differently to exogenous ABA. The
adaxial \( g_a \) was less sensitive than the abaxial one to exogenous ABA in treated plants, and as a consequence the total \( g_a \) was not affected or was even slightly higher in the leaves treated with 17.0 \( \mu M \) PB at a wide ABA concentration range (Fig. 2).

It is known that a highly sensitive relationship exists between guard cells and their environment. The sensitivity of stomata on the abaxial and adaxial surfaces are different to auxin (Pemadasa 1982a). The guard cells respond to IAA by opening in the presence of appropriate K\(^+\) concentration which may vary in different parts of the leaves. ABA counteracts the effect of IAA on stomatal opening in a concentration dependent manner (Dunlavy and Ladley 1995). We found higher IAA contents in the blades of PB-treated leaves which may interfere with the effects of exogenously applied ABA. The guard cells respond to the concentration changes of ABA in the apoplast, but depending on the apoplastic pH, ABA can be readily trapped in the cytoplasm or in the chloroplasts of the surrounding cells (Hartung and Slovik 1991) which decreases the effect of ABA on stomatal closure. This process is highly enhanced by light when the light reactions of photosynthesis increase the pH of the stroma. The sequestration of the hormone among cell compartments may be changed by the retardant and the capacity of mesophyll or epidermal cells to metabolize ABA can also modify the amount of the hormone arriving to guard cell plasma membranes in PB-treated plants.

As calculated from the slope of the water loss curves immediately after excision, there was a significant increase in the transpiration rate on a unit area basis at 17.0 \( \mu M \) PB concentration. The effect could be attributed to the extremely high stomatal frequencies, but the water loss of whole primary leaves decreased significantly in PB-treated plants (Table 3). This suggests that, under well-watered conditions, the effect of PB on the transpiration rate is a very complex phenomenon comprising the actual hormonal status, water potential and stomatal density changes of leaves. The retardant enhanced the difference between the stomatal frequencies and between conductances of the two leaf surfaces but the higher stomatal density was not necessarily coupled with higher \( g_a \) on the abaxial surface. Moreover, the heterogeneity of various stomatal characteristics on the same leaf surface results in fluctuations in \( g_a \), thus the amount of transpired water is primarily regulated by the reduction of leaf area in PB-treated leaves, as a result plants able to limit water loss.

Fig. 1. Effects of 8.5 or 17.0 \( \mu M \) paclobutrazol treatment on adaxial and abaxial stomatal conductances of 15-d-old bean primary leaves in the light period (Means \( \pm \) SE for 3 pots with 10 plants in each). Means denoted by * and ** are significantly different from the appropriate control at \( P \leq 0.05 \) and 0.01 as determined by Student’s \( t \)-test.

Fig. 2. Effects of 0.1 - 1000 \( \mu M \) abscisic acid on adaxial and total stomatal conductances in bean primary leaves treated with 8.5 or 17.0 \( \mu M \) paclobutrazol. Stomatal conductances were measured two hours after the beginning of the light period. Means \( \pm \) SE (for 3 pots with 10 plants in each). Means denoted by *, ** and *** are significantly different from the untreated control at \( P \leq 0.05 \), 0.01 and 0.001 as determined by Student’s \( t \)-test.

References

Asamoah, T.E.O., Atkinson, D.: The effects of (2RS, 3RS)-1-(4-chlorophenyl) 4, 4-dimethyl-2-(1H-1, 2, 4 triazol-1-yl) pentan-3-ol (paclobutrazol;PP333) and root pruning on the growth, water use and response to drought of Colt Cherry rootstocks. - Plant Growth Regul. 3: 37-45, 1985.


