Annals of Botany **80**: 485–489, 1997

Jasmonates Induce Intracellular Alkalinization and Closure of *Paphiopedilum* Guard Cells

C. A. GEHRING*†, H. R. IRVING‡, R. McCONCHIE*§ and R. W. PARISH*‖

* *School of Botany, La Trobe University, Bundoora, Melbourne, Victoria 3083 and* ‡ *Department of Pharmaceutical Biology and Pharmacology, Victorian College of Pharmacy, Monash University, 381 Royal Parade, Parkville, Melbourne, Victoria 3052, Australia*

Received: 14 February 1997 Accepted: 30 May 1997

Jasmonates (jasmonic acid or methyl jasmonate) promote stomatal closure in *Paphiopedilum Supersuk* (RHS, 1973) and *P. tonsum* (Rchb.f) Stein. Studies on guard cells loaded with pH dependent fluorescent dyes show that jasmonates cause intracellular alkalinization of up to 0·5 pH units within 5 to 15 min. Jasmonate-induced alkalinization always preceded stomatal closure and where alkalinization was not detected no closure occurred. Propionic acid inhibited jasmonate-induced stomatal closure, suggesting that jasmonate-induced intracellular alkalinization is involved in guard cell movements.

Key words: BCECF, confocal microscopy, cytosolic pH, guard cells, jasmonic acid, methyl jasmonate, *Paphiopedilum Supersuk* (R. H. S.), Snarf-1, stomatal movements.

INTRODUCTION

Jasmonates, like abscisic acid (ABA), induce a number of physiological responses ranging from stomatal closure to growth inhibition and promotion of senescence related events. This has led to suggestions that these two hormones act alike and have similar functions in plant growth and development, particularly in response to stresses such as desiccation (Sembdner and Parthier, 1993). Methyl jasmonate (JA-Me) has been reported to cause stomatal closure in oats (Satler and Thimann, 1981) but not barley (Horton, 1991). Stomatal opening is inhibited by JA-Me in *Commelina benghalensis* (Raghavendra and Reddy, 1987) and olive leaves (Sanz *et al*., 1993).

Closure of stomatal pores is associated with an efflux of K^+ and anions from the guard cells to reduce their turgor (Ward, Pei and Schroeder, 1995). In response to ABA, outward K^+ channels are activated and inward K^+ channels are inactivated in guard cells (Blatt, 1990). These responses to ABA are preceded by increases in cytosolic pH (Irving, Gehring and Parish, 1992; Blatt and Armstrong, 1993).

The stomata of *Paphiopedilum* open to produce a pore a few microns wide (Rutter and Willmer, 1979) in response to blue light (Zeiger, Assmann and Meidner, 1983), auxin, kinetin, fusicoccin and acetic acid (Irving *et al*., 1992). Closure can be induced by ABA (Mayo and Ehret, 1980; Irving *et al*., 1992) showing that control mechanisms similar to other species operate in the achlorophyllous guard cells of *Paphiopedilum*. For these reasons, we used *Paphiopedilum* guard cells to monitor intracellular pH changes with fluorescent indicator dyes by confocal microscopy. We demonstrated that jasmonates increase the intracellular pH of *Paphiopedilum* guard cells and that this increase preceded stomatal closure.

MATERIALS AND METHODS

Measurement of stomatal movements

Epidermal strips from *Paphiopedilum Supersuk* (R. H. S. hybrid; 1973) or *Paphiopedilum tonsum* (Rchb.f.) Stein var *curtisfolium* were peeled from the abaxial surface of young leaves as previously described (Irving *et al*., 1992). Over 70% of the stomata on freshly peeled strips were closed. To promote stomatal opening, epidermal strips were submerged for at least 1 h at 20–25 °C in 'opening buffer' (10 mM Pipes (Sigma), 50 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$ and 100 μM $CaCl_2$, pH 6·3) under blue light ($\lambda = 430$ nm at 35 Wm^{-2}) and aerated with house air passed through a column of self-indicating soda lime ('Carbosorb', BDH). The pore width of stomata was assessed for several separate epidermal strips per treatment. Stomata with a pore width greater than 1·5–2 μm were considered open in this study.

Intracellular pH measurements

Blue light pre-treated epidermal strips were incubated in opening buffer containing acetoxymethyl esters of either carboxy-semi-naphthorhodafluor-1 (Snarf-1) or 2′,7′-bis(2-carboxyethyl)-5-(and-6) carboxyfluorescein (BCECF) at 20 and 10 μM respectively, for 30 min at 20–25 °C in the dark (Irving *et al*., 1992). Loading was terminated by extensively rinsing the epidermal strips in fresh opening buffer to remove extracellular dye in the cleaved or acetoxymethyl

‖ For correspondence.

† Present address: School of Biological and Chemical Sciences, Deakin University, Geelong, Victoria 3217 Australia and § School of Environmental Biology & Horticulture, University of Technology Sydney, Gore Hill campus, Sydney NSW 2065 Australia.

0305-7364/97/100485+05 $25.00/0 bo970471

ester form. Images were acquired and processed with a CLSM-FLUOVERT System with an inverted confocal microscope (Leica Lasertechnik Gmbh, Heidelberg, Germany) as previously detailed (Gehring, Irving and Parish, 1994). Intracellular calibration of Snarf-1 pH dependent fluorescence in guard cells is difficult. In the most successful strategy, an epidermal strip was incubated in buffer in the presence of 10 μM nigericin and 50 mM KCl for 10 min before collecting emission signals; the strip was then transferred to the next buffer for 2 min before viewing a guard cell pair until the emission ratio was stable; this was repeated with the next buffer (see inset Fig. 2A). Single excitation measurements with BCECF are detailed in Irving *et al.* (1992). In each experiment using either Snarf-1 or BCECF loaded epidermal strips, five separate small regions (approx. 2·5 μm^2) within the cytoplasm were selected from each guard cell pair. The areas were selected only if the signal was homogenous within the region and thus likely to be cytoplasmic. The signal from these separate regions was collected at each time-point of the experiment for analysis of cytosolic pH changes. Vacuolar, oil body, nuclear and other distinctly non-cytosolic regions were excluded from this analysis.

RESULTS AND DISCUSSION

Paphiopedilum spp. have very thick guard cells and only open their stomatal pores to a small degree (Rutter and Willmer, 1979; Fig. 1A). In this study, stomata with a pore width greater than 1·5 to 2 μM were considered open (Fig. 1A). When the stomatal pores were opened in response to blue light, closure was induced with either 10 nM or 100 nM jasmonic acid (JA; Table 1, Fig. 1B). In a separate experiment, stomata also closed in response to JA-Me at 40 nM (Table 1) or 100 μM ABA (Table 1; Irving *et al.*, 1992). The minimum concentration of JA required to close the stomata was 10 nM as concentrations of 1 nM were without effect (Table 1).

Closure of stomata is preceded by alkalinization (Irving *et al.*, 1992; Blatt and Armstrong, 1993) which directly activates the outward K^+ channel in *Vicia* guard cells and this is inhibited by 'clamping' the cytosolic pH in the presence of butyrate (Blatt and Armstrong, 1993). Weak acids permeate the cell in the undissociated form and thereby 'clamp' the cytosolic pH as they reach equilibrium with the dissociated form. Previously, we have shown that *Paphiopedilum* stomata open in the presence of 5 mM acetate (Irving *et al.*, 1992) which corresponds to approximately 0·2 mM acetic acid (using the Henderson-Hasselbalch equation). Propionate (approx. 0·4 mM propionic acid present) induced stomatal opening and overrode JA-Me mediated closure (Table 1). The acid load achieved with propionate in these experiments clamped the cytosolic pH between 6·9 and 7·0 (data not shown). This was similar to the clamping observed by Blatt and Armstrong (1993) in *Vicia* where cytosolic pH was maintained at pH 7·0 using 10 mM butyrate. Under such conditions, ABA could not activate the outward K^+ channel. Since JA-Me was ineffective at inducing closure in the presence of propionate, the hormone may resemble ABA by inducing increases in intracellular pH that represent a component of the signal(s) causing stomatal closure.

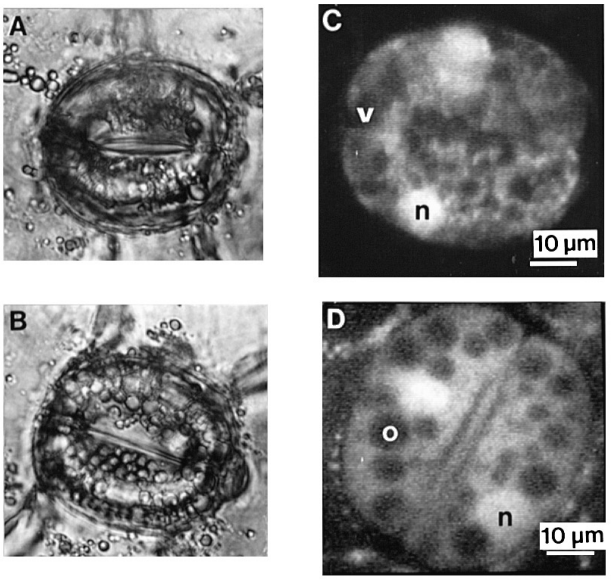


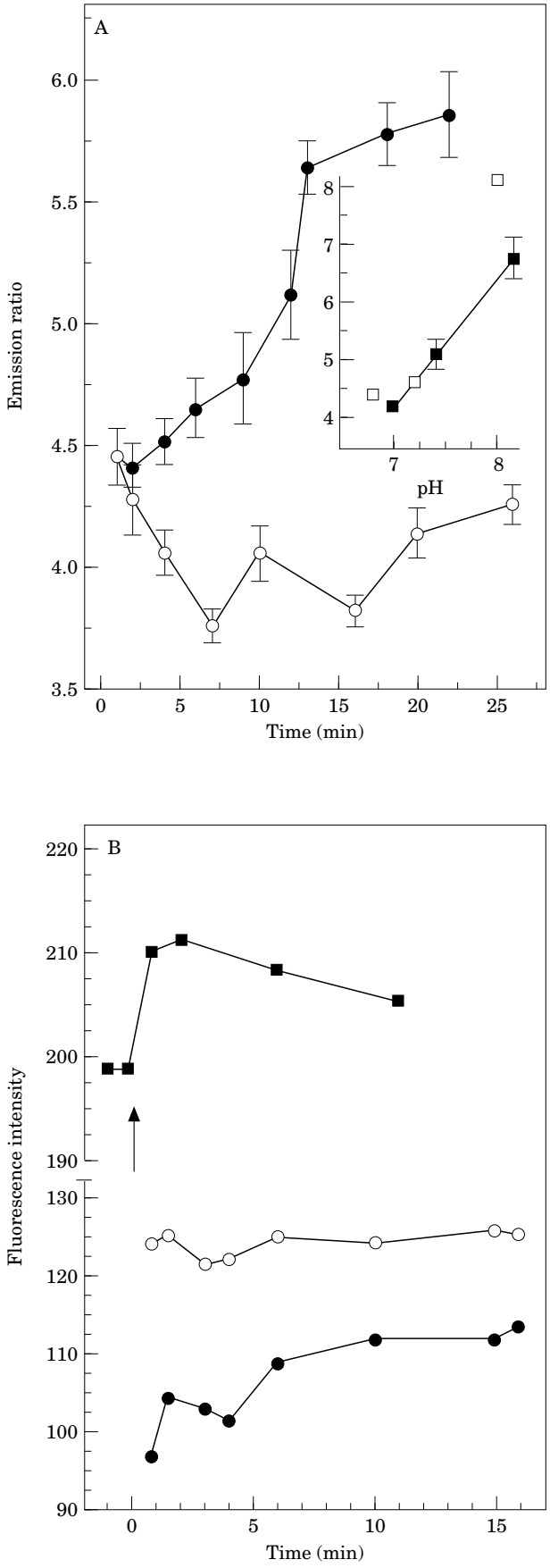
FIG. 1. Intracellular location of fluorescent dyes in *Paphiopedilum* guard cells. Light micrographs of stomata of *Paphiopedilum Supersuk* taken with Nomarski optics to demonstrate the range of opening and closure observed under our experimental conditions (A and B). Typical open stoma with a pore width of approx. 3 μm (A) and a typical closed stoma with a pore width of 0·5 μm (B). Fluorescence image of a *P. tonsum* stoma loaded with Snarf-1 (C). The segment was viewed (objective: PL fluotar 40/1·3 oil), excited at 514 nm and emission images collected above 610 nm to demonstrate dye distribution following 30 min loading. Fluorescence image of a *P. tonsum* stoma loaded with BCECF (D). The segment was viewed (objective: PL fluotar 10/0·3), excited at 488 nm and images collected to demonstrate dye distribution following 30 min loading. Bars = 10 μm and increased brightness corresponds to increased fluorescence intensity (C and D); e, epidermal cell; n, nucleus; o, oil droplet; v, part of vacuole.

TABLE 1. *Effect of jasmonates on closure of stomatal pores in abaxial epidermal strips of* P. Supersuk

Experiment	Treatment	% Closed stomata
1	Control	$32·2 \pm 1·1^a$
	1 nM JA	$37·8 \pm 2·2^a$
	10 nM JA	$65·5 \pm 4·8^b$
	100 nM JA	$75·5 \pm 2·9^b$
2	Control	$44·4 \pm 8·4^a$
	40 nM JA-Me	$74·4 \pm 5·4^b$
	100 μM ABA	$81·7 \pm 6·5^b$
3	Control	$43·3 \pm 0·05^b$
	10 mM propionate	$24·4 \pm 0·07^a$
	40 nM JA-Me	$66·7 \pm 0·04^c$
	JA-Me + propionate	$25·5 \pm 0·04^a$

Results are expressed as mean ± s.e. Values followed by a different superscript are significantly different ($P < 0·05$; Dunnett's test).

Two pH dependent fluorescent dyes, Snarf-1 and BCECF, were loaded in the acetoxymethyl form that is hydrolysed to release active dye by endogenous esterases. Such loading has proved successful in cell suspension cultures of *Asparagus*, *Catharanthus* and *Gossypium* (Sakano, Yazaki and Mimura, 1992; Roos, 1992; Crawford *et al.*, 1994) and



Paphiopedilum epidermal strips (Irving *et al.*, 1992). Typical fluorescence images of guard cells loaded with either Snarf-1 (Fig. 1C) or BCECF (Fig. 1D) have distinct non-fluorescing spherical bodies that correspond to the oil droplets present in guard cells (Fig. 1A and B). Such oil droplets were not an atypical feature of guard cells although *Paphiopedilum* spp. do contain a large number (Rutter and Willmer, 1979) which appear to be generally smaller in *P. Supersuk* than *P. tonsum* (cf. Fig. 1B and D). The thickness of the guard cells and number of oil bodies present, made resolution of other organelles difficult but allowed the epidermal cells to be mainly out of the confocal plane (Fig. 1C and D). Occasionally some fluorescence of the epidermal cells was observed which was brighter towards the outer regions of the cell (Fig. 1D). The vacuole of *Paphiopedilum* guard cells was relatively small when compared to those in other cell types (Rutter and Willmer, 1979; Fig. 1C). Since vacuolar pH is typically below 6 (Roos, 1992) the vacuole fluoresces little in BCECF loaded tissue (Fig. 1D) although some dye may accumulate in the vacuole as up to 10% of BCECF loaded in *Catharanthus* cells collected in vacuoles over 2 h (Sakano *et al.*, 1992). The regions of punctate fluorescence could be due to dye sequestration in small organelles such as endoplasmic reticulum or mitochondria (Fig. 1C and D). The nucleus fluoresced highly with both dyes (Fig. 1C and D) and this region was not used in assessing changes in cytosolic pH. Whether this reflects dye accumulation in the nucleus or in endoplasmic reticulum surrounding the nucleus, attachment of the dye to the membranes of these organelles, or an elevated pH of the compartment is unclear. However, all regions of highly punctate fluorescence were excluded and only homogenous areas representing the cytoplasm were selected for analysis of pH changes.

Guard cells of open stomata in epidermal strips from *Paphiopedilum* (loaded with Snarf-1) were imaged and their response to JA-Me monitored over at least a 15 min period ($n = 12$). We observed three types of response to JA-Me ranging from a slight increase or no change ($n = 5$) to a steady alkalinization of 0·4 pH units over 10 min ($n = 4$; Fig. 2A) to a relatively rapid initial alkalinization followed by a slower increase of approx. 0·2–0·4 pH units to eventually reach a plateau ($n = 3$). To confirm these results with Snarf-1 loaded guard cells, we also loaded strips containing open stomata with BCECF and treated these cells with JA-Me. We consistently observed increases in fluorescence signal within 15 min of application of either 50 nM JA-Me or 100 nM JA which corresponded to an alkalinization of approximately 0·2 pH units (six experiments, $n = 8$; Fig.

FIG. 2. Effect of methyl jasmonate on intracellular pH of *Paphiopedilum* stomata. Guard cells in epidermal strips loaded with Snarf-1 were excited at 514 nm (A). The emission ratios (mean ± s.e. of five cytoplasmic regions within stoma) for stoma from epidermal strips treated with 45 nM JA-Me (●) or buffer (○) were determined (A). The inset (A) shows the intracellular pH calibration estimation determined for guard cells (■) in epidermal strips of *Paphiopedilum* loaded with Snarf-1 and the extracellular microscopic calibration (□). Guard cells in epidermal strips loaded with BCECF were excited at 488 nm (B). Fluorescence intensity of stoma from two separate epidermal strips treated with 50 nM JA-Me (●, ■) or the control strip (○) is shown.

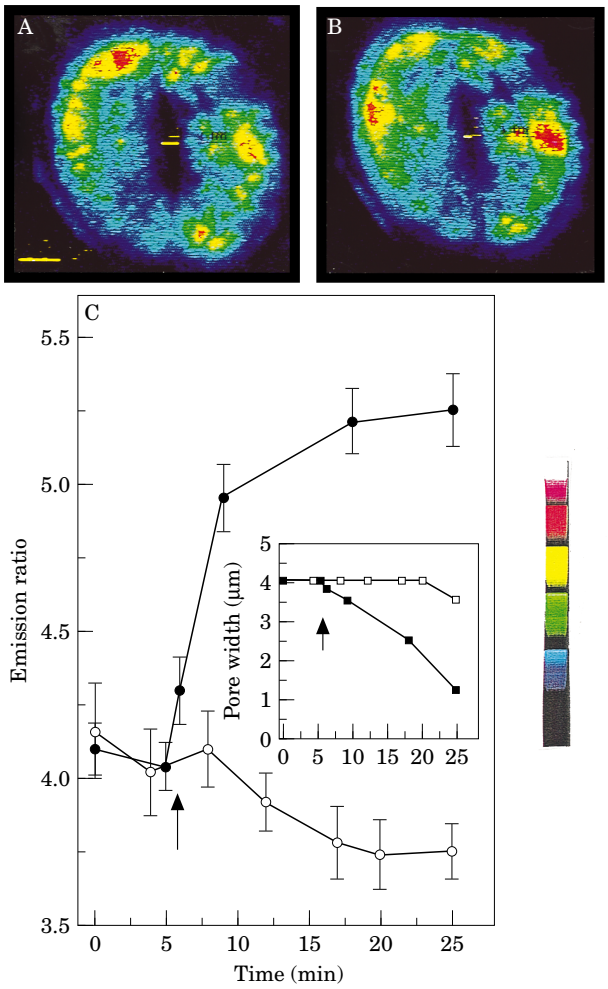


FIG. 3. Effect of methyl jasmonate on closure and intracellular pH of a *P. Supersuk* stoma. Fluorescent images (signal above 610 nm) using the relative fluorescence intensity colour scale indicated (alongside graph) of a stoma in an epidermal strip loaded with Snarf-1 were taken to show the pore region of the stoma (A and B). The stoma was viewed (objective: PL fluotar 10/0·3), excited at 514 nm and fluorescent emission images collected above 610 nm (shown) and between 555 and 580 nm. A, Fluorescent image of the open stoma showing the pore (scale 4 μm). The scale bar on the left of the stoma is 10 μm. B, Fluorescent image of the same stoma following treatment with 45 nM JA-Me for 13 min showing decreased pore aperture (scale 2 μm). C, Graphical representation of the emission ratios (mean $\pm$ s.e. of five cytoplasmic regions within the stoma) of the stoma depicted above (●) or a stoma in a control epidermal strip (○). The inset shows the pore width for the stoma treated with JA-Me (■) and the control stoma (□). Two emission ratios were collected before 45 nM JA-Me was added to the epidermal strip at 5·5 min (indicated by the arrow).

2B). Control strips, on the other hand, showed little change or a slight decrease in fluorescence over the same period (Fig. 2B). Since fluorescence changes that corresponded to increases in intracellular pH occurred with both dyes, we are confident that JA-Me does indeed induce increases in intracellular pH in *Paphiopedilum* guard cells.

To test whether dye loaded guard cells also close in response to JA-Me, opened stomata were monitored for 5 min, exposed to JA-Me (45 nM) or additional buffer (control) for the remainder of the imaging period and the degree of closure of the stoma determined ($n = 4$; Fig. 3). The pore remained open (pore approx. 3·5–4 μm wide; Fig. 3A) during the pre-treatment period and the emission ratio was constant. Following addition of JA-Me, the pore was still open within the first minute (pore 3·75 μm wide) and the fluorescence emission ratio began to rise (Fig. 3C). The rise in emission ratio continued and reached a plateau by 13 min and at this stage the pore was approx. 2·5 μm wide (Fig. 3B and inset C). The pore was closed (less than 1·5 μm wide) 20 min after JA-Me treatment (inset Fig. 3C). The changes in fluorescence intensity that occurred as the guard cell closed (cf. Fig. 3A and B) were due to the movement of the specimen although as the ratio markedly increased (Fig. 3C) this does reflect a true increase in intracellular pH of 0·4 pH units. Control cells imaged over 25 min exhibited little change in emission ratio (Fig. 3C) and the pores remained open (3–4 μm wide). In a separate experiment, the basal emission peaks were collected for seven open stomata (pore width 3 to 4 μm) in an epidermal strip which was then exposed to 50 nM JA-Me for 25 min. Of these stomata, five had closed and the emission ratio data corresponded to alkalinization of 0·2 to 0·5 pH units. The remaining two stomata neither closed discernibly, nor alkalinized (data not shown).

We have demonstrated that addition of JA-Me leads to increases in intracellular pH in stomatal guard cells of *Paphiopedilum* (Fig. 2) and that this alkalinization preceded stomatal closure (Fig. 3). The alkalinization observed in response to JA-Me (Figs 2 and 3) was similar in extent and magnitude to that observed in response to ABA in *Paphiopedilum* (Irving *et al.*, 1992) and *Vicia* (Blatt and Armstrong, 1993). Alkalinization, such as occurred in *Vicia* guard cells in response to ABA, has been shown to directly activate the outward rectifying K^+ channel that is a prerequisite of induction of closure (Blatt and Armstrong, 1993). Our results provide further support that increases in intracellular pH are an important component of the signal pathway inducing stomatal closure. However, as the operation of several channels would be involved, changes in cytosolic pH would form only one component of a complex pathway of interactions regulating these channels (see Discussion, Blatt and Armstrong, 1993; Ward *et al.*, 1995).

ACKNOWLEDGEMENTS

We thank Dr C. A. Ryan (Washington State University, Pullman, Washington, USA) for kindly supplying the methyl jasmonate. We thank Drs G. Stephenson, S. H. Cody, P. J. Harris and C. K. Pallaghy for helpful discussions and Mr T. Phillips for processing the photographic material. This work was supported by the Australian Research Council and Monash Research Fund.

LITERATURE CITED

Blatt MR. 1990. Potassium channel currents in intact stomatal guard cells: rapid enhancement by abscisic acid. *Planta* **180**: 445–455.

Blatt MR, Armstrong F. 1993. K^+ channels of stomatal guard cells: Abscisic-acid-evoked control of the outward rectifier mediated by cytoplasmic pH. *Planta* **191**: 330–341.

Crawford LA, Bown AW, Breitkreuz KE, Guinel FC. 1994. Synthesis of γ-aminobutyric acid in response to treatments reducing cytosolic pH. *Plant Physiology* **104**: 865–871.

Gehring CA, Irving HR, Parish RW. 1994. Intracellular pH changes precede gibberellic acid induced elongation in maize coleoptiles. *Planta* **194**: 532–540.

Horton RF. 1991. Methyl jasmonate and transpiration in barley. *Plant Physiology* **96**: 1376–1378.

Irving HR, Gehring CA, Parish RW. 1992. Changes in cytosolic pH and calcium of guard cells precede stomatal movements. *Proceedings of the National Academy of Sciences USA* **89**: 1790–1794.

Mayo JM, Ehret D. 1980. The effects of abscisic acid and vapor pressure deficit on leaf resistance of *Paphiopedilum leeanum*. *Canadian Journal of Botany* **58**: 1202–1204.

Raghavendra AS, Reddy KB. 1987. Action of proline on stomata differs from that of abscisic acid, G-substances, or methyl jasmonate. *Plant Physiology* **83**: 732–734.

Roos W. 1992. Confocal pH topography in plant cells–acidic layers in the peripheral cytoplasm and apoplast. *Botanica Acta* **105**: 253–259.

Rutter JC, Willmer CM. 1979. A light and electron microscopy study of the epidermis of *Paphiopedilum* spp. with emphasis on stomatal ultrastructure. *Plant Cell and Environment* **2**: 211–219.

Sakano K, Yazaki Y, Mimura T. 1992. Cytoplasmic acidification induced by inorganic phosphate uptake in suspension cultured *Catharanthus roseus* cells. Measurement with fluorescent pH indicator and ^{31}P-nuclear magnetic resonance. *Plant Physiology* **99**: 672–680.

Sanz LC, Fernández-Maculet JC, Gómez E, Vioque B, Olías JM. 1993. Effects of methyl jasmonate on ethylene biosynthesis and stomatal closure in olive leaves. *Phytochemistry* **33**: 285–289.

Satler SO, Thimann KV. 1981. Le jasmonate de méthyle: nouveau et puissant promoteur de la sénescence des feuilles. *C R academie scientique Paris series III* **293**: 735–740.

Sembdner G, Parthier B. 1993. The biochemistry and the physiological and molecular actions of jasmonates. *Annual Review of Plant Physiology and Plant Molecular Biology* **44**: 569–589.

Ward JM, Pei Z-M, Schroeder JI. 1995. Roles of ion channels in initiation of signal transduction in higher plants. *Plant Cell* **7**: 833–844.

Zeiger E, Assmann SM, Meidner H. 1983. The photobiology of *Paphiopedilum* stomata, opening under blue but not red light. *Photochemistry and Photobiology* **38**: 627–630.