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*Communication*

# Action of Proline on Stomata Differs from That of Abscisic Acid, G-Substances, or Methyl Jasmonate[1]

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## ABSTRACT

**Methyl jasmonate (MJ) and a mixture of $G_1$, $G_2$, and $G_3$ (G-substances) inhibited stomatal opening in abaxial epidermis of *Commelina benghalensis* and complete closure occurred at $10^{-6}$ molar MJ, or $10^{-3}$ molar G-substances compared to $10^{-5}$ molar abscisic acid (ABA). Proline, even at $10^{-3}$ molar caused only a partial stomatal closure. Apart from ABA, other endogenous plant growth regulators do regulate stomata. Reduction in the stimulation by fusicoccin and complete stomatal closure, at 30 millimolar KCl or less, were affected by ABA, MJ, or G-substances, but not by proline. The action of MJ or G-substances was similar to ABA in decreasing proton efflux and the levels of potassium, malate, or reducing sugars. Proline, however, interfered with starch-sugar interconversion but had no effect on proton efflux or potassium content of epidermis.**

The hormonal control of stomatal movement is one of the impressive adaptations of plants to water stress (1, 16). However, among several plant growth regulators, only a few exert a marked influence on stomata in isolated epidermis. Stomatal opening is suppressed by ABA (4) and promoted by cytokinins (3) or auxins (14). Fusicoccin, a fungal toxin, enhances remarkably stomatal opening (5).

Proline accumulates during water stress in leaves of many plants (19). G-substances,[2] extracted from *Eucalyptus* leaves, suppress seed germination, coleoptile growth, rooting and transpiration (2, 8). Methyl jasmonate isolated from extracts of leaves and stem of wormwood, *Artemisia absinthium*, can promote senescence (17, 18). A decrease in stomatal conductance by proline (10) or MJ (13) has been reported but it is not known whether these substances exert, if at all, a direct action on stomata. We therefore studied the effects of proline, G-substances, and MJ on stomatal movement in abaxial epidermis of *Commelina benghalensis*. The mechanism of action of these new test compounds is compared with that of ABA and FC, well known regulants of stomatal movement.

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[2] Abbreviations: G-substances, a mixture of equal proportions of $G_1$, $G_2$, and $G_3$ (Ref. 9); FC, fusicoccin; MJ, (±)-methyl jasmonate (methyl-3-oxo-2-(2′-*cis*-pentenyl)-cyclopentane-1-acetate).

## MATERIALS AND METHODS

**Plant Material.** Cuttings of *Commelina benghalensis* L. were raised in 30 cm diameter seed pans on soil supplemented with fertilizer (N:P:K = 20:20:20, w/w). They were grown outdoor in a natural (approximately 12 h) photoperiod at average temperatures of 30°C day/20°C night. Second to fourth fully developed leaves (from the top) were picked from 6- to 8-week-old plants.

**Preparation of Epidermal Strips.** Strips of 1 × 0.5 cm were prepared from the abaxial (lower) epidermis of leaves (20). The underside of the strips was brushed with a zero size painting brush to remove the adhering mesophyll cells (9). The strips were exposed to ultrasonication (5 strips in 10 ml of 25 mM Mes-NaOH [pH 7.0] with 0.05 mM $Ca[NO_3]_2$ at 25 mamp for three

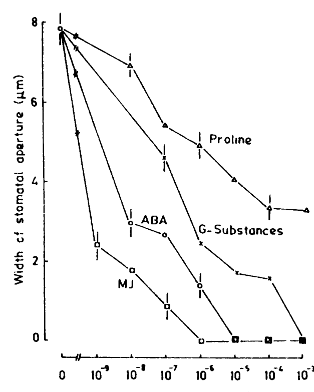


Fig. 1. The effect of ABA, G-substances, MJ, or proline on the degree of stomatal opening in abaxial epidermis. The values represent the width of stomatal aperture at the end of 4 h incubation.

Table I. *Interaction of Proline, MJ, G-Substances or ABA, with FC, during Stomatal Opening in Abaxial Epidermis of C. benghalensis*

The values represent the width of stomatal aperture in µm after incubation for 4 h. Figures in parentheses represent the changes induced by FC.

| | Stomatal Aperture | |
|---|---|---|
| | None | $+10^{-5}$ M FC |
| | µm | |
| Control | 8.2 ± 1.3 | 18.8 ± 1.4 (10.6) |
| $10^{-4}$ M Proline | 4.4 ± 1.2 | 14.9 ± 1.1 (10.5) |
| $10^{-6}$ M MJ | 0 | 8.0 ± 0.7 (8.0) |
| $10^{-3}$ M G-substances | 0 | 9.2 ± 1.0 (9.2) |
| $10^{-5}$ M ABA | 0 | 8.8 ± 0.7 (8.8) |

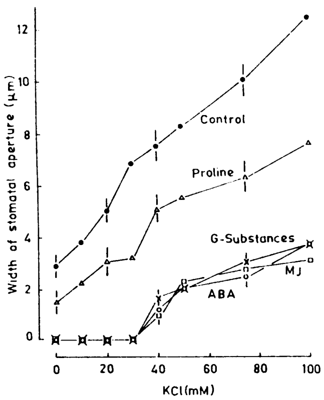


FIG. 2. The responses of stomata in abaxial epidermis to the concentration of KCl in the incubation medium in presence or absence of ABA, G-substances, MJ, or proline. The values represent the width of stomatal aperture after incubation for 4 h.

20 s duration) to kill the epidermal and subsidiary cells, checked by the uptake of 0.01% (w/v) neutral red (11). The sonicated strips were rinsed briskly in ice cold distilled $H_2O$.

**Stomatal Movement and Epidermal Components.** Fifteen to twenty strips were placed in 5 cm diameter Petri dishes containing 20 ml of 25 mM Mes-NaOH buffer (pH 7.0) with 0.05 mM $Ca(NO_3)_2$, 0.1% Tween 80, 30 mM KCl, 0.01% (v/v) ethanol and the test chemicals at the mentioned final concentrations. At the end of 4 h incubation at 30 ± 2°C under illumination with a bank of incandescent bulbs at 200 W $m^{-2}$ (a 15 cm thick water filter prevented heating), the stomatal aperture was measured with the help of a precalibrated ocular micrometer (9).

Proton efflux, potassium levels, malate, and carbohydrate contents of epidermis were determined as described earlier (11).

**Chemicals.** ABA, Mes, proline, and Tween 80 were from Sigma. The sources of FC, G-substances, and MJ are as indicated in "Acknowledgments."

## RESULTS AND DISCUSSION

Stomatal opening in abaxial epidermis was suppressed not only by ABA but also MJ and G-substances (Fig. 1). Stomata remained partially open in the presence of proline even at $10^{-3}$ M. Stomata were completely closed at $10^{-5}$ M ABA, $10^{-6}$ M MJ, and $10^{-3}$ M G-substances.

Although proline accumulates in large quantities in leaves of water stressed plants, it is not as striking as ABA in controlling stomatal aperture. Our results, support the view that proline may primarily function as a solute for intracellular osmotic adjustment of water potential (15) or as a protectant of metabolic machinery (6).

A naturally occurring volatile oil related to jasmonic acid, MJ, proved to be a powerful inhibitor of stomatal opening, nearly 10 times more effective than ABA (Fig. 1). G-substances were less inhibitory than ABA but were still able to suppress completely the opening of stomata. The present investigation indicates that, apart from ABA, other naturally occurring endogenous compounds in plants can effectively control stomata *in vivo* and implies a regulation of transpirational water loss *in situ*. G-substances which exhibit antitranspirant activity on *Eucalyptus* leaves (8) occur in considerable quantity in leaves of *Eucalyptus* as well as other members of Myrtaceae (2). Several compounds similar to MJ in their structure as well as in their senescence promoting activity are widely distributed in the plant kingdom (17, 18). Since MJ is an odoriferous oil, it is possible that stomatal aperture is regulated by volatile substances in the intercellular spaces of leaf.

Proline was far less effective in reducing the stimulation of stomatal opening by FC than that by ABA, MJ, or G-substances (Table I). The stomatal response to varying concentrations of KCl further revealed the distinct nature of action of proline compared to that of ABA, MJ, or G-substances. Stomatal opening at low KCl (30 mM or less) was completely suppressed by ABA, MJ, or G-substances, while inhibition by proline was partial at any level of KCl (Fig. 2).

FC enhanced proton efflux and the level of potassium or sugars but decreased the starch content (Table II). G-substances or MJ, like ABA, reduced proton efflux, potassium content, and restricted starch hydrolysis. On the other hand, proline inhibited hydrolysis of starch into sugars, decreased marginally the level of malate, but had no effect on potassium content or proton efflux.

During stomatal opening potassium uptake is facilitated by ATP dependent proton efflux (7, 21). Proton expulsion raises the pH inside the guard cells, activates phosphoenolpyruvate carboxylase, and leads to malate synthesis. During opening, starch hydrolysis produces soluble sugars (12) contributing to the osmotica of guard cells along with potassium malate.

The primary effect of ABA or FC was to regulate $H^+$ efflux/$K^+$ influx (21), although ABA was also reported to suppress starch breakdown in guard cells (4). In epidermis of *Commelina*, FC enhanced not only $H^+$ efflux but also reducing sugar content (11). The action of MJ and G-substances was similar to that of ABA in suppression of proton efflux, reduction in the level of potassium, malate, or reducing sugars of epidermis as well as counteraction against FC. G-substances, although not as effective as ABA in reducing proton efflux, could, however, suppress

Table II. *Proton Efflux and the Levels of Malate or Carbohydrates in Relation to Stomatal Opening in Abaxial Epidermis of C. benghalensis*

After a 4 h-incubation in light, the degree of opening was measured and the strips were extracted with hot 80% ethanol to determine the levels of malate and carbohydrate.

| Condition/Addition | Stomatal Aperture | Proton Efflux | Malate | Potassium | Carbohydrates: Starch | Reducing sugars[a] | Nonreducing sugars[b] | Total sugars | Sugars/ Malate Ratio |
|---|---|---|---|---|---|---|---|---|---|
| | *μm* | *pmol mm⁻²* | | *neq mm⁻²* | | | | | *pmol osmotica mm⁻²* |
| At the start of the experiment | 1.2 | | 42.1 | 2.8 | 368.5 | 72.3 | 20.5 | 92.8 | 0.73 |
| After incubation | | | | | | | | | |
| Control | 7.8 | 12.4 | 57.9 | 4.3 | 247.2 | 87.2 | 28.8 | 116.0 | 0.67 |
| Fusicoccin $10^{-5}$ M | 18.2 | 38.7 | 72.9 | 4.9 | 127.2 | 135.0 | 43.5 | 178.5 | 0.82 |
| ABA $10^{-5}$ M | 0 | 1.6 | 39.5 | 2.1 | 403.3 | 68.9 | 17.4 | 86.3 | 0.73 |
| MJ $10^{-6}$ M | 0 | 1.0 | 37.6 | 2.0 | 406.7 | 69.9 | 17.1 | 87.0 | 0.77 |
| G-substances $10^{-3}$ M | 0 | 4.4 | 40.2 | 2.5 | 380.6 | 71.1 | 20.8 | 91.9 | 0.76 |
| Proline $10^{-4}$ M | 4.9 | 12.2 | 52.8 | 4.1 | 346.7 | 72.2 | 21.2 | 93.4 | 0.59 |

[a] pmol glucose eq $mm^{-2}$. [b] pmol sucrose eq $mm^{-2}$.

completely stomatal opening at 30 mM or less KCl. We conclude that MJ or G-substances interfere with proton efflux from guard cells.

Proline differed from ABA in its failure to counteract FC (Table I). Further, proline affected primarily starch-sugar interconversion (which might have restricted malate formation) and could not suppress opening completely, even at low KCl. The inability of proline to suppress proton efflux and potassium uptake by epidermal tissue might be the reason for its being not a powerful inhibitor of stomatal opening.

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## LITERATURE CITED

1. Aspinall D 1980 Role of hormones in adaptation to water stress. *In* NC Turner, PJ Kramer, eds, Adaptation of Plants to Water and High Temperature Stress. Wiley Interscience, New York, pp 155–172
2. Dhawan AK, DM Paton, RR Willing 1979 Occurrence and bioassay responses of G: a plant growth regulator in Eucalyptus and other Myrtaceae. Planta 146: 419–422
3. Incoll LD, GE Whitelam 1977 The effect of kinetin on stomata of the grass, *Anthephora pubescens* Nees. Planta 137: 243–245
4. Mansfield TA, RS Jones 1971 Effects of abscisic acid on potassium uptake and starch content of stomatal guard cells. Planta 101: 147–158
5. Marrè E 1979 Fusicoccin: a tool in plant physiology. Annu Rev Plant Physiol 30: 273–288
6. Nash D, LG Paleg, JT Wiskich 1982 Effect of proline, betaine, and some other solutes on the heat stability of mitochondrial enzymes. Aust J Plant Physiol 9: 47–57
7. Outlaw WH Jr 1983 Current concepts on the role of potassium in stomatal movements. Physiol Plant 59: 302–311
8. Paton DM, AK Dhawan, RR Willing 1980 Effect of *Eucalyptus* growth regulators on the water loss from plant leaves. Plant Physiol 66: 254–256
9. Raghavendra AS 1981 Energy supply for stomatal opening in epidermal strips of *Commelina benghalensis*. Plant Physiol 67: 385–387
10. Rajagopal V 1981 The influence of exogenous proline on the stomatal resistance in *Vicia faba*. Physiol Plant 52: 292–296
11. Reddy KB, KR Rao, AS Raghavendra 1983 Stomatal metabolism in abaxial and adaxial epidermis of *Commelina benghalensis*: variation in proton efflux and adenosine triphosphates activity. Z Pflanzenphysiol 111: 401–412
12. Robinson N, J Preiss 1985 Biochemical phenomena associated with stomatal function. Physiol Plant 64: 141–146
13. Satler SO, KV Thimann 1981 Methyl jasmonate: new and powerful promoter of leaf senescence. C R Acad Sci Paris Ser III 293: 735–740
14. Snaith PJ, TA Mansfield 1982 Control of the $CO_2$ responses of stomata by indol-3-ylacetic acid and abscisic acid. J Exp Bot 33: 360–365
15. Stewart CR, JA Lee 1974 The role of proline accumulation in halophytes. Planta 120: 279–289
16. Turner NC, JE Begg 1981 Plant-water relations and adaptation to stress. Plant Soil 58: 97–131
17. Ueda J, J Kato 1980 Isolation and identification of a senescene promoting substance from wormwood (*Artemisia absinthium* L.). Plant Physiol 66: 246–249
18. Ueda J, J Kato, H Yamane, N Takahashi 1981 Inhibitory effect of methyl jasmonate and its related compounds on kinetin-induced retardation of oat leaf senescence. Physiol Plant 52: 305–309
19. Waldren RP, JD Teare 1974 Free proline accumulation in drought stressed plants under laboratory conditions. Plant Soil 40: 689–692
20. Weyers JDB, AJ Travis 1981 Selection and preparation of leaf epidermis for experiments on stomatal physiology. J Exp Bot 32: 837–850
21. Zeiger E 1983 The biology of stomatal guard cells. Annu Rev Plant Physiol 34: 441–475