Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8992–8995, December 1986
Botany

Indole-3-acetic acid and fusicoccin cause cytosolic acidification of corn coleoptile cells

(auxins/elongation growth/membrane potential/pH microelectrodes)

HUBERT FELLE*, BENNO BRUMMER†, ADAM BERTL*, AND ROGER W. PARISH†‡

*Botanical Institute I, Justus Liebig University, Senkenbergstrasse 17-21, D-6300 Giessen, Federal Republic of Germany; and †Plant Biology Institute, University of Zürich, Zollikerstrasse 107, 8008 Zürich, Switzerland

Communicated by Winslow R. Briggs, July 23, 1986§

ABSTRACT **Microelectrodes were used to measure simultaneously the effects of indole-3-acetic acid (IAA) on membrane potential and cytosolic pH of corn coleoptile cells. IAA caused an initial depolarization followed by hyperpolarization, the latter displaying rhythmic oscillations. The extent of the changes in membrane potential was dependent on IAA concentration, and hyperpolarization, but not depolarization, could be detected with concentrations of IAA as low as 10 nM. Membrane hyperpolarization was preceded by a decrease in cytosolic pH. The decrease commenced ≈5 min after adding IAA and continued for 15–20 min before reaching a new steady state ≈0.1 pH unit lower than the original pH. The decrease in pH was readily detectable after treatment with 0.1 μM IAA. Fusicoccin and acetate, which, like IAA, induce elongation growth, caused a similar drop in cytosolic pH and subsequent membrane hyperpolarization, the decrease in pH commencing within seconds. The addition of fusicoccin to IAA-treated cells resulted in a further cytosolic acidification and membrane hyperpolarization. The two substances probably change cytosolic pH via different mechanisms. The results imply that one of the primary effects of auxins in coleoptiles is to lower cytosolic pH.**

The addition of auxins such as indole-3-acetic acid (IAA) to stems or coleoptile tissue of seedlings dramatically stimulates cell elongation (1). One of the earliest known responses to such treatments is membrane hyperpolarization, which occurs within minutes (2–8). This hyperpolarization is due to stimulation of the outwardly directed electrogenic proton pump present in the plasma membrane. Because acid solutions also induce growth, the cell wall acidification following stimulation of the proton pump is thought to explain the effect of auxins on growth (9–14). However, there are inconsistencies in the wall-acidification theory (15–22). Weak acids may induce growth because they penetrate the plasma membrane and acidify the cytosol (23, 24). Certainly, the degree of growth stimulation by acids is positively correlated with the extent to which they acidify the cytosol and thereby stimulate the proton pump (23).

Auxins may stimulate the pump by acidifying the cytosol (20, 25, 26)—i.e., by increasing the substrate levels (27). The fungal toxin fusicoccin also hyperpolarizes the membrane potential of plant cells and induces elongation growth in a variety of tissues (28, 29). Using pH microelectrodes, we found that fusicoccin induced cytosolic acidification of *Zea mays* (30) and *Sinapis alba* (31) root cells within seconds. Membrane hyperpolarization commenced some time after this change. We used pH indicators to measure the cytosolic pH of corn coleoptile cells and showed that fusicoccin caused acidification (30). IAA caused cytosolic acidification after a lag of 5 min. However, the pH-indicator method is relatively insensitive and difficult to calibrate, and we have now succeeded in inserting pH microelectrodes into coleoptile cells. Our results indicate that one of the primary effects of auxins in these cells is to acidify the cytoplasm.

MATERIALS AND METHODS

Plant Material. *Z. mays* (Orla 264) seeds were soaked for 8–12 hr in tap water. They were then placed in moist vermiculite in a plastic box for 4–5 days at 25°C in the dark. The apical 3 mm of the coleoptiles was removed, the following 10 mm was excised, and the primary leaves were also removed. The segments were washed with distilled water and buffer, and fixed in a Plexiglas chamber (30). The buffer used in all experiments was 5 mM Tris/2-(*N*-morpholino)ethanesulfonic acid (Mes)/1 mM KCl/1 mM NaCl/0.1 mM $CaCl_2$. The pH of each experiment is indicated in the figures.

Measurement of Membrane Potential. A standard electrophysiological apparatus was used (29–33). Micropipettes were pulled on a Getra instrument (vertical) from fiber glass-filled borosilicate tubing (Hilgenberg, Malsfeld, F.R.G.) and filled by capillary deplacement with 0.5 M KCl. Tip diameters were 0.3–0.5 μm. Membrane potentials were recorded from maize coleoptile cells in a Plexiglas chamber that was continuously perfused by the test buffer and allowed horizontal approach by the microelectrodes (29). The measurements were made in the first cell penetrated by the microelectrodes, and this appeared always to be an epidermal cell.

Fabrication of pH-Sensitive Microelectrodes. Fabrication was as described (33). Briefly, micropipettes were dipped with the blunt end into a mixture of 0.1% dimethyldichlorosilane/chloroform and baked at 180°C for 30 min to provide a water-repellent interior surface. For further stabilization of the tips, 0.1% polyvinylchloride dissolved in tetrahydrofuran was sucked into the tip. The proton exchanger resin (Fluka, no. 82500) was backfilled into the tip, as was the remainder of the capillary with 0.5 M KCl/0.1 M Mes. These electrodes had resistances of $5–8 \times 10^{10}$ Ω and displayed a slope of 56–58 mV per pH unit between pH 4 and 9. The rigid and turgor-sensitive tip is suitable for impaling cells with walls (31).

Recording of Intracellular pH. The pH electrode always recorded a sum of membrane potential plus the electromotive force of the pH difference. A second electrode, placed in the same cell, detected the membrane potential only. A high impedance (10^{15} Ω) differential amplifier (WPI Instruments, Waltham, MA; FD 223) recorded and subtracted the signals from the two electrodes, which were monitored on a pen

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: IAA, indole-3-acetic acid.

‡To whom reprint requests should be addressed.

§Communication of this paper was initiated by A. Frey-Wyssling and, following his illness, completed by Winslow R. Briggs.

chart (Kontron W + W 314). Resting potential was measured for 30 min before IAA was added to the external solution.

Localization of the Electrodes. The pH values are generally found to be slightly alkaline. This means the electrode is located within the cytoplasm. Theoretically, it is possible that the voltage electrode is located within the vacuole while the pH electrode measures the cytoplasmic pH. An electrical potential drop of +15 to +20 mV can be assumed across the tonoplast, which is equivalent to 0.3 to 0.4 pH unit, in which case the reported cytoplasmic pH would in fact be too alkaline. This is highly unlikely for two reasons. First, both electrodes were always identically pulled; therefore, there is no obvious reason why the two electrodes should impale two different compartments. Moreover, it is most unlikely that the voltage electrode would always be measuring within the vacuole, since the pH values show that the pH electrode always measures within the cytoplasm. Second, there is no turgor across the tonoplast, and it is difficult to impale a membrane without a pressure gradient.

RESULTS

Effects of IAA on Membrane Potential. The addition of IAA to corn coleoptiles resulted in membrane hyperpolarization followed by oscillations of potential in the hyperpolarized phase (Fig. 1). These oscillations were found at all IAA concentrations large enough to cause detectable hyperpolarization. A short treatment with IAA (2 min) was sufficient to cause the membrane potential to hyperpolarize to a maximum value ≈15 min after IAA had been removed from the external solution. This was followed by depolarization to the original potential (Fig. 1). (Note: the hyperpolarization following the short treatment was faster than when IAA was not removed; the reasons are unknown.)

When IAA was continuously present, membrane hyperpolarization reached a maximum after 25–30 min, and the extent of hyperpolarization was dependent on IAA concentration (Fig. 2). The maximum hyperpolarization increased sharply by treatment with 1 mM IAA. This was probably due to direct acidification of the cytosol by the hormone, which acts as a weak acid at high concentrations, causing an additional stimulation of the proton pump.

Initial depolarization immediately following IAA addition was observed at hormone concentrations of 1 μM and above (occasionally even at 0.1 μM) (Fig. 2). The extent of depolarization was directly dependent on IAA concentration, and the time taken to recover to the initial potential was inversely dependent on IAA concentration.

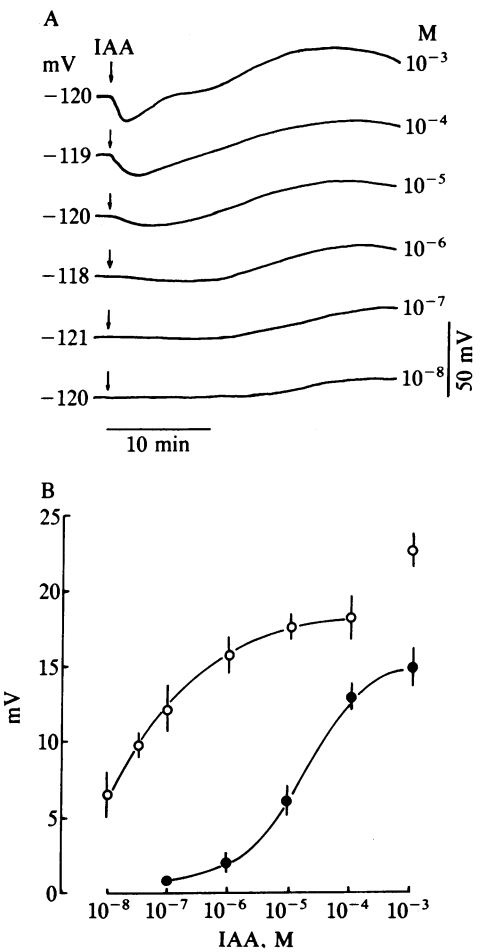

FIG. 2. (*A*) Effect of IAA concentration on membrane potential. (*B*) Dependency of maximum depolarization (●) and hyperpolarization (○) values on IAA concentration.

Cytosolic Acidification Caused by IAA and Fusicoccin. IAA treatment resulted in acidification of the cytosol (Fig. 3). This acidification was first detected after 4–5 min and preceded membrane hyperpolarization. The pH drop was rapid with 1

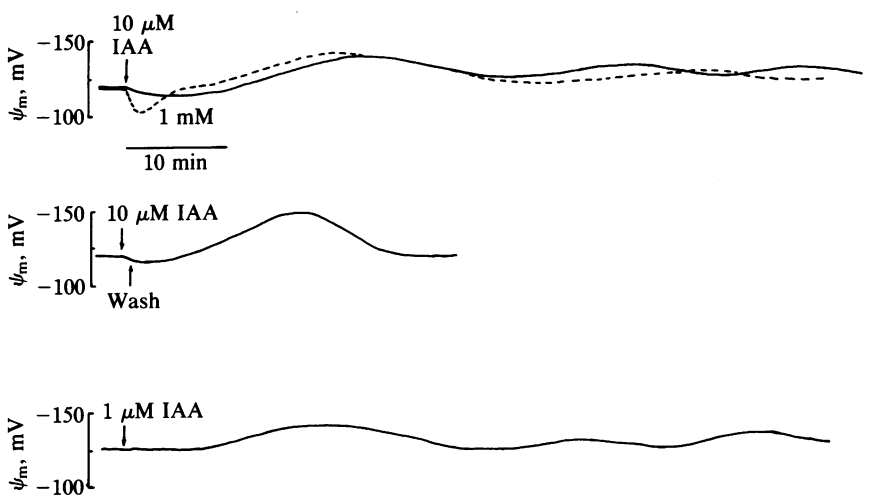

FIG. 1. Oscillations in membrane potential (ψ_m) following treatment of corn coleoptiles with different IAA concentrations. Middle trace shows the effect of removing IAA after 2 min.

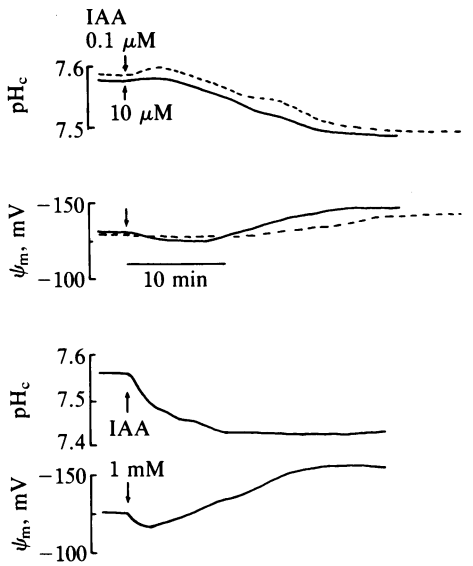

FIG. 3. Effect of IAA concentration on cytosolic pH (pH_c) and membrane potential (ψ_m) of coleoptile cells. The incubation solution was buffered at pH 6.

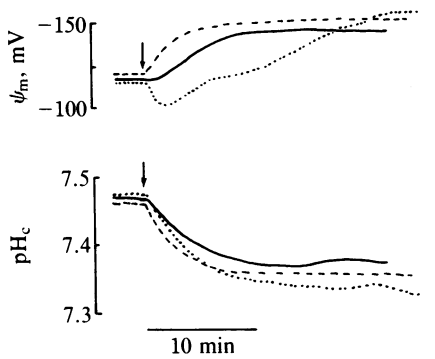

FIG. 5. Comparison of effects of 1 mM acetate (——), 2.5 μM fusicoccin (——), and 1 mM IAA (·····) on cytosolic pH (pH_c) and membrane potential (ψ_m). The incubation solution was buffered at pH 5.2.

mM IAA due to direct acidification of the cytosol by the hormone. The pH decreased by ≈0.1 unit with IAA concentrations from 0.1 μM to 0.1 mM. At low concentrations, a weak alkalinization preceding the acidification was occasionally detected.

The addition of 2.5 μM fusicoccin led to a rapid cytosolic acidification, which also preceded the membrane hyperpolarization (Fig. 4*A*). When fusicoccin was added to coleoptiles treated with 10 μM IAA, there was a further decrease in the cytosolic pH followed by membrane hyperpolarization (Fig. 4*B*). As expected, 1 mM IAA caused an additional drop in cytosolic pH (Fig. 4*B*).

A direct comparison of the effects of 1 mM IAA, fusicoccin, and acetic acid on cytosolic pH and membrane potential is shown in Fig. 5. Like 1 mM IAA, acetic acid rapidly enters and acidifies the cytosol (weak acid effect). However, the membrane potential curves for the two acids are different.

DISCUSSION

IAA-induced hyperpolarization of corn coleoptile cells occurred after a lag, which varied according to hormone concentration but was not less than 10 min. Similar results have been obtained with a number of tissues (2–8), although continuing depolarization following IAA treatment has also been reported (34). We consistently observed oscillations in the membrane potential that were IAA specific. Oscillations have previously been found in corn coleoptiles when potential was discontinuously measured with microelectrodes (i.e., by repeated puncturing of new cells for each value) (34, 35). However, these oscillations were all in the depolarization phase and had an amplitude of 20–25 mV. Continuous measurements on wheat coleoptiles showed no oscillations (34). Bates and Goldsmith (8) failed to detect oscillations of potential in *Avena* coleoptile cells. We have not yet examined species other than corn.

IAA has also been found to cause an initial depolarization prior to the sustained hyperpolarization (5, 6, 8). *Avena* coleoptiles responded to 1 μM IAA with a depolarization of 20–25 mV (8). This lasted ≈10 min and was followed by repolarization during which the potential became 20–25 mV more negative than the original potential. Depolarization was less with higher IAA concentrations. Micromolar concentrations of butyric and benzoic acids also caused an initial depolarization but no subsequent hyperpolarization. The authors concluded that hyperpolarization but not the depolarization, was a specific hormonal effect (8). Although we also found initial depolarization, this increased with increasing IAA concentrations and showed different kinetics to hyperpolarization (Fig. 2*B*). The reasons for the depolarization are unknown, although the $IAA^-/2H^+$ cotransport system (36) may be involved. In addition, passive diffusion of undissociated IAA into cells also occurs (37) and may increase membrane conductivity, thereby causing depolarization. Subsequent stimulation of the pump by cytosolic acidification would reverse the depolarization. Acetate did not cause depolarization (Fig. 5), where butyric and benzoic acids do (8). The change in membrane conductivity is dependent on the weak acid used, and these also have different effects on metabolism (benzoic acid is rapidly toxic) (H.F., unpublished observations).

The pH microelectrode permitted direct and continuous measurement of cytosolic pH. Moreover, cytosolic pH and membrane potential were measured simultaneously in the same cells. Our reasons for believing both electrodes are

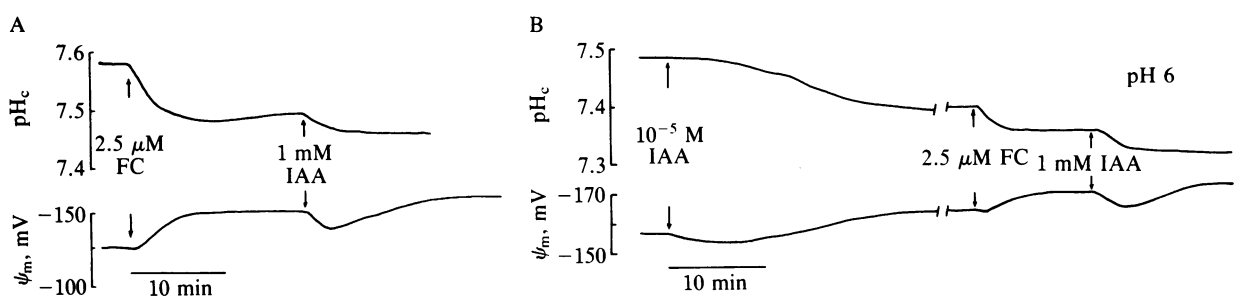

FIG. 4. (*A*) Effects of fusicoccin (FC) on cytosolic pH (pH_c) and membrane potential (ψ_m). (*B*) Effects of consecutive addition of IAA and fusicoccin on cytosolic pH and membrane potential. The weak acid effect of 1 mM IAA is also shown in both experiments.

placed within the cytosol are discussed in *Materials and Methods* and in a previous publication (31).

IAA lowered the cytosolic pH, the decrease being measurable after ≈5 min. Using a dual wavelength absorbance technique to measure cytosolic pH in corn coleoptiles, we found a similar lag before IAA-induced acidification commenced (31). However, the method is less sensitive than the pH microelectrode and is difficult to calibrate. The microelectrode measurements in the present paper showed that a change of ≈0.1 pH unit occurred and was readily detectable even at low IAA concentrations. IAA cytosolic acidification always preceded membrane hyperpolarization, indicating that IAA stimulates the proton pump by lowering the cytosolic pH.

Similar cytosolic acidification is induced by 0.1 and 10 μM IAA (see Fig. 3), although the rate of membrane hyperpolarization and the maximum hyperpolarization obtained are different. The reasons for this are not clear, but membrane conductivity changes may be involved.

Relating the changes in membrane potential and cytosolic pH to growth response suggests that membrane hyperpolarization accompanied by a pH decrease, but without the preceding depolarization (see Fig. 2), could in some way regulate growth. The increasing depolarization obtained with higher IAA concentrations may become increasingly inhibitory to growth. These criteria are met by the concentration range between 10 nM and 10 μM, which goes roughly from threshold to saturation for growth.

High concentrations of IAA (1 mM) caused a rapid cytosolic acidification (Fig. 5). However, a lag prior to growth induction still occurs (ref. 37; B.B., unpublished observations). No such lag occurs with fusicoccin or acetate (23, 29, 38). The explanation is probably that the latter substances also rapidly induce hyperpolarization, whereas there was a lag of 8 min following 1 mM IAA treatment before hyperpolarization commenced (Fig. 5).

Our measurements were made on epidermal cells, and there is increasing evidence that the epidermis is the primary target for the early growth-promoting action of IAA (39). We have not as yet peeled the coleoptiles and made measurements on meosphyll cells.

The oscillations in membrane potential detected following IAA treatment may result from a feedback loop involving cytosolic pH and other factors (e.g., free Ca^{2+} levels).

The fungal toxin fusicoccin induces elongation growth in many plant tissues (28, 29). Fusicoccin causes rapid membrane hyperpolarization and, in root tissue, we have shown that this is due to cytosolic acidification, which then results in pump stimulation (30, 31). An identical effect was seen with corn coleoptiles (Fig. 4*A*). The cytosolic acidification was rapid (commencing within seconds) and, when fusicoccin was added to coleoptiles already hyperpolarized by IAA treatment, a further hyperpolarization associated with an additional drop in cytosolic pH occurred. These results suggest that IAA and fusicoccin lower cytosolic pH via different mechanisms. The IAA response may involve a second messenger such as Ca^{2+} (20, 40), whereas fusicoccin may stimulate acid-producing metabolism more directly (30, 31). Even when IAA was removed after 2 min, the hyperpolarization took place and persisted for 15–20 min after IAA removal.

If cytosolic Ca^{2+} is regulated by auxin, this, combined with effects on cytosolic pH and membrane potential, would have pleiotropic effects on metabolism (38, 40). Changes in transmembrane ion gradients brought about by pump stimulation could act, like free Ca^{2+}, as a second messenger (20, 40) and influence processes such as secretion, protein synthesis, and gene transcription (41, 42). Cell wall acidification resulting from pump stimulation may be at least partly involved in elongation growth, although evidence is accumulating against the acid-growth theory (9–22). Weak acids probably stimulate growth because they acidify the cytosol and stimulate the proton pump rather than via direct acidification of the wall (23, 24).

Microelectrodes can be used to study the effects of IAA on cytosolic free Ca^{2+} levels and to discover whether these constitute the second messenger regulating cytosolic pH.

1. Cleland, R. E. (1980) in *Plant Growth Substances 1979*, ed. Skoog, F. (Springer, Berlin), pp. 71–78.
2. Etherton, B. (1970) *Plant Physiol.* **45**, 527–528.
3. Lüttge, U., Higinbotham, N. & Pallaghy, C. K. (1972) *Z. Naturforsch. Teil B* **27**, 1239–1242.
4. Marrè, E., Lado, P., Ferroni, A. & Ballarain-Denti, A. (1974) *Plant Sci. Lett.* **2**, 257–265.
5. Cleland, R. E., Prins, H. B. A., Harper, J. R. & Higinbotham, N. (1977) *Plant Physiol.* **59**, 395–397.
6. Nelles, A. (1977) *Planta* **147**, 293–298.
7. Mizuno, A., Katuo, K. & Ohamoto, H. (1980) *Plant Cell Physiol.* **21**, 395–403.
8. Bates, G. W. & Goldsmith, M. H. M. (1983) *Planta* **159**, 231–237.
9. Hager, A., Menzel, H. & Krauss, A. (1971) *Planta* **100**, 47–75.
10. Cleland, R. E. (1971) *Planta* **99**, 1–11.
11. Rayle, D. L. (1973) *Planta* **114**, 68–73.
12. Cleland, R. E. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3092–3093.
13. Cleland, R. E. (1976) *Plant Physiol.* **58**, 210–213.
14. Jacobs, M. & Ray, P. M. (1976) *Plant Physiol.* **58**, 203–209.
15. Penny, P., Dunlop, J., Perley, E. & Penny, D. (1975) *Plant Sci. Lett.* **4**, 35–40.
16. Vanderhoef, L. N., Findley, J. S., Burke, J. J. & Blizzard, W. E. (1977) *Plant Physiol.* **59**, 100–103.
17. Pope, D. G. (1977) *Ann. Bot. (Rome)* **41**, 1069–1071.
18. Vesper, M. J. & Evans, M. L. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 6366–6370.
19. Cleland, R. E. (1983) *J. Exp. Bot.* **34**, 676–680.
20. Brummer, B. & Parish, R. W. (1983) *FEBS Lett.* **161**, 9–13.
21. Brummer, B., Potrykus, I. & Parish, R. W. (1984) *Planta* **162**, 345–352.
22. Kutschera, U. & Schopfer, P. (1985) *Planta* **163**, 483–493.
23. Brummer, B., Felle, H. & Parish, R. W. (1984) *FEBS Lett.* **174**, 223–227.
24. Hager, A. & Moser, I. (1985) *Planta* **163**, 391–400.
25. Göring, H. & Bleiss, W. (1982) in *Plasmalemma and Tonoplast: Their Function in the Plant Cell*, eds. Marmé, D., Marrè, E. & Hertel, R. (Elsevier, Amsterdam), pp. 263–269.
26. Brummer, B. & Parish, R. W. (1985) *FEBS Lett.* **188**, 181–183.
27. Raven, J. A. (1985) *Sci. Prog. (Oxford)* **69**, 495–509.
28. Marrè, E. (1979) *Annu. Rev. Plant Physiol.* **30**, 273–288.
29. Felle, H. (1982) *Plant Sci. Lett.* **25**, 219–225.
30. Brummer, B., Bertl, A., Potrykus, I., Felle, H. & Parish, R. W. (1985) *FEBS Lett.* **189**, 109–114.
31. Bertl, A. & Felle, H. (1985) *J. Exp. Bot.* **36**, 1142–1149.
32. Felle, H. (1981) *Biochim. Biophys. Acta* **602**, 151–160.
33. Bertl, A., Felle, H. & Bentrup, F. W. (1984) *Plant Physiol.* **76**, 75–78.
34. Göring, H., Polevoy, V. V., Stahlberg, R. & Stumpe, G. (1979) *Plant Cell Physiol.* **20**, 649–656.
35. Stahlberg, R. & Polevoy, V. V. (1979) *Dokl. Bot. Sci.* **247**, 74–76.
36. Hertel, R. (1983) *Z. Pflanzenphysiol.* **112**, 53–67.
37. Evans, M. L. (1985) *CRC Crit. Rev. Plant Sci.* **2**, 317–365.
38. Hanson, J. B. & Trewavas, A. J. (1982) *New Phytol.* **90**, 1–18.
39. Pope, D. G. (1982) *Ann. Bot. (Rome)* **49**, 493–501.
40. Parish, R. W., Felle, M. & Brummer, B. (1985) in *Molecular and Cellular Aspects of Calcium in Plant Development*, ed. Trewavas, A. J. (Plenum, New York), pp. 288–296.
41. Walker, J. C., Legocka, J., Feldman, L. & Key, J. L. (1985) *Plant Physiol.* **77**, 847–850.
42. Hagen, G. & Guilfoyle, T. J. (1985) *Mol. Cell. Biol.* **5**, 1197–1203.