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CALCIUM IONS PROMOTE THE RESPONSE OF *CITRUS LIMON* AGAINST FUNGAL ELICITORS OR WOUNDING

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Key Word Index—*Citrus limon*; Rutaceae; lemon; elicitors; calcium; defense response; wounding.

Abstract—Lemon seedlings treated with 1 μM $CaCl_2$ increased phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activity 1 hr earlier than when treated with fungal elicitors from *Alternaria alternata*, or when wounded. The calcium dependent response was suppressed by EGTA and Verapamil, and was mimicked by the calcium ionophore A23187.

INTRODUCTION

Plant defense against pathogen attack includes many different strategies. The defense capacity of higher plants, mainly observed in incompatible reactions, has been associated with inducible mechanisms, which include accumulation of phytoalexins [1], activation of pathogenesis-related (PR) proteins such as chitinases and β-glucanases [2], synthesis of proteinase inhibitors [3], accumulation of hydroxyproline-rich proteins in the cell wall [4], and deposition of lignin [5]. These defense responses can be induced not only by pathogens but also by UV light, wounding, heavy metals and elicitors [6], and require specific recognition [7, 8] and efficient transduction mechanisms of the initial signal or stimulus.

Citrus limon seedlings inoculated with conidia from *Alternaria alternata* or treated with fungal elicitors (FE) increase phenylpropanoid metabolism [9, 10] and synthesize umbelliferone and scoparone as part of the developed hypersensitive response [11]. The transduction of the initial signal produced by oligomers of galacturonic acid that contain 19 sugar residues [9], is unknown in this plant species. The participation of calcium ions in signal transduction has been reported in several plant systems [12, 13], as well as its role as a second messenger [14–16].

This work reports the participation of calcium ions and calcium channels in early steps of the transduction of the signal produced by FE of *A. alternata* or by wounding of *C. limon* seedlings.

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RESULTS AND DISCUSSION

Effect of calcium ions and of the calcium ionophore A23187 on the kinetics of PAL induction

The increase of phenylalanine ammonia-lyase (PAL) activity produced by wounding in seedlings of *C. limon* after 8 hr treatment [10], took place 1 hr before (at 7 hr) when 10 μM $CaCl_2$ was added, and was slightly higher than the one observed to wounding. Also, the presence of a new maximum PAL activity at 3 hr after treatment with Ca^{2+} was observed (Fig. 1). On the other hand, the increase of PAL activity in wounded seedlings at 4 hr, in response to FE [10], was also advanced in 1 hr when these elicitors were replaced by the same $CaCl_2$ concentration (Fig. 1), i.e. the increase of PAL activity was observed after 3 hrs treatment; although the magnitude of the increase of PAL activity at these Ca^{2+} concentrations was smaller than the one obtained in response to FE. The advancement in these responses suggests that Ca^{2+} might be participating in the transduction of the signal produced by elicitors or by wounding, in order to produce two time-dependent responses: one that has to be accomplished at short times, related to the plant defense against FE; and the other, to be accomplished at longer times than the former and related to wounding, to repair the damage produced.

To test the involvement of Ca^{2+} in the observed response, $CaCl_2$ was replaced by 10 nM of the Ca^{2+} ionophore A23187 (Fig. 2). The increase in PAL activity was larger than the one observed with 10 μM $CaCl_2$, suggesting that the internal concentration of Ca^{2+} depends not only on the external concentration, but also on an active mobilization of those inside the

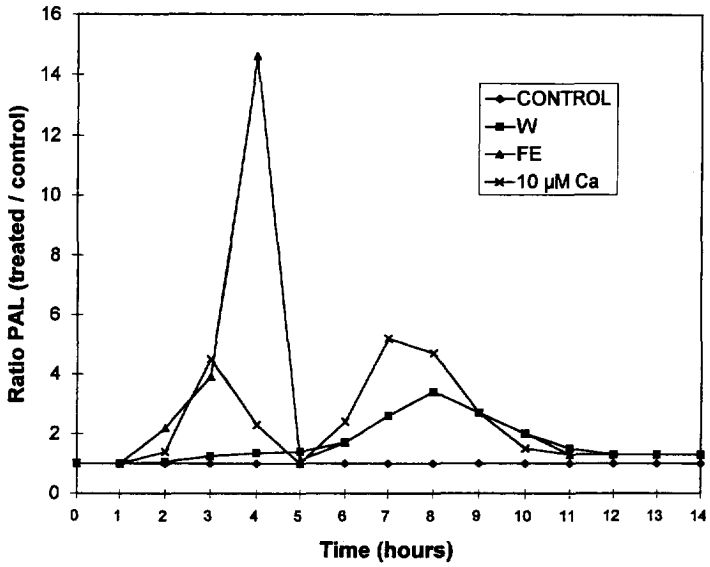

Fig. 1. Time course increase of PAL activity ratio in lemon seedlings after different treatments. (■) Wounding; (▲) wounding plus FE; (×) wounding plus 10 μM $CaCl_2$; (◆) control (intact seedlings): 5 pkat mg^{-1} protein of basal PAL activity. S.D. did not exceed 10%.

cell. Thus, the presence of the ionophore could produce a mobilization of larger amounts of Ca^{2+}, most probably from the plant cell wall. The effect of the ionophore A23187, in the absence of added Ca^{2+}, reinforces the idea of the participation of this ion in the internalization of the signal. Similar results have been reported in carrot cell suspension cultures, where the production of phytoalexins was induced by the ionophore A23187, both in the presence and in the absence of the elicitor [17].

Several reports published recently prove that Ca^{2+} can act as second messenger in plant cells [14–16]; however, it has been difficult to determine if the increase of cytosolic Ca^{2+} occurs by an influx from the extracellular solution into the cytoplasm, or by an efflux from intracellular organelles.

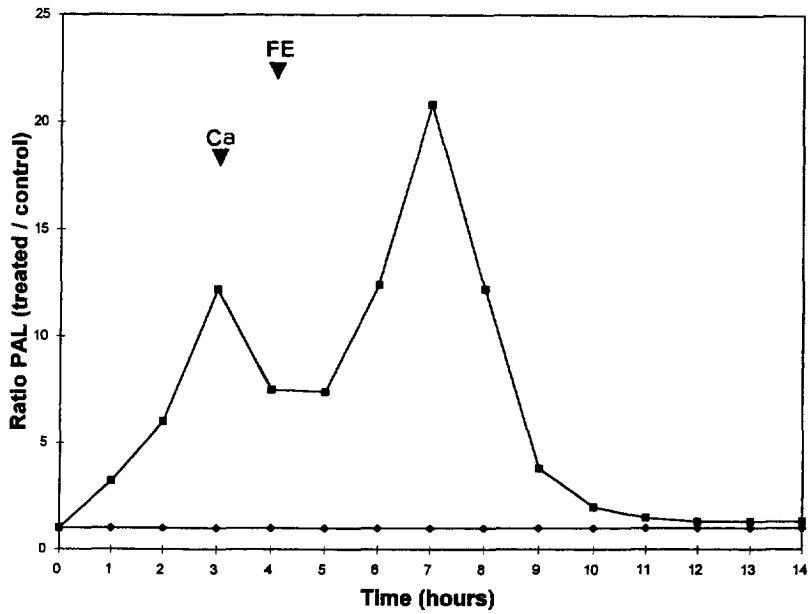

Fig. 2. Time course increase of PAL activity ratio in wounded lemon seedlings treated with 0.1 μM calcium ionophore A23187 (■). The FE-arrow shows the time period for maximal PAL activation after treatment of wounded seedlings with fungal elicitors. The Ca-arrow shows the time period for maximal PAL activation after treatment of wounded seedlings with 10 μM $CaCl_2$. Control (◆). S.D. did not exceed 10%.

The only fact that appears clear is that the response to a Ca^{2+} signal depends on the change in the concentrations of this ion [15] and on the nature of the stimulus [18].

When different $CaCl_2$ concentrations were tested for their effect in increasing PAL activity as a response to addition of the ion to wounded seedlings, it was found that the optimal external concentration to achieve the largest increase in PAL activity was 1 μM $CaCl_2$, both at 3 hr and at 7 hr after treatment. The increase over basal enzyme activity was 8- and 6.7-fold, for the respective time periods. The different $CaCl_2$ concentrations, added to the PAL assay medium, did not alter the enzyme activity, suggesting that Ca^{2+} ions are involved in the cellular response and not in the activity of the enzyme. 50% maximal PAL activation was obtained at $CaCl_2$ concentrations of 0.2 μM (3 and 7 hr) and 10 μM (3 hr) or 15 μM (7 hr). Nevertheless, the internal Ca^{2+} concentration reached after treatment of seedlings with 1 μM $CaCl_2$ might be much lower. It has been reported recently that calcium cytosolic concentrations in resting cells are *ca* 150 nM, reaching excitatory levels of 300 to 400 nM or higher [15, 19]. These concentrations could be reached by external influx of calcium ions from solutions or by its mobilization from the plant cell wall.

The increase of PAL activity both at 3 hr and at 7 hr after treatment, was also dependent on the concentration of the ionophore A23187, following a similar pattern to that observed for Ca^{2+}: a maximal PAL activation was obtained with 10 nM of the ionophore, with 50% maximal PAL activation at 1 nM (3 and 7 hr) and 15 μM (3 hr) or 6.4 μM (7 hr) of this molecule. The increase over basal enzyme activity was 12.5-fold (3 hr) and 21-fold (7 hr). As for Ca^{2+} ions, the ionophore did not alter PAL activity when added to the assay medium.

The ratio for the increase of PAL activity obtained when the effect of Ca^{2+} was compared with the ionophore A23187, at their optimal PAL activation concentrations (1 μM Ca^{2+}/10 nM^{-1} ionophore), was of 0.64 (3 hr) and of 0.32 (7 hr) suggesting that 10 nM ionophore might be producing a larger influx of Ca^{2+} than the one produced by 1 μM of the same added ion. Then, external Ca^{2+} ions (or from the cell wall) could be responsible for the change of timing of the plant response against FE or wounding, although these experiments cannot discard the possibility that simultaneous eflux of Ca^{2+} from organelles could also occur.

Effect of EGTA and of Verapamil on the calcium-dependent response

The increase in PAL activity observed as a response to Ca^{2+} treatment (3 hr) or to wounding (7 hr), was reduced when increasing EGTA concentrations were added to wounded seedlings in the presence of 1 μM $CaCl_2$ (Table 1). These EGTA concentrations did not affect PAL activity, indicating that no response is obtained if external Ca^{2+} are unavailable due to their chelation by EGTA and that Ca^{2+} is not necessary for PAL activity. Thus, Ca^{2+} eflux from organelles to the cytoplasm, if occurring, is probably not involved in the signal transduction. The need of extracellular Ca^{2+} to induce an increase in PAL and in CHS activities has been reported for soybean suspension cultures, treated with elicitors from *Phytophthora megasperma f.* sp. *glycinea* [13]. In this case, the chelation of extracellular Ca^{2+} by EGTA suppressed the elicitor-mediated synthesis of phytoalexins, a situation that was reversed by new additions of Ca^{2+}.

Table 1. Effect of EGTA in PAL activity ratio elicited by 1 μM $CaCl_2$ in wounded lemon seedlings

[EGTA] (mM)	PAL ratio after Ca^{2+} treatment* (treated/control)	
	3 hr	7 hr
0	8.0	6.8
0.25	3.9	3.2
0.50	3.1	2.6
1	2.5	2.0
2	1.3	1.0
3	0.9	0.8

*PAL activity was measured after 3 and 7 hr treatment of wounded lemon seedlings with 1 μM $CaCl_2$. S.D. did not exceed 10%.

Since extracellular Ca^{2+} was apparently involved in the response of lemon seedlings to FE or wounding, we tested the effect of Verapamil, which has been described as one of the best calcium channel blockers in plant cells [20, 21]. 50 μM Verapamil completely suppressed the increase of PAL activity in wounded lemon seedlings in response to FE (4 hr after treatment). This same Verapamil concentration did not affect PAL activity. This Ca^{2+} channel blocker also suppressed the response to Ca^{2+} at 3 and 7 hr in wounded seedlings, as well as the one of lemon seedlings to wounding (8 hr), with a $I_{0.5}$ of 25 μM. Therefore, Verapamil is blocking both the response to wounding and the one elicited by FE or Ca^{2+}, suggesting that in both types of response the participation of Ca^{2+} channels might be involved. It has been described that Verapamil is able to bind specifically to maize coleoptile membranes [22] and to carrot protoplasts [21], blocking Ca^{2+} channels. Hence, the influx of extracellular Ca^{2+} [23, 24], could be accomplished through these type of channels in lemon seedlings, as has been discussed for guard cells [25]. After an increase of the cytoplasmic Ca^{2+} concentration, other mechanisms of signal transduction that require the presence of high Ca^{2+} concentrations can be triggered, initiating a cascade of events leading to the complex response of lemon seedlings to mechanical wounding and fungal elicitation.

EXPERIMENTAL

All reagents were analytical grade and were purchased from Sigma and Merck.

Biological. Lemon seedlings were grown from seeds, as described [9]. *A. alternata* was isolated from *Citrus* trees infected with sooty molds [26], and their conidia obtained as recommended by AOAC [27]. Fungal cell walls were prepared from cultures of the fungus in liquid Mandels medium [28], following the procedure of ref. [29].

Treatment of lemon seedlings. Lemon seedlings were wounded [9] and analysed at different time intervals. Wounding was performed by halving hypocotyls lengthwise with a surgical blade. Cotyledons were also sliced into 2 sections. When seedlings were treated with elicitors a suspension of fungal cell walls (fungal elicitors) was carefully spread on top of the wounded tissue as described in ref. [10]. Alternatively, the appropriate soln was carefully spread on the wounded tissue.

The following treatments were performed immediately after wounding: i) fungal elicitors; ii) $CaCl_2$ (0 to 100 μM); iii) ionophore A23187 (0–100 μM); iv) $CaCl_2$ 1 μM plus 0–3 mM EGTA; v) $CaCl_2$ 1 μM plus 0–50 μM Verapamil. Homogenates from the samples were prepared [9] to quantify PAL activity [30] and protein concn [31]. Results are expressed as the ratio of PAL activity in treated/control seedlings, and are the mean of four different experiments run in duplicates. All results are referred to intact seedlings. Controls (without treatments) were run both with intact (control 1) and wounded (control 2) lemon seedlings.

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REFERENCES

1. Darwill, A. G. and Albersheim, P. (1984) *Annu. Rev. Plant Physiol.* **35**, 243.
2. Mauch, F. and Staehelin, A. (1989) *The Plant Cell* **1**, 447.
3. Farmer, E. and Ryan, C. (1990) *Proc. Natl Acad. Sci. USA* **87**, 7713.
4. Roby, D., Toppan, A. and Esquerré-Tugayé, M. (1985) *Plant Physiol.* **77**, 700.
5. Moerschbacher, B., Noll, U., Gorrichon, L. and Joachim Reisener, H. (1990) *Plant Physiol.* **93**, 645.
6. Kuc, J. and Rush, J. (1985) *Arch. Biochem. Biophys.* **236**, 455.
7. Sharp, J., McNeil, M. and Albersheim, P. (1984) *J. Biol. Chem.* **259**, 11321.
8. Cheong, J. and Hahn, M. (1991) *The Plant Cell* **3**, 137.
9. Roco, A., Castañeda, P. and Pérez, L. M. (1993) *Phytochemistry* **33**, 1301.
10. Pérez, L. M., Chiong, M., Roco, A. and Castañeda, P. (1993) *Fitopatologia* **28**, 86.
11. Pérez, L. M., Pavani, M., Quaas, A. and Roco, A. (1994) *Fitopatologia* **29**, 94.
12. Köhle, H., Jeblick, W., Poten, F., Blaschek, W. and Kauss, H. (1985) *Plant Physiol.* **77**, 544.
13. Stäb, M. and Ebel, J. (1987) *Arch. Biochem. Biophys.* **257**, 416.
14. Kurosaki, F. and Nishi, A. (1993) *Arch. Biochem. Biophys.* **302**, 144.
15. Trewavas, A. and Gilroy, S. (1991) *Trends Genetics* **7**, 356.
16. Schwacke, R. and Hager, A. (1992) *Planta* **187**, 136.
17. Kurosaki, F., Tsurusawa, Y. and Nishi, A. (1987) *Phytochemistry* **26**, 1919.
18. Knight, M., Campbell, A., Smith, S. and Trewavas, A. (1991) *Nature* **352**, 524.
19. Schroeder, J. and Thuleau, P. (1991) *The Plant Cell* **3**, 555.
20. Andrejauskas, E., Hertel, R. and Marmé, D. (1985) *J. Biol. Chem.* **260**, 5411.
21. Graziana, A., Fosset, M., Ranjeva, R., Hetherriton, A. and Lazdunski, M. (1988) *Biochemistry* **27**, 764.
22. Harvey, H., Venis, M. and Trewavas, A. (1989) *Biochem. J.* **257**, 95.
23. Bolwell, G., Coulson, V., Rodgers, M., Murphy, D. and Jones, D. (1991) *Phytochemistry* **30**, 397.
24. Vögeli, U., Vögeli-Lange, R. and Chappell, J. (1992) *Plant Physiol.* **100**, 1369.
25. Schroeder, J. and Hagiwara, S. (1990) *Proc. Natl Acad. Sci., USA* **87**, 9305.
26. Pérez, L. M., Mettifogo, S., Zaldívar, M. and Musalem, M. (1991) *Fitopatologia* **26**, 28.
27. A.O.A.C. (1980) *Official Methods of Analysis of the Association of Official Analytical Chemists.* 13th Edn (Horwitz, W., ed.), p. 61.
28. Mandels, M., Hontz, L. and Nystrom, J. (1974) *Biotech. Bioeng.* **14**, 1471.
29. Anderson-Prouty, A. J. and Albersheim, P. (1975) *Plant Physiol.* **56**, 286.
30. Zucker, N. (1965) *Plant Physiol.* **40**, 779.
31. Bradford, M. (1976) *Analyt. Biochem.* **72**, 248.