

# IP<sub>3</sub> production in the hypersensitive response of lemon seedlings against *Alternaria alternata* involves active protein tyrosine kinases but not a G-protein

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## ABSTRACT

IP<sub>3</sub> increase and *de novo* synthesis of scoparone are produced in the hypersensitive response (HR) of lemon seedlings against the fungus *Alternaria alternata*. To elucidate whether a G-protein and/or a protein tyrosine kinase (PTK) are involved in signal transduction leading to the production of such a defensive response, we studied the HR in this plant system after treatment with G-protein activators alone and PTK inhibitors in the presence of fungal conidia. No changes in the level of IP<sub>3</sub> were detected in response to the treatment with the G-protein activators cholera toxin or mastoparan, although the HR was observed in response to these compounds as determined by the scoparone synthesis. On the contrary, the PTK inhibitors lavendustin A and 2,5-dihydroxy methyl cinnamate (DHMC) not only prevented the IP<sub>3</sub> changes observed in response to the fungal inoculation of lemon seedlings but also blocked the development of the HR. These results suggest that the IP<sub>3</sub> changes observed in response to *A. alternata* require a PTK activity and are the result of a G-protein independent Phospholipase C activity, even though the activation of a G-protein can also lead to the development of a HR. Therefore, it appears that more than one signaling pathway may be activated for the development of HR in lemon seedlings: one involving a G-protein and the other involving a PTK-dependent PLC.

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## INTRODUCTION

The infection of resistant plants by pathogens triggers the development of the hypersensitive response (HR): a series of biochemical and cellular events that take place at or near the affected site and help to defend plants by limiting the spread of infection (Beffa et al., 1995). The HR consists of a rapid cell death forming necrotic lesions, the production of antifungal compounds called phytoalexins, the deposition of cell wall compounds such as lignin and callose, and the production of pathogenesis related (PR) proteins. Lemon seedlings inoculated with *Alternaria alternata* develop an

HR which is characterized by the induction of the phenylpropanoid pathway and its key enzyme Phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5) (Roco et al., 1993), the increase in umbelliferone concentration that results in the synthesis of the phytoalexin scoparone (Pérez et al., 1994) and the expression of new chitinases and  $\beta$ -1,3-glucanases (Ortega and Pérez, 1999).

The development of an appropriate defensive response depends upon an effective and rapid signaling process that includes the perception of the external signal by cell surface receptors, the transduction of this information through the plasma membrane into the cell by the action of transducers and the amplification of the signal by the activation of specific chain reactions that lead to the ultimate cellular response (Munnik et al., 1998).

The phosphoinositide-signaling pathway is involved in the transduction of several different signals in animal cells (Munnik et al. 1998). Once this signaling system is activated, phospholipase C (PLC, EC 3.1.4.11) hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>), forming the products inositol-1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG), which act as secondary intracellular messengers. IP<sub>3</sub> triggers the release of Ca<sup>2+</sup> from intracellular stores, regulating calcium and calmodulin-dependent enzymes and channels while DAG remains in the membrane to activate protein kinase-C. The downstream target for DAG in plants is unclear (Wang, 2004), and it is believed that DAG is immediately phosphorylated by DAG-kinase to form phosphatidic acid (PA) (Testerink et al., 2004; Wang et al., 2004) so in plants PLC can be considered to generate IP<sub>3</sub> and PA rather than DAG.

There are currently five different families of PLC: PLC $\beta$  are activated by G $\alpha$  subunits of the G $\alpha$  family of heterotrimeric G-proteins, and some of them can be activated by G $\beta\gamma$  (Wing et al., 2003); PLC $\gamma$  are activated by phosphorylated receptor tyrosine kinases (Munnik et al., 1998, Rebecchi and Pentylala 2000, Wing et al., 2003); PLC $\delta$ , whose activation could be dependent on its interaction with a Gh type G-protein (transglutaminase II) (Wing et al., 2003, Ochocka and Pawelczyk 2003); PLC $\epsilon$ , which is thought to be activated by small monomeric GTPases of the Ras and Rho families and by heterotrimeric G proteins (Song et al., 2001; Wing et al., 2001; Wing et al., 2003), and the recently identified PLC $\zeta$ , whose regulatory mechanism is still unknown (Wing et al., 2003; Swann et al., 2004). Heterotrimeric G-proteins consist of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits and act as molecular switches that control many important cellular responses and relay information from cell surface receptors to intracellular effectors such as PLC, phospholipase D (PLD, EC 3.1.4.4), cyclases, ion channels, phosphodiesterases, etc. (Law et al., 1994; Legendre et al., 1992). When a cell surface receptor is activated, the G-protein  $\alpha$ -subunit bound GDP is replaced by GTP, which causes the dissociation of the  $\alpha$ -GTP from de $\beta\gamma$ -complex allowing the transmission of the original signal by their interaction with downstream effector ion channels and enzymes (Bourne 1997; Munnik et al., 1995; Seo et al., 1997). The signaling pathway remains activated until the intrinsic GTPase activity of the G-protein causes GTP hydrolysis to GDP. In its GDP-bound form, the  $\alpha$ -subunit binds to the  $\beta\gamma$ -complex again and the G-protein becomes ready to react to another cell surface receptor activation (Legendre et al., 1992). Cholera toxin is a multimeric protein that catalyzes the ADP-ribosylation of the G $\alpha$  subunit of trimeric G-proteins; this reversibly blocks the GTPase activity of the G-protein leading to a sustained activation of the downstream signaling pathway in which this transducer is involved (Beffa et al., 1995). Mastoparan is a cationic amphiphilic tetradecapeptide isolated from wasp venom that activates G-proteins by mimicking the region of an activated receptor and binding to the amino terminus of the G $\alpha$  subunit (Cho et al., 1995).

Protein tyrosine kinases (PTK) have also been involved in signaling processes either participating as receptors or as part of kinase cascades (Schenk and Snaar-Jagalska, 1999) such as MAPK, which are involved in plant defense (Zhang and Klessig, 2001). Lavendustin A is a potent and selective tyrosine kinase inhibitor that is competitive with ATP and non-competitive with its peptide substrate. It is able to inhibit the phosphorylation of a synthetic peptide representing the tyrosine phosphorylation site of PLC<sub>γ1</sub> (Hsu et al 1991). Methyl-2,5-dihydroxycinnamate (DHMC) is an erbstatin analogue, and both compounds are tyrosine kinase inhibitors. Specifically, DHMC inhibits the EGF receptor kinase competitively with the peptide and non-competitively with ATP (Koch et al 1996; Umezawa et al., 1990).

The G-protein activators and protein tyrosine kinase inhibitors mentioned above have been used to establish the participation of these elements in signal transduction events both in animal and plant systems (Bach and Seitz, 1997; Beffa et al., 1995; de Vrije and Munnik, 1997; Munnik et al. 1995). The participation of a G-protein and PTKs has been established in the signaling for the development of the HR in lemon seedlings using this approach (Ortega et al., 2002). Furthermore, IP<sub>3</sub> increases have been detected within the early events of transduction in this same plant system in response to inoculation with *A. alternata* (Ortega and Pérez, 2001), but the formation of this second messenger through a G-protein or a protein tyrosine kinase receptor-dependent PLC activity has not been established.

This work describes the use of the G-protein activators cholera toxin and mastoparan and of the PTK inhibitors Lavendustin-A and DHCM to investigate whether a G-protein and/or a PTK are involved in the IP<sub>3</sub> production for the HR development in lemon seedlings after fungal inoculation.

## **METHODS**

### *Chemicals*

All reagents were analytical grade and were purchased either from Amersham, Merck or Sigma.

### *Biologicals*

Lemon seedlings were grown from seeds at 28°C in a dark chamber until they reached 5 cm length of hypocotyl (Roco et al., 1993). They were then placed under a 16 / 8-hour photoperiod until they were used for the experiments. *A. alternata* was isolated from lemon trees infected with sooty molds (Pérez et al., 1991), grown on PDA and their conidia obtained as recommended by AOAC (1980).

### *Plant treatment*

Lemon seedlings were cut in half lengthwise and inoculated by carefully spreading 1 mL of a suspension of fungal conidia ( $2 \times 10^6$  conidia/mL), or 1 mL of cholera toxin (1 µg/mL), or 1 mL of mastoparan (25 mM) per gram of fresh weight of seedlings on top of the wounded tissue (Pérez et al., 1994). Where indicated, 1 mL of the protein tyrosine kinase inhibitors, lavendustin-A (7.5 nM), or 2,5-dihydroxymethylcinnamate (0.5 µM) were spread 5 minutes earlier and pre-incubated before the fungal inoculation. Controls were performed by spreading the appropriate solutions in the absence of fungal conidia. Addition of the fungal conidia is taken as zero time. Treated and control

seedlings were incubated at 28°C. Experiments were run in duplicates and were repeated at least three times.

#### *Protein concentration and PAL activity*

Homogenates from control or treated seedlings were obtained after 4 hours of treatment with fungal conidia (Castañeda et al., 1996; Roco et al., 1993) or after 1 hour of treatment with cholera toxin or mastoparan (Ortega and Pérez, 1999). These homogenates were used to estimate protein concentration (Bradford, 1976) and to assay PAL activity using L-Phe as substrate (Zucker, 1965). Results are expressed as PAL ratio of treated/control seedlings, or as PAL activity (pkat/mg proteins), and correspond to the mean of at least three different experiments run in duplicates  $\pm$  SEM.

#### *Analysis of the phytoalexin scoparone*

Experiments were conducted under the same conditions used for the PAL induction assay, except that 42 h after PAL induction (Pérez et al., 1994), seedlings were frozen in liquid nitrogen and directly extracted with Ethyl acetate (2mL per gram of fresh weight of seedlings). The organic extract was reduced to one third of its original volume and 120  $\mu$ L were applied to TLC plates (silica gel GF254). These were developed in Toluene: Ethyl acetate (1:1). The presence of scoparone was visualized under UV light (254 and 320 nm) (Roco et al., 1993) and compared to commercial standards. The results correspond to the mean of at least three different experiments  $\pm$  SEM.

#### *IP<sub>3</sub> quantitation*

IP<sub>3</sub> was extracted and quantified at different time periods (0-30 minutes) after inoculation with fungal conidia, or after treatment with the G-protein activators or with the protein tyrosine kinase inhibitors plus fungal conidia. In the case of the inhibitors, seedlings were incubated with Lavendustin A or DHMC 5 minutes before fungal inoculation. At the appropriate times, the plants were frozen in liquid nitrogen and the phosphoinositides were extracted (Chandok and Sopory, 1994). IP<sub>3</sub> was quantified as recommended by the manufacturer of the IP<sub>3</sub> quantitation kit TRK 1000 (Amersham, UK). Results are expressed as pmoles of IP<sub>3</sub> per gram of fresh weight of seedlings, and correspond to the mean of at least four different experiments  $\pm$  SEM.

#### *Statistical analysis*

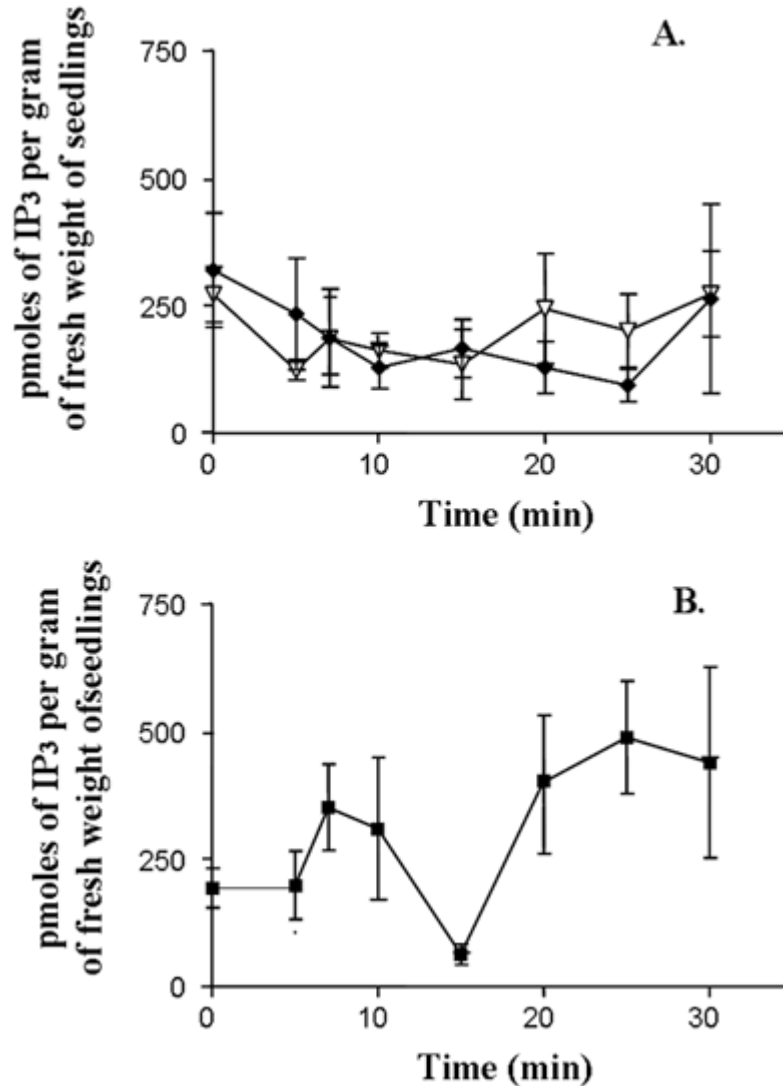
Data was analyzed using the Dunnett test at  $p < 0.05$ .

## **RESULTS**

#### *IP<sub>3</sub> changes, PAL activity and scoparone synthesis in response to the G-protein activators cholera toxin and mastoparan*

We have previously demonstrated that the inoculation of lemon seedlings with conidia from *A. alternata* induces time-dependent changes in the IP<sub>3</sub> levels of the plant (Ortega and Pérez 2001). In contrast to this earlier observation, IP<sub>3</sub> time-dependent changes were not detected in lemon seedlings that were treated with cholera toxin or

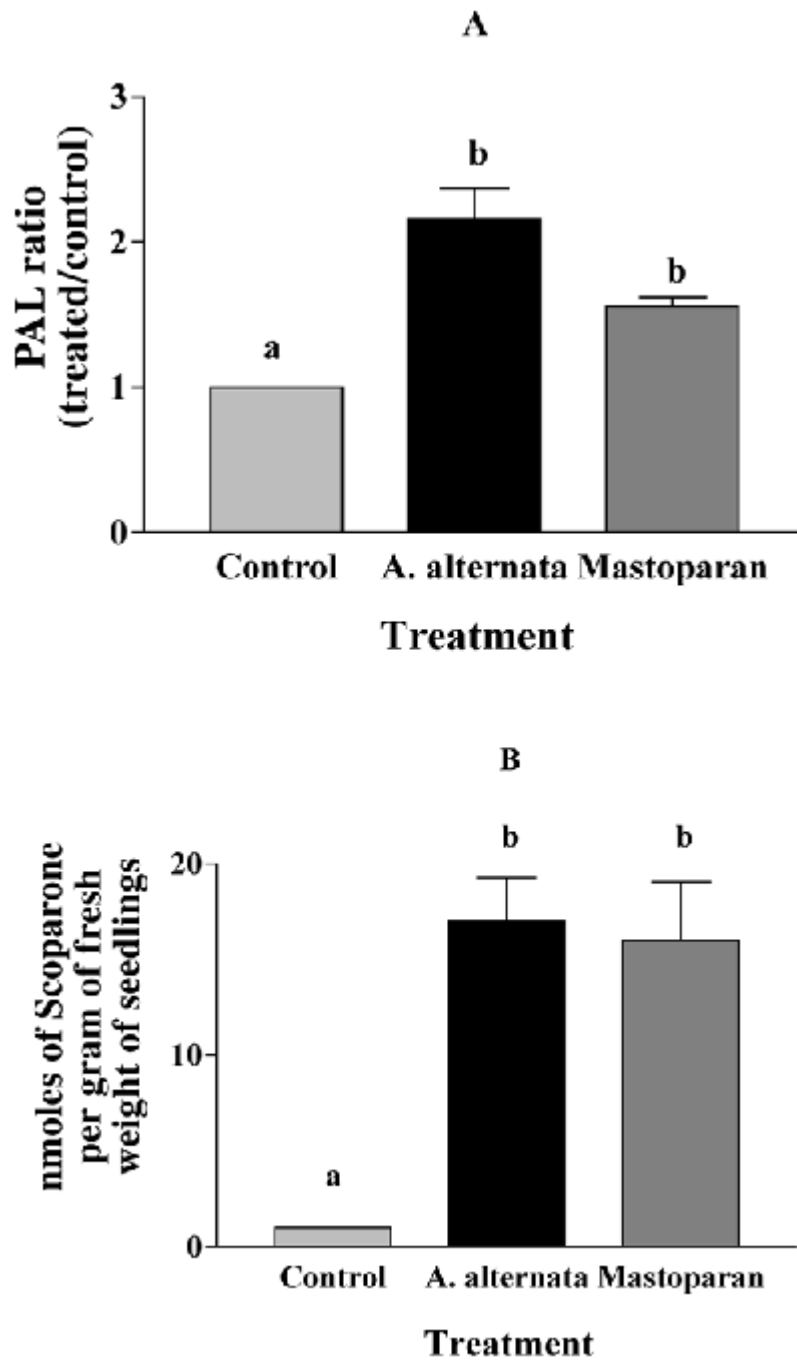
mastoparan in the absence of conidia from *A. alternata* (Fig. 1). Nevertheless, we have shown that this plant system induces PAL activity and the synthesis of the phytoalexin scoparone in response to cholera toxin (Ortega et al., 2002). These results suggest that lemon seedlings are able to develop their HR in response to this G-protein activator in the absence of IP<sub>3</sub> changes.



**Figure 1.** Time course changes in IP<sub>3</sub> concentration in wounded lemon seedlings: **A.** after treatment with Cholera toxin (◆), or with mastoparan (▽). **B.** after treatment with conidia from *A. alternata* (■) (for comparison, taken from Ortega and Pérez, 2001), as described in Experimental Procedures. Bars represent ± SEM. IP<sub>3</sub> concentration values at different time periods within experiments run with the same G-protein activator, were not significantly different to controls at  $p < 0.05$ .

Mastoparan, which activates G-proteins through a mechanism other than that of cholera toxin (Cho et al., 1995; den Hartog et al., 2001), also induced PAL activity and the synthesis of scoparone in lemon seedlings (Fig. 2), in a similar time-dependent

way as cholera toxin (Ortega et al., 2002) and in a similar magnitude as *A. alternata* (Roco et al., 1993). This gives additional support to the possible participation of G-proteins in the development of the HR of lemon seedlings against *A. alternata*.



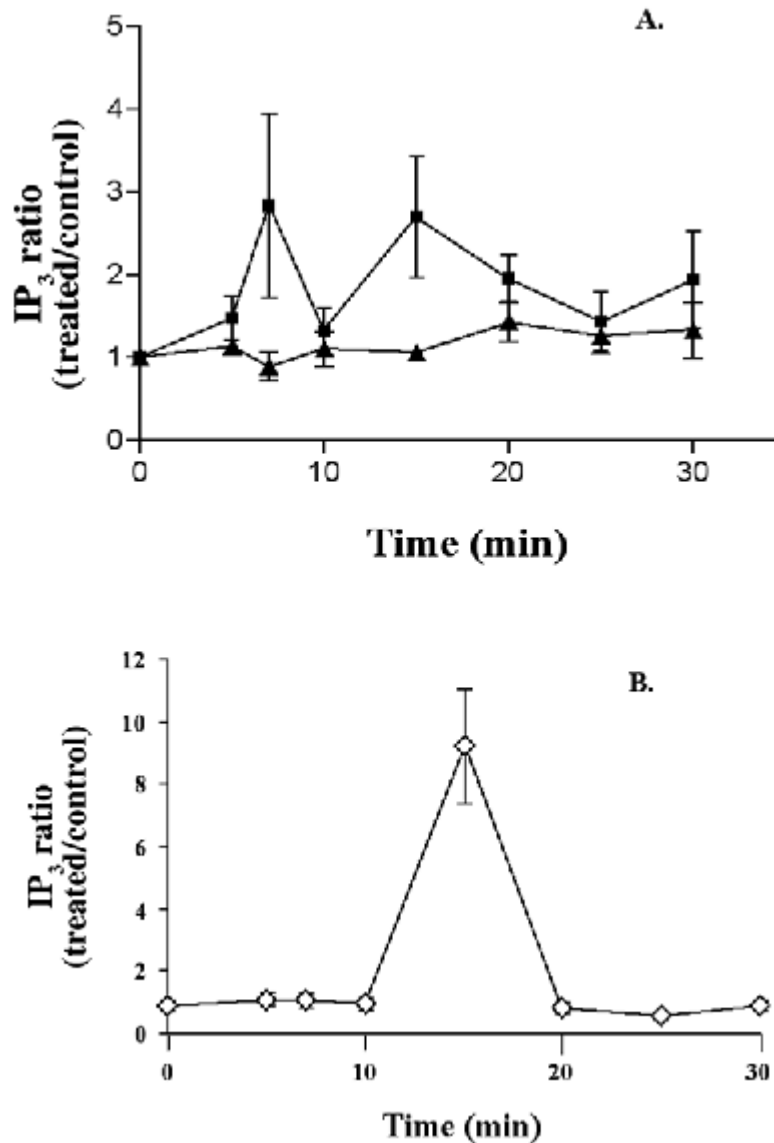
**Figure 2.** PAL activity (A) and Scoparone concentration (B) found in wounded lemon seedlings in the absence or after treatment with *A. alternata* or mastoparan. Results correspond to the mean of at least three different experiments. See Experimental procedures. Bars represent

± SEM. Significant differences are represented by different letters.

*IP<sub>3</sub> changes, PAL activity and scoparone synthesis in response to the protein tyrosine kinase inhibitors lavendustin A and 2,5-dihydroxymethyl cinnamate.*

As shown previously, IP<sub>3</sub> increases were observed after 7 and 25 minutes of inoculation of lemon seedlings with *A. alternata* ([Fig. 1B](#), from Ortega and Pérez, 2001). These increases in IP<sub>3</sub> levels were not observed when this plant system was treated with 0.5 µM DHMC 5 minutes before fungal inoculation ([Fig. 3A](#)). In these experimental conditions, only constant basal IP<sub>3</sub> levels were observed along the time period studied. This protein tyrosine kinase inhibitor also prevents PAL induction, scoparone synthesis and protein tyrosine phosphorylation in this same system (Ortega et al, 2002).

On the other hand, pre-treatment of the lemon seedlings with lavendustin A before fungal inoculation resulted in a single increase in IP<sub>3</sub> levels at 7 minutes post-inoculation ([Fig. 3A](#)). Control experiments in which lemon seedlings were inoculated with lavendustin A in the absence of fungal conidia allowed us to establish that the IP<sub>3</sub> increase observed at 15 minutes corresponds to the plant response toward Lavendustin A in the absence of *A. alternata* ([Fig. 3B](#)).



**Figure 3.** Time course changes in IP<sub>3</sub> concentration in wounded lemon seedlings: **A.** inoculated with *A. alternata* after 5 minutes treatment with lavendustin A (■) or with DHMC (▲), **B.** after 5 minutes treatment with Lavendustin A in the absence of *A. alternata* (◇), as described in Experimental Procedures. Bars represent  $\pm$  SEM. IP<sub>3</sub> concentration values at different time periods within experiments run with DHMC, were not significantly different to controls at  $p < 0.05$ .

## DISCUSSION

There is evidence supporting the participation of G-proteins in the development of defensive responses in many different plant systems. Experiments conducted in French bean suspension cultures using cholera toxin established the participation of a G-protein in the PAL induction that is observed in response to fungal elicitors (Bolwell et



al., 1991). The expression of cholera toxin in transgenic tobacco plants or its microinjection into plant cells induces the expression of PR and increases their resistance to *P. tabaci* (Beffa et al., 1995). Also, GTP-binding proteins participate in the development of the oxidative burst in soybean cells (Legendre et al., 1992), a process that is involved in plant defense responses against pathogens (Chrispeels et al., 1999), although IP<sub>3</sub> quantitation was not considered in that study. Another example is the synthesis of the phytoalexin  $\beta$ -thujaplicin in *Cupressus lusitanica* cell cultures, which can be stimulated by the incubation with mastoparan, mellitin or cholera toxin alone or in combination with a yeast elicitor, and inhibited by the G-protein inhibitor suramin (Zhao and Sakai, 2003)

The participation of G-protein has been also demonstrated in tobacco cells in response to mastoparan, although an oxidative burst could not be observed in these cells. In this case, an increase in cytosolic calcium concentration and activation of protein kinases was detected as a result of the phosphoinositide turnover (Takahashi et al., 1998). Also, the involvement of a G-protein in the activation of a PLC and a PLD has been described after mastoparan or its active analogue treatment of *Vicia sativa* (den Hartog et al., 2001), where mastoparan simulates the effect of the Nod factor-induced phosphatidic acid and DAG pyrophosphate formation. Anaerobiosis of rice roots results in IP<sub>3</sub> increases that maximize at 5 minutes of anoxia; these can be suppressed by the PLC inhibitor neomycin or simulated by the addition of aluminum fluoride, another G-protein activator (Reggiani and Laoreti, 2000). In this latter case, increases in IP<sub>3</sub> appear to be related to G-protein activation, although the plant response is against anoxia and not against a fungal pathogen. These results suggest that even though G-proteins are involved in several plant responses, their participation does not always involve the activation of a PLC to account for changes in IP<sub>3</sub> concentration in response to stimulus. In fact, no G-protein activated PLC has been described in plants (Jones and Assmann, 2004). Plant PLCs were previously thought to be of the  $\delta$  type, but they lack the PH domain present in this type of isoenzymes and now it is known that their structure resembles the recently discovered PLC  $\zeta$  isoenzyme (Wang 2004; Swann et al., 2004), whose regulation is still not well understood, rather than the G-protein dependent PLC isoenzymes. This would be the case of lemon seedlings, where G-protein activators cannot simulate the IP<sub>3</sub> changes observed after inoculation with *A. alternata* (Ortega and Pérez, 2001) although they are able to develop their HR. The fact that PAL is induced and scoparone is synthesized in lemon seedlings in response to the G-protein activators cholera toxin and mastoparan in the absence of any IP<sub>3</sub> changes suggests that more than one signaling pathway could be present in lemon seedlings for the development of their HR. In this case, the G-protein activated pathway appears to be independent of changes in the IP<sub>3</sub> concentration. *Arabidopsis* has seven PLC genes, but none of their sequences seem to correspond to the G-protein dependent  $\alpha$ ,  $\beta$  or  $\epsilon$  isoforms (Jones and Assmann, 2004). This agrees with our results in which we did not find a G-protein dependent IP<sub>3</sub> production in lemon seedlings. In tomato plants the activation of G $\alpha$  increases the open probability of Ca<sup>2+</sup> channels, which is also enhanced by fungal elicitors (Jones and Assmann, 2004), suggesting that plants could sense fungal pathogens through G-protein-dependent Ca<sup>2+</sup> channels. As we have previously demonstrated, calcium ions participate in the signaling events leading to the development of the plant HR (Ortega et al., 2001), and therefore the activation of a G-protein dependent Ca<sup>2+</sup> channel could be part of the defensive response of lemon seedlings to *A. alternata*.

The presence of independent signaling pathways for the development of HR in lemon seedlings could be also explained through the different timings observed for PAL induction and scoparone synthesis after their treatment with G-protein activators or with fungal

conidia. While an early PAL induction is observed 1 hour after treatment with cholera toxin (Ortega et al., 2002) or with mastoparan (data not shown), PAL induction was detected only after 4 hours of treatment with *A. alternata* (Roco et al., 1993). The different timing of the plant HR response to the different stimulus and of the IP<sub>3</sub> changes observed in response to fungal inoculation reinforce the idea that the plant could cope in a partially independent way with different initial stimulus (G-protein activator or *A. alternata*) for the development of its HR.

Protein kinases are also described as part of the signaling pathways that lead to the development of defensive responses in plants. DHMC has been described as a competitive inhibitor of the peptide substrate of tyrosine kinases (Koch et al., 1996; Umezawa et al., 1990), and therefore its inhibitory effect on the lemon system could be explained in a similar way. Whether the protein substrate(s) correspond to a receptor or to other protein(s) within the signaling pathway cannot be deduced from these results, but they do allow us to propose that the inhibited protein tyrosine kinase is located upstream of the lemon PLC.

The absence of the 25-minute change in IP<sub>3</sub> observed after the fungal treatment could be explained through the inhibition of one type of tyrosine kinase located upstream of a PLC, which could be participating in signaling for PAL induction and scoparone synthesis in response to *A. alternata* due to the fact that both are suppressed by this PTK inhibitor as we have previously shown (Ortega et al., 2002). The 7-minute change in IP<sub>3</sub> could correspond to the activity of a different PLC, independent of PTK activation. This agrees with the fact that lavendustin A is not able to completely suppress protein tyrosine phosphorylation in response to *A. alternata* (Ortega et al., 2002). The differential behaviors observed for the two tyrosine kinase inhibitors may be explained through the involvement of more than one protein tyrosine kinase in signal transduction, these having different sensitivities toward the inhibitors as has been described for other kinases (Schenk and Snarr-Jagalska, 1999). The differences in behavior could be also due to the different mechanisms used by DHMC and lavendustin A to inhibit PTKs. While DHMC is a competitive inhibitor of the peptide substrate, lavendustin A is a competitive inhibitor of ATP (Hsu et al., 1991). Therefore, if these seedlings have high ATP levels, inhibition could be less effective for a group of lavendustin A-sensitive tyrosine kinases, as opposed to the case of DHMC, where the substrate(s) are one or more specific proteins whose level could be very low. Therefore, it may be suggested that the activation of a protein tyrosine kinase is necessary for the downstream activation of a PLC that leads to the IP<sub>3</sub> production in response to *A. alternata* inoculation of lemon seedlings. As expected, the lemon seedlings that did not show protein tyrosine phosphorylation or changes in IP<sub>3</sub> neither induced PAL nor synthesized the phytoalexin scoparone after the fungal inoculation, confirming that a protein tyrosine kinase is necessary to activate the HR in lemon seedlings in response to *A. alternata* (Ortega et al., 2002). Moreover, this PTK could be located down-stream from the *A. alternata* receptor or be a receptor with tyrosine kinase activity, and in any case, it should be located up-stream of the PLC responsible for the IP<sub>3</sub> increases in our plant system.

In conclusion, our results support the idea that more than one signaling pathway is present in lemon seedlings for the development of its HR: one activated through a G-protein that is not coupled to a PLC, and the other dependent on a PTK (or a receptor with tyrosine kinase activity, RTK) coupled to a PLC. In fact, it is generally accepted that most regulatory interactions with PLC $\gamma$  involve receptor or non-receptor tyrosine kinases (Katan, 1998). The presence of multiple signaling pathways for the development of the HR could be useful to provide plants with different systems to counteract the attack by microorganisms that use

different strategies to penetrate plant tissues. Markham and Hille (2001) described the infection of plants by microorganisms that use toxins to kill plant tissue directly or to inhibit the secretion of inhibitory compounds that prevent fungal development. If any toxin from *A. alternata* or other phytopathogens (Rotem, 1994; Markham and Hille, 2001) could activate a G-protein, the G-protein dependent signaling pathway could account for the defensive response against this kind of microorganisms. The effect of fungal toxins on G-protein activation in plants has not been described and cannot be ruled out. On the other hand, the PTK-dependent signaling pathway could be the one involved in the HR response that has been described for lemon seedlings toward different plant or fungal-derived elicitors (Roco et al, 1993) involving the participation of the second IP<sub>3</sub> messenger for its development (Ortega and Pérez, 2001). Until recently it was generally accepted that only PLC<sub>δ</sub>-related proteins are present in plants (Rebecchi and Pentylala, 2000), but now a new PLC<sub>ζ</sub> more closely related to plant PLC (Swann et al., 2004). The regulation mechanism of this new PLC<sub>ζ</sub> is still unknown, so the fact that IP<sub>3</sub> production and PTK activation are related in lemon seedlings could provide a clue to understanding the properties of the PLC that is involved in signal transduction in the defensive response of plant systems against pathogens.

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