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Calcium Requirement for Ethylene-Dependent Responses

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Ethylene, a gaseous plant hormone, plays a role in plant development, defense, and climacteric fruit ripening. Both genetic and biochemical evidence suggest that the response of plants to ethylene is mediated by a specific ethylene receptor. The signal emanating from the receptor–effector complex is then presumably transduced via an unknown cascade pathway. We have used the plant pathogenesis response, exemplified by the induction of the pathogenesis-related gene chitinase, as a paradigm to investigate ethylene-dependent signal transduction in the plant cell. We showed that calcium is necessarily involved in the ethylene-mediated pathogenesis response. Blocking calcium fluxes with chelators inhibited ethylene-dependent induction of chitinase accumulation, but not ethylene independent induction. Artificially increasing cytosolic calcium levels by treatments with the calcium ionophore ionomycin or the calcium pump blocker thapsigargin stimulated chitinase accumulation. Plants grown in calcium-poor soil showed a 10-fold reduction in leaf extractable calcium. Their leaves exhibited a reduced pathogenesis reaction to ethylene and were impaired in another hormone response mediated by calcium, i.e., abscisic acid–controlled closure of guard cells. The addition of calcium to leaves excised from calcium-deficient plants restored their sensitivity to ethylene. Ethylene participates in the control of seedling growth, promoting the so-called "triple response" that results in distinct morphological development, such as hypocotyl hook formation. This effect, similar to the ethylene-promoted pathogenesis response, was found to be calcium dependent. The results indicate that calcium is required for a variety of ethylene-dependent processes.

INTRODUCTION

Ethylene evolution is associated with stress and is involved in modulating a broad spectrum of physiological processes such as pathogenesis, senescence, flowering, fruit ripening, and the morphogenic response in seedlings called the "triple response" (Goeschl et al., 1966; Yang and Hoffman, 1984). Ethylene biosynthesis is rapidly increased during plant–pathogen interaction or application of chemical elicitors (Yang, 1985; Lotan and Fluhr, 1990a). Ethylene subsequently induces a multitude of gene arrays collectively called the pathogenesis response (Van Loon and Antoniw, 1982; Ecker and Davis, 1987; Eyal and Fluhr, 1992). Pathogenesis-related (PR) proteins are encoded by gene families that are coordinately expressed as part of this response and are examples of the nonspecific host reaction to pathogen invasion (Meins and Ahl, 1989; Bol et al., 1990). Some of the PR proteins have well-defined enzymatic activities such as chitinase (Legrand et al., 1987), which catalyzes the hydrolysis of chitin, a major component of cell walls of many filamentous fungi (Aronson, 1965). Indeed, transgenic plants constitutively expressing chitinase show increased resistance to a fungal pathogen (Broglie et al., 1991). Chitinase expression, as a subset of the pathogenesis response, is induced by the presence of pathogens and directly by ethylene. In this study, we used expression of chitinase as a molecular paradigm for ethylene-dependent responses.

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At least two different pathways regulate pathogenesis response (Lotan and Fluhr, 1990a). One pathway is ethylene dependent and is exemplified by ethylene itself (Boller et al., 1983; Ecker and Davis, 1987). It also includes elicitors, such as α-aminobutyric acid (α-AB), that promote ethylene production and require ethylene in their mode of action (Lotan and Fluhr, 1990b; Eyal and Fluhr, 1992). The other pathway is ethylene independent (Mauch et al., 1984) and is exemplified by the fungal endoxylanase. The enzyme apparently degrades β-1,4-xylan linkages in the plant cell wall and is an exceptionally potent inducer of chitinase accumulation. It also promotes ethylene evolution (Fuchs et al., 1989); nevertheless, ethylene is not required in its mode of action (Lotan and Fluhr, 1990b).

Cellular transduction of external signals provided by hormones is typically a chain of consecutive events. In the case of ethylene, intermediates for the transduction pathway have not been elucidated. In other cases, extracellular hormonal or environmental signals were shown to alter cytosolic levels of calcium, which is considered to be a primary event in triggering cellular responses. In plant cells, the concentration of free calcium in the cytosol is very low (100 nM), whereas in extracellular and intracellular pools it is 10^4 to 10^5 times higher (Hepler and Wayne, 1985). Release of free calcium from these sources to the cytosol can play the role of a second messenger in signal transduction. In animal cells, elevated levels of cytosolic free calcium were shown to regulate target proteins directly

or via Ca^{2+} binding proteins (Williamson and Monck, 1989). In plant cells, there is a growing list of stimulus-response couplings in which cytosolic calcium appears to play an essential role. Examples are stomata opening, gravitropism, and certain light-stimulated chloroplast movements (Hepler and Wayne, 1985; Gilroy et al., 1987; Pooviah and Reddy, 1987; Trewavas and Gilroy, 1991). In this study, we showed that calcium is essential for the ethylene-dependent pathway of PR protein induction but not for the ethylene-independent pathway. Indeed, direct elevation of cytosolic calcium induced chitinase accumulation in the leaf. We further implicated calcium involvement in ethylene-promoted morphogenic responses in seedlings.

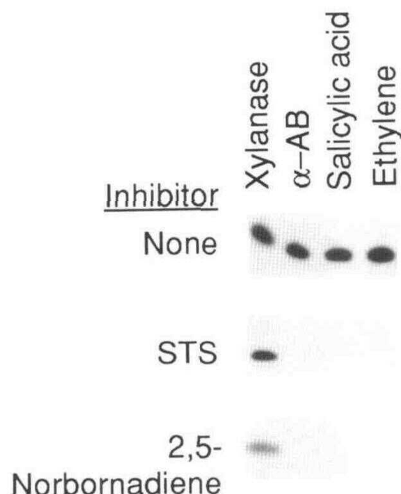

Figure 1. Immunoblot Analysis of Protein Extracts from Elicitor-Induced Leaves.

Leaves were treated with the elicitors xylanase, α-AB, salicylic acid, or ethylene in the presence or absence of ethylene action inhibitors. Top panel: Elicitors applied without the addition of inhibitors. Middle panel: Elicitors applied with the addition of 50 μM STS. Lower panel: Elicitors applied with the addition of 1250 ppm of 2,5-norbornadiene. Proteins were subjected to SDS-PAGE and immunoblotted with anti-chitinase antibody.

RESULTS

Ethylene-Dependent and Non-Ethylene-Dependent Pathways

Differentiation between ethylene-dependent and the ethylene-independent PR protein induction pathways was previously demonstrated in tobacco leaves using ethylene inhibitors (Lotan and Fluhr, 1990b). We wished to further characterize these pathways with respect to salicylic acid, a novel elicitor of systemic acquired resistance (Ward et al., 1991; Yalpani et al., 1991). Xylanase, salicylic acid, α-AB, and ethylene are efficient inducers of a 25-kD polypeptide identified as chitinase in immunoblots, as shown in Figure 1. With each elicitor, the induced immunoreactive polypeptide can be resolved into two coordinately expressed isoelectric components (PR-P and PR-Q; Bol et al., 1990; Lotan and Fluhr, 1990a, 1990b). Silver thiosulfate (STS) and 2,5-norbornadiene are considered to be ethylene action inhibitors (Yang, 1985). In the presence of these inhibitors, the accumulation of chitinase induced by salicylic acid as well as α-AB and ethylene was completely inhibited (Figure 1). However, the accumulation of chitinase induced by xylanase was not affected (Figure 1). The results support the existence of two independent pathways for the induction of PR genes. One pathway is induced directly by ethylene or by elicitors like α-AB and salicylic acid that depend on ethylene for their action. The second pathway characterized by xylanase is ethylene independent.

Induction of Chitinase Accumulation by the Ethylene-Dependent Pathway Is Calcium Dependent

The possible involvement of calcium in mediating the pathogenesis response was investigated by treating intact tobacco leaves with different elicitors together with application of 10 mM EGTA. EGTA chelates calcium and can specifically reduce the availability of extracellular calcium (Gilroy et al., 1986; Pooviah and Reddy, 1987). The induction of chitinase accumulation by ethylene or α-AB was inhibited by EGTA, as shown in Figure 2. The accumulation of chitinase induced by xylanase was not affected by the same treatment (Figure 2). Thus, under our experimental conditions, EGTA did not exert pleiotropic inhibition on cellular physiology. The results further support the existence of at least two independent pathways to plant pathogenesis response and lend credence to the possibility that calcium is involved in cellular reaction to ethylene. We next examined the involvement of calcium using agents that directly perturb intracellular calcium levels. Ionomycin, a calcium ionophore (Liu and Hermann, 1978), was shown to increase cytosolic calcium in an EGTA-dependent manner in animal and plant cells (Ozaki and Kume, 1988; Gilroy et al., 1989). The application of ionomycin to intact tobacco leaves induced chitinase expression in a concentration-dependent manner, as shown in Figure 3A. Application of 100 nM ionomycin induced chitinase accumulation to levels achieved by ethylene. The induction of chitinase by ionomycin was more than 90% inhibited by concomitant application of EGTA (average of three experiments); however, it was not blocked by ethylene action inhibitor STS (Figure 3A).

Thapsigargin was shown to inhibit a ubiquitous endomembrane localized Ca-ATPase pump present in animal cells, compelling an intracellular rise in calcium (Takemura et al., 1989; Thastrup et al., 1990). This drug has not been studied in plants; however, treatment of leaves with thapsigargin induced chitinase accumulation (Figure 3B). In the presence of EGTA, chitinase accumulation was inhibited by 30% (average of three experiments; Figure 3B). The range of effective

concentration used with both chemicals was well within the concentrations that have been used in other systems (Ozaki and Kume, 1988; Lodish, 1990; Schönthal et al., 1991). The results are consistent with ionomycin and thapsigargin both playing a similar role in plant and animal cells. They suggest that an artificial increase in cytosolic calcium leads to induction of chitinase accumulation in leaves. This induction is abrogated by blocking calcium flux into the cytosol during EGTA-motivated calcium depletion, demonstrating the essential role of calcium in the ethylene-dependent PR protein induction pathway.

Calcium-Deficient Plants Exhibit Aberrant Pathogenesis Response

The physiological role of calcium in promoting the pathogenesis response was further examined using calcium-poor plants. Plants were grown in perlite and watered with growth solution without calcium. The plants developed normally but displayed a doubling of generation time when compared to plants potted in soil. The leaves of the calcium-poor plants contained more than 10-fold less extractable calcium than normal leaves, as determined by flame absorption spectrophotometry and shown in Figure 4A. Calcium-deficient leaves, similar to normal leaves, contained very low to nondetectable levels of chitinase (Figure 4B). The addition of 10 mM calcium chloride imbibed through the petiole replenished extractable calcium levels in the leaves (Figure 4A) and resulted in a variable but detectable level of basal chitinase accumulation. The basal level ranged from 10 to 40% relative to that achieved with elicitor-induced normal leaves, as shown in Figures 4B and 5. A similar observation of enhanced basal level accumulation was noted in calcium treatment of normal leaves as well (data not shown). Petioles of calcium-poor plants imbibed with

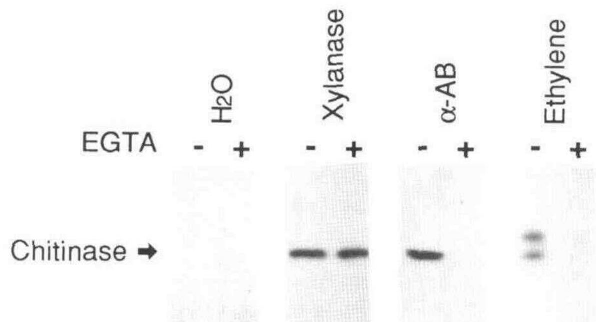

Figure 2. Immunoblot Analysis of Protein Extracts from Elicitor-Induced Leaves in the Presence of EGTA.

Leaves were treated with water, xylanase, α-AB, or ethylene in the presence (+) or absence (−) of 10 mM EGTA. Proteins were subjected to SDS-PAGE and immunoblotted with anti-chitinase antibody. An additional immunoreactive band of slower mobility and unknown origin was frequently observed only in ethylene treatments.

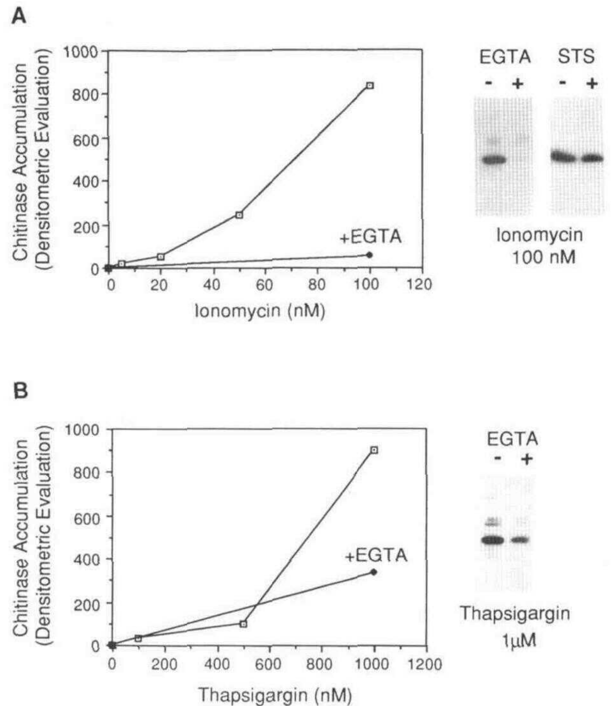

Figure 3. Chitinase Accumulation Induced by Ionomycin and Thapsigargin.

(A) Effect of ionomycin on chitinase induction. Leaves were treated with increasing concentrations of ionomycin (2, 20, 50, and 100 nM). Protein extracts were subjected to SDS-PAGE and immunoblotted with anti-chitinase antibody. Densitometric evaluations were taken from the immunoblot and plotted in arbitrary units. Shown at right are immunoblots of leaves treated with 100 nM ionomycin in the presence (+) or absence (−) of 10 mM EGTA and an immunoblot of leaves treated with 100 nM ionomycin in the presence (+) or absence (−) of 50 μM STS.
(B) Effect of thapsigargin on chitinase induction. Leaves were treated with increasing concentrations of thapsigargin (100, 500, and 1000 nM). Protein extracts were subjected to SDS-PAGE and immunoblotted with anti-chitinase antibody. Densitometric evaluations were taken from the immunoblot and plotted in arbitrary units. Shown at right is an immunoblot of leaves treated with 1 μM thapsigargin in the presence (+) or absence (−) of 10 mM EGTA.

50 mM calcium chloride resulted in a higher basal level of chitinase accumulation in the leaves, which was comparable to levels achieved after elicitor application (135% relative to that achieved with elicitor applied to normal leaves, as shown in Figure 4B). The induction by calcium was abrogated by the presence of EGTA (Figure 4C) but was not blocked by ethylene inhibitors (data not shown).

Significantly, the application of α-AB to calcium-deficient leaves promoted only a very low level of response compared to normal leaves (Figure 4B). When α-AB was applied together with calcium to calcium-deficient plants, induction of high levels of chitinase accumulation was restored irrespective of the starting basal level (Figures 4B and 5). Calcium-deficient leaves

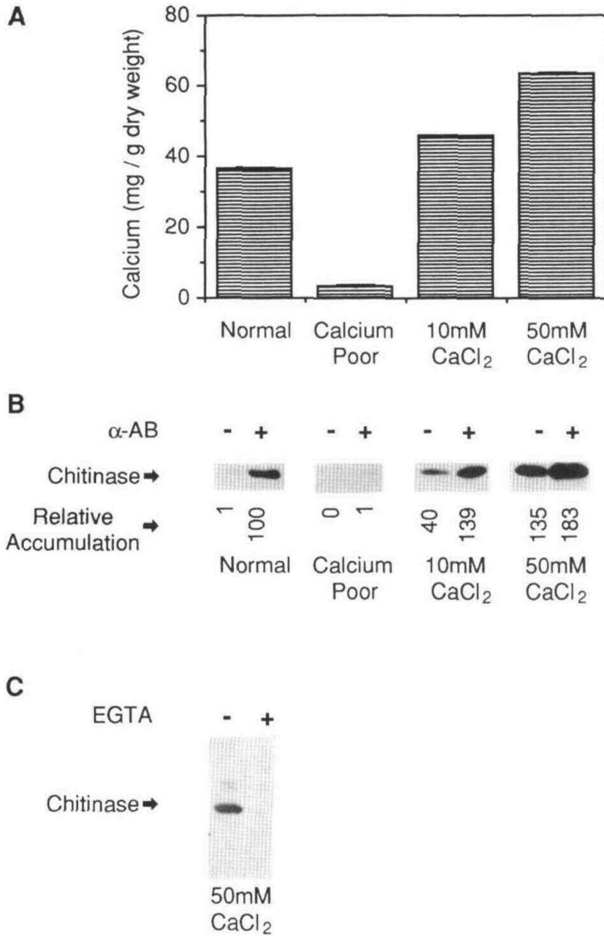

Figure 4. Chitinase Accumulation in Calcium-Poor Plants.

(A) Total calcium concentration in normal leaves and calcium-poor leaves. Calcium-deficient leaves were imbibed with 10 or 50 mM $CaCl_2$ for 24 hr prior to extraction.

(B) Immunoblot analysis of protein extracts from normal or calcium-deficient leaves. Calcium-poor leaves were imbibed with water or with 10 or 50 mM $CaCl_2$ for 24 hr, and treated with (+) or without (−) α-AB elicitor. Proteins were subjected to SDS-PAGE and immunoblotted with anti-chitinase antibody. Numbers represent the densitometric evaluation of the accumulation of chitinase relative to the induction obtained in the normal leaf.

(C) Effect of EGTA on calcium-induced chitinase accumulation. Leaves were imbibed through the petiole with 50 mM $CaCl_2$ in the presence (+) or absence (−) of 10 mM EGTA applied directly to the leaf surface. Proteins were subjected to SDS-PAGE and immunoblotted with anti-chitinase antibody.

may show an impaired ability to synthesize characteristic amounts of PR proteins; therefore, other elicitors of chitinase accumulation were tested (Figure 5). We noted that the ability of xylanase to elicit PR proteins in calcium-deficient leaves remained intact; however, the leaves showed reduced sensitivity to α-AB, ethylene, and salicylic acid (Figure 5). Hence, plants deficient in calcium can synthesize PR proteins when elicited by the ethylene-independent pathway but show reduced induction capability when elicitors of the ethylene-dependent pathway are used.

Calcium is thought to be involved in abscisic acid (ABA)–mediated closing of guard cells (for reviews, see McAinsh et al., 1991; Trewavas and Gilroy, 1991). We, therefore, examined the ability of calcium-deficient plants to respond to ABA by measuring changes in stomatal conductance in excised leaf discs after the application of ABA. Excised leaves from soil-grown plants showed the expected rapid increase in stomatal resistance in the presence of 10 μM ABA, as shown in Figure 6. Excised leaves from calcium-deficient leaves did not respond to ABA. However, the addition of 10 mM calcium to the leaves resulted in a rapid rise in stomatal resistance, indicative of guard cell closure (Figure 6). These results suggest that another calcium-mediated system was concomitantly affected by calcium deficiency. They are in contrast to experiments performed in Commelina that showed only a marginal effect on stomatal opening as a result of growth in medium with varying levels of calcium (Atkinson et al., 1989). We noted that the lowest concentration of calcium used in those experiments was two to three orders of magnitude higher than that used here.

Morphogenic Manifestation of the Ethylene Response

The results thus far indicate a role for calcium in the leaf response to ethylene during pathogenesis. We wished to examine aspects of plant growth that are thought to be directly influenced by ethylene. The triple response of etiolated seedlings consists of distinct morphological changes in seedling shape. Prominent among them are exaggerated hook formation and repressed hypocotyl and root elongation reported in pea and Arabidopsis (Goeschi et al., 1966; Guzmán and Ecker, 1990).

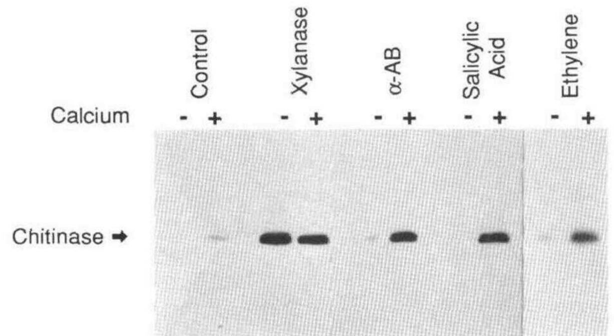

Figure 5. Immunoblot Analysis of Protein Extracts from Elicitor-Induced Calcium-Deficient Leaves.

The elicitors xylanase, α-AB, salicylic acid, and ethylene were applied to calcium-deficient leaves imbibed in the presence (+) or absence (−) of 10 mM calcium. Proteins were subjected to SDS-PAGE and immunoblotted with anti-chitinase antibody.

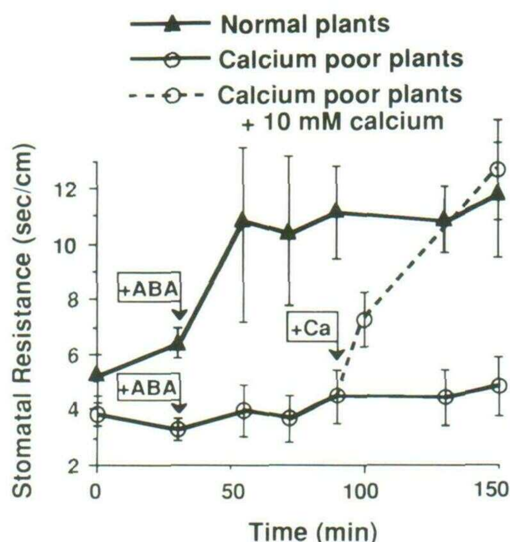

Figure 6. Stomatal Resistance in Calcium-Poor Plants.

Discs were excised from normal and calcium-deficient plants and floated on water. After 30 min, ABA was added (arrow), and, after 90 min, 10 mM calcium was added as indicated (arrow). Experimental points are averages and SE of abaxial side measurements of six discs in three independent experiments.

Tobacco seeds were germinated in the dark in the presence of air or ethylene in an attempt to visualize the triple response. Air-grown etiolated seedlings displayed expanded cotyledons without hypocotyl hook; however, the ethylene-treated seedlings showed repressed cell elongation in both root and stem sections and exhibited a pronounced hypocotyl hook and folded cotyledons, as shown in Figures 7 and 8. When etiolated seedlings were grown in the presence of ethylene without calcium or in medium supplemented with EGTA, the prevalence of hook formation was reduced from 70% of seedlings to 4 and 0% of seedlings, respectively (Figure 8). Root and shoot length were inhibited by ethylene as well; however, our experimental procedures to reduce calcium inhibited cell elongation even in the absence of ethylene (Figure 7C). This observation prevents us from drawing straightforward conclusions about the necessity for calcium in the ethylene-motivated inhibition of elongation.

DISCUSSION

Here and in previous studies, we have shown that elicitors of the pathogenesis response utilize two different induction pathways. One is ethylene independent and can be induced by xylanase and certain pathogens (Lotan and Fluhr, 1990b). The other pathway is ethylene dependent and is characterized by α-AB (Lotan and Fluhr, 1990b), ethylene (Eyal and Fluhr, 1992), and salicylic acid (this paper). Salicylic acid is thought to be a natural systemic signal in the pathogenesis response promoted by tobacco mosaic virus infection (Malamy et al., 1990). Exogenous application of salicylic acid induces an increase in endogenous salicylic acid and PR proteins (Van Loon and Antoniw, 1982; Ward et al., 1991; Yalpani et al., 1991; Enyedi et al., 1992). Salicylic acid was previously suggested to operate further downstream from ethylene action based on temperature shift experiments (Van Loon and Antoniw, 1982). However, our results showed that salicylic acid–induced chitinase accumulation is clearly ethylene dependent. Its effect is blocked by ethylene biosynthesis inhibitors like 1-aminoethoxyvinylglycine (data not shown) and ethylene action inhibitors like STS and 2,5-norbornadiene.

In this work, we have depicted the expression of chitinase. The possibility exists that gene members of the chitinase class are differentially expressed by calcium and elicitors. However, PR proteins generally accumulate coordinately (Bol et al., 1990; Lotan and Fluhr, 1990a, 1990b). We monitored, in addition to chitinase, the acidic and basic PR-1 protein families. As expected, EGTA inhibited the ethylene-based induction of these proteins. Similarly, addition of calcium or application of calcium ionophore induced their accumulation (data not shown).

The results presented in this paper indicate an essential role of calcium in the ethylene-dependent induction of chitinase accumulation; however, in the ethylene-independent pathway, we could not show a requirement for calcium. Blocking calcium fluxes with EGTA or depletion of calcium during growth caused inhibition of response to ethylene, which can be

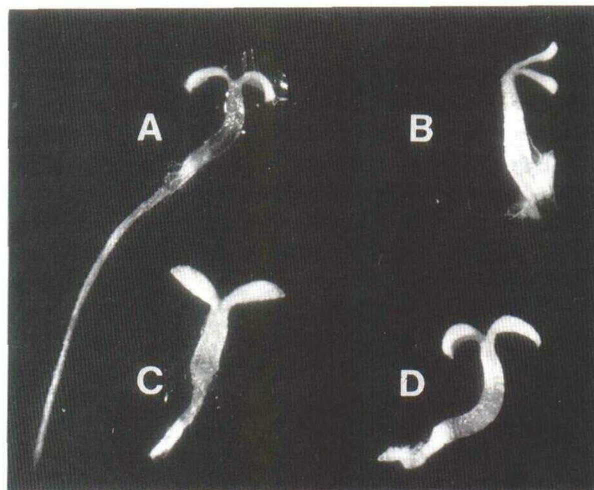

Figure 7. Morphological Features of Etiolated Seedlings Germinated in the Presence of Ethylene.

(A) Air-grown etiolated seedling.

(B) Ethylene-treated etiolated seedling.

(C) Air-grown etiolated seedling grown in the presence of 1 μM EGTA.

(D) Ethylene-treated etiolated seedling grown in the presence of 1 μM EGTA.

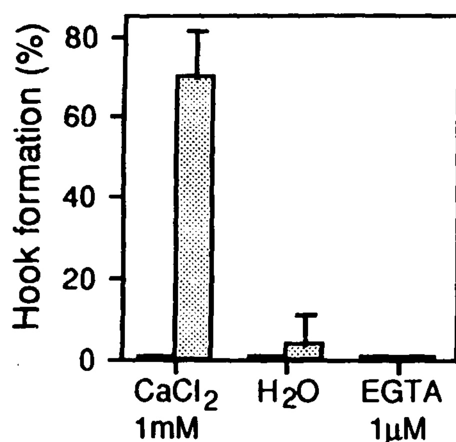

Figure 8. Statistical Analysis of Hook Formation in Etiolated Seedlings Germinated in the Presence of Ethylene.

Seeds were plated on agarose plates containing 1 mM calcium, water, or 1 μM EGTA and germinated as described in Methods. Hook formation was monitored in air-grown seedlings (black bars) and in ethylene-grown seedlings (stippled bars). Averages and their SE are of three independent experiments, and 100 total seedlings were measured.

restored by returning calcium to the plant. Exogenous application of calcium to plants not only restored sensitivity to elicitors but stimulated elicitor-independent chitinase accumulation. Moreover, chitinase accumulation could be stimulated directly by artificially increasing cytosolic calcium using ionomycin and thapsigargin. Both inducers were fully or partially inhibited by EGTA, respectively. Ionomycin is thought to mobilize extracellular stores of calcium and is thus inhibited efficiently by EGTA (Ozaki and Kume, 1988), whereas thapsigargin increases cytosolic calcium mostly from internal stores (e.g., the endoplasmic reticulum) that would be less accessible to EGTA (Thastrup et al., 1990). Possibly, the interactive effects of both potential calcium stores will prove to be important for normal signal transduction, as seems to be the case in animal systems (Mason et al., 1991). Although our experiments did not include direct kinetic measurements of cellular calcium fluxes, they are, however, consistent with the scenario that ethylene induces perturbations in intracellular calcium levels. The fact that chitinase accumulation induced by either ionomycin or high levels of calcium was not affected by ethylene action inhibitors is compatible with this order of events. Recently, using transgenic plants transformed with calcium-sensitive aequorin, it was shown that a proteinaceous elicitor of phytoalexins (a related pathogenesis response) elicits cytosolic calcium (Knight et al., 1991).

We demonstrated that EGTA prevents hypocotyl bending in the presence of ethylene. A trivial explanation would be that EGTA simply blocks all cell elongation and consequently hook formation. However, cotyledon opening, which involves elongation, does take place in the presence of EGTA (Figure 7C). Furthermore, ethylene itself also prevents axial elongation in roots and hypocotyl, yet promotes hook formation (Figure 7B). These observations argue for a specific effect of EGTA on ethylene-motivated hook formation. The results are evocative of experiments that showed loss of gravitropic sensitivity in maize roots after tip application of calcium chelators (Lee et al., 1983). This suggests that the slower ethylene-dependent morphogenic responses may be linked to similar calcium dependency as the rapid gene activation response.

It is conceivable that certain classes of ethylene-sensitive mutations described in Arabidopsis, such as the constitutive response (*ctr*) or hook formation (*his*), can be a result of lesions in promoting or recognizing ethylene-initiated calcium fluxes (Guzmán and Ecker, 1990). The requirement for calcium in the ethylene-based triple response proved to be complex because normal hypocotyl elongation required the presence of calcium. Calcium has been shown to be stimulatory to root elongation in pea and corn as well (Takahashi et al., 1992). Interestingly, analysis of Arabidopsis mutants insensitive to ethylene (*ein*) or that lack the ability to form hooks (*his*) suggests that hypocotyl hook formation and elongation of hypocotyl and root are independent ethylene responses.

Phototropism, gravitropism, and ABA- or cytokinin-induced guard cell movement are linked to calcium fluxes that induce rapid physiological or slower morphological processes (Hepler and Wayne, 1985; Gilroy et al., 1987; Trewavas and Gilroy, 1991). In this work, we implicated calcium as being required for the induction of pathogenesis-related gene arrays. Direct gene activation by calcium-based signaling processes have been described in animal systems where calcium ionophores were shown to induce accumulation of the endoplasmic reticulum-localized immunoglobin binding protein chaperone and nuclear-localized proteins (Watowich and Morimoto, 1988; Lodish, 1990; Schönthal et al., 1991). The induction of chitinase accumulation as a paradigm for ethylene-dependent processes has provided useful insight into additional aspects of the ethylene response. The use of intact leaves in these studies circumvents the difficulties and aberrations that have been encountered in measuring controlled pathogenesis responses in cell culture and, of course, makes the direct whole plant physiological ramifications self-evident. The elucidation of a calcium requirement for ethylene-mediated pathogenesis response provides molecular insight to the well-noted but poorly understood pleiotropic effects of calcium in plant resistance to disease (Zook et al., 1987; Volpin and Elad, 1991). Direct visualizations of ethylene-motivated calcium fluxes and their subcellular origin are future tasks, as is examining how cells differentiate between potential calcium fluxes arising from different hormonal inputs.

METHODS

Plant Material

Nicotiana tabacum cv Samsun NN plants were grown in the greenhouse in 18-hr day (26°C) and 6-hr night (22°C) diurnal cycles. Calcium-deficient plants were prepared by replanting plantlets with

one to two true leaves in washed mineral-free perlite (Cooke and Dunsby, 1978) containing 10 g/L 20/20/20 (potassium, nitrogen, and phosphate mix; Haifa Chemicals, Haifa, Israel). Plants were watered with distilled water. Experiments with normal or calcium-deficient plants were performed in the greenhouse using young potted plants with three to five leaves at least 10 cm in length or under Grolux (Sylvania) fluorescent lamps (2.5 to 3.0 nE cm^{-2} sec^{-1}). Experiments were carried out on excised leaves imbibed with distilled water or $CaCl_2$ where indicated. For triple response experiments, tobacco seeds were plated on 0.8% agarose plates containing water and where stated supplemented with 1 mM $CaCl_2$ or 1 μM EGTA. Plates were kept in the light for 24 hr, followed by dark incubation for 10 days either in the air or in an ethylene box with a constant stream of 20 ppm of ethylene.

Induction of PR Proteins in Leaves

Accumulation of pathogenesis-related (PR) proteins was induced by brushing leaves with 1-mL solutions of 5 mM α-aminobutyric acid (D,L-2-amino-*n*-butyric acid; α-AB), 5 mM salicylic acid, 10 mM EGTA, pH 7.0, ionomycin, or thapsigargin, as specified in the text. Purified β-1,4-xylanase was a gift of Jim Anderson, United States Department of Agriculture (Beltsville, MD). It was prepared as a solution of 10 ng/mL and was injected into the extracellular leaf spaces as described by Lotan and Fluhr (1990b). Ethylene treatments were carried out in a constant stream of 20 ppm of ethylene in a glass box housing the plants or excised leaves. Leaves were incubated for 24 hr in the presence of elicitors. Leaf discs were excised from treated sites and homogenized in solubilization buffer as described by Lotan and Fluhr (1990b).

Electrophoresis and Immunoblotting

Prior to gel electrophoresis, sample buffer was added to protein extract. Boiled proteins (20 μg) were fractionated on 12% SDS–polyacrylamide gels and then transferred to nitrocellulose blots. Blots were immunoblotted with anti-chitinase antibody prepared against the acidic PR-3 class (formerly classified PR-P, Q; Lotan and Fluhr, 1990b) and visualized with ECL reagents (Amersham International).

Measurements of Calcium and Stomatal Resistance

Detached leaves were imbibed with water or $CaCl_2$ as indicated. After 24 hr, leaves were oven dried and weighed. Leaves were digested with HNO_3 overnight. The mixture was heated until the production of red NO_2 fumes ceased. A few drops of 70% $HClO_4$ were added, and the mixture was reheated. Samples were diluted in distilled water, and calcium concentration was measured using the flame absorption spectrophotometer (5100 PC; Perkin-Elmer Corp., Norwalk, CT). Diffusive resistance of normal leaves and calcium-deficient leaves was measured with a transient porometer (LI-700; Li-Cor, Lincoln, NE) using 1.5-cm-diameter excised leaf discs floating on water, with or without added calcium or abscisic acid. Discs were thoroughly damped dry, and the diffusive resistance of the abaxial side was measured.

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