Ethylene Signal Is Transduced via Protein Phosphorylation Events in Plants

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A plethora of abiotic and biotic environmental stresses exert their influence on plants via the gaseous hormone ethylene. In addition, aspects of plant development and climacteric fruit ripening are regulated by ethylene. Sensitivity to ethylene is presumably mediated by a specific ethylene receptor whose activation signal is then transduced via an unknown cascade pathway. We have used the plant pathogenesis response, exemplified by the induction of pathogenesis-related (PR) genes, as a paradigm to investigate ethylene-dependent signal transduction in the plant cell. Ethylene application induced very rapid and transient protein phosphorylation in tobacco leaves. In the presence of the kinase inhibitors H-7 and K-252a, the transient rise in phosphorylation and the induced expression of PR genes were abolished. Similarly, these inhibitors blocked the response induced by an ethylene-dependent elicitor, α-AB. Reciprocally, application of okadaic acid, a specific inhibitor of phosphatases type 1 and type 2A, enhanced total protein phosphorylation and by itself elicited the accumulation of PR proteins. In the presence of H-7 and K-252a, PR protein accumulation induced by okadaic acid was blocked. In contrast to the action of ethylene and α-AB, xylanase elicits the accumulation of PR protein by an ethylene-independent pathway. Xylanase-induced PR protein accumulation was not affected by H-7 and K-252a. The results indicate that responsiveness to ethylene in leaves is transduced via putative phosphorylated intermediates that are regulated by specific kinases and phosphatases.

INTRODUCTION

The hypersensitive reaction, resulting in local lesions of dead cells, is one of the many manifestations of a plant defense response against pathogens. The exact role of the lesions in containing pathogen spread is not clear, but the lesions indicate that the pathogen was recognized and that additional plant pathogenesis responses have been triggered. Among those responses are the activation of pathogenesis-related (PR) protein genes and accumulation of their products. Some of the PR proteins have well-defined antipathogen enzymatic activities, such as chitinase (Legrand et al., 1987) that 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). Accumulation of PR proteins can also be triggered by the application of various hormones and chemical elicitors in a process that does not involve the hypersensitive response. Because the expression of PR genes is indicative of the pathogenesis state and may play a role in the general defense mechanism of the plant, they have been used as molecular paradigms for the response of plants to pathogens.

An important mediator of the pathogenesis response is the plant hormone ethylene (for review, see Enyedi et al., 1992).

Ethylene biosynthesis rapidly increases during plant–pathogen interaction or application of chemical elicitors (Yang and Hoffman, 1984; Lotan and Fluhr, 1990). It has been implicated in the induction of defense-related gene arrays (Ecker and Davis, 1987) and both acidic- and basic-type PR proteins (Van Loon and Antoniw, 1982; Brederode et al., 1991; Eyal et al., 1992; Raz and Fluhr, 1992). However, not all pathogenesis responses depend on ethylene (Boller et al., 1983). Using inhibitors of ethylene biosynthesis and action, it was suggested that elicitors and pathogens utilize at least two different pathways for PR gene activation (Lotan and Fluhr, 1990). One pathway is ethylene independent (Mauch et al., 1984) and is exemplified by the elicitor endoxylanase, an enzyme of fungal origin that can degrade β-1-4-xylan linkages in the plant cell wall and is a potent inducer of chitinase accumulation (Lotan and Fluhr, 1990). The other pathway is ethylene dependent and is exemplified by ethylene itself (Boller et al., 1983; Ecker and Davis, 1987) and other elicitors such as α-aminobutyric acid (α-AB), and salicylic acid (Lotan and Fluhr, 1990; Eyal et al., 1992; Raz and Fluhr, 1992).

Cellular transduction of the external signal provided by ethylene is presumed to be an ordered process of consecutive events. One component in the pathway was recently shown to require calcium (Raz and Fluhr, 1992). Other possible components such as protein phosphorylation are thought to play a key role in diverse biological signal transduction systems

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(for reviews, see Cohen, 1982; Hardie, 1990). In plants, protein phosphorylation was shown to play a direct regulatory role in light response (for reviews, see Bennett, 1991; Allen, 1992) and circadian rhythms (Carter et al., 1991). Correlations between protein phosphorylation and physiological response were demonstrated in pollen embryogenesis (Kyo and Harada, 1990) and fungal elicitor treatment of cultured cells (Grab et al., 1989; Dietrich et al., 1990; Grosskopf et al., 1990; Felix et al., 1991; Schwacke and Hager, 1992). Here, with the use of specific kinase and phosphatase inhibitors, we show that phosphorylation events are essential for the ethylene-mediated pathogenesis response in tobacco plants.

RESULTS

Ethylene-Triggered Transient Changes in Protein Phosphorylation Profiles

The possible involvement of phosphorylation in the plant response to ethylene was directly monitored by visualizing the
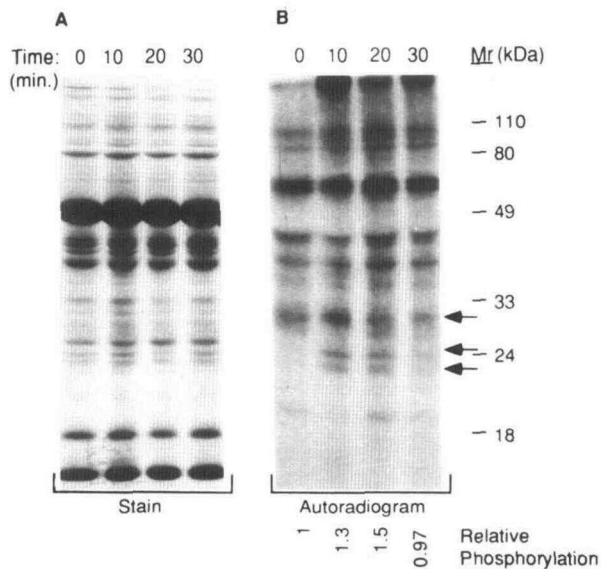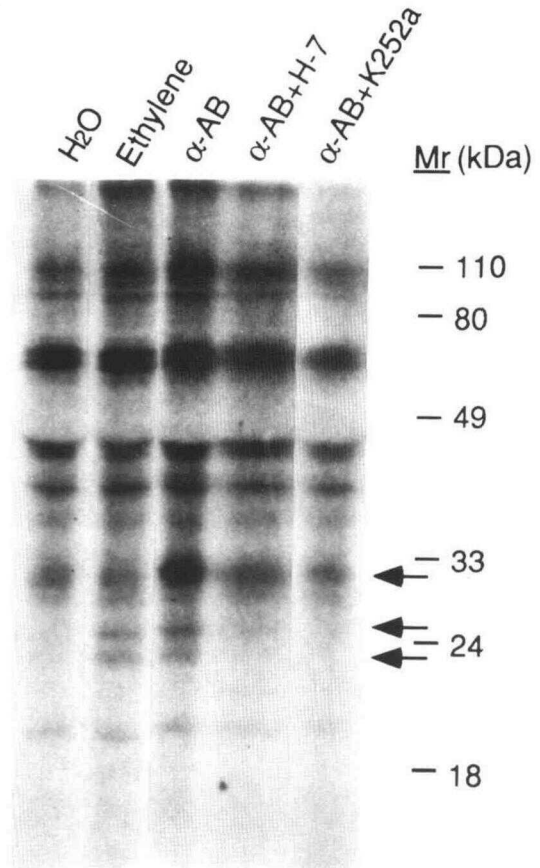

Figure 1. In Vivo Protein Phosphorylation in the Presence of Ethylene.

Leaf discs were labeled with ^{32}P-orthophosphate for 4 hr and then treated with 20 ppm ethylene for the indicated times. Proteins were subjected to fractionation on SDS–polyacrylamide gels.
(A) Coomassie blue staining.
(B) Autoradiograph of the gel in **(A)**. Proteins whose phosphorylation state was enhanced due to ethylene treatment are indicated by arrows. The relative phosphorylation ratio is indicated below each lane. It was determined by dividing the incorporated counts per min by 10 mg of proteins and normalizing each time to "0" time. Molecular mass markers at right are given in kilodaltons.

Figure 2. In Vivo Protein Phosphorylation in the Presence of Elicitors and Kinase Inhibitors.

Autoradiograph of ^{32}P-labeled leaf extracts fractionated on SDS–polyacrylamide gels as given in Figure 1. Discs were treated for 20 min with the following elicitors: water (control); 20 ppm of ethylene; 5 mM α-AB; 5 mM α-AB and 100 μM H-7; 5 mM α-AB and 100 nM K-252a. Molecular mass markers at right are given in kilodaltons.

general state of protein phosphorylation in the leaf. Leaf discs were labeled with ^{32}P-orthophosphate for 4 hr to achieve a steady state level of protein phosphorylation. After 4 hr, leaf discs were exposed to ethylene, in the continued presence of orthophosphate, and the phosphorylation pattern was monitored for 30 min, as shown in Figure 1. When protein aliquots were fractionated (Figure 1A), a transient increase in total relative phosphorylation (radioactivity/protein) was apparent during ethylene treatment and new or more heavily phosphorylated polypeptides were detected (Figure 1B, see arrows). These phosphoproteins were apparent after 10 min, reached a maximum level after 20 min, and returned to the basal level after 30 min. While a transient increase in phosphorylation after exposure to ethylene was always detectable, the time for achieving maximum phosphorylation varied between 10 and

20 min. The results indicate that very rapid protein phosphorylation is induced by ethylene. However, they do not necessarily mean that the specific polypeptides shown in Figure 1B participate in the signal transduction pathway. If the specific pattern of transient phosphorylation shown in Figure 1B is common to ethylene-activated processes, we asked whether α-AB, which is known to induce pathogenesis by an ethylene-requiring pathway, would stimulate phosphorylation as well. As shown in Figure 2, α-AB appears to enhance the same subset of phosphorylated proteins as does ethylene.

We further asked if the rapid ethylene-triggered phosphorylation events would result in rapid gene expression activation, as has been shown for some hormones in animal systems (Kruijer et al., 1984). Ethylene is an effective elicitor of PRB-1b, a basic-type PR-1 protein in tobacco plants. After ethylene application, specific PRB-1b RNA and protein accumulate within 1 and 24 hr, respectively (Eyal et al., 1992). To test for the presence of PRB-1b transcript at earlier times after induction, we employed the highly sensitive RNA-dependent reverse transcriptase–polymerase chain reaction (Robinson and Simon, 1991; Dilworth and McCarrey, 1992) using primers specific to PRB-1b gene. As shown in Figure 3 (top section), transcript can be detected within 10 min, and levels increased during the course of the experiment.

Kinase Inhibitors Prevent Pathogenesis Response

1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H-7) and K-252a are potent inhibitors of mammalian protein kinases and can
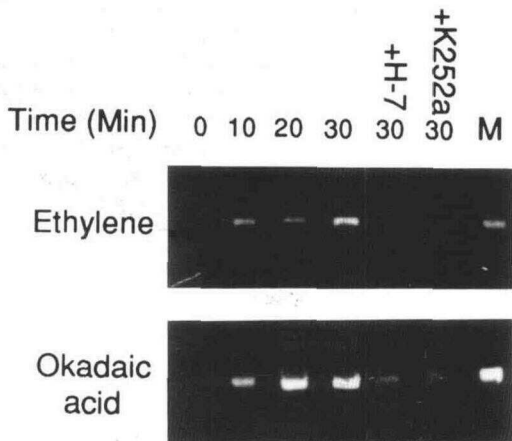
Figure 3. Quantitative RNA-Dependent Reverse Transcriptase–Polymerase Chain Reaction of PRB-1b Transcript.

Total RNA was extracted from ethylene-treated leaves (top panel) or okadaic acid–treated leaves (bottom panel) at 10-min intervals as indicated in each lane and subjected to reverse transcriptase–polymerase chain reaction. In lanes marked H-7 and K-252a, leaves were treated with inhibitors and ethylene or okadaic acid for 30 min. The lane marked M is a direct PCR product from cloned PRB-1b DNA.
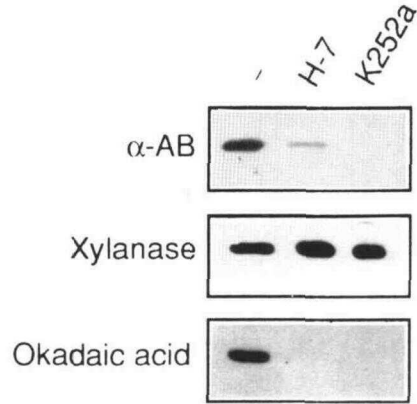
Figure 4. Immunoblot Analysis of Protein Extracts from Elicitor-Induced Leaves in the Presence of Kinase Inhibitors.

Leaves were treated with 5 mM α-AB, 10 ng/mL xylanase, or 0.5 μM okadaic acid in the presence or absence of kinase inhibitors as indicated. Left lanes, elicitors applied without the addition of inhibitors; middle lanes, elicitors applied with the addition of 100 μM H-7; right lanes, elicitors applied with the addition of 100 nM K-252a. Proteins were fractionated on SDS–polyacrylamide gels and immunoblotted with anti-chitinase antibody.

be indicative of protein kinase C–like activity (Kawamoto and Hidaka, 1984; Kase et al., 1986; Gat-Yablonski and Sagi-Eisenberg, 1990). K-252a was shown to reduce protein phosphorylation when added to plant cell culture (Grosskopf et al., 1990). Application of these drugs to leaves, at concentrations that elicit a specific response in animal systems, inhibited α-AB–induced protein phosphorylation (Figure 2). This indicates that K-252a and H-7 are effective inhibitors of plant protein kinases in leaves. The possible effects of these kinase inhibitors on the ethylene-triggered pathogenesis response were tested by monitoring the accumulation of PRB-1b transcript. As shown in Figure 3 (top section), application of kinase inhibitors together with ethylene treatment blocked the rapid increase in transcript levels.

It was of interest to establish whether the ethylene-dependent and ethylene-independent pathways for pathogenesis response differed with respect to their sensitivity to kinase inhibitors. α-AB represents a class of elicitors that activates the ethylene-dependent pathway, whereas xylanase represents an elicitor class that induces pathogenesis response via an ethylene-independent pathway (Lotan and Fluhr, 1990; Raz and Fluhr, 1992). When PR protein accumulation was monitored by immunoblot detection, kinase inhibitors blocked α-AB–induced accumulation of chitinase while xylanase-induced chitinase accumulation was not affected, as shown in Figure 4. The fact that induction of PR protein accumulation by xylanase was unaffected by H-7 and K-252a also indicates that the inhibitors do not exert pleiotropic effects on gene activation.

Application of α-AB induces the concomitant accumulation of ethylene, PR proteins, and formation of microlesions (Lotan
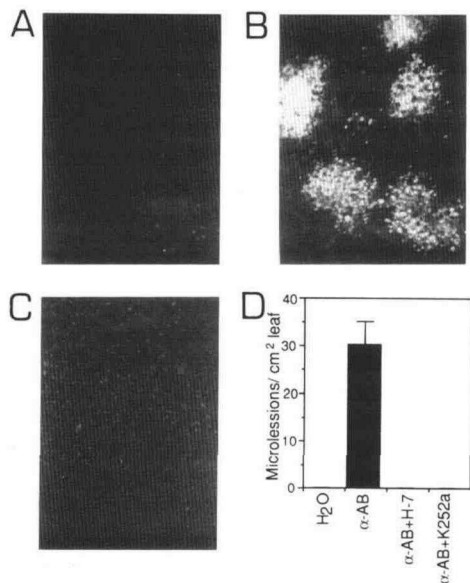
Figure 5. Epifluorescent Visualization of Microlesions.

Leaves were treated with α-AB in the presence or absence of kinase inhibitors for 24 hr. Panel widths represent 2 mm of leaf surface.

(A) Water control.

(B) Leaves treated with 5 mM α-AB.

(C) Leaves treated with 5 mM α-AB and 100 mM H-7.

(D) Statistical analysis of microlesion formation in the presence of water, 5 mM α-AB, 100 mM H-7, or 100 nM K-252a.

substrate specificity through interaction with specific control proteins (Cohen, 1989). The application of specific inhibitors of phosphatases and kinases is one means to study the effect of these enzymes on particular biological systems. Thus, if a kinase inhibitor blocks a particular response, the reciprocal use of a phosphatase inhibitor may activate the response. The core catalytic component of PP1 and PP2A can be specifically inhibited by the polyether compound okadaic acid (MacKintosh and Cohen, 1989; Cohen et al., 1990). Leaves treated with okadaic acid showed a twofold increase in relative protein phosphorylation within 10 min, as shown in Figure 6. The general increase in the phosphorylation level observed is consistent with the inhibition of a pivotal cellular phosphatase. Examination of RNA from okadaic acid–treated leaf extracts showed a rapid increase in PRB-1b transcript (Figure 3). This increase was blocked in the presence of kinase inhibitors H-7 or K-252a. The capability of okadaic acid to elicit coordinate expression of additional PR protein classes was examined by immunodetection. Leaves treated with okadaic acid showed accumulation of glucanase (PR-2), chitinase (PR-3), acidic-type PR-1, and basic-type PRB-1b, as shown in Figure 7. As shown in Figure 4, the okadaic acid–elicited accumulation of chitinase was blocked in the presence of H-7 or K-252a.

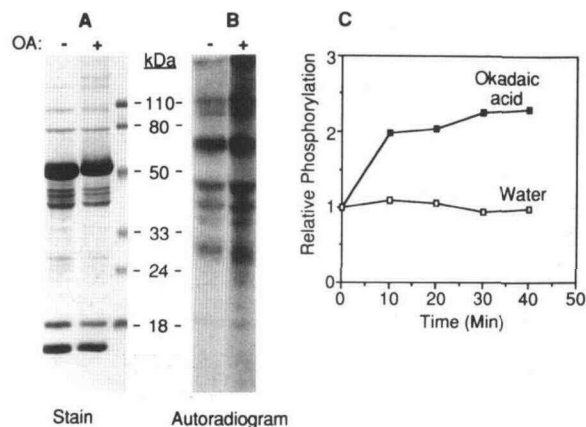Okadaic acid induced an array of PR proteins and can be classified as an elicitor of the pathogenesis response. The
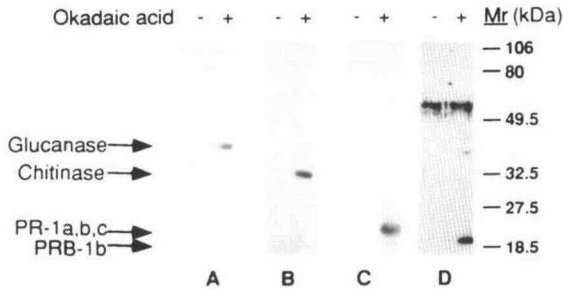
and Fluhr, 1990). Application of α-AB together with the ethylene production inhibitor 1-aminoethoxyvinylglycine or with ethylene-action inhibitors silver thiosulfate and norbordiene completely blocks PR protein accumulation (Lotan and Fluhr, 1990) and microlesion formation (data not shown). It was of interest to see whether the additional aspects of the pathogenesis response induced by α-AB were similarly affected by the inhibitors. As shown in Figure 5, microlesion development after α-AB application is demonstrated by the appearance of UV autofluorescence, probably associated with lignification processes occurring in surrounding cells (compare Figures 5A and 5B). Application of α-AB in the presence of H-7 and K-252a completely blocked microlesion formation and associated UV fluorescence (compare Figures 5B and 5C).

Figure 6. In Vivo Protein Phosphorylation in the Presence of Okadaic Acid.

Leaf discs were labeled with ^{32}P-orthophosphate for 4 hr and then treated with 0.5 mM okadaic acid (OA) for the times indicated. Proteins were fractionated on SDS–polyacrylamide gels. Molecular mass markers are shown between **(A)** and **(B)** and are given in kilodaltons.

(A) Coomassie blue staining of extracts from leaves 0 min (−) and 20 min (+) after induction.

(B) Autoradiograph of lanes shown in **(A)**.

(C) Time course of protein phosphorylation induced by okadaic acid. The relative phosphorylation was determined by the densitometric evaluation of radioactivity in proteins subjected to fractionation on SDS–polyacrylamide gels normalized to time 0.

Induction of PR Proteins Accumulation by a Phosphatase Inhibitor

Protein phosphatases type 1 (PP1) and type 2A (PP2A) are ubiquitous, broad specificity phosphatases that have recently been characterized in plants (MacKintosh and Cohen, 1989; MacKintosh et al., 1990). They are believed to achieve their

Figure 7. Immunoblot Analysis of Protein Extracts from Okadaic Acid–Induced Leaves.

Leaves were treated with control (–) or 0.5 mM okadaic acid (+). Proteins were fractionated on SDS–polyacrylamide gels and immunoblotted with various antisera.

(A) Anti-glucanase antibody.
(B) Anti-chitinase antibody.
(C) Anti–acidic-type PR-1 antibody.
(D) Anti-PRB-1b antibody.
Molecular mass markers are given at right in kilodaltons.

range of effective concentration used (50 to 500 nM) was well within the concentrations that have been used in other in vivo systems (Heystead et al., 1989; Nagamine and Ziegler, 1991). When applied in vitro, okadaic acid can differentiate between the two classes of phosphatase. It inhibits type 2A phosphatase at the picomolar range and type 1 phosphatase at the nanomolar range (Cohen, 1989). However, concentrations necessary for work in intact leaf tissue (50 to 500 nM) make it impossible to differentiate between the two potential phosphatase targets.

Okadaic acid may stimulate plant pathogenesis response by inducing ethylene production. In that case, it would not intervene directly through the signal transduction pathway, and the results obtained above could be explained on the basis of ethylene action. Alternatively, okadaic acid may stimulate the signal transduction pathway for PR gene expression downstream to ethylene. To differentiate between these possibilities, okadaic acid was applied together with the ethylene inhibitor silver thiosulfate, which is thought to inhibit ethylene action. The inhibitor blocked ethylene-dependent chitinase accumulation but did not affect chitinase accumulation induced by okadaic acid, as shown in Figure 8. We concluded that the phosphatase involved in the regulation of PR genes expression operates downstream, in the transduction pathway, relative to ethylene action.

DISCUSSION

Labeling experiments in leaves showed that specific protein phosphorylation events are induced by ethylene. α-AB, which induces pathogenesis response in a pathway that

requires ethylene, induced profiles of transient polypeptide phosphorylation similar to that obtained with ethylene. The phosphorylation of these polypeptides was inhibited by kinase inhibitors K-252a and H-7. In addition, K-252a and H-7 inhibited the ethylene-induced accumulation of PRB-1b transcript and chitinase protein. Thus, rapid polypeptide phosphorylation is associated with ethylene application, and phosphorylation events are necessary for ethylene-activated signal transduction that results in the induction of PR gene expression and microlesion formation. The fact that both K-252a and H-7 had the same effect on PR protein expression and protein phosphorylation may indicate that both drugs block kinase activity of the same enzyme, as has been suggested for their inhibition of protein kinase C activation of serotonin secretion in animal cells (Gat-Yablonski and Sagi-Eisenberg, 1990). Protein phosphorylation of cultured plant cells was shown to be affected by fungal elicitor treatments (Dietrich et al., 1990; Grosskopf et al., 1990; Felix et al., 1991; Schwacke and Hager, 1992). The kinetics of phosphorylation induced by the elicitors was rapid (within minutes), transient (Dietrich et al., 1990), and negatively regulated by K-252a (Grosskopf et al., 1990; Felix et al., 1991). To what extent nondifferentiated cell cultures reflect normal signal transduction is unknown. Indeed, in tobacco, the regulation of PR proteins must be studied in leaves as they are constitutively expressed in cell cultures (Antoniw et al., 1981).

Okadaic acid, a specific inhibitor of animal and plant PP1 and PP2A, was shown here to increase the general level of protein phosphorylation in intact leaves, similar to its effect in animal cell cultures (Cohen, 1989; Heystead et al., 1989). In addition, it elicited the rapid accumulation of a PR-specific transcript and PR proteins in intact leaves. In animal systems, okadaic acid has been shown to be capable of inducing specific genes at the transcriptional level (Nagamine and Ziegler, 1991; Guy et al., 1992). In leaves, the induction of PR gene expression by okadaic acid was blocked by the presence of
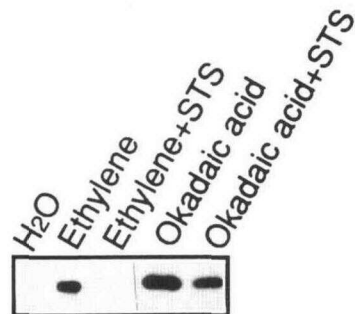
Figure 8. Immunoblot Analysis of Protein Extracts from Elicitor-Induced Leaves in the Presence of Ethylene Inhibitor.

Leaves were treated with ethylene or 0.5 μM okadaic acid in the presence or absence of 50 μM silver thiosulfate (STS). Proteins were fractionated on SDS–polyacrylamide gels and immunoblotted with anti-chitinase antibody.
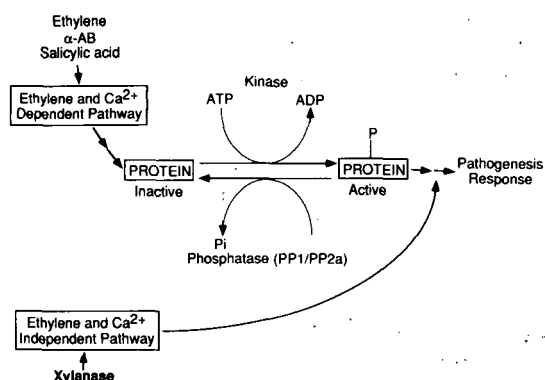
Figure 9. A scheme for the Involvement of Phosphorylation Events in the Induction of Ethylene-Dependent Pathogenesis Response.

Ethylene, α-AB, and salicylic acid utilize the ethylene- and calcium-dependent pathway while xylanase utilizes a different pathway (Raz and Fluhr, 1992). The transducing activity of a putative control protein is determined by its phosphorylation level. The level of phosphorylation is determined by the kinetics of kinase to phosphatase activity.

kinase inhibitors, suggesting that the transduction events requiring kinase and phosphatase activity are probably acting through the same pathway. Two types of protein phosphorylation kinetics have been detected, a stable increase induced by okadaic acid and a transient increase induced by ethylene (Figures 1 and 6). The transient increase is consistent with attenuation of an initial receptor response to a signal molecule, while the stable increase is expected from a constitutive biochemical type of inhibition. Hence, at least one stage of the transduction pathway requires kinase activity, which is modified by a mechanism that maintains phosphorylation equilibrium, as shown schematically in Figure 9. Alternatively, or in addition, the induction of PR proteins by ethylene could be transduced via negative regulation of PP1/PP2A phosphatase activity.

Elicitors of pathogenesis utilize at least two pathways for the induction of PR proteins, which differ in their requirement for ethylene (Lotan and Fluhr, 1990) and calcium (Raz and Fluhr, 1992). Here we show that the ethylene-independent pathway, which is induced by xylanase, was not affected by the kinase inhibitors we used. Thus, these pathways differ in their initial cellular signal-transducing machinery as well. Apparently, downstream from the transduction events depicted here, the pathways leading to gene activation converge to give, in each case, coordinate regulation of PR protein accumulation (Figure 9).

Our studies implicate the ubiquitous cellular PP1/PP2A phosphatases in PR protein induction. A component in the transduction pathway of ethylene has been recently isolated using Arabidopsis mutants. It was shown to be a Raf-like protein kinase that may negatively regulate ethylene-motivated triple response (Kieber et al., 1993). The possibility of isolating mutants in the core PP1/PP2A complex itself using genetic

methods is unlikely, due to its participation in the regulation of many pathways. Future studies should involve differentiating between the two phosphatases and looking for their specific substrate interactions.

METHODS

Plant Material

Greenhouse grown *Nicotiana tabacum* cv Samsun NN plants were subjected to 18-hr day (26°C) and 6-hr night (22°C) diurnal cycles. Experiments were performed in the greenhouse using young potted plants with three to five leaves of at least 10 cm in length or under Grolux (Sylvania) fluorescent lamps (2.5 to 3.0 nE cm^{-2} sec^{-1}).

Induction of PR Proteins and Microlesions in Leaves

Accumulation of pathogenesis-related (PR) proteins was induced by brushing leaves with 1-mL solutions of 5 mM α-aminobutyric acid (α-AB; D, L-2-amino-*n*-butyric acid) or 0.5 μM okadaic acid. Purified β-1-4-xylanase was a gift from Dr. Jim Anderson (U.S. 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 previously (Lotan and Fluhr, 1990). Ethylene treatments were performed in a constant stream of 20 ppm ethylene in a glass box housing whole plants, excised leaves, or leaf discs. Leaves were incubated for the times indicated in the presence of elicitors. Leaf discs were excised from treated sites and homogenized in solubilization buffer as described previously (Lotan and Fluhr, 1990). Microlesions were visualized under 365-nm exciter light with a 395-nm dichromatic beam splitter and 420-nm barrier filter (Carl Zeiss, Oberkochen, Germany).

Leaf Labeling

Leaf discs (1-cm-diameter) were incubated for 4 hr with 100 μCi/mL carrier-free ^{32}P-orthophosphate (Amersham International) and treated with the different elicitors and drugs described for PR protein induction. Protein extraction was performed at 4°C, which prevented detectable phosphatase and kinase activity in vitro. After the indicated times, discs were transferred into liquid nitrogen and homogenized in solubilization buffer containing 50 mM Hepes, pH 7.5, 25 mM sucrose, 5 mM EDTA, 5 mM EGTA, 10 mM NaF, 14 mM β-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 1 μg leupeptin, and 50 μg aprotinin. Protein extracts were cleaned over P-6 columns (Bio-Rad).

Reverse Transcription–Polymerase Chain Reaction Determination of Transcript Levels

Leaves were treated with elicitors as described for PR protein induction for the times indicated. RNA was prepared as described previously (Logemann et al., 1987) and treated with DNase I before being subjected to the reverse transcriptase reaction as described by Dilworth and McCarrey (1992). Primers for the polymerase chain reaction (PCR) were chosen from the coding region and a region of the 3′ nontranslated end that showed no homology to other basic-type PR-1 genes. The 5′ primer was from nucleotides 195 to 216 and the 3′ primer was from 657 to 678, according to the published sequence (Eyal et al., 1992).

PCR amplification was performed under limiting input RNA (50 ng) to keep all other substrates in excess as described by Robinson and Simon (1991). Conditions were as follows: 40 sec at 95°C, 40 sec at 66°C, and 40 sec at 75°C, all for 30 cycles. The amount of RNA in the reaction was first titered. Above 200 ng of total RNA, a detectable amount of PCR product was observed in untreated leaves. Amounts of transcript were quantitated by using in vitro–synthesized RNA transcripts of the PRB-1b cloned gene. PCR products were linear at 20 cycles from 1 to 100 pg of RNA and at 30 cycles from 0.1 to 10 pg.

Electrophoresis and Immunoblotting

Prior to gel electrophoresis, sample buffer was added to protein extract. Boiled proteins (20 µg) were subjected to electrophoresis on 12% SDS–polyacrylamide gels followed by transfer to nitrocellulose sheets. Immunoblot techniques and antibodies for acidic chitinase, acidic glucanase, acidic PR-1, and their specificities have been described by Lotan and Fluhr (1990). Antibody for the basic PR-1b and its specificity have been described by Eyal et al. (1993). Immunoblots were visualized with ECL reagents (Amersham International). Gels with ^{32}P-labeled proteins were stained for 10 min with Coomassie Brilliant Blue R and destained in 20% methanol, 12% acetic acid for 16 hr. Gels were photographed, dried, and autoradiographed.

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